Investigation of biomarker determinants of treatment efficacy of fulvestrant +/- the RET inhibitor vandetanib in oestrogen receptor positive breast cancer

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Thesis summary

ER+ breast cancer affects millions of women worldwide. Disease relapse is common after initial treatment and causes significant morbidity and mortality. Many treatments focus on targeting the endocrine receptor but over time resistance to endocrine therapies emerges. The fulvestrant and vandetanib in advanced aromatase inhibitor resistant breast cancer (FURVA) clinical trial aimed to address this by adding vandetanib (a RET inhibitor) to a hormone directed backbone (fulvestrant).

The work presented in this thesis documents the investigation into biomarker determinants of response to treatment. Three key areas are investigated (i) RET expression as determined by immunohistochemistry (IHC), (ii) presence of single nucleotide variants (SNVs) by NGS and digital droplet PCR (ddPCR) and (iii) copy number alterations determined by ddPCR. Tissue and plasma samples were collected during trial participation and allowed investigations of both primary tumour, represented by formalin fixed paraffin embedded tissue samples and metastatic disease, represented by circulating free DNA (cfDNA) extracted from plasma.

Both high total-RET (t-RET) and phosphorylated-RET (p-RET) expression by IHC correlated with longer progression free survival (PFS) in participants in the FURVA clinical trial irrespective of treatment received. In addition, patients with no detectable circulating tumour DNA (ctDNA) in plasma samples during trial participation had a longer PFS. There were notable negative findings; increased RET expression did not appear to be related to SNVs in *RET* and copy number alterations in *FGFR1* or *MYC* did not correlate with PFS but were detectable using ddPCR technology.

In conclusion, this thesis has shown that high RET expression correlates with longer PFS and that detection of ctDNA during treatment correlates with shorter PFS when patients are treated with fulvestrant +/- vandetanib.

Statements and declaration

This thesis is being submitted in partial fulfilment of the requirements for the degree of PhD.

This work has not been submitted in substance for any other degree or award at this or any other university or place of learning, nor is it being submitted concurrently for any other degree or award (outside any formal collaboration agreement between the University and a partner organisation).

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Declaration

This thesis is the result of my own independent work, except where otherwise stated, and the views expressed are my own. Other sources are acknowledged by explicit references. The thesis has not been edited by a third party beyond what is permitted by Cardiff University's Use of Third Party Editors by Research Degree Students Procedure.

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Meeting presentations and abstracts related to this thesis

Meeting presentations

Jones R, Casbard A…Hudson Z…Beresford M; LBA20 Vandetanib plus fulvestrant versus placebo plus fulvestrant after relapse or progression on an aromatase inhibitor in metastatic ER positive breast cancer (FURVA): A randomised, double-blind, placebo-controlled, phase II trial. Oral virtual presentation at ESMO 2020. https://doi.org/10.1016/j.annonc.2020.08.2248

Abstracts

Hudson Z, Gee J, Jones R et al; Total RET (t-RET) expression in formalin-fixed paraffin-embedded (FFPE) clinical breast cancer samples and correlation with signalling biology and clinical outcomes. Poster presentation at NCRI 2019 Congress, Glasgow, UK.

Smith J, Hudson Z… Jones R et al; Fulvestrant +/- Vandetanib in advanced aromatase inhibitor resistant breast cancer. Poster presentation at NCRI 2017 Congress, Liverpool, UK

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Commonly used abbreviations

ABC	advanced breast cancer
AGO1	protein argonaute-1
AKT1	protein kinase B-1
ARTN	artemin
AWGL	All Wales Genetics Laboratory, Heath Park, Cardiff, CF14 4XW
BASE	blood sample taken at trial entry
BEAMing	beads, emulsion, amplification, magnetics PCR technology
CCDN1	cyclin D-1
CDK	cyclin dependant kinase
cfDNA	circulating free DNA
CI	chief Investigator
ctDNA	circulating tumour DNA
CTR	Centre for Trials Research
CU	Cardiff University
DCIS	ductal carcinoma in situ
DNA	deoxyribonucleic acid
DRFS	distant relapse free survival
EGFR	epidermal growth factor receptor
EOT	blood sample taken at end of trial treatment
ER	oestrogen receptor
ESR1	oestrogen receptor-1

ET	endocrine therapy
FA	Dr Fouad Alchami, Consultant Histopathologist
FFPE	formalin fixed paraffin embedded
FURVA	fulvestrant and vandetanib in advanced aromatase inhibitor resistant breast cancer
GCP	good clinical practice
GDNF	glial derived neurotrophic factor
GFRα	glial derived neurotrophic factor family alpha receptor
H&E	haematoxylin and eosin
H2O2	hydrogen peroxide
HeLa	Henrietta Lacks (uterine, cervical cancer cell variety, named after patient)
HER	human epidermal growth factor receptor
HER2 (ERBB2)	human epidermal growth factor receptor-2
HR	hazard ratio
HTA	The Human Tissue Authority
ICF	informed consent forms
ICH	International Conference on Harmonisation
IGFR	insulin like growth factor receptor
IGFR	insulin like growth factor receptor
IHC	immunohistochemistry
kDa	kilodaltons (unit of molecular mass)
LTED	long term oestrogen deprived
MHRA	Medicines and Healthcare products Regulatory Agency
MSK-IMPAKT	Memorial Sloane Kettering integrated mutation profiling of actionable cancer targets

mTOR	mammalian target of rapamycin
NHS	National Health Service
NRTN	neurturin
OS	overall survival
PBS	phophate buffered saline
PBS-T	phosphate buffered saline with tween
PFS	progression free survival
PI3K	phosphatidylinositol 3-kinase
PIP	Phosphatidylinositol 4,5-bisphosphate
PIS	participant information sheet
PR	Progesterone receptor
P-RET	Phosphorylated RET (rearranged during transfection) protein
PSPN	persephin
PTEN	phosphatase and tensin homolog
QA	quality assurance
RB1	retinoblastoma protein 1
REC	research ethics committee
RECIST	response evaluation criteria in solid tumours
RET	rearranged during transfection
RTK	receptor tyrosine kinase
RT-PCR	real time polymerase chain reaction
SERD	selective oestrogen receptor downregulator
SFK	SRC family kinase
SOP	standard operating procedure

SRC	sarcoma
T47D	endocrine-responsive, luminal A breast cancer cells
TBS	triphosphate buffered saline
TBS-T	triphosphate buffered saline with tween
TCGA	the cancer genome atlas
TMF	trial master file
TMG	trial management group
T-RET	Total RET (rearranged during transfection) protein
UHW	University Hospital Wales, Heath Park, Cardiff, CF14 4XW
VEGFR	vascular endothelial growth factor receptor
WCB	Wales Cancer Bank, Heath Park, Cardiff, CF14 4XW
WES	whole exome sequencing
WGS	whole genome sequencing
WSP	Welsh School of Pharmacy, King Edward VII Avenue, Cardiff, CF10 3NB
ZH	Zoe Hudson, PhD Candidate

1 Introduction

Chapter overview

This introductory chapter will set the scene for this thesis with background about breast cancer, the mechanisms by which endocrine resistance develops and the **Fu**lvestrant and **Va**ndetanib in endocrine resistant breast cancer (FURVA) trial; the source of the research samples for this project. This will be followed by an overview of techniques in use for biomarker investigation in the field of oestrogen receptor positive (ER+) breast cancer.

1.1 Breast Cancer

1.1.1 Introduction

In 2014 55,000 people received a diagnosis of invasive breast cancer, representing 15% of all cancer diagnoses in the UK that year. The incidence of breast cancer in the UK is predicted to rise at 2% per year meaning that by 2035 there will be an estimated 71,000 new cases of breast cancer each year (Smittenaar et al. 2016) this equates to nearly 200 new diagnoses per day.

While there are many treatment options available for patients with breast cancer it remains the second most common cause of cancer death in women in the UK with over 11,000 deaths per year (CRUK) (https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/breast-cancer, accessed 7/2/2019)

Clinical sub-typing of breast cancers is based on receptor status for oestrogen, progesterone and HER2 as determined by immunohistochemistry (IHC). The addition of fluoro in situ hybridisation (FISH) testing is required if examination of HER2 receptor status by IHC has resulted in borderline positivity (RCPath 2016)(https://www.rcpath.org/uploads/assets/693db661-0592-4d7e-9644357fbfa00a76/G148_BreastDataset-lowres-Jun16.pdf. Accessed 18/7/2021)

The majority of breast cancers express oestrogen receptor (ER+), many express progesterone receptor (PR+) and a minority (~20%) overexpress human epidermal growth factor two (HER2+). Based on the expression pattern of these protein receptors tumours can be termed 'ER+' (ER+, PR+/-, HER2-), 'triple positive' (ER+, PR+, HER2+), 'HER2+' (ER+/-, PR+/-, HER2+) or 'triple negative' (ER-, PR-, HER2-) with each subtype showing different patterns of disease development, metastatic spread and prognosis {Cardoso, 2020 #377}. However, this categorisation does not incorporate the subtleties of the molecular characteristics at a genomic level or the histological subtype e.g. ductal, lobular or inflammatory.

1.1.2 Molecular characterisation

With the advent of molecular characterisation technologies such as gene expression profiling more detailed subdivisions have been possible. Perou et al categorised the gene expression of 8102 genes using RNA microarrays of 65 samples from 42 patients with breast cancer to reveal four major subgroups; basal like, HER2+, normal-breast like and luminal epithelial ER+ (Perou et al. 2000). The original paper was followed by a paper which subcategorised the luminal epithelial group into three. Sørlie et al proposed that the luminal A breast cancers strongly expressed ER and GATA3 amongst other genes while luminal B and C breast cancers showed low expression of the luminal A subtype associated genes. Luminal C breast cancers could be differentiated from luminal B breast cancers by the presence of a novel set of genes which could also be noted in basal-like and HER2+ subtypes.

Clinical outcome data showed that patients with luminal A breast cancers had the best prognosis. Basal-like subtype was associated with the poorest clinical outcomes while normal-like, luminal B and C had an intermediate prognosis (Sørlie et al. 2001). When the microarrays used were refined and a further set of 115 malignant breast tumours were studied tumours could be categorised into five groups with four groups correlating with prognosis; basal-like, HER2+, luminal A and luminal B (Sorlie et al. 2003) as shown in Figure 1.

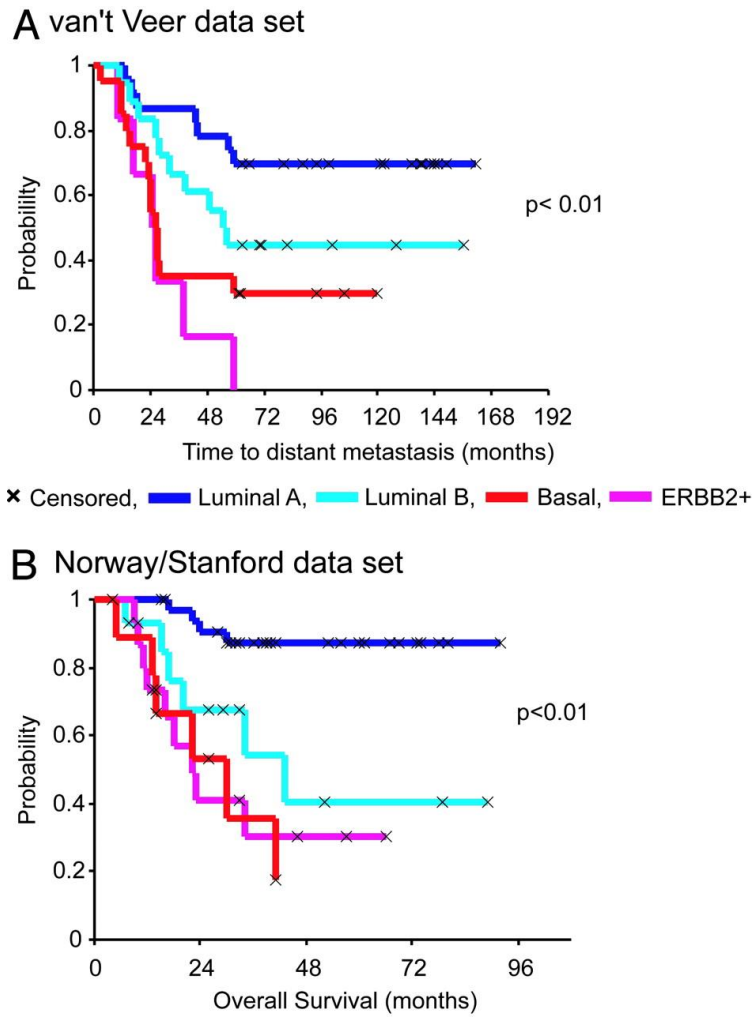


Figure 1 Kaplan Meier curves showing A) time to distant relapse and B) overall survival by molecular subtype. Reproduced from Sørlie et al, PNAS, 2003. It is worth noting that patients with HER2+ disease would not have received HER2 directed therapies as would be standard of care today.

Molecular subtyping of breast cancers is now possible in the clinic with use of the PAM50 gene expression test (Parker et al. 2009). Luminal A breast cancers typically express both ER and PR and have a low level of proliferation, their prognosis is often excellent. Luminal B cancers again express ER and much less commonly PR but have higher proliferation markers and when compared to luminal A cancers tend to carry a worse prognosis (Russnes et al. 2017). Although not routinely used in UK clinical practice the PAM50 subtype is frequently used in research cohorts and so will be referred to in this thesis. The tumours of participants in the FURVA trial are likely to be a mixture of luminal A and luminal B subtypes although luminal subtype was not tested. Where comparative

studies or cohorts of patients are sort and categorisation of tumours is possible then comparison will be made to subgroups of samples that resemble the cohort of patients in the FURVA clinical trial as closely as possible i.e. patients with ER+, HER2- breast cancer.

This thesis is specifically concerned with patients with ER+/HER2- breast cancer who have advanced breast cancer (ABC) and have developed endocrine resistance; Luminal A and Luminal B cancers by molecular subtype, all of which can be referred to by the umbrella term 'ER+ breast cancer'.

More recently increasingly complex molecular classifications have been offered based on our increased understanding of the underlying genomics of the disease. Using the METABRIC dataset, split into a discovery and validation cohort, Russnes et al identified 10 clusters based on gene expression driven by copy number alterations. Of the 10 clusters identified clusters 1, 2, 3, 4, 6,7,8 and 9 were associated with ER+ breast cancer and helped differentiate between good prognosis and poor prognosis ER+/HER2- disease. Cluster 2 denoted poor prognosis, clusters 1 and 9 correlated with intermediate survival and clusters 3,4,7 and 8 showed good prognosis based on mean clinical follow up of 10 years (Russnes et al. 2017). This method of classification is only in use in selected research settings and will not be used in this thesis.

1.1.3 Oestrogen receptor positive breast cancer

Around 70% of breast cancers overexpress ER and are termed ER+.

If patients are diagnosed with early stage breast cancer (disease that has not spread beyond the breast or axillary lymph nodes) they will usually be offered treatment with surgery, radiotherapy and adjuvant endocrine therapy with the aim of cure. If the disease is more advanced at presentation (e.g. is a large tumour or there is evidence of spread to local lymph nodes) they may also be treated with chemotherapy to try and increase the chance of long term survival.

If patients are diagnosed with metastatic disease (often termed 'secondary breast cancer' or 'advanced breast cancer') then a sequential combination of chemotherapy and endocrine therapy is used to control the disease. In this thesis the term advanced breast cancer (ABC) will be used.

Endocrine therapiess either reduce circulating oestrogen in the body by preventing peripheral conversion of testosterone to oestrogen e.g. aromatase inhibitors (AIs), through interactions with the oestrogen receptor e.g. tamoxifen, a selective oestrogen receptor modulator (SERM), or by inducing downregulation of the oestrogen receptor e.g. fulvestrant, a selective oestrogen receptor down regulator (SERD).

Despite the initial efficacy of these drugs tumours may either have inherent resistance (primary resistance) or may acquire resistance during treatment (secondary resistance). The Advanced Breast Cancer Consensus Meeting 2 (ABC2) agreed on the following definitions to clarify the term endocrine resistance (Cardoso et al. 2014):

- **Primary (intrinsic) resistance** – recurrence during the first two years of adjuvant endocrine therapy OR progression during the first six months of primary endocrine therapy in the setting of breast cancer presenting as de novo metastatic disease.
- **Secondary (acquired) resistance** – recurrence after the first two years or within 12 months of completing adjuvant endocrine therapy OR after the first six months of primary endocrine therapy in the setting of breast cancer presenting as de novo metastatic disease.

The terms primary and secondary resistance will be used in this thesis when referring to endocrine resistance.

At the point that endocrine therapy is no longer controlling patient's disease there are, broadly speaking, two therapeutic approaches; to give an alternative endocrine therapy ideally with another targeted agent e.g. a CDK4/6 inhibitor or to use chemotherapy. While both approaches have potential side effects, the unwanted effects of chemotherapy are often more severe due to its indiscriminate effects on rapidly dividing cells. Adding a further drug to endocrine therapy is an

appealing therapeutic strategy as there is potential for overcoming the resistance mechanism that has developed. Often drugs used in this setting are oral rather than intravenous and so along with potential prognostic benefits they are frequently more tolerable and appealing to patients and their pursuit of quality of life. Combinations of treatments often prolong life but ultimately the cancer becomes resistant to all therapies and the patient dies.

1.2 Mechanisms of endocrine resistance

1.2.1 Introduction

When endocrine resistance occurs, be this within the primary tumour or later in the course of disease it is ultimately the mechanism by which the cancer escapes treatment control. Currently, despite advances in molecular profiling it is not possible to clinically identify patients who have intrinsic endocrine resistance although potential biomarkers are being explored. Attempts have been made such as using change in Ki67 level after neo-adjuvant endocrine therapy (Ellis et al. 2017). However, currently in UK clinical practice all patients with ER+ disease are offered adjuvant endocrine therapy. A proportion of patients will develop metastatic disease while still taking their adjuvant endocrine treatment while others will relapse many years after (Pan et al. 2017). The reason as to why this happens is one of the key questions in the field of ER+ breast cancer.

There are many mechanisms of endocrine resistance that have been characterised over the past twenty years (see Figure 2). Focus will be placed on those which have been best characterised and are most relevant to this project with a brief summary of other hypotheses.

This section begins with a review of RET and its role in the development of endocrine resistance as this is the key to the scientific rationale of the FURVA trial. Following this focus turns to those pathways downstream from RET (PI3K/AKT/mTOR and MAPK pathways). Additionally, mechanisms of resistance that will affect response to fulvestrant such as pathogenic genetic variants in *ESR1* are reviewed. Furthermore, mechanisms of escape from normal cell cycle regulation are important to

consider with focus on TP53 and CDK4/6. Finally, attention turns to less well explored but potentially interesting findings from recent studies exploring endocrine resistance such as variants in *LYN* and copy number changes in *MYC* and *FGFR1*. This is summarised in Figure 2.

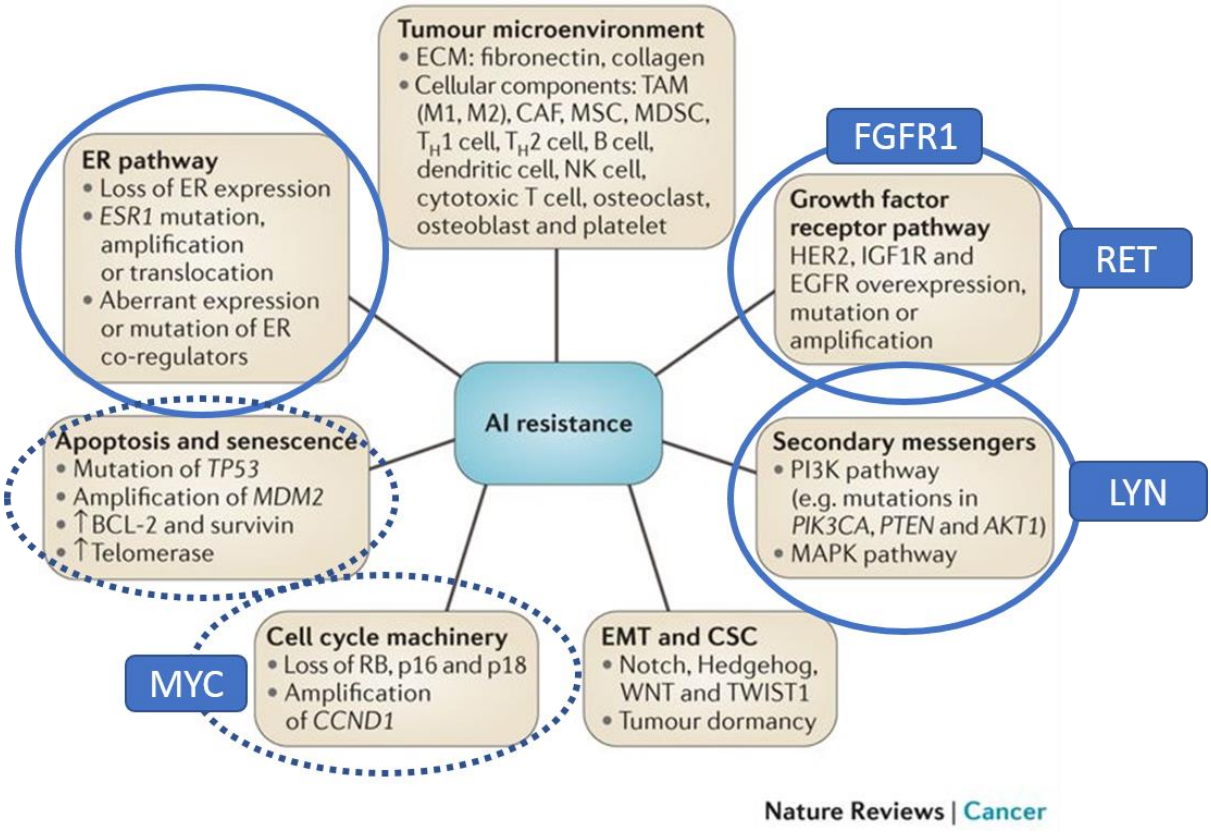


Figure 2 Summary of potential mechanisms of resistance to aromatase inhibitors reproduced from Ma et al, Nature Reviews Cancer, 2015. Superimposed on the original graphic are the additional genes of interest which will be investigated in this project. Full circles indicate pathways that are the focus of this project with dotted lines indicating those that are also investigated. The tumour microenvironment along with epithelial to mesenchymal transition (EMT) and cancer stem cells (CSC) will not be investigated.

1.2.2 RET

1.2.2.1 *Overview*

Along with fulvestrant targeting the ER the other main target in the FURVA study is RET which is targeted with vandetanib. All samples used in this project have been donated by participants in the FURVA trial.

The structure of **RE**arranged during **T**ransfection (RET) is well characterised. It's role in breast cancer has been established more recently. RET is a transmembrane receptor protein encoded by the gene *RET* which is localized to 10q11.2. It comprises 1114 amino acids and a has a molecular weight of 124kDa (Stelzer et al. 2016). The name is derived from the discovery of the *RET* oncogene which is comprised of the fusion of two separate DNA sequences that come together during transfection (Arighi et al. 2005). It is a tyrosine kinase receptor which has three domains; an extracellular ligand binding domain, a hydrophobic transmembrane domain and an intracellular tyrosine kinase domain. At release from the endoplasmic reticulum RET is only partially glycosylated and of 150 kDa, when fully glycosylated it is of 170kDa. RET has three isoforms (RET51, RET43 and RET9), the number signifies the number of amino acids in the carboxy terminus. RET has four cadherin like domains (GFRα1-4) each with their own ligand (glial derived neurotrophic factor (GDNF), neurturin (NTRN), artemin (ARTN) and persephin (PSPN)). Ligand binding to its preferred co-receptor leads to dimerization of RET and subsequent downstream signalling pathway activation (Morandi et al. 2011).

In health, functional RET is essential for organogenesis of the kidney, the development of neural crest derived cells such as neurons found in the nervous system and for the correct projection of hind-limb-innervating axons (Plaza-Menacho et al. 2014). Dysfunctional RET is the cause of Hirschprungs disease, where the bowel develops without enteric parasympathetic neurons causing severe constipation in the new-born as faecal material is unable to move along the bowel (Kapur 2009).

Germline variants in *RET* are implicated in several genetic syndromes; such as multiple endocrine neoplasia 2A and 2B, which can be inherited or occur as a result of sporadic genetic variants. The majority of the features of this genetic syndrome are malignancies but it also causes non-malignant pathology such as parathyroid hyperplasia. In sporadic cases the causative genetic variants are usually single nucleotide polymorphisms (SNPs) in *RET* (Plaza-Menacho et al. 2006).

In malignant disease it is thyroid cancer that is most commonly associated with either germline or somatic variants in *RET*. Germline variants in *RET* are implicated in the development of around 25% of medullary thyroid cancers. The most commonly documented variant in familial medullary thyroid cancer is M918T (Cote et al. 2017) with patients with MEN2B syndrome making up the majority of cases. Somatic variants are found in *RET* in around 65% of cases of medullary thyroid cancers, again with M918T being most common (Drilon et al. 2018).

RET fusion proteins have been noted in diseases such as papillary thyroid cancer and non-small cell lung cancer and are potentially highly targetable. The creation of fusion proteins occurs when rearrangement of the genome creates proteins with the intracellular components of RET but with replacement of the N-terminal domain with that of unrelated proteins such as CCDC6-RET and NCOA4-RET in papillary thyroid cancer and KIF5B-RET in non-small cell lung cancer (Drilon et al. 2018).

Meanwhile in non-small cell lung cancers it has been established that RET fusions are present in 1-2% of all cases, although this increases to 16-17% in never smokers. Three fusions have been identified; KIF5B-RET, CCDC6-RET and TRIM33-RET (Wang et al. 2012).

RET protein expression has been identified as a potential prognostic biomarker in both breast cancer and squamous cell carcinoma (SCC) of the head and neck in both disease types high RET expression correlated with larger tumours and more advanced tumour stage. In SCC of the head and neck it was an independent prognostic marker for overall survival(Lin et al. 2016). While in breast cancer high RET expression correlated with metastases free survival and overall survival but it was not stated if this association was independent of its correlation with larger, more advanced tumours (Gattelli et al. 2013).

1.2.2.2 *RET in oestrogen resistant breast cancer*

The case for RET expression as a mechanism for endocrine resistance was raised partially when it was noted that the frequency of expression was higher in patient samples that had been exposed to

adjuvant tamoxifen treatment and gone on to develop recurrent disease compared to samples taken from unselected patients with breast cancer (25% vs. 56%) (Plaza-Menacho et al. 2010). Furthermore, it had been noted that ER+ cell lines such as MCF7 and T47D expressed high levels of mRNA encoding RET and its co-receptor GFRA1 (Esseghir et al. 2007; Boulay et al. 2008).

Following on from these early studies, it was demonstrated that RET was targetable and when knocked down resulted in increased sensitivity to tamoxifen. When knockdown of RET was performed in tamoxifen resistant cells it restored sensitivity to the drug offering evidence that RET was potentially involved in endocrine resistance and that inhibiting it could restore sensitivity to endocrine therapy (Plaza-Menacho et al. 2010).

Mouse models were developed to test potential RET inhibitors such as NVP-AST487 (Gattelli et al. 2013; Andreucci et al. 2016). Overall, while both studies demonstrated convincing inhibition of downstream targets in cell lines; this failed to translate into differences in tumour growth in the mice when treated with fulvestrant, a RET inhibitor or a combination of both. However, all treatments performed better than a control in the mice. It is noted that the cell lines injected into the mice did not represent endocrine resistant disease where one might expect higher RET expression to result in hyper stimulation of downstream targets.

A further mouse model was developed by Spanheimer et al, this time using vandetanib as the RET inhibitor in combination with tamoxifen. Again, the model was not representative of endocrine resistant disease but on this occasion those mice treated with the combination of endocrine therapy plus a RET inhibitor convincingly demonstrated reduced tumour growth compared to those mice treated with control or tamoxifen or vandetanib as a single agent (Spanheimer et al. 2014). In addition to reduced tumour growth the combination therapy also reduced the chance of disease progression after 10 days of treatment; 87.5% of mice in the single agent groups progressed and only 33% in the combination group did. For those receiving no active drug there was 100% rate of progression. However, progression was described as any increase in tumour size beyond that which

was recorded prior to treatment, no detail is given on how these measurements were made or if they were duplicated or assessors blinded.

In the same paper the combination therapy was tested in cell lines. Cell lines representative of both hormone sensitive (MCF7) and hormone resistant disease (BT-474) had RET inhibited by either vandetanib or siRNA knockdown of RET. BT-474 is an ER+, HER2+ cell line so may not be fully representative of classical endocrine resistance. Both manners of disabling RET showed a reduction in the number of viable cells but those inhibited with vandetanib both in the endocrine sensitive and endocrine resistant cell lines showed the most dramatic reductions in cell viability with a 30% reduction with tamoxifen alone and a 67% reduction with tamoxifen and vandetanib. In endocrine resistant cell lines, the comparison was more marked; from 5% cell viability with tamoxifen alone to 40% with combination. The data presented by Spanheimer et al give the most convincing rationale for the FURVA clinical trial.

RET expression is strongly linked to mRNA expression (Plaza-Menacho et al. 2010) but it is not known what results in this overexpression. During the course of this project it has become clear that it is not due to gain of function genetic variants in *RET*. Exploring large genomic breast cancer datasets using CBioPortal (Gao et al. 2013) such as those by Razavi et al show that *RET* variants are present in just 1% of patients with ER+/HER2- breast cancer (Razavi et al. 2018). A detailed analysis of all RET variants in breast cancer confirmed that the overall frequency of *RET* alterations (rearrangements, missense variants and amplifications) was 1.2% in a cohort of 9693 breast cancer samples taken from women with a mixture of primary and advanced breast cancer (Paratala et al. 2018). This would strongly suggest that genetic variants in *RET* are very infrequently, if ever, responsible for the increase in mRNA and protein expression which occurs in around 30% of ER+ breast cancers and up to 50% of endocrine resistant breast cancers.

1.2.2.3 *Summary*

Activation of RET (and other growth factor receptors) activates the oestrogen receptor via multiple pathways (see Figure 3). The activation of the oestrogen receptor results in increased proliferation, survival and scattering of ER+ breast cancer cells and is the key driver of ER+ breast cancer. RET induced activation of ER occurs via the MAPK pathway (Boulay et al. 2008) and the PI3K pathway (Esseghir et al. 2007). These findings were confirmed by inhibiting each pathway in turn suggesting that the PI3K pathway was likely to be the dominant mechanism by which upregulation of ER occurred (Plaza-Menacho et al. 2010).

There is limited evidence that RET may also feed into the JAK/STAT pathway as *in vivo* inhibition of RET resulted in lower phosphorylated FAK and STAT3 levels (Gattelli et al. 2013) this may be due to the proposed interaction between RET and IL6. Variants in the JAK/STAT pathway have been identified in patients with metastatic ER+ breast cancer suggesting that it is a pathway of some importance in endocrine resistance (Yates et al. 2017).

Taken together this body of literature illustrates that RET signals downstream to the ER via the MAPK, JAK/STAT and most strongly by the PI3K signalling pathways (shown in Figure 3). Collectively it has been demonstrated that inhibiting RET decreases cell growth in representative ER+ cell lines, including cell lines representative of endocrine resistant disease and slows tumour growth in ER+ xenograft models (only when vandetanib is used; other RET inhibitors such as NVP-AST487 did not show a meaningful difference). This evidence provides the rationale for the FURVA trial which will be discussed in more detail in section 1.3.

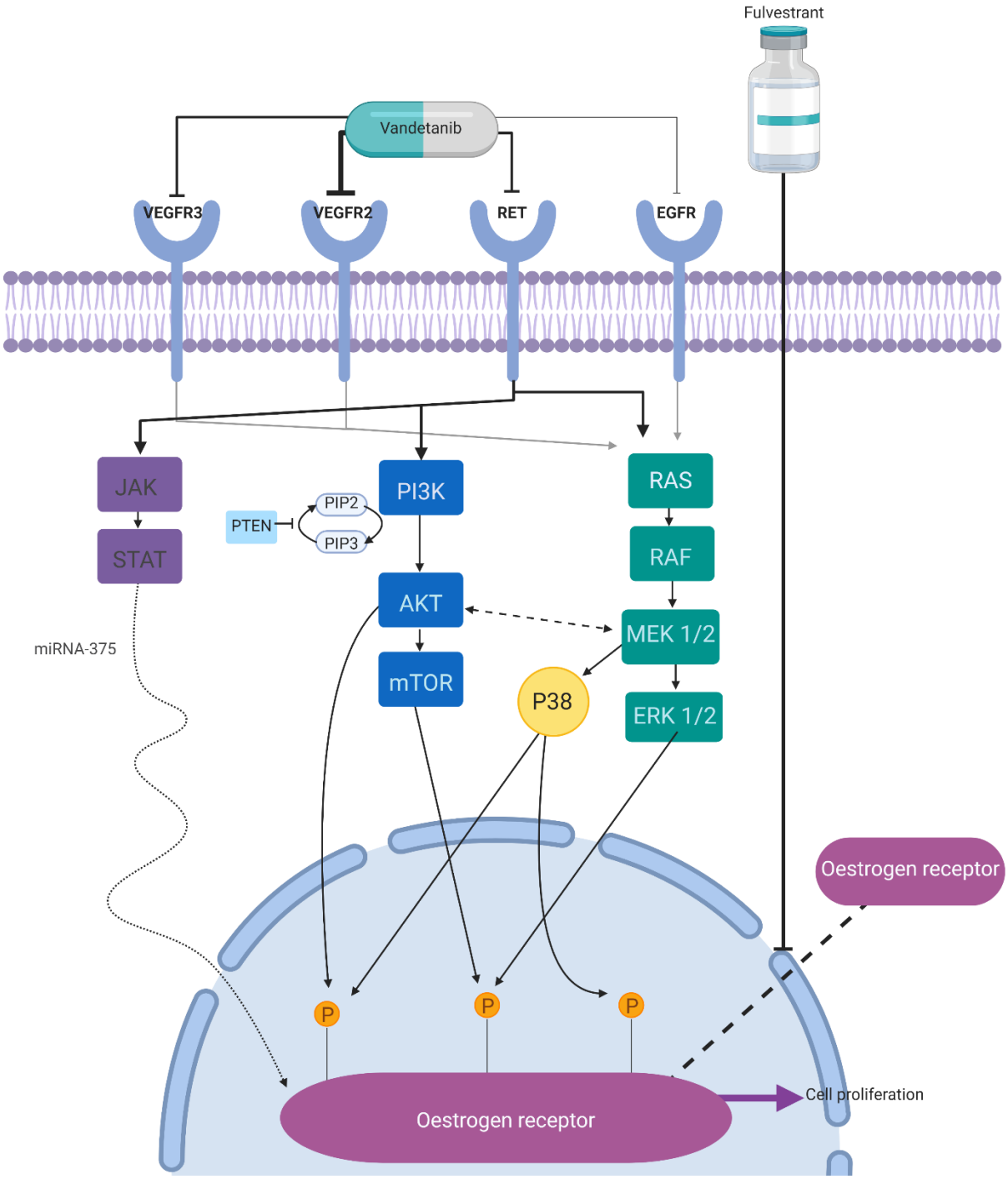


Figure 3 Inhibition of the oestrogen receptor using Vandetanib and Fulvestrant. Vandetanib targets multiple tyrosine kinases as shown by the varying weight of arrows reflecting the IC50 for each receptor. This figure focuses on its role as a RET inhibitor, but we must keep in mind that it is also a potent inhibitor of VEGFR2 and VEGFR3. In contrast fulvestrant acts more directly on the oestrogen receptor (ER) by preventing its migration from the cytoplasm to the nucleus. It has been shown that RET activates the PI3K and MAPK and possibly the JAK/STAT pathway (as indicated by the dotted line). Even this simplified diagram shows that there is crosstalk between pathways and that multiple nodes within the pathway can activate different phosphorylation sites within the ER. Figure produced using biorender.com.

1.2.3 Focus on the PI3K/AKT/mTOR pathway (hereafter referred to as the PI3K pathway)

1.2.3.1 *Background*

The PI3K pathway is frequently upregulated in ER+ breast cancer. Activation of the pathway occurs via growth factor receptors such as insulin like growth factor receptor 1 (IGFR1), fibroblast like growth factor receptor 1 (FGFR1) and HER family receptors (including EGFR (HER1) and HER2-4). Activation of any one of these growth factors located within the cell membrane results in downstream activation of a cascade of intracellular transducer enzymes with three key enzymatic steps; PI3K, AKT and mTOR. The components of the PI3K/AKT/mTOR pathway play a key role in the regulation of protein synthesis alongside cell survival, migration, proliferation and glucose metabolism. The pathway has inbuilt regulation by PTEN which dephosphorylates PIP3. PIP3 is a key intermediary between PI3K activation and AKT phosphorylation (Yang et al. 2016). These pathways are shown in Figure 4 (Dienstmann et al. 2014).

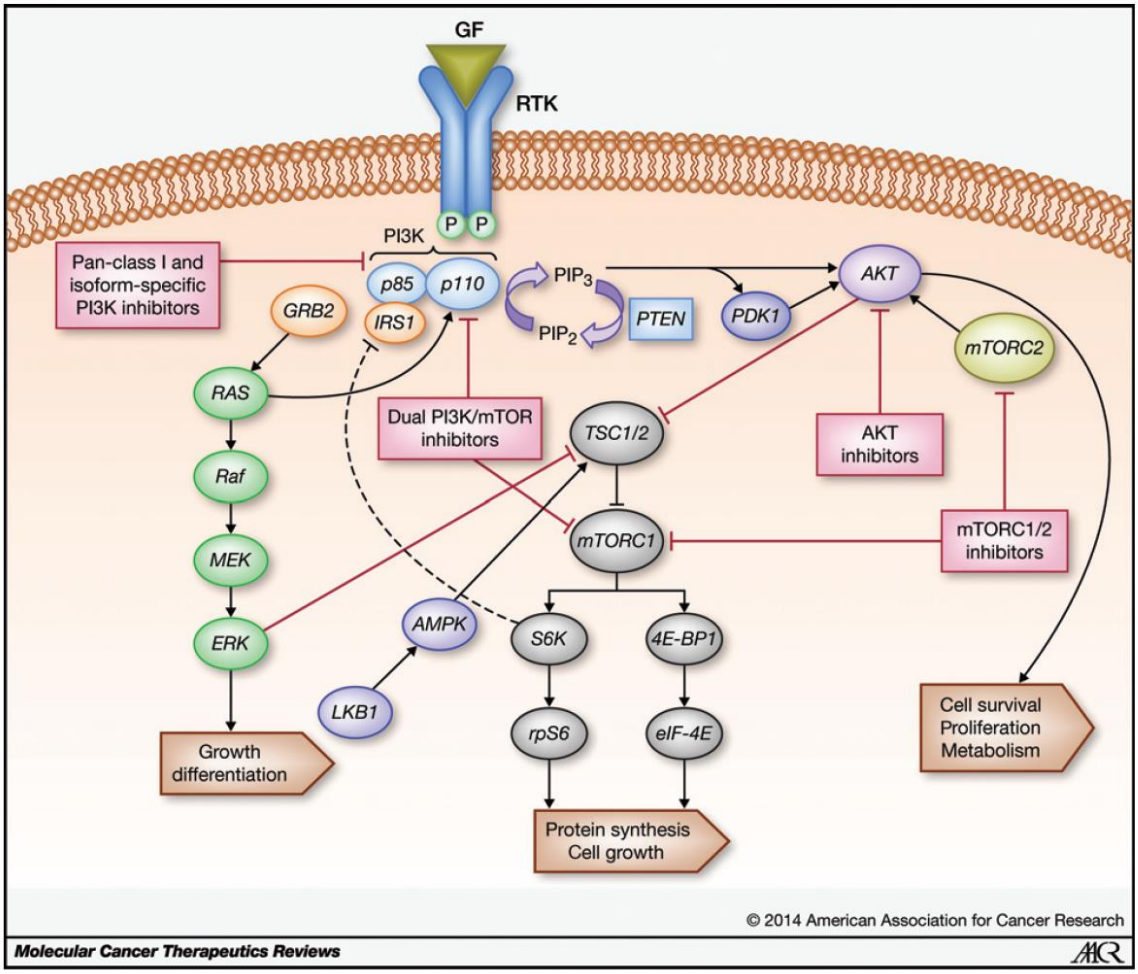


Figure 4 The PI3K and MAPK pathway along with potential points of inhibition along the PI3K pathway Figure reproduced with permission from Dienstmann et al, 2014.

By developing long term oestrogen deprived (LTED) cell lines and performing gene expression analyses comparing them to endocrine sensitive cell lines, upregulation of the PI3K pathway was identified as a route to the development of endocrine resistance (Miller et al. 2010). Hyper activation of the pathway can be linked to increased tyrosine phosphorylation in RTKs such as IGFR, INSR, EGFR, HER2 and HER3 (Miller et al. 2010). Key to this theory was the demonstration that PI3K activity led to oestrogen independent ER activation In mouse models it was demonstrated that mice with an activating PIK3CA variant develop mammary tumours (Lauring et al. 2013). The following section will examine the role of each key component of the PI3K pathway.

1.2.3.2 *PIK3CA*

Variants in *PIK3CA* were first noted in the early 2000s (Samuels et al. 2004) and it is now one of the most investigated genes in breast cancer and has recently been reviewed by the author of the original paper (Arafeh and Samuels 2019). Variants cluster in the helical domain ('exon 9') and the kinase domain ('exon 20'). Hotspot variants cause activation of downstream signalling pathways without the need of activation by growth factor receptors on the cell surface. Various cell line and genetically engineered mouse models have shown that the presence of these variants results in tumour growth, development and resistance to treatment (Bader et al. 2006). Bader et al used cell line models harbouring common variants to show that H1047R transfected cells showed the highest levels of phosphorylated AKT followed by E545K and finally E542K transfected cells. The difference in 'potency' of the two variants may relate to the different ways in which they allow activation of the downstream signalling pathway in the absence of normal regulatory signals. Those in the helical domain result in the p85 nSH2 domain not being able to bind a 'switch off' downstream signalling (Miled et al. 2007). While those in the kinase domain still require p85 binding to cause downstream activation (Zhao and Vogt 2008).

In many datasets where presence of variants in *PIK3CA* has been correlated with clinical outcomes the presence of *PIK3CA* variants seems to result in a progression free survival benefit. This is eloquently discussed in a pooled analysis of 19 studies where the presence of a *PIK3CA* mutation correlated with better invasive disease-free survival (HR=0.77, 95% CI 0.71-0.84), distant disease free survival (HR 0.79 95% CI 0.72-0.86) and overall survival (HR 0.90 95% CI 0.82-0.99). Data from 10,319 patients was analysed with all breast cancer subtypes represented. The authors acknowledge limitations such as heterogeneity amongst datasets and a possible bias towards positive results, as the study only included published datasets. When the results were adjusted for other prognostic factors such as age, grade and tumour size only correlation with invasive disease free survival remained statistically significant (HR 0.88 95% CI 0.78-1.00, p=0.043) (Zardavas et al. 2018). It

appears, although is not explicitly stated, that the variants were identified from tissue samples and no distinction was made between variants in the helical or kinase domain.

Over 40% of the pooled data came from a single study by Sabine et al where the authors acknowledged that while *PIK3CA* variants correlated with improved 5-year distant relapse free survival (DRFS) they were also associated with lower grade, node negative luminal breast cancers which in themselves are good prognostic factors. The authors acknowledge that *PIK3CA* variant status is not an independent prognostic marker for DRFS (Sabine et al. 2014). There is a significant area of uncertainty over how pathogenic variants can be demonstrated as oncogenic yet, if anything, predict for positive clinical outcome, or at least good prognosis in the first 5 years after diagnosis.

This highlights the complexity of breast cancer genomics. The idea of *PIK3CA* variants being the key to many breast cancers was quickly quashed. Not only is the presence or absence of a *PIK3CA* variant important, it is also important which variant is present and whether it occurs alone or with other variants either in *PIK3CA* or another gene. In individual patients it is likely to also be important at what frequency it occurs. This complexity is not accounted for in the clinical series mentioned above and may offer explanation as to why there is no definite conclusion reached about the presence of variants in *PIK3CA* as a prognostic biomarker.

Despite the mixed data regarding whether *PIK3CA* variant status is an independent prognostic marker or not it is a good target for therapy. Strategies for targeting PI3K are developing at pace. Two promising agents entered late phase clinical trials before limited clinical benefit was established halting their further development. Pictilisib was tested in combination with fulvestrant in the phase II FERGI trial and did not demonstrate PFS when compared to placebo, the study also showed that non selective inhibition of PI3K had significant toxicity (Krop et al. 2016). The BELLE-3 study was a phase III placebo-controlled trial comparing buparlisib and fulvestrant with fulvestrant and placebo. It met its primary endpoint demonstrating a PFS advantage [3.9 months vs. 1.8 months HR 0.57, 95% confidence interval 0.53-0.84, $p<0.001$]. The study included testing for *PIK3CA* genetic variants and

found a weak correlation between PFS improvement in the treatment arm and *PIK3CA* mutant status (HR 0.46, 95% CI 0.29-0.73, p=0.11), PIK3CA status was determined by a mixture of tissue analysis using PCR and circulating tumour DNA (ctDNA) analysis using BEAMing technology the paper requires an assumption that only variants in exon 9 and 20 were tested for (Di Leo et al. 2018).

Refinement of the specificity of PI3K inhibitors has recently resulted in the first positive trial of a PI3K inhibitor with a potentially meaningful PFS benefit. The SOLAR-1 study used alpelisib, an alpha selective PI3K inhibitor and combined it with fulvestrant in patients with endocrine resistant ABC. The trial demonstrated an improvement in PFS in patients with tumours harbouring genetic variants in PIK3CA (E542K, E545X and H1047X only[1]); PFS 11.0 months (95% CI 7.5-14.5 months) in patients with $PIK3CA^{mut}$ tumours receiving the drug compared to 5.7 months (95% CI 3.7-7.4 months) for patients receiving fulvestrant and placebo . No benefit was seen for patients whose tumours were $PIK3CA^{WT}$ (André et al. 2019). In a further study using the PI3K inhibitor taselisib no extra clinical benefit was noted in patients with variants in *PIK3CA* although this was a small phase II study (Dickler et al. 2018). Phase 3 results are in circulation showing a small improvement in PFS in patients with a *PIK3CA* variant, the formal publication of the results is awaited (Baselga et al. 2018).

1.2.3.3 *AKT1*

AKT1 is activated by PI3K and in turn activates mTOR resulting in activation of genes controlling resistance, survival, proliferation and invasion pathways. It can be inactivated by PTEN via PIP3.

The *AKT1* E17K variant is by far the most common *AKT1* variant detected in breast cancer with a frequency of 5-6% (Razavi et al. 2018) . It occurs in the pleckstrin homology domain (PHD). The variants presence allows PI3K independent activation of AKT1 in cell lines and can induce leukaemia in mice (Carpten et al. 2007). *AKT1* E17K variants also occur in benign papillomas of the breast; in a small study *AKT1* E17K variants were found in 15/28 papillary lesions with no evidence of atypia

[1] X used to show that several different amino acid changes are possible at the same position depending on the SNV present

(Troxell et al. 2010). Later studies continued to explore the role of *AKT1* E17K variants in tumorigenesis but contrary to the work by Carpten et al a subsequent study showed that E17K variants did not cause a significant increase in downstream signalling in cell lines (Lauring et al. 2010). Furthermore, it was shown that the presence of an *AKT1* E17K variant did not result in tumour development in mice (Mancini et al. 2016). Both these studies did use an ER- cell line which could be a criticism as it is unclear if the results may have been different in an ER+ or potentially a long term oestrogen deprived (LTED) cell line.

Further studies have examined the role of *AKT1* E17K variants in breast cancers. In one study survival data was available for 104/701 patients with ER+ breast cancer who had been tested for the presence of *AKT1* E17K variants. At the time of study closure *AKT1* E17K variants were linked to poorer survival even when adjusted for age and disease tier HR 0.23, 95% CI 0.07-0.75, p=0.015. However, only 22 patients had died and the cause of death was not known (Rudolph et al. 2016). More recently through international collection of combined genomic and clinical outcome data the *AKT1* E17K variant has been demonstrated to predict for good responses to mTOR directed therapy and when evaluated against patients with matched *AKT1* WT tumours showed no difference in overall survival (Smyth et al. 2020)

Again, like PIK3CA, AKT1 is an attractive druggable target. The compound MK-2206 was combined with hormonal therapy in phase I (Ma et al. 2016) and anastrazole neo-adjuvantly in a phase II study (Ma et al. 2017). The pre-clinical evidence reviewed suggest that AKT1 inhibition could be a successful treatment strategy, however in the phase II study of MK-2206 the recommendation was that MK-2206 should not be studied further in ER+ breast cancer after significant toxicity in the form of fatigue and rash developed alongside evidence that AKT pathway inhibition was incomplete (Ma et al. 2017).

AZD5363 has been tested in a phase I study which included a cohort of *PIK3CA*^{mut} patients with breast and gynaecological cancers. AZD5363 was more successful in phase I than MK-2206 with

demonstration of tumour shrinkage in 12/26 *PIK3CA*^{mut} breast cancer patients treated with the recommended phase 2 dose. However only 1 patient met RECIST criteria for partial response (>30% change in tumour size from baseline) (Banerji et al. 2018). All patients gave samples for testing for the presence of *AKT1* variants. The results of the *AKT1*^{mut} patients have not been included in the current published results. The results of the phase II FAKTION trial showed a significant PFS benefit for patients treated with fulvestrant and AZD5363 (Jones et al. 2019) and a phase III trial is now ongoing, however no subgroup based on activation of the PI3K pathway showed evidence of increased efficacy.

1.2.3.4 *mTOR*

Variants in *MTOR* are rare in breast cancer with only around 1% of cases containing a genetic variant in CBioPortal (Gao et al. 2013). MTOR can be effectively targeted with everolimus. The BOLERO-2 trial treated post-menopausal patients who had progressed on first line ET with a combination of exemestane (an AI) and everolimus. The trial demonstrated a significant PFS benefit [central review: 11.0 versus 4.1 months, respectively; hazard ratio = 0.38 (95% CI 0.31-0.48); log-rank P < 0.0001] (Yardley et al. 2013). This study was one of the first to show that targeting the PI3K pathway in postmenopausal patients could bring meaningful clinical benefit.

1.2.3.5 *PTEN*

PTEN is a key regulator of the PI3K pathway as shown in Figure 4 where when active it dephosphorylates PIP3 causing inhibition of AKT activation. Thus, any change that results in loss of PTEN will allow for unchallenged activation of the PI3K pathway. In addition, PTEN may play a role in the stability of TP53 and the DNA damage repair mechanisms by recruitment of RAD51 (Carbognin et al. 2019). Like *PIK3CA*, the data around whether loss of *PTEN* (assessed by loss of protein expression) is an independent prognostic marker in breast cancer is mixed. A meta-analysis where 25/27 studies used IHC to assess PTEN status showed that *PTEN* loss was higher in breast cancer than normal tissues and was more likely to be found in larger, more advanced and poorly differentiated tumours.

It was also more common in ER- tumours, and TNBC. Overall, *PTEN* loss was associated with poorer overall survival (HR 1.41, 95% CI 1.04-1.73) although no test was applied to determine whether this association was independent of its association with well-established poor prognostic characteristics (Li et al. 2017b). Germline gain of function variants in *PTEN* are known to result in cancer predisposition syndromes such as Cowden syndrome(Stemke-Hale et al. 2008). Somatic variants in *PTEN* are detected in around 5-10% of breast cancers using next generation sequencing (NGS) and it is likely there is also significant epigenetic regulation of the gene (Razavi et al. 2018).

1.2.4 Focus on the MAPK pathway

The MAPK pathway has been studied extensively in tumour types such as lung cancer and colorectal cancer and often contains key drivers of disease such as variants in *KRAS* and *EGFR*. In breast cancer it has often played second fiddle to the more commonly upregulated PI3K pathway. However, a recent large scale NGS based study of over 1500 patients with ER+ breast cancer has brought its role to light in the development of endocrine resistance (Razavi et al. 2018). Unlike previous large genomic studies, the key focus in this study was on the genomic changes that had occurred over the course of treatment by comparing genomic profiles of primary and metastatic breast cancers and focusing on those genes which more commonly contained variants in the metastatic rather than primary setting.

Razavi et al demonstrated that variants in the MAPK pathway (including variants in *HER2, NF1, EGFR, HER3, KRAS, BRAF, MAP2K1* and *HRAS*) were present in around 13% of patients who had been treated with hormonal therapy. Changes in the MAPK signalling pathway were mutually exclusive with the presence of *ESR1* variants. The presence of a pathogenic variant in the MAPK pathway predicted for shorter progression free survival on an aromatase inhibitor when compared to samples that were $MAPK^{WT}$ n=302, p=<0.0001. The genes within the pathway most commonly harbouring variants were *ERBB2* (5.8%), *NF1* (4.6%) and *EGFR* (1.7%).

1.2.4.1 *ERBB2 and its role in endocrine resistance*

Growth factor receptors such as HER2 are well studied in breast cancer. In oestrogen receptor positive breast cancers, most tumours will not co-overexpress HER2 in addition to ER. However, there are several ways in which the HER2 receptor can contribute to endocrine resistance. Long term oestrogen deprivation of cell lines such as MCF7 has demonstrated that increased HER2 expression results in downstream activation of the MAPK pathway causing increased expression of the ER (Martin et al. 2003). During this study the investigators used a MEK inhibitor to block the MAPK pathway, however this did not cause complete loss of ER phosphorylation suggesting, as expected, that there are other pathways involved in the regulation of ER.

Genetic variants identified in the *ERBB2* receptor have been postulated as a mechanism for upregulation of HER2 and thus oestrogen resistance. Often the variants are identified in tumours that are 'HER2 negative', i.e. they do not overexpress HER2 on immunohistochemical testing which is standard clinical practice in breast cancer samples (Connell & Doherty 2017). Variants in the extracellular domain (ECD), transmembrane domain (TMB) or tyrosine kinase domain (TKD) can activate the receptor even in the absence of copy number variation or protein overexpression. Within breast cancer most variants are found in the protein tyrosine kinase domain, although the frequency of mutations in all breast cancers is only 1.8%. However, for patients with these activating mutations there are drugs available that target HER2 and may provide a treatment strategy in this cohort such as neratinib as shown in the SUMMIT trial (Hyman et al. 2016).

1.2.5 Genetic changes in the ESR1

The ER is encoded by *ESR1* which contains activating genetic variants in around 20% of ER+ ABC which have been treated with aromatase inhibitors. This suggests that variants in *ESR1* are a key potential mechanism of resistance to endocrine therapy. Variants come in several forms; point mutations, translocations and amplifications (Angus et al. 2017). The point mutations cluster in the ligand binding domain with D538G and Y537S variants being the most common. D538G and Y537S

variants have been studied in depth both with regard to frequency, as they are detectable in blood using ddPCR, and to function. Toy et al identified that cell lines which contain D538G or Y537S variants demonstrate ligand (oestrogen) independent activation, likely due to the bonding of the variant amino acid to D531 which favours the oestrogen receptor to take its agonist form resulting in cell proliferation (Toy et al. 2013). The Y537S variant has been modelled in LTED MCF7 cell lines showing that ligand independent activation of the ER occurs in cell lines with the Y537S variant present (Martin et al. 2017).

ESR1 translocations have also been reported, in particular *ESR1/YAP1* translocations have been demonstrated to induce oestrogen independent activation of ESR1 in a similar manner to the induction by the point mutations described previously (Li et al. 2013).

Furthermore, amplifications in *ESR1* have been reported (Holst et al. 2007). There has been controversy over the frequency of the amplification largely due to a variety of different techniques and scoring systems used to establish whether it is present (Holst et al. 2012) (Ooi et al. 2012). On balance there is good data to suggest that amplification occurs, but there is ongoing discussion about the frequency of the amplification with estimates ranging from 5% (Ooi et al. 2012) to 35% (Moelans et al. 2013) in primary breast tumours. To investigate the correlation between *ESR1* amplification and prognosis 61 patients who had relapsed less than four years after initial treatment were compared with 48 patients who had relapsed at least seven years after initiation of adjuvant tamoxifen. In a Cox analysis of overall survival *ESR1* amplification demonstrated a hazard ratio of 3.8 ($p<0.0048$) suggesting that the presence of an *ESR1* amplification is a poor prognostic factor (Nielsen et al. 2011). Using publicly available databases the frequency of *ESR1* amplifications, when tested for using large scale genomic sequencing, is low with <1% of samples in the MSK-IMPKAT breast cancer dataset accessed via CBioPortal (Gao et al. 2013).

Overall, it is clear that the emergence of *ESR1* variants is an event that occurs most frequently in tumours subjected to long term oestrogen deprivation (Schiavon et al. 2015). While their

appearance renders endocrine inhibition with an AI ineffective patients still appear to derive benefit from fulvestrant (Fribbens et al. 2016; Spoerke et al. 2016). The validity of *ESR1* variants as prognostic biomarkers varies between studies; Spoerke et al found no difference in PFS in patients treated with fulvestrant +/- pictisilib (a Pi3K inhibitor) although they did limit *ESR1* variants to those in the LBD only whereas, in a secondary analysis of the BOLERO-2 trial (treatment with exemestane +/- everolimus the presence of an ESR1 variant correlated with poorer OS in a multivariate analysis (Chandarlapaty et al. 2016).

1.2.6 Dysregulation of the cell cycle as a mechanism of endocrine resistance

Control of the cell cycle is key for normal cell function and is frequently disrupted in cancer. In ER+ breast cancer genetic variants are commonly found in key genes involved in the control of the S into G1 phase in the cell cycle. Figure 5 shows the machinery involved in the regulation of the cell cycle.

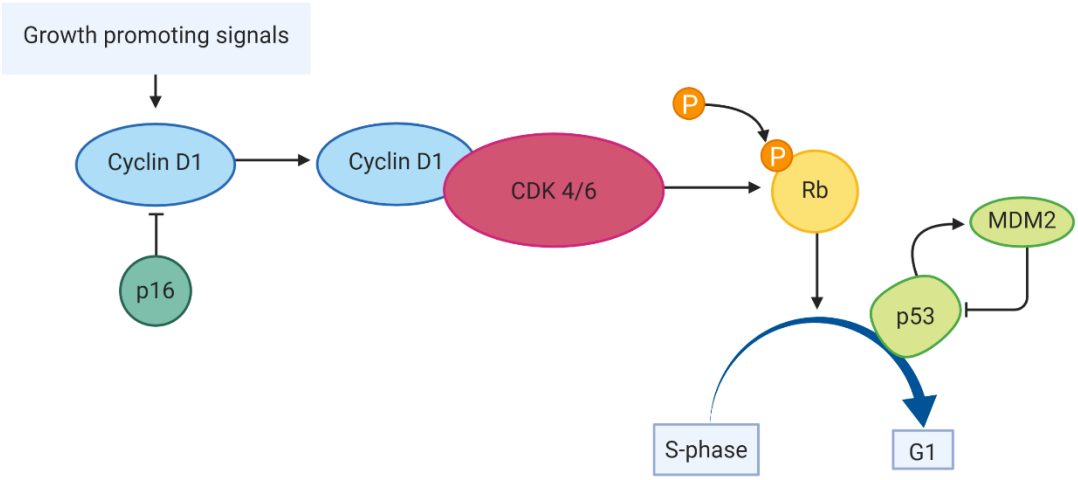


Figure 5 Overview of regulation of the cell cycle. Cyclin D1 (CCND1) binds to CDK4/6. This complex allows the phosphorylation of Rb. Phosphorylated Rb allows transition of the cell from S phase to G1. CCND1 is usually switched off by

p16 (encoded by the gene CDKN2A) unless a cell needs to divide. Alongside Rb p53 is also a key gatekeeper of the S to G1 phase transition. Rb = retinoblastoma protein.

There are multiple ways that this pathway is dysregulated in cancer; firstly, increased growth factor signals can stimulate CCND1 such as those from the PI3K or MAPK pathway. *CCND1* itself can also be amplified leading to increased activation of its downstream proteins. Secondly, pathogenic variants in *CDKN2A* can cause structural changes in p16 resulting in impairment of its ability to inhibit the pathway. Thirdly, pathogenic variants in *RB1* can result in it permanently being phosphorylated thus allowing unlimited progression through the cell cycle. These three key mechanisms will be discussed in detail in section 1.2.6.1. Finally, variants in *TP53* can stop it performing its usual role of preventing genetically unstable cells entering G1 phase (discussed in section 1.2.6.2).

1.2.6.1 *CDK4/6, p16, CCND1 and RB1*

One of the key steps in transition from S phase into G1 during the cell cycle is the binding of CCND1 to CDK4/6 which, when activated, allows phosphorylation of retinoblastoma protein (Rb) which permits progression of the cell cycle from S phase into G1 (de Groot et al. 2017). Rb acts as a key brake on the cell cycle, phosphorylation by CDK4/6 switches the inhibition off allowing the cell to move into G1 phase. CCND1 is inhibited by the presence of p16 which is encoded by the gene *CDKN2A*. *CDKN2A* contains genetic variants in ER+ breast cancer although not at high frequencies; deletions of the gene occur in around 3% of ER+ breast cancers (Pereira et al. 2016). As understanding increased in the role of the cyclins in ER+ breast cancer drugs inhibiting CDK4/6 progressed from early phase clinical trials to the clinic.

The largest evidence base for the use of CDK4/6 inhibitors in ER+ metastatic breast cancer is in the first-line setting combined with either an AI or fulvestrant. The MONALEESA trials (Ribociclib) (Tripathy et al. 2018), MONARCH trials (Abemaciclib) (Sledge et al. 2017) and the PALOMA trials (palbociclib) (Cristofanilli et al. 2016) have all shown significant PFS benefit and more recently an OS benefit for patients (Im et al. 2019; Sledge et al. 2019) . Based on these successes and study of the

potential patterns of resistance CDK4/6 inhibitors are now also being studied in other breast cancer types, as well as in combination with PI3K inhibitors and in the neo-adjuvant setting. Efforts to identify predictive biomarkers for CDK4/6 inhibition have been extensive yet fruitless with neither *CCND1* amplification nor *PI3KCA* variant status or loss of p16 being predictive of response (Ribnikar et al. 2019).

Copy number amplification of *CCND1* is a common finding in ER+ metastatic breast cancer (Razavi et al. 2018). This may be another mechanism by which the normal control of cell cycle progression is over-ridden in breast cancer. However, it is not an independent prognostic marker and may not correspond to an increase in mRNA (Callegari et al. 2016). Variants, most commonly SNVs in *CDKN2A* (~1%) and *RB1* (~2%) are also noted in ER+ breast cancer adding weight to the importance of the CDK axis as a potential mechanism of endocrine resistance (Razavi et al. 2018).

Overall, the regulation of the cell cycle is key in all cancers and there is evidence to suggest that changes in the CDK axis are of particular relevance in ER+ breast cancer. However, like so many other individual changes there is little evidence that any of the changes discussed here aside from variants in *TP53* will act as biomarkers in isolation.

1.2.6.2 *TP53*

TP53 is the gene that encodes for p53 a key regulator of the cell cycle and one of the key 'tumour suppressor genes'. Pathogenic variants in *TP53* are common in breast cancer (Razavi et al. 2018). They are strongly associated with poor survival and to date cannot be targeted therapeutically although many strategies have been tried. Control of cell death by p53 is essential to many normal physiological processes and variants in *TP53* in cancer cells are one of the key drivers of cancer cell immortality (Brown et al. 2009; Babikir et al. 2018). Before the advent of large scale sequencing it was already known that the presence of *TP53* variants was a poor prognostic factor in breast cancer (Andersen et al. 1993). Subsequently, after many smaller studies, a large dataset exploring the prevalence of *TP53* variants in breast cancer patients was generated using sanger sequencing

(Silwal-Pandit et al. 2014). The authors collected variant data, along with clinical outcome data from 1420 patients with breast cancer. All samples were from primary breast tumours, the overall frequency of variants in *TP53* was 402/1420 (28%). 73% of variants were single nucleotide variants (SNVs). 81% of the variants were within the DNA binding domain (exons 5-8). In patients with ER+ breast cancer the presence of a *TP53* variant was strongly predictive of poor survival. When ER+ tumours were categorised by PAM50 subtype it was noted that patients with luminal B cancers had poorer overall survival if a *TP53* variant was present (n=375, HR 1.66 (95% CI 1.14-2.42, p=0.007) whereas patients with luminal A cancers did not (n=499, p=0.761 -HR not given). One could hypothesise that there are additional protective effects in luminal A tumours that override the *TP53* variant present. No differences were detected in survival dependant of type of *TP53* variant (Silwal-Pandit et al. 2014).

Several studies have shown that the frequency of *TP53* variants is higher in metastatic samples than in primary tumours (Yates et al. 2017; Razavi et al. 2018). While this does not necessarily mean they have occurred as a result of treatment with endocrine therapy it may be that either a sub clone present at very low levels in primary tumour has been able to expand to detectable levels or that a spontaneous variant has occurred later in the course of disease. Either way the presence or development of a variant in *TP53* is likely to promote an aggressive phenotype that can escape control of endocrine therapy.

1.2.7 *MYC*

c-MYC is a key protein involved in many cellular processes including growth and survival it is encoded by *MYC*. *MYC* amplification occurs in around 10% of patients with breast cancer (Razavi et al. 2018). High c-MYC expression is associated with poor prognosis in breast cancer.(Green et al. 2016).

While the mechanism by which c-MYC is such a key regulator of many cellular functions is not completely understood it has been established as being intertwined with the development of

endocrine resistance in breast cancer (Miller et al. 2011; Chen et al. 2015; Green et al. 2016). After identifying a gene signature which included *MYC* and MYC associated genes from comparing parental MCF7 cell lines with a long term oestrogen deprived MCF7 cells Miller et al used this signature to demonstrate worse relapse free survival in several cohorts of breast cancer patients. 85% of the cohort were patients with ER+ luminal A and B breast cancers. Furthermore, they demonstrated that siRNA knockdown of *MYC* resulted in inhibition of cell growth (Miller et al. 2011).

In exploring how MYC expression contributes to a more aggressive breast cancer phenotype Chen et al identified that cross talk between ER and HER2 were important for increased MYC expression. In particular, they demonstrated that a possible mechanism of upregulation of MYC was via HER2 derived activation of the MAPK pathway. They also explored the role of MYC in glutamine metabolism (Chen et al. 2015). Glutamine metabolism is an essential part of the TCA cycle and there is evidence to suggest that ER+ breast cancer cells show 'glutamine addiction' (Wise et al. 2008). Chen et al demonstrated that glutamine transport was upregulated in LTED cells adding weight to the hypothesis generated by Wise et al that one of the mechanisms of resistance to endocrine therapy may involve glutamine dependence. Furthermore, Chen et al offered insight into how treatment with fulvestrant might be used to treat cells that were reliant on increased glutamine uptake mediated, in part, by c-MYC. However, caution should be noted as LTED cell lines modified to silence *MYC* were still able to proliferate albeit at a reduced rate while those that were endocrine sensitive were not. This suggests that additional mechanisms are at play in endocrine resistant disease and that if targeting c-MYC was possible then it should not be targeted in isolation.

The role of c-MYC in breast cancer remains unclear but there are suggestions it is a key mechanism in both endocrine sensitive and perhaps even more so in endocrine resistant disease. In detailed *in vivo* studies in other tumour types e.g. lung cancer it has been shown as a key player in the structure of the tumour microenvironment and may have a role in creating an environment of immune suppression to allow unchallenged cellular proliferation (Kortlever et al. 2017).

1.2.8 Increased activity of FGFR1 and its ligands

FGFR1 is another commonly amplified gene in ER+ breast cancer (Drago et al. 2019) and is often amplified alongside some of the genes encoding its ligands such as *FGF19*, *FGF3* and *FGF4* . Amplification of the gene as determined by chromogenic in situ hybridisation (CISH) correlated with shorter disease free survival and overall survival in patients with ER+ breast cancer (Elbauomy Elsheikh et al. 2007). This work was expanded on in 2010 when the relationship between *FGFR1* copy number (as determined by CISH) and *FGFR1* gene expression was examined. In a clinical series of 87 breast cancer samples taken from patients who had received adjuvant treatment with tamoxifen, strong correlation between *FGFR1* amplification and over-expression was demonstrated. In turn it was shown that over-expression of FGFR1 resulted in increased downstream signalling in AKT and ERK1/2 (in cell lines) and poorer disease-free survival (Turner et al. 2010). FGFR1 over-expression was significantly ($p=0.0004$ by $\chi 2$) more common in luminal B type tumours than others (as determined using the van de Vijver dataset) (Turner et al. 2010).

In the neo-adjuvant setting a cohort of patients with early stage ER+ breast cancer who had received 2 weeks of neo-adjuvant letrozole were used to demonstrate that those patients where the Ki67 remained elevated (>7.4%, arguably not the standard cut off in the UK) had a higher frequency of *FGFR1* amplification as determined by fluorescence in situ hybridisation (FISH) than those in which the Ki67 fell (Formisano et al. 2017). Here high Ki67 as determined by IHC is used as a surrogate marker for endocrine resistant disease. 21/72 tumours retained a high Ki67 level and of these 43% had evidence of *FGFR1* amplification while in those tumours where the Ki67 fell the rate of *FGFR1* amplification was only 7.5%. By subsequently developing patient derived xenografts it was possible to demonstrate that it was possible to target *FGFR1* amplifications with lucitanib, a tyrosine kinase inhibitor of FGFR1. Inhibition resulted in a reduction in tumour size of >50% in all mice treated with a combination of fulvestrant and lucitanib (n=8/32 mice all with the same PDX).

Furthermore, by using genomic data in combination with information regarding *FGFR1* amplification as determined by FISH it was shown that FGFR1 amplified tumours were more likely to have co-occurring *TP53* variants (40% vs 20% for non-amplified tumours) but less likely to have tumours containing *PIK3CA* variants (10% vs 40% for non-amplified tumours). The overall frequency of *FGFR1* amplification as determined by FISH assay on FFPE tissue was 30/110 (27%) in this cohort of patients with ER+ metastatic breast cancer. With access to information on the treatments patients had received the authors were able to demonstrate that the progression free survival of patients taking endocrine therapy was significantly shorter in patients with *FGFR1* amplified tumours than those without (p=0.009 by log rank testing n=73) (Drago et al. 2019).

1.2.9 Lyn as a potential mediator of endocrine resistance

Lyn (encoded by *LYN*) and its associated Src family kinases are key signalling proteins which exert control over many cellular processes. Lyn is one of nine Src Family Kinases (SFK), the others being Src, Lck, Hck, Fyn, Yes, Fgr, Blk, and Frk (Elsberger et al. 2010). Lyn has two splice-variants; p53 and p56 kDa isoforms which vary by a 20 amino acid region in the SH4 domain that includes a pY motif (pY32). Lyn is located in the subcellular region and interacts with other proteins either via protein binding in the SH2 and SH3 domain or by altering phosphorylation status. Lyn can act as both a positive and negative regulator of downstream signalling targets, in addition it has key roles in the regulation of a number of haemopoetic cells including stem cells and may act as an oncogene in a number of haematological malignancies (Ingley 2012).

Members of the SFK family play key roles in integrin signalling and thus cell adhesion and migration. In a study where a 200 gene signature related to epithelial-mesenchymal transition (EMT) was investigated in breast cancer cell lines *LYN* was identified as the top ranked EMT signature gene (Choi et al. 2010). Knockdown of *LYN* inhibited cell migration and invasion. *LYN* mRNA expression correlated with a triple negative breast cancer phenotype. Using a tissue micro array (TMA) of breast cancer samples Lyn was overexpressed in 133/939 (14.2%) of cases and overexpression was an

independent prognostic factor in a multivariate analysis. However, these findings were not replicated in an additional study where Lyn expression was also determined using immunohistochemistry on breast cancer samples; Lyn protein expression was higher than in the study by Choi et al (34% in the nucleus and 35% of samples in the cytoplasm) but did not correlate with disease free survival (Elsberger et al. 2010).

Studies have specifically examined the role of Lyn in ER+ breast cancer and proposed a role for Lyn in endocrine resistance. In array studies of endocrine responsive and resistant cell lines Lyn was found to persist at high levels once endocrine resistance had occurred (Gee et al. 2006). These cell line findings were reproduced as part of a larger study examining the role of Lyn in ER+ breast cancers showing evidence of endocrine resistance; in this case determined by the persistence of a high Ki67 score after two weeks of endocrine therapy in the neo-adjuvant setting (Schwarz et al. 2014). In addition to the cell line work Schwarz et al identified a novel *LYN* variant; D189Y. This variant was then transduced into MCF7 cells and showed increased cell growth in the absence of oestrogen compared to WT cells.

Variants in *LYN* or Lyn overexpression are not commonly noted in the literature around ER+ breast cancer. However, following on from the work performed by Gee et al and Schwarz et al the next generation sequencing part of the project will be designed so that it is possible to detect genetic variants in *LYN* if they are present.

1.2.10 Other considerations

In this chapter focus has been placed on the potential mechanisms of endocrine resistance and key genes and proteins that may determine response to fulvestrant and vandetanib that will be investigated in this thesis. However, there are many additional factors that are also important to consider in the development of endocrine resistance, two are discussed, in brief, here. Whole theses could be written on minutiae in each topic, so the overview presented here merely acknowledges the existence of these fascinating fields.

1.2.10.1 *Epigenetics*

Epigenetics refers to changes in DNA that do not arise from alterations in the DNA sequence. In cancers this is due to changes in methylation status, histone modification or changes in higher order chromatic structures. These changes are controlled by other elements in the genome such as non-coding RNAs. Epigenetics can be influenced by environment, inheritance and biological processes such as ageing (Drake and Søreide 2019). Broadly speaking while there are considerable genomic drivers of cancer which are relatively 'fixed' targets, the epigenome is constantly evolving in response to internal and external stimuli and may offer explanation as to why simply targeting genomic changes rarely represents a cure when treating solid cancers.

Of the three main epigenetic changes DNA methylation has been most frequently studied in breast cancer. DNA methylation changes studied in the TCGA breast cancer cohort revealed 5 DNA methylation groups with group 3 having considerable overlap with patients with luminal B breast cancers. These patients were noted to have a lower than expected rate of *PIK3CA* and *MAP3K1* variants (TCGA 2012). Furthermore, changes in methylation status of key genes such as ESR1 could offer an alternative mechanism of endocrine resistance that is independent of changes in the gene itself (Martínez-Galán et al. 2014). Epigenetic changes are also implicated in drug resistance for example phosphorylation of *KMT2D* is implicated in the activation of ER and is not inhibited when anti PIK3CA or AKT1 drugs are used (Toska et al. 2017).

1.2.10.2 *The tumour microenvironment (TME)*

The tumour microenvironment has been hypothesised to contribute to endocrine resistance via mechanisms that allow uncontrolled cell growth, evasion of apoptosis and promotion of EMT. Cancer associated fibroblasts present in the TME secrete proteins that play a key role in tumour growth and angiogenesis e.g. vascular endothelial growth factor A (VEGFA), platelet derived growth factor (PDGF) and hepatocyte growth factor (HGF). Increased production of these factors can then result in upregulation of key growth pathways such as the MAPK and PI3K pathway. Additional factors can directly result in EMT such as tissue growth factor beta (TGFβ). Furthermore, E-cadherin,

a protein that normally promotes geographical stability of cells, has been shown to be reduced in endocrine resistant ER+ breast cancer (Ma et al. 2015).

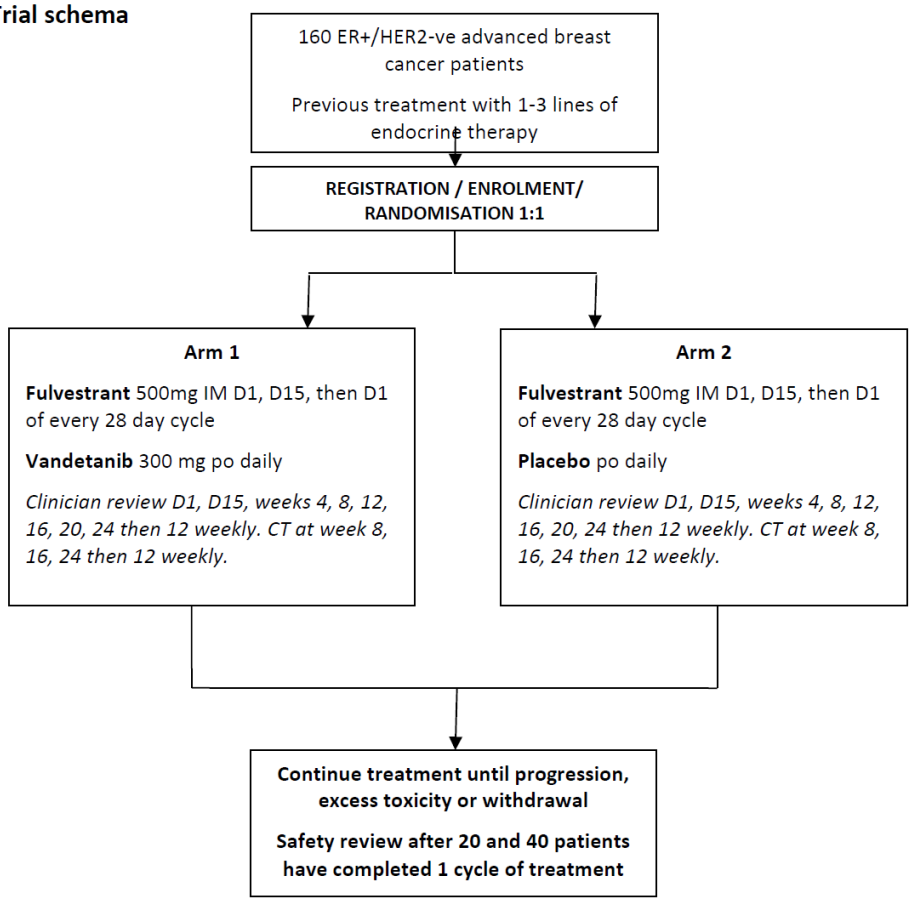
1.3 The FURVA trial

The FURVA trial was designed to investigate whether treating patients with aromatase inhibitor resistant breast cancer with a combination of vandetanib (a tyrosine kinase inhibitor with action against RET) and fulvestrant results in a longer progression free survival than treating patients with fulvestrant and placebo. The rationale for targeting RET is has been discussed in section 1.2.2. The FURVA trial is the primary source of the tissue and plasma samples used in this thesis (REC number 14/WA/1219 (Wales REC 3). Details of samples used including number of tissue and plasma samples are given in Chapter 2.

1.3.1 Overview

The FURVA trial is a prospective randomised phase II clinical trial. Post-menopausal women with ABC that had progressed on an aromatase inhibitor were recruited to the trial and randomised in a 1:1 ratio to receive either fulvestrant and vandetanib or fulvestrant and placebo. The primary endpoint of the trial was progression free survival. Secondary endpoints encompass safety, tolerability and feasibility of use; objective response rate, clinical benefit, overall survival and an exploratory analysis of the influence of the RET signalling pathway on vandetanib activity. The trial schema is detailed in Figure 6).

1.0 Trial schema



Primary outcome measure:

- Progression-free survival (PFS - time to event) based on Response Evaluation Criteria in Solid Tumours (RECIST) v1.1. - Time from randomisation to any progression and/or death (from any cause).

Secondary outcome measures:

- Safety, tolerability (side effects) and feasibility of use (number of participants requiring dose delays or reductions and/or treatment withdrawal).
- Objective response rate and clinical benefit rate as assessed by RECIST v1.1.
- Overall survival (OS), time from enrolment to death with those still alive censored at date last seen.
- Exploratory analysis: The influence of RET signalling pathway components expression on vandetanib activity.

Figure 6 FURVA Trial schema taken from trial protocol version 3.0 09 December 2016

1.3.2 Vandetanib

1.3.2.1 *Mechanism of action*

Vandetanib is an oral tyrosine kinase inhibitor with activity against multiple receptors. Table 1 shows the IC50 levels for its targets (Morandi et al. 2011). Vandetanib is a potent inhibitor of RET with additional activity against VEGFR-2 and VEGFR-3 as shown in Table 1 and Figure 7.

Target	**IC50 (nM)**
RET	130
VEGFR-1	1600
VEGFR-2	40
VEGFR-3	110
EGFR	500
PDGFR	1100

Table 1 Vandetanib targets and their associated IC50 values

Vandetanib is a quinazoline which was originally developed as a VEGFR inhibitor (Wedge et al. 2002). It is currently licenced for the treatment of aggressive and symptomatic medullary thyroid cancer in patients with locally advanced or metastatic disease. The use in medullary thyroid cancer is based on a phase III clinical trial where the primary endpoint of increased progression free survival was met with a HR of 0.46 (95% CI 0.31-0.69 p<0.01) (Wells et al. 2012). There appeared to be greater benefit in patients whose tumours harboured pathogenic *RET* variants as determined by amplification-refractory mutation system (ARMS)-PCR for M918T and direct sequencing of *RET* exons 10,11 and 13-16. However, no comment was made on the limits of detection of the methods used to determine the presence or absence of *RET* variants.

It should be noted that the VEGF and EGFR pathways are also implicated in the pathogenesis of medullary thyroid cancer and that these pathways may also have been inhibited by vandetanib. Genetic variants in neither *VEGF1-3* nor *EGFR* were studied. The magnitude of benefit derived in

patients who were *RET*^{mut} is likely to have been driven from those who were *RET*^{M918T} positive (35/40 *RET*^{mut} positive patients had a M918T variant and were treated with vandetanib in the trial).

Vandetanib was not effective against all variants in *RET*; the variant V804M (commonly found in both sporadic and MEN2 associated medullary thyroid cancers) conferred resistance to vandetanib as it controls access to the binding pocket used by vandetanib (Knowles et al. 2006).

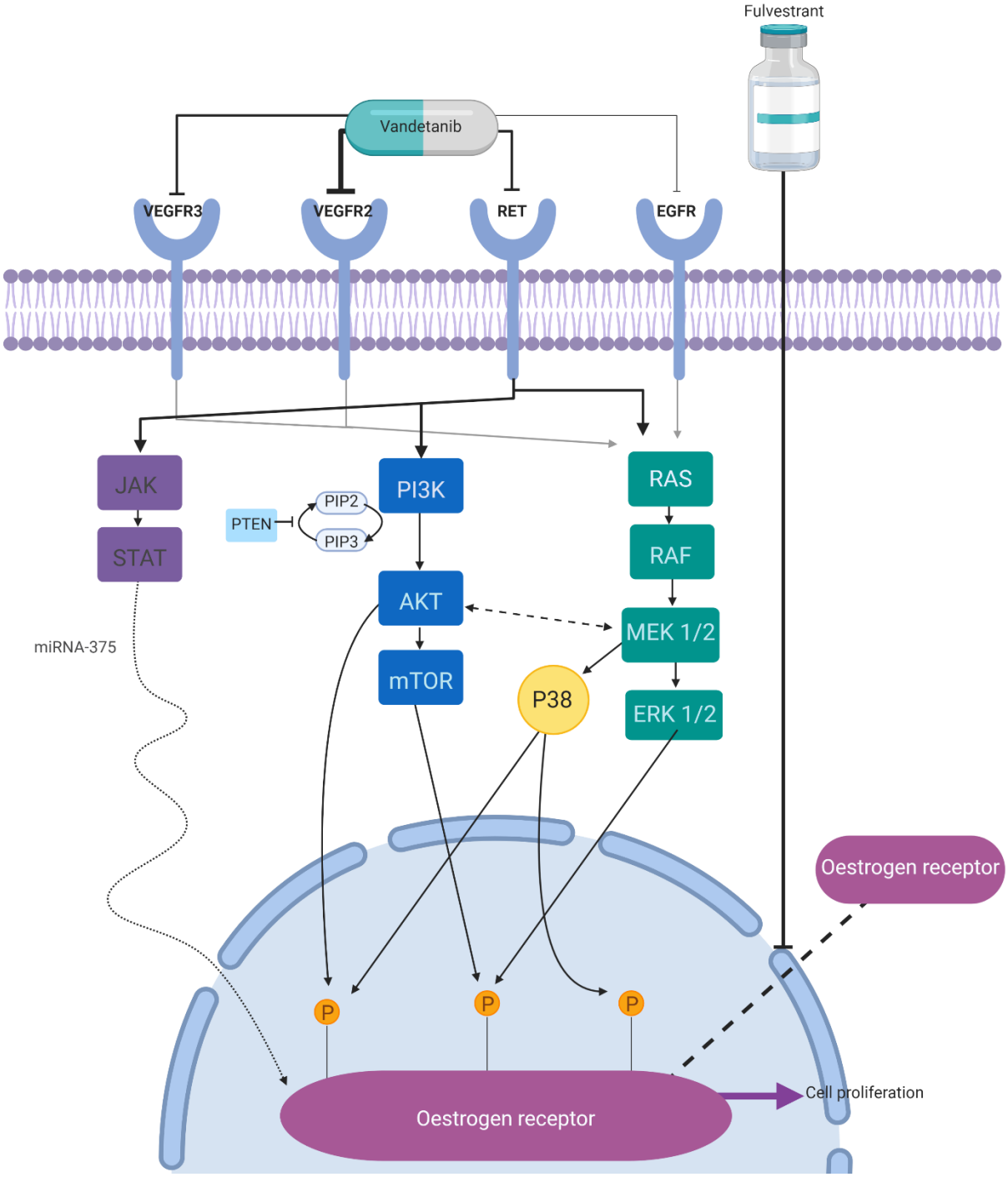


Figure 7 The mechanism of action of Vandetanib. Vandetanib is a multi-kinase inhibitor with varying affinity for the growth factor receptors VEGFR3, VEGFR2, RET and EGFR. Between the four main target receptors three downstream pathways may be inhibited; the JAK/STAT pathway, the PI3K pathway and the MAPK pathway, all of which are implicated in the development of endocrine resistance in breast cancer. The infographic also shows the inhibition of the ER directly by fulvestrant.

1.3.2.2 *Previous use of vandetanib in breast cancer*

Two clinical trials have been reported where vandetanib has been used to treat patients with breast cancer. A small randomised phase II study of 64 patients offered patients treatment with docetaxel +/- vandetanib in the first line metastatic breast cancer setting. The study was able to confirm the safety and tolerability of the combination but did not demonstrate a PFS benefit. The authors acknowledge that the study size, design and execution meant that no firm conclusions could be drawn about the efficacy of vandetanib in breast cancer (Boér et al. 2012).

Secondly, the ZAMOBNEY study (Clemons et al. 2014). The primary endpoint of this phase II randomised study was a biomarker analysis using urine *N*-telopeptide as a marker of response to bone targeted therapies. In this study vandetanib 100mg PO OD was combined with fulvestrant in post-menopausal patients with hormone receptor positive metastatic breast cancer with bone predominant metastatic disease. There was no difference in biomarker response, PFS or OS between the two arms.

Other RET inhibitors such as LOXO-292 and BLU-667 have not been formally tested in breast cancer patients in clinical trials although ongoing studies are using both drugs for RET activated cancers in a tissue agnostic approach. For example, the LIBRETTO-001 study is using LOXO-292 in patients with advanced solid tumours, RET fusion positive solid tumours and medullary thyroid cancer (ClinicalTrials.gov Identifier: NCT03157128). The trial team recently announced that LOXO-292 had shown significant activity resulting in prolongation of PFS in patients with non-small cell lung cancer (NSCLC) with RET fusions and are planning a randomised phase III study (Drilon et al. 2019).

1.3.3 Fulvestrant

1.3.3.1 *Mechanism of action*

Fulvestrant is a selective oestrogen receptor down regulator (SERD). The ER is predominately found in the nucleus and controls many genes involved with transcription thus contributing to proliferation, invasion, survival and angiogenesis in benign and malignant cells. ER activation by

oestradiol results in the dissociation of heat shock proteins from the ER followed by receptor dimerization. This dimerised complex then binds to specific DNA sequences allowing control of transcription via two regions of the ER; activation function 1 and 2 (AF1 and AF2). AF1 activation is controlled by the MAPK pathway, while AF2 is under the control of oestrogen. Two types of ER have been identified; ERα and ERβ. ERα exerts control of the oestrogen related genes in breast cancer and where ER is used to define the oestrogen receptor it is ERα that is referred to unless otherwise stated. Fulvestrant is a synthetic analogue of 17β oestradiol which acts as a competitive antagonist to the ER. Once bound it inhibits dimerization and the relay of the ER from cytoplasm to the nucleus. This complex has inactive AF1 and AF2, resulting in inherent instability and heightened degradation of the ER (Osborne et al. 2004).

Fulvestrant is administered as an intramuscular injection every four weeks. In the FALCON study it proved to be effective as monotherapy and demonstrated a small progression free survival benefit when compared with an aromatase inhibitor in the first line locally advanced or metastatic setting (HR 0.797; 95% CI 0.637-0.999, p=0.0486). The overall progression free survival benefit was measured in short months, although was greater in a subgroup analysis of patients with no evidence of visceral disease (Robertson et al. 2016). Currently in the UK fulvestrant is not recommended over aromatase inhibitors as the first line choice patients with ER+ unresectable locally advanced or metastatic breast cancer.

In the endocrine resistant setting fulvestrant has successfully been combined with a number of agents including CDK4/6 inhibitors and PI3K inhibitors (as shown in Table 2). In this setting, albeit within the context of clinical trials, the progression free survival is approximately 5 months with fulvestrant alone. All trials listed in Table 2 recruited patients at the point of endocrine resistant disease, so these populations are comparable to the patients recruited into the FURVA study. This is not an exhaustive list of trials in this setting but demonstrates agreement that the median PFS for

patients with ER+ endocrine resistant ABC is around 5 months when patients are treated with fulvestrant.

Clinical Trial	Progression free survival in fulvestrant only arm	Reference
PALOMA-3 (fulvestrant and placebo vs. fulvestrant and palbociclib (a CDK4/6 inhibitor))	4.6 months (95% CI 3.5-5.6)	(Cristofanilli et al. 2016)
FERGI (fulvestrant and placebo vs. fulvestrant and pictisilib (a PI3K inhibitor))	5.1 months (95% CI 3.6-7.3)	(Krop et al. 2016)
BELLE-2 (fulvestrant and placebo vs. fulvestrant and buparlisib (a pan PI3K inhibitor))	5.0 months (95% CI 4.0-5.2)	(Baselga et al. 2017)

Table 2 Clinical trials comparing fulvestrant to fulvestrant PLUS investigatory medicinal product (IMP) in patients with endocrine resistant breast cancer i.e. at the same time point as patients have been recruited to the FURVA study.

There is also 'real world' data available regarding the use of fulvestrant as endocrine therapy after treatment with an anti-oestrogen. In a retrospective review of 263 women with secondary breast cancer that had been treated with fulvestrant after disease progression on at least one endocrine therapy PFS was found to be around 10 months with a gradual decrease depending on which line of treatment fulvestrant represented. In the first line setting, after tamoxifen or an aromatase inhibitor or both the median PFS was 11.5 months (95% CI 9.5-19.8). Even in the 4th line setting the median PFS was 8.5 months (95% CI 6.5-11.1). 50% of the patients in the study were stage II at primary diagnosis, while 86% of patients in the metastatic setting did not have visceral disease, this means that patients in this study already had potentially good prognosis disease. This study suggests that outside the context of a clinical trial fulvestrant still has activity in the setting of endocrine resistance and may even offer longer PFS than previously thought for certain patients (Blancas et al. 2018).

1.3.4 Summary of the FURVA trial design

The rationale for treating patients with fulvestrant is strong and the idea of combining it with another agent designed to target one of the potential mechanisms of resistance to aromatase inhibition is logical. Fulvestrant is active in this patient group and vandetanib has shown activity in combination with fulvestrant in mouse models. However, previous trials using vandetanib in breast cancer have not shown clinical benefit, although their trial design and statistical analysis could be questioned (Boér et al. 2012). It is important to remember that although the scientific rationale for this project focusses on vandetanib as a RET inhibitor it may also have effects on other receptors such as VEGF-2 and VEGF-3.

1.4 Biomarker discovery in breast cancer clinical trials; introducing the techniques in use in this thesis

Simple biomarkers have been used in breast cancer treatment for many years to predict clinical outcome. For example, tumour grade, tumour size and lymph node involvement are all independent prognostic variables. More recently increasingly complex predictive tools have been developed to help not only prognosticate but also to predict benefit from adjuvant chemotherapy e.g. Oncotype Dx, a 21 gene mRNA expression panel (Nicolini et al. 2018).

Furthermore, some drugs used in modern breast cancer treatment have their own predictive biomarkers such as HER2 status and the use of trastuzumab and ER status and the use of anti-hormonal therapies. More recently alpelisib has become the first PI3K inhibitor to be approved with the use of a companion test for *PIK3CA* variant status (André et al. 2019).

There are many potential predictive biomarkers in breast cancer; expression of single genes or multigene signatures, single or multiple micro-RNAs, circulating tumour cells, circulating tumour DNA (ctDNA) detectable genetic variants or signatures comprised of multiple genetic variants, tumour mutational burden, tumour infiltrating lymphocytes; this list is by no means exhaustive.

With the cost of bringing a new drug to market and the potential for harm if a drug is given to a patient who is unlikely to benefit biomarker discovery has become an essential but complex part of clinical trial design (Wilhelm-Benartzi et al. 2017). The REMARK guidelines have been developed to try and improve biomarker development (McShane et al. 2005; Sauerbrei et al. 2018).

Perez-Garcia et al have written eloquently regarding the lack of defined clinical trial methodology around identifying and then prospectively validating predictive biomarkers in clinical trials (Perez-Gracia et al. 2017). Their paper details the discovery and subsequent validation of eight key biomarkers that are in clinical oncology use today. Three of the eight were identified from a retrospective analysis (hormone receptors in breast cancer, *EGFR* variants in lung cancer and *KRAS* variants in colorectal cancer). While HER2 expression, *C-KIT* variants, *ALK* translocations. *BRAF* variants and *BRCA1* and *2* variants were identified in prospective studies. Of the eight, two did not have preclinical evidence at the time of discovery (*EGFR* and *KRAS*). The authors demonstrate that there are many approaches to biomarker discovery and many variables that need to be considered. They conclude by making several recommendations including:

- retrospective designs for biomarker studies are a useful tool
- single agent studies make for simpler biomarker identification than multi-agent studies
- response rate is a useful endpoint for biomarker studies
- there is a knowledge gap regarding how best to calculate sample size in biomarker studies, in particular those performed using genomics
- timing and type of sample acquisition is key

While there are many potential techniques to look for thousands of different potential biomarkers this section will focus on the techniques that are to be used in this thesis with a brief overview of other techniques considered but ultimately not used within the scope of this project. This section is

intended to provide background to the detailed description of the techniques selected for use in this project in the methods chapter (Chapter 2).

1.4.1 Immunohistochemistry (IHC) in breast cancer – investigating protein expression

IHC is one of the most commonly used techniques in the pathological assessment of breast cancers. Testing of tumours for the expression of ER, PR and HER2 is all assessed using IHC. Despite many technological advances it is still IHC that is the basis for many clinical treatment decisions. Modern IHC techniques involve the application of commercially available primary antibodies to antigens present in tissue samples. Using a secondary antibody with attached enzyme and subsequent exposure to a detection agent the presence of the target antigen in the tissue can be assessed. IHC not only allows a binary assessment of whether the antibody is present or absent but allows quantitative measurement of the amount of antigen present and the tissues it is present in. With the use of microscopy further detail can be gathered regarding the location of the antigen within the cell and the variability of this location between tissues.

In the clinic IHC is used to determine ER, PR and HER2 status while in the laboratory it is an extremely useful technique to evaluate the presence of specific proteins including many that are relevant to endocrine resistance such as PTEN, RET and LYN. In this project it will be used to examine tissue sections for the presence of both total RET (t-RET) and phosphorylated or active RET (p-RET). IHC has great potential as a biomarker technique as it is well established, cheap, quick and can easily be performed locally and reviewed centrally if required. However, its success relies on the underlying assay being sufficiently well tested and validated, which, in turn, relies on the antibody used being well developed.

1.4.2 DNA based biomarkers in breast cancer

1.4.2.1 *Next generation sequencing (NGS)*

Next generation sequencing in breast cancer has rapidly enhanced our understanding of the key drivers in tumour biology. With larger and larger studies being published over the past 10 years and

the advent of national genomic medicine programs and direct to consumer testing available it would be fair to argue that NGS has revolutionised our knowledge of breast cancer biology and is here to stay. There are several issues that have become pressing ethical, logistical and scientific dilemmas as we try to manage the volume of data that is being created.

Genomic sequencing from tissue samples remains the gold standard investigation with cleaner genomic data produced from fresh frozen biopsy samples than formalin fixed paraffin embedded (FFPE) tissue samples. However, fresh frozen samples are practically challenging and involve invasive procedures for a test which, currently, may not significantly change the management of the patient. Using FFPE tissue samples is more practical but the quality of the DNA sequenced has often been impaired by the fixation process. Furthermore, using historic biopsy samples in order to spare patients an additional invasive procedure may not give the most useful and up to date genomic makeup of the tumour. Serial biopsies are impractical and thus there has been significant interest in the development of a 'liquid biopsy' where circulating free DNA (cfDNA) is extracted from blood and sequenced. A proportion of cfDNA will be circulating tumour DNA (ctDNA)

All cells in the body, both malignant and non-malignant will release DNA molecules into the blood. These free DNA molecules are detectable from plasma samples extracted from whole blood. cfDNA refers to all the DNA detected while ctDNA refers to DNA which is known to come from a malignant cell. This is usually determined by the presence of a pathogenic variant {Schwarzenbach, 2011 #270}.

The 'liquid biopsy' now offers a viable alternative to traditional tissue biopsy but still has limitations such as low DNA yield limiting depth of coverage with larger panels (Buono et al. 2019). Furthermore, additional work is needing to fully understand the factors influencing the secretion or shedding of ctDNA into the blood stream.

Early series such as those by Nik Zainal et al went from exploring the life history of 21 breast cancers (Nik-Zainal et al. 2012) to whole genome sequencing of 560 breast cancer tumours (Nik-Zainal et al. 2016) in just 4 years. By 2018 we were presented with detailed targeted sequencing data from over

1900 patients with endocrine resistant breast cancers (Razavi et al. 2018). The repeated detection of the same key variants is good evidence that the genomic landscape of breast cancer from the point of single nucleotide variants (SNVs) has been established. What is much more challenging is using this information to make informed clinical decisions and target therapy with the overall aim of preventing breast cancers and treating them more effectively when they occur. This new knowledge has led to an explosion of investigation into key variants and gene targets.

1.4.2.2 *Identification of genetic variants in breast cancer samples; single nucleotide variants (SNVs)*

NGS has been used to explore the changes in the genomic landscape between primary and metastatic breast cancer samples. One of the largest published datasets managed to compare over 600 primary ER+ breast cancer tumours with metastatic samples (Razavi et al. 2018). The samples were not matched by patient but the numbers are large enough that cohort comparison is applicable. Figure 8 summarises the changes between primary and metastatic tumours with the largest increase in variants seen in *ESR1*. Variants in *TP53, KMT2C, ERBB2, FAT1* and *ATR* were also statistically significantly increased in metastatic samples compared to primary tumour These increases may indicate mechanisms of resistance to therapy driven by these genes or simply the overall increase in mutational burden as disease advances.

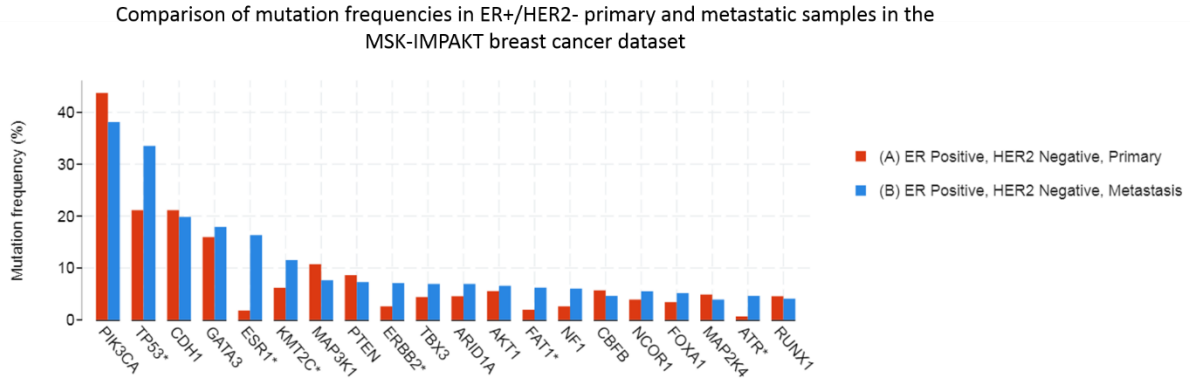


*Figure 8 Comparative variant frequencies between primary and metastatic tumours in the MSK-IMPAKT breast cancer dataset accessed through CBioPortal. Graph created using the 'groups' function. *indicates a p value of <0.05 as determined using Fisher's exact test.*

Other groups have also explored the changes in genetic variant profile in samples over time e.g. (Meric-Bernstam et al. 2014; Schleifman et al. 2014; Wheler et al. 2014). Numbers of patients included in these studies are much smaller, generally <100 patients. In the work by Meric-Bernstam et al genes that were more commonly altered in metastatic samples compared to primary tissue included *PIK3CA, MDM2* and *CDK4*. The changes in *MDM2* and *CDK4* tended to be copy number variations as detected using targeted NGS sequencing. Wheler et al looked specifically at patients treated with anastrazole and everolimus and examined the relationship between clinical response and the presence of genetic variants. They found that patients were responding to treatment despite the presence of genetic alterations in tissue as assessed using targeted NGS. Although only 3 patients had either a partial or complete response; their tumours contained variants such as *PTEN* loss, *CCNE1, IRS2, MCL1, CCND1, FGFR1* and *MYC* amplifications, rearrangement in *PRKDC*, variants in *PIK3CA* (H1047R), *PIK3R1* (G376R) and *TP53* (I195T) suggesting that the presence of variants does not equate to resistance to treatment. While Schliefman et al looked specifically at *PIK3CA* and *AKT1* variants and *PTEN* loss across paired primary and metastatic tumour samples finding that there was minimal change over time (n=75 participants).

1.4.2.3 *Identification of genetic variants; copy number variations (CNVs)*

Gene amplification or copy number gain has been associated with prognosis and response to treatment in breast cancer since the use of fluorescent in situ hybridisation (FISH) to investigate HER2 amplification in breast cancers. With the advent of NGS it is now possible to establish copy number variation in tumours using genetic sequencing techniques. Bioinformatically these techniques rely on the presence of a reference gene that will always have two copies or large enough datasets that expected CNV can be calculated by pooling sample data. Through these sequencing projects it has been noted that CNVs change during the disease course. As detailed in Figure 9 there are many genes that are more frequently amplified in metastatic samples than in primary tumour. The most common amplification is in *CCND1*, followed by amplifications of *FGF19*, 4

and 3. *FGFR1, PAK1* and *MYC* are also frequently amplified but without a significant change over time. This is demonstrated in Figure 9.

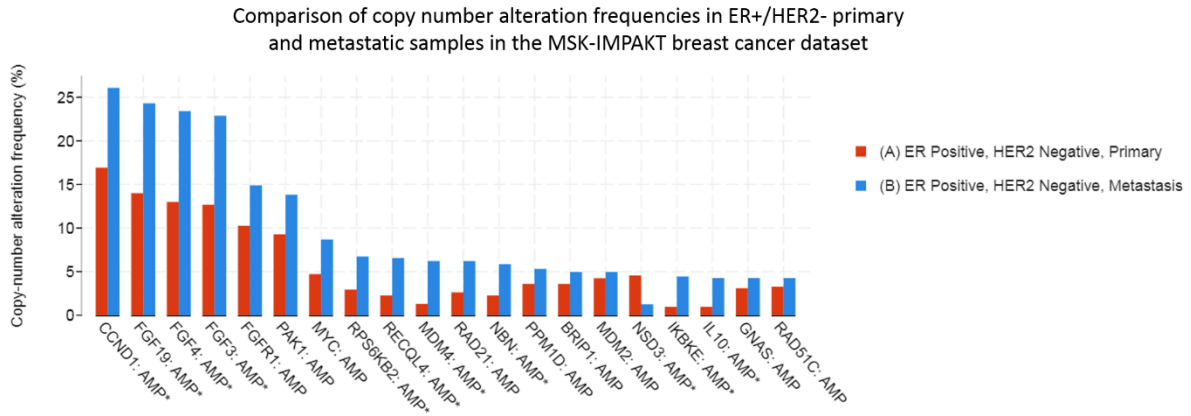


*Figure 9 Comparative copy number variation frequencies between primary and metastatic tumours in the MSK-IMPAKT breast cancer dataset accessed through CBioPortal. Graph created using the 'groups' function. *indicates a p value of <0.05 as determined using Fisher's exact test.*

1.4.2.4 *Droplet digital PCR (ddPCR)*

While next generation sequencing technologies offer the ability to search the genome for variants in many genes from a single sample there are drawbacks in terms of cost, complexity and time. For genes where hotspot variants have been identified ddPCR can offer a quick and more sensitive way to establish whether a variant is present. In ER+ breast cancer this technology has most often been applied to the presence of hotspot variants in *AKT1, PIK3CA* and *ESR1*. Assays can be designed to identify single nucleotide variants or can be multiplexed to allow the identification of up to 4 different variants in one experiment (Hrebien et al. 2016). Assays can also be developed to identify changes in copy number such as in *ERBB2* (Gevensleben et al. 2013).

DdPCR can also be useful when a single variant is identified from next generation sequencing and is then 'tracked' over the course of the patients' disease. DdPCR can also be used to confirm the presence of variants detected using NGS (Lopez-Knowles et al. 2019).

A good example of the use of ddPCR technology in the field of endocrine resistant breast cancer comes from the FERGI study (a randomised phase II study comparing a pan PI3K inhibitor with fulvestrant against fulvestrant and placebo in the setting of aromatase inhibitor resistant ABC) where samples were evaluated for the presence of *PIK3CA* and *ESR1* variants using BEAMing ddPCR technology and standard ddPCR technology using the Biorad CX200 system. Using both tissue and plasma samples the authors explored the presence of variants in both genes and were able to track these variants during the course of the disease. In several patients they were able to demonstrate the variant allele frequency (VAF) falling in response to treatment and then rising as the disease escaped control (Spoerke et al. 2016).

1.4.3 Other potential biomarker techniques not used in this project

1.4.3.1 *Tumour mutational burden (TMB) in breast cancer*

TMB is a measurement of the number of variants in tumour samples (different methodologies exist, some including synonymous variants and others counting non-synonymous variants only) and is used as a predictive and prognostic biomarker for response to immunotherapies in tumours such as melanoma and non-small lung cancer (Marra et al. 2019). Traditionally it was calculated from whole exome sequencing but more recently it has also been calculated effectively from smaller panels of around 300 genes. TMB is likely to be linked to failure of DNA damage repair and problems with accurate DNA replication. There is a strong link between the presence of *TP53* variants and high TMB (Chalmers et al. 2017). Breast cancer is not traditionally a cancer with high TMB but one could hypothesise that if variants occurred in key genes responsible for DNA repair and replication such as *TP53, MSH2, MLH1* then TMB could be a potential biomarker in ER+ endocrine resistant breast cancer. The accurate calculation of TMB relies on a volume of data greater than that which will be generated using a small targeted panel in this project and thus will not be calculated. Had circumstances and timing been different a larger panel may have been used to allow exploration of TMB as a biomarker with the hypothesis that therapy with fulvestrant and vandetanib may not be

suitable for patients with high TMB, as these tumours may respond better to immunotherapy or direct DNA damaging agents due to the inherently unstable nature of the tumours DNA.

1.4.3.2 *Mutational signatures in breast cancer*

Genomics research in breast cancer often focusses on frequently occurring genetic variants in the exonic regions of the genome. Genetic signatures are derived from bioinformatic examination of data from WES or WGS and use data from both coding and non-coding regions of the genome. Signatures are described in a review by Nik-Zainal and Morganella as "the scars of biological processes that have gone awry during cancer development" (Nik-Zainal and Morganella 2017). Five signatures have been frequently identified in breast cancer (Signature 1B, 2, 3,8 and 13) these relate to age at diagnosis, APOBEC and BRCA1/2 deficiency; the associations with other signatures have not yet been identified (Alexandrov et al. 2013). APOBEC refers to a collection of cytosine deaminases that are part of the immune response. BRCA 1/2 signatures correlate with defects in homologous recombination (HR), this can either be from genetic variants in the genes themselves or a more complex signature resulting in 'BRCAness' a term given to tumours exhibiting failure in HR. In a more detailed study of 560 breast cancer samples an additional 7 signatures were identified (Nik-Zainal et al. 2016).

While there is some overlap between histological subtype, molecular characterisation and signature pattern e.g. triple negative, basal like and signatures 3 and 8 (HR deficiency) this relationship is not present in a significant minority of cases. For patients with ER+/HER2- or luminal A or B disease signatures 1,2 and 5 are more common although around 10% of cases will have evidence of HR deficiency linked signatures (statistics extrapolated from Figure 5 (Nik-Zainal et al. 2016)). The fact that these cancers are more frequent in older patients correlates with the presence of signature 1 and 5. Signature 2 relates to the presence of dysfunctional APOBEC enzymes that contribute to the immune response.

Mutational signatures offer a fascinating insight into the aetiology of tumours. However, they do not have strong independent prognostic value in ER+ breast cancer and thus will not be explored as potential biomarkers in this project.

1.4.3.3 *RNA based biomarkers in breast cancer*

Gene expression signatures using data generated from extracted RNA were shown to be predictive of clinical outcome in the early 2000s (see section 1.1.2). Subsequently RNA analysis has been used to explore the role of micro-RNAs (miRNA) as both prognostic and predictive biomarkers. MiRNA are non-coding RNA molecules that act as 'switches' to regulate gene expression and cellular processes (Zelli et al. 2020). Many miRNAs have been identified in breast cancer and in individual studies can act as both prognostic and predictive biomarkers, however there is a lack of consensus over the best candidates to take forward for further study. Studies that have had both discovery and validation cohorts have shown that miRNA signatures (made by combining two or more miRNAs) can differentiate metastatic from locally advanced luminal breast cancers. For example, miR-331 and miR-195 were analysed in 74 patient samples (22 metastatic samples, 31 local samples and 21 healthy control samples). It was noted by the authors that the two candidate miRNAs targeted HER2 and E2F1 amongst other genes (McAnena et al. 2019). MiRNAs can be extracted from blood and sequenced using NGS and are appealing as a potential biomarker in all stages of disease. In this project a decision was made to focus on DNA based biomarkers but in future projects exploration of miRNAs during treatment would be of great interest.

1.5 Thesis overview

The key question this thesis sets out to answer is:

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*Can **potential** biomarkers of clinical response to treatment with vandetanib and fulvestrant be identified from tissue and/or plasma samples from patients with ER+ breast cancer who have developed endocrine resistant disease? Which biomarkers, if any, are candidates for prospective validation?*

The idea of *potential* biomarkers is important here as this will be a retrospective analysis and thus will be hypothesis generating with any potential biomarkers requiring prospective analysis and validation before implementation in the clinic. The identification of biomarkers for any new potential new treatment is important because good patient selection spares those that will not benefit from unnecessary toxicities and enables those patients, who are likely to respond, to receive treatments better suited to their situation. Investigation of predictive biomarkers for clinical response to fulvestrant and vandetanib, in combination, have not been previously studied.

In addition to this the use of circulating biomarkers in breast cancer remains under investigation, particularly in the arena of ER+ breast cancer where disease burden and thus circulating tumour DNA (ctDNA) levels may be low. The samples that form the basis of this thesis were taken early in the disease course after patients developed advanced breast cancer and represent a distinct cohort of patients that are often only studied as part of a more heterogeneous group of patients with advanced breast cancer. Furthermore, the investigation into assessing copy number variation in commonly amplified genes such as *MYC* and *FGFR1* by ddPCR offers new data around the feasibility of this technique in ER+ advanced breast cancer.

These strategies relate to a key hypothesis and a number of overarching aims of the thesis.

Key Hypothesis

High expression of RET (t-RET, p-RET or both) will act as a prognostic biomarker and by inhibiting RET (and other targets of vandetanib) RET expression will also act as a predictive biomarker for response to vandetanib; with high expressers experiencing greater clinical benefit from RET inhibition.

Thesis Aims

1. To establish protein expression of RET in FFPE primary tumour samples using immunohistochemistry and to correlate protein expression with clinical outcomes in patients receiving treatment with fulvestrant +/- vandetanib
2. To examine the presence of genetic variants (SNVs and CNVs) in both FFPE and ctDNA tumour samples using NGS and ddPCR. The presence or absence of genetic variants will be correlated with clinical outcomes with a focus on variants that could influence response to fulvestrant and vandetanib.
3. Methodology will be developed to examine CNVs by ddPCR in the genes MYC and FGFR1 in FFPE and cfDNA. Clinical outcome data will be used to determine whether clinically meaningful cut points can be identified to support the use of these tests as prognostic biomarkers.

Each chapter will include a clear statement of its individual aims and objectives and conclude with a discussion as to whether these have been met. Each key technique will form the basis of the three main results chapters (chapters 3,4 and 5). Then in chapter 6 the potential biomarkers will be evaluated alongside clinic-pathological outcome data from the FURVA clinical trial. Finally, a general discussion will look at the results in context of a clinical and scientific world where our knowledge of

the genomic basis and biological underpinnings of ER+ endocrine resistance has increased rapidly during the time course of this project.

1.6 Chapter summary

Despite the relatively good prognosis of ER+ breast cancer between 10 and 40% of patients diagnosed with early stage ER+ breast cancer will go on to develop ABC (Pan et al. 2017) meaning each year roughly 12,000 people in the UK will be diagnosed with ER+ ABC each year and many will die from their disease.

The mechanisms by which endocrine resistance develops and thus treatments fail is complex and is likely to vary between patients and even within metastatic deposits and clonal populations in the same patient. Developing new treatments to prolong life for this group of patients is essential as even with advances in screening, early diagnosis and treatment there will always be people living with ABC. A one size fits all approach will not work here due to the different mechanisms of endocrine resistance involved and the FURVA trial seeks to target a group of patients in whom endocrine resistance may be driven by RET overexpression.

The focus of this thesis is to try and determine whether there are biomarkers that can predict response to treatment with vandetanib and/or fulvestrant that have potential to be validated in prospective studies. To determine this, samples collected as part of the clinical trial will be tested for various potential biomarkers, selected based on the best available evidence at the time of development of the project, using a variety of techniques. Not only will this strategy try to answer the main aim of the thesis but will also contribute to the further understanding of potential mechanisms of endocrine resistance and how best to identify these using both well established and newer technologies such as liquid biopsy.

2 Materials and methods

2.1 Introduction

2.1.1 Chapter Overview

This chapter documents the techniques used to investigate potential biomarkers within the context of the FURVA trial using both targeted and semi-unbiased approaches. The targeted approach involved interrogation of the presence and functionality of RET protein in tissue samples using immunohistochemistry (IHC) for both total (t-RET) and phosphorylated/activated RET (p-RET) respectively. At a DNA level, a targeted next generation sequencing (NGS) panel was selected to investigate key variants in a panel of genes of potential interest in the setting of oestrogen resistant metastatic breast cancer. Finally, droplet digital PCR (ddPCR) was used to investigate the prevalence of ESR1 variants and copy number changes in MYC and FGFR1 in circulating free DNA representative of aromatase inhibitor resistant disease.

This chapter will cover the samples used, the methods employed and the method development of the different techniques.

2.1.2 Chapter Aim

To document the techniques used in this thesis and subsequently the development and validation of the methods selected to explore potential biomarkers in the FURVA study at both a protein and DNA level.

2.1.3 Chapter Objectives

- To develop and validate immunohistochemistry assays suitable for the detection of t-RET and p-RET in freshly cut sections of historic formalin fixed paraffin embedded (FFPE)

tumour blocks. The assays will then be used to evaluate t-RET and p-RET in the FURVA trial breast cancer sample series.

- To develop a complementary scoring system to allow grouping of the immunostained samples into t-RET and p-RET 'high' and 'low' samples.
- To select an appropriate targeted next generation sequencing panel for exploration of variants in genes of interest in advanced aromatase inhibitor resistant breast cancer
- To establish a limit of detection (for both DNA input and variant allele frequency (VAF)) for the selected targeted next generation sequencing panel
- To establish limits of detection for ddPCR probes for *ESR1* variant detection
- To establish methodology for measuring copy number gain in *MYC* and *FGFR1* using ddPCR technology.

2.2 Materials

2.2.1 Ethical approval

All patient samples were obtained from patients recruited to the FURVA trial (EduraCT Number: 2014-001208-23, Sponsor: Velindre NHS Trust). Trial participants had specifically consented for their samples to be used in translational research. All samples were determined relevant material and thus were stored at premises licenced in accordance with the Human Tissue Act 2004. The trial is registered with a multi-centre research ethics committee (MREC). The REC reference number is 14/WA/1219 (Wales REC 3) (Appendix document 1). See Appendix document 2 for Patient Information Sheet and Consent Form.

Historic FFPE tissue samples used for IHC assay development and validation work were clinical breast cancer samples collected during diagnosis and treatment in the 1980s-1990s by teams at Nottingham City Hospital (University of Nottingham, Prof IO Ellis and Dr A Green) and held under material transfer arrangement in the School of Pharmacy, Cardiff University. The use of these long

term stored samples for studies in endocrine resistance under the guidance of Dr J Gee in the school has been approved based on continuous rolling ethical approval from Nottingham REC2: C2020313 with generic consent for the research use of the samples.

2.2.2 Patient Characteristics

All participants in the FURVA study were post-menopausal women with histological evidence of ER+, HER2- breast cancer alongside clinical confirmation of locally advanced or metastatic disease where surgical resection with possibility of cure was not feasible. Participants had experienced disease progression or relapse while taking an aromatase inhibitor such as exemestane, letrozole or anastrozole. Participants could have received up to three lines of endocrine therapy but no more than one line of cytotoxic chemotherapy for ABC prior to participation in the FURVA trial.

2.2.3 Samples

2.2.3.1 *FURVA FFPE samples*

Centres participating in the FURVA trial were requested to provide a diagnostic tissue block from each participant for analysis, it was not specified whether this should be primary tumour or a metastatic biopsy. For IHC analysis all available FFPE samples were cut into 3 micron sections and placed onto charged glass slides. All slides were cut by the same technician in the same laboratory. Slides were then transported in batches to the laboratory where the immunohistochemistry analysis took place. The slides were assayed as soon as practically possible for both t-RET and p-RET. Samples were either from primary tumour, lymph node or metastatic deposit (lung, liver, skin or bone). Detail on the tissue of origin was obtained from the accompanying pathology report and reviewed during the scoring of each sample by a consultant pathologist (Dr Fouad Alchami (FA)). All samples were assayed, and analysis was performed in groups depending on the tissue of origin. On review by Zoe Hudson (ZH) and FA a small number of tissue blocks were found to not contain any cancer cells or were from a cytology sample; these samples were excluded from all analyses. More detail on this is given in Chapter 3.

For analysis of the genomic landscape of tumours prior to treatment with fulvestrant +/- vandetanib sections from all FFPE blocks available were cut to a thickness of 5 microns onto non charged slides. Tumour samples were macro dissected based on a stained slide identifying the area with maximal tumour content by trained NHS staff working in the All Wales Genetics Laboratory (AWGL). DNA was extracted from 6 sections per patient by trained NHS staff using the Maxwell 16 FFPE Plus LEV DNA purification kit (Promega, Madison, WI, USA) according to the manufacturers protocol. This automated process allows DNA extraction in batches of 16 samples using magnetic bead based purification of DNA from FFPE samples. The three basic steps comprise; break down of the cell and its structures to form a lysate, separation of cell debris and insoluble material from the desired DNA end product and elution of purified DNA from the magnetic beads. After extraction the concentration of the final elute was determined using the Qubit High Sensitivity Kit (Thermofisher Scientific, Waltham, MA, USA).

115 primary FFPE samples were analysed using immunohistochemistry. NGS sequencing data was available from 48 primary FFPE samples.

2.2.3.2 *cfDNA samples*

As part of the trial protocol participants were asked to have a blood sample collected at trial entry, after 8 weeks of trial treatment and at end of trial treatment. These samples will subsequently be referred to in this thesis as ‘Baseline’, ‘8 week’ and ‘EOT’ respectively. Where samples were collected as per protocol 10mls of whole blood collected in CellSave tubes was sent to the AWGL. Upon arrival (and within 96 hours of collection) the tube was spun at 2000g for 10 minutes at 4°C. Plasma and buffy coat were separated from the red blood cells (RBC) and spun again at 2000g for 10 minutes to ensure no red or white blood cells remained in the plasma sample. Plasma and buffy coat were stored in 1ml aliquots at -80°C until DNA extraction. This initial processing step was performed by trained NHS employees in the AWGL.

41 patients treated with fulvestrant and vandetanib had cfDNA samples analysed using NGS. Between 61 and 67 patients treated with fulvestrant +/- vandetanib had a cfDNA sample analysed by ddPCR for the presence of *ESR1* variants or copy number variations in *MYC* or *FGFR1*. The number of patients included varied between assays due to sample availability.

2.3 Methods

Each method will be presented in turn. Following this, detail on methodological development and validation experiments will be detailed in section 2.4.

2.3.1 Immunohistochemistry (IHC) materials and methods

This section will focus on the techniques used to assess the presence of RET protein in tissue samples from participants in the FURVA trial. Assays measuring both t-RET and p-RET are detailed, along with background to IHC and reasons for its selection in this project.

2.3.1.1 Background

IHC is an antibody based semi-quantitative method for determining the presence of biomarkers in tissue samples. The procedure has many advantages such as the ability to detect and semi quantify protein expression and/or activity and localise the protein within the cell. Furthermore, when tissue contains multiple types of cell expression patterns can be compared between cell types. In addition, in larger cut sections heterogeneity of expression can be assessed. Finally, it is a relatively simple and versatile technique that can be used to study diverse samples including FFPE material.

Nevertheless, since its inception in the late 1960s (Kawarai and Nakane 1970) immunohistochemistry has also had some limitations in terms of its reproducibility and standardisation. This became most relevant when clinical treatment decisions were made based on the results of the IHC assays. For example, to select for the use of hormone receptor blockade in patients whose breast cancers stained positively for the oestrogen receptor protein (ER+ breast cancer).

Fortunately, over time, technology has improved both the specificity of the antibodies available for IHC and the detection methods resulting in assays that can perform well both in reproducibility and quality. Use of antigen retrieval during assays has in many instances also improved assay performance in FFPE material. Routine clinical assays are now often automated with commercially produced controls. There has also been instigation of a national quality control scheme (UK NEQAS) for routinely monitored clinical markers such as ER. However, where new assays are being developed for research purposes, experimental assays are more likely to be optimised in house for the specific tissue and preparation they will be used with and then run manually with internal control samples, as was the situation in this project.

The basic methodology of any IHC assay begins with the removal of tissue from the patient which is then processed by fixation (usually using formalin) and embedded into a paraffin block. The FFPE tissue is then sectioned into thin (3-5µm) slices and dried onto charged glass slides. This section of sample is then analysed using a number of steps optimised for the retrieval of a specific protein of interest that include antigen retrieval, blocking of any non-specific sites or endogenous enzymes, application of primary antibody specific to the human protein under evaluation, antibody incubation (again optimised for the protein of interest), application of a secondary antibody system that recognises the primary antibody species and is linked to horseradish peroxidase (HRP) and finally the use of a chromogen/substrate (usually DAB/hydrogen-peroxide) detection to visualise the antigen/primary antibody/secondary antibody binding on the sample. This is followed by the application of counterstain to reveal the tissue morphology of any IHC-negative cells and the dehydration and cover slipping of the sample ready for semi-quantitative staining assessment using a light microscope, interpretation and long-term storage of the slides.

Immunohistochemistry was selected as the best method to assess RET protein expression in this project for several reasons. Firstly, it ensures staining analysed is in the plasma membrane/cytoplasm of breast tumour epithelial cells, a localisation previously reported for the RET

protein in this tissue type and in keeping with its potential signalling function (Esseghir et al. 2007). Secondly it allows analysis of the range of staining patterns and levels of expression in the tumour epithelial cells. This is particularly important if there is any significant heterogeneity of RET expression within the cancer cells present in the sample or between samples. It also allows the observation of RET expression in other tissues such as normal breast tissue or non-invasive disease which may be included in the sample so these can be eliminated from analysis of its tumour prevalence. Furthermore, IHC is a common and well understood technique in histopathology meaning that should RET expression as determined by IHC demonstrate potential as a biomarker it could be a good candidate for further validation for clinical practice.

Limitations of using IHC to evaluate RET in breast cancer samples include the subjectivity of the semi-quantitative sample scoring and the challenges of reproducibility in assays that have been performed over the duration of the study, sub optimal staining if there is compromised antigenicity in any FFPE samples and non-specific staining may also be encountered. The IHC assay will be designed to limit these issues using antigen retrieval and protein or peroxidase blocking respectively. Maximal sensitivity will be needed since the studies will be performed in FFPE material, and so for this purpose the sensitive Envision immunoperoxidase detection system (Agilent, Santa Clara, CA, USA) has been chosen. The basic principles of this system are illustrated in Figure 10.

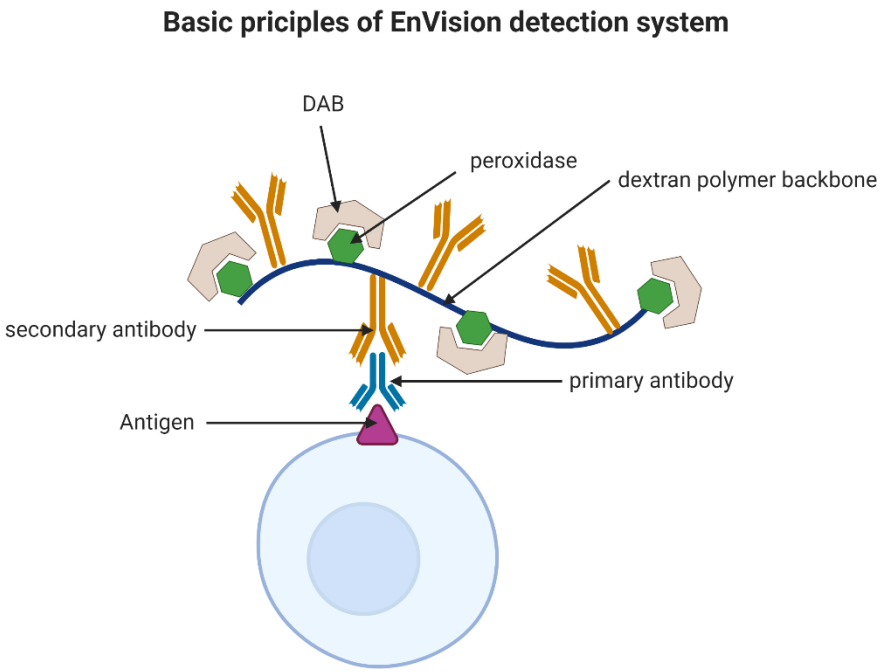


Figure 10 Overview of the EnVision detection system. DAB = 3'diaminobenzidine tetrahydrochloride

Furthermore, commercially produced primary antibodies can vary in concentration between batches and for less commonly used antibodies the inter batch variability can potentially be significant. The study duration meant that multiple antibody batches were required over a period of 3 years. To mitigate for the extended time period over which the assays were performed all slides were stained by ZH thus limiting operator variance. Batches all contained an internal control sample to ensure minimal inter batch variability.

2.3.1.2 IHC materials used and final protocols for T-RET and P-RET assays

Table 3 details the materials used in the IHC experiments.

Buffers	
0.01M Phosphate buffered saline (PBS)	42.5g Sodium Chloride (Fisher 11984051) 7.15g di-potassium hydrogen orthophosphate anhydrous K2HPO4 (Fisher 10375760) 1.25g Potassium dihydrogen orthophosphate KH2PO4 (Fisher 10783611) Make up to 5L with distilled water and pH check aiming for a pH of 7.2-7.4
PBS - Tween	Add 150µL of Tween 20 (Sigma P2287) to 500mls PBS made as above to give a solution of 0.03% Tween solution
Triphosphate buffered saline (TBS) 0.5%	Add 60.55g Tris Base and 45g sodium chloride to 250mls distilled water. Add 5M hydrochloric acid dropwise to achieve pH of 7.5 Make up to 500mls with distilled water.
TBS-Tween 0.5%	Add 1.5mls of Tween 20 (Sigma P2287) to TBS 0.5%
TBS-Tween 0.05%	Add 50mls of TBS Tween 0.5% to 450mls distilled water
EDTA buffer (pH9)	Add 1.21g Tris Base and 0.37g EDTA to 1L of distilled water. pH should measure 9 without adjustment.
Sodium Citrate buffer (pH6)	Add 2.94g sodium citrate to 1L distilled water then add 5M hydrochloric acid dropwise until pH 7.6 reached.
Peroxidase blocking	
3% Hydrogen Peroxide	Dilute 3ml 30% Hydrogen peroxide (Fisher 10736291) in 27mls distilled water

0.18% Hydrogen Peroxide	Add 3ml 6% hydrogen peroxide (Fisher 10502002) solution to 97mls distilled water
Protein blocking	
Protein block	Dako (X0909) a serum free block with casein
Antibodies	
Primary antibody (t-RET)	Abcam Ab134100 a recombinant anti rabbit IgG monoclonal antibody. Aliquotted on arrival and stored at -20°C until use. Stock concentration 0.649mg/mL.
Primary antibody (p-RET)	Abcam Ab51103 an anti-rabbit IgG polyclonal antibody to phospho site Y1062. Aliquotted on arrival and stored at -20°C until use.
Secondary Antibody	Dako Envision+ System HRP labelled polymer Anti Rabbit (K4009)
Detection and counterstain	
DAB detection kit	Dako Liquid DAB+ substrate chromogen system (K3468)
Methyl Green 0.05% counterstain	Dissolve 0.05g Methyl Green (Sigma M8884) in 100mls distilled water

Table 3 Materials required for IHC experiments in FFPE breast cancer samples

The final optimised assay for FFPE breast cancer samples for t-RET expression is outlined in Table 4.

Step	Timings
Dewaxing and rehydration of FFPE sections	2 x 7 minutes in xylene 2 x 2 minutes in 100% ethanol 2 x 2 minutes in 90% ethanol 2 x 2 minutes in 70% ethanol Rest in distilled water for 5 minutes
Antigen Retrieval	1 minute at microwave on full power (power level 10, 950W) 9 minutes at microwave power level 6 (560W) in pH 9 EDTA buffer
Blocking steps	1 drop 0.18% H2O2 for 20 minutes then wash in PBS-Tween buffer followed by 2 drops Dako Protein Block for 20 minutes
Primary antibody application	50-70µL[2] Abcam Ab134100 diluted to 1:60-1:100 in TBST 0.05% (see detail in 2.1.1.1) applied directly to slides and incubated at 23°C overnight
Secondary antibody application	After washing twice in PBS-Tween buffer in a slide bath 1 or 2 drops Dako Envision Rabbit secondary applied for 60 minutes
DAB detection	50-70µL Dako Envision DAB chromogen/substrate detection system applied for 10 minutes
Counterstain, dry and coverslip	After washing in distilled H_2O (dH_2O) 0.05% methyl green applied for 2 minutes. Wash with dH_2O and dry at 40°C for 1 hour, coverslip using DPX.

Table 4 Final optimised protocol for T-RET staining assay on breast cancer sections

[2] 50µL for core biopsy samples or sections <1cm^2, 70 µL for larger sections.

The final optimised assay protocol used to examine p-RET expression in the FURVA samples is shown in Table 5.

Step	Timings
Dewaxing	2 x 7 minutes in xylene 2 x 2 minutes in 100% ethanol 2 x 2 minutes in 90% ethanol 2 x 2 minutes in 70% ethanol Rest in distilled water for 5 minutes
Antigen retrieval	1 minute at microwave on full power (power level 10, 950W) 9 minutes at microwave power level 6 (560W) in pH 6 sodium citrate buffer
Blocking steps	3% H2O2 for 5 minutes then wash in PBST 2 drops of protein block for 20 minutes
Primary antibody application	50-70µL[3] Abcam Ab55103 diluted to 1:50 in TBS 0.05% buffer applied directly to slides and incubated at 23°C overnight
Secondary antibody application	After washing twice in PBST Dako Envision Rabbit secondary applied for 120 minutes incubated at 23°C
DAB detection	Dako Envision DAB chromogen/substrate detection system applied fresh for 8 minutes
Counterstain, dry and coverslip	After washing in distilled H_2O (dH_2O) 0.05% methyl green applied for 2 minutes. Wash with dH_2O and dry at 40°C for 1 hour, coverslip using DPX.

Table 5 Protocol for p-RET staining assay on breast cancer sections

[3] 50µL for core biopsy samples or sections <1cm^2, 70 µL for larger sections.

2.3.1.3

2.3.1.3 Scoring of t-RET and p-RET immunostaining

Both assays were scored in each sample using an H-scoring method (Fedchenko and Reifenrath 2014). Scorers (ZH and FA) were blinded to treatment received. Slides were viewed at x10 magnification with areas of interest viewed at x20, in particular to observe the staining pattern or to determine the cell type stained. The whole section was then reviewed at x 10 magnification with brown staining in the cytoplasm of invasive tumour epithelial cells scored as positive. Samples were scored by ZH initially. Samples were then reviewed by FA who calculated his score. Where there was disparity in the scores from the two assessors further review was undertaken, and consensus reached on the final score for the sample. Whole samples were scored, these being either primary tumour, lymph node or metastatic lesion. All tumour epithelial cells in the sample were allocated to a score of either 0 (negative), 1 (low positive staining intensity), 2 (moderate positive staining intensity) or 3 (high positive staining intensity). Estimating the percentage of tumour cells staining positively for each intensity level permitted the calculation of the final H-score using the following formula H=1Xx+ 2y +3z, with a maximum score of 300 (all tumour cells demonstrating strong staining intensity). The intensity levels were as follows.

x = percentage of tumour cells staining at low intensity

y = percentage of tumour cells staining at moderate intensity

z = percentage of tumour cells staining at high intensity

Where there was difficulty in ascertaining tumour from non-tumour component an H&E stained slide was reviewed. Normal breast or non-invasive ductal carcinoma in situ (DCIS), if present, was not included in the scoring. Total H-score and breakdown by intensity category were tabulated in an Excel spreadsheet and subsequently in an SPSS workbook ready for further statistical analysis and correlation of the immunoscoring with clinicopathological data. Note was made of the origin of the sample; whether primary tumour or metastases and whether the section was from a core biopsy or

a tumour section. The size of the section was also noted. Samples that were from cytology specimens or had <100 tumour cells were excluded from further analysis.

A number of methods for determining a cut point between high and low expression were explored (discussed in section 2.4.3). Finally, a cut point determined using the maxstat method {Hothorn, 2008 #191} was selected for use in Chapter 6: Clinical Correlation. T-RET samples were deemed to have high expression if the H-score was ≥166 and P-RET samples showed high expression if the H-score was ≥85.

2.3.2 Next Generation Sequencing (NGS) for detection of genetic variants in DNA extracted from FFPE and plasma samples

2.3.2.1 Background

Next generation sequencing is an umbrella term referring to the sequencing of DNA using technologies that have evolved from initial early methods such as Sanger sequencing. The 'next generation' technologies allow for quicker, cheaper and deeper sequencing of either the whole genome (WGS), whole exome (WES) or regions of interest (ROI) within the genome using targeted gene or hotspot panels.

Many variables must be considered in choosing how best to incorporate next generation sequencing into a project. The aim of the project must align with the technology chosen; for example, sequencing the whole genome or exome would be appropriate if looking for novel mutations related to a specific pathology where there is a paucity of data currently in the literature. If the aim is to identify whether a specific genetic variant is present at a frequency of > 20% or absent to guide a treatment decision then this can be achieved with older technologies such as Sanger sequencing, for example KRAS mutation status predicting for response to VEGF inhibitors in colorectal cancer. Between WGS and simple Sanger sequencing lies a myriad of technologies all with a variety of pros and cons. With good use of the existing literature base it is possible to identify ROI specific to a

research question, in this situation targeted panels can be used allowing for greater depth of sequencing thereby improving the limit of detection.

Whole exome sequencing falls between WGS and targeted panels and offers a reduction in time and cost when compared to WGS but due to the vast number of megabases sequenced the overall coverage of the exome will be less than can be achieved with targeted panels. Large datasets, such as those generated by WGS or WES or even some of the larger targeted panels can be difficult to work with and require significant bioinformatic and manpower to review potential variants and assign significance. With any of these approaches long term storage of patient data also has to be considered; the storage requirements increasing with the complexity of the technology.

	Next (or second) generation sequencing			First generation sequencing
	WGS	WES	Targeted Panels	Sanger Sequencing
Time sequencing	+++	++	+	+
Time analysing	+++	++	+	+
Bases sequenced	3.3 billion	40-50 million	Varies	200bp of region of interest
Average depth of coverage	15-30 reads per base	100-150 reads per base	500-5000 reads per base	NA

Table 6 Comparison of sequencing technologies. Compiled using data from a review by Horak et al (Horak et al. 2016). Based on the assumption that 50 samples of each type will be run.

When applying the overview in Table 6 to the samples used in this study; both FFPE samples from historical tumour blocks and cfDNA extracted from plasma there are a number of specific considerations. Firstly, that the amount of DNA that can be extracted from each sample type is finite. Secondly, that the aim of the project is to detect somatic variants that indicate the presence

of ctDNA. Thirdly, that there are financial restrictions and fourthly, that there is limited bioinformatic support and long-term data storage. Taking all this into consideration a targeted panel approach was selected for this project.

A further benefit to using a targeted panel is the lower requirements for amount of input DNA. The FURVA trial had pragmatically not required a biopsy of metastatic disease therefore the only DNA available to determine genetic variants present in ABC was cfDNA extracted from blood samples. The amount of DNA that can be extracted from cfDNA in ER+ breast cancer patients is highly variable, even at the upper limits of DNA concentration it may not have been technologically feasible to attempt WGS or WES on these samples with the technology available at the time in the laboratory.

Being able to perform WGS or WES on cfDNA was under investigation at the time of technology selection; Adalsteinsson et al demonstrated that it was possible using cfDNA samples from patients with metastatic breast and prostate cancer. They used a two-step approach designed to identify samples with >10% tumour content which then proceeded to WES. In their metastatic breast cancer samples >30% of samples and over 40% of patients had sufficient DNA for WES but no comment was made about the type of breast cancers and the point in the patient's treatment pathway the samples had been taken. However, given that this was the first major report and that many patients in the study had provided up to 14 samples to ensure an adequate sample was available it was felt that this technology was not at a stage where it could be considered for this project (Adalsteinsson et al. 2017).

Even at the early stages of the project (and exponentially since) there was a large volume of publicly available data describing the genomic landscape of breast cancer. At the beginning of the project whole genome data from Nik Zainal et al was available first for the life history of 21 breast cancers (Nik-Zainal et al. 2012), followed by WGS from 560 breast cancers (Nik-Zainal et al. 2016). This was further supplemented by whole exome data from 103 breast cancer patients (Banerji et al. 2012),

817 patients with invasive lobular and ductal breast cancer (Ciriello et al. 2015) and 216 patients with metastatic breast cancer (Lefebvre et al. 2016). Ellis et al published details of a combination of WGS and WES data for 77 ER+ patients and correlated their findings with response to aromatase inhibition (Ellis et al. 2012). Projects using targeted approaches had also published data such as Pererira et al who sequenced 173 genes in 2433 primary breast cancers (Pereira et al. 2016). This meant that at the time of deciding which technology to use in Summer 2017 there was already a large amount of data to help refine which genes and even variants could be of interest in ER+ breast cancer. These large studies used a mixture of DNA extracted from fresh frozen and FFPE samples. Studies from Lefebvre and Ellis only used fresh frozen samples.

Subsequent to selection of technology for this project larger and more specific datasets became publicly available. For example, 1918 samples from ER+ breast cancer patients, some with multiple samples sequenced using a 468 gene targeted panel (Razavi et al. 2018) have been published and added to publicly searchable online data repositories such as CBioPortal (Gao et al. 2013).

By carefully interrogating existing datasets with particular reference to the genomic landscape of ER+ breast cancers and if possible those that had showed endocrine resistance it was possible to build up a picture of which genes frequently contained pathogenic variants and thus could be of interest in determining how different variants may affect clinical outcomes.

Therefore, the main requirement of the targeted panel was maximum coverage of the genes of interest while being suitable to run of a reasonable group of patients without becoming prohibitively costly or time consuming for the available samples.

This meant the two broad options were to either custom design a panel as has been done in a number of trials to date or to use an ‘off the shelf’ panel, either designed for general cancer research or specifically for breast cancer research.

2.3.2.2 NGS materials used

Table 7 details the materials required for the extraction of cfDNA and subsequent library preparation, quantification and sequencing.

cfDNA extraction		
QIAamp circulating nucleic acids kit	Qiagen	Cat ID 55114
Library preparation		
Ion Ampliseq Library Kit 2.0	Thermofisher	Cat ID 4475345
IonExpress Barcode Adaptors	Thermofisher	Cat ID e.g. 4474521
Library quantification		
Qubit dsDNA HS kit	Thermofisher	Cat ID Q32854
Agilent High Sensitivity DNA kit	Agilent	Cat ID 5067-4626
Sequencing reagents		
Ion PI Hi Q Sequencing 200 Kit	Thermofisher	Cat ID 26772
Ion PI Hi Q Chef Kit	Thermofisher	Cat ID A27198
Ion PI Chip Kit v3	Thermofisher	Cat ID A26770
Bioinformatic software		
Torrent Suite	Thermofisher	Version: 5.8.0
Ion Reporter	Thermofisher	Version: 5.10.5.0

Table 7 Materials required for NGS experiments

2.3.2.3 NGS methods

Two targeted hotspot panels were used for the NGS work in this project. The first was the Cancer Hotspot Panel V2 (Thermofisher, Waltham, MA, USA). The second panel was a custom panel.

Online software developed by Thermofisher allows design of custom panels. As determined from the current literature variants in *ESR1* and *GATA3* were common and of interest in ER+ ABC but were not

covered in the CHPv2 panel. In addition, the coverage of *RET* in the CHPv2 panel was limited to 63/3345 bases covered. Furthermore, although *LYN* variants had not been prevalent in sequencing studies to date it would not have been sequenced in many targeted panels and there was rationale for investigating whether *LYN* variants could be detected in the FURVA trial participant samples. Therefore, a customised panel was designed. An initial design was created by ZH and was optimised by the in-house development team at Thermofisher and allowed coverage of 158/171 (92%) hotspots of interest in *RET, LYN, GATA3* and *ESR1*. Those hotspots which were not covered all lay within *GATA3*.

All libraries were prepared by ZH. Both panels were used as per manufacturer's instructions. Briefly, up to 12µL of DNA extracted from either FFPE or plasma samples was added to reagents for library preparation. During initial amplification 20 PCR cycles were used for the CHPv2 panela and 24 for the CUSTOM panel. Following amplification amplicons underwent partial digestion prior to purification and final library analysis. The final library concentration was determined using Qubit High Sensitivity Flurometer. Samples were then pooled to create a library of ~100pM concentration. Final preparation for sequencing was then performed using the IonChef. Finally, pooled libraries were sequenced using the IonTorrent Sequencer. BAM and BAI files were automatically uploaded to Ion Reporter for variant analysis.

Ion Reporter (Thermofisher) offers a graphical user interface allowing the user to design a fully customised workflow for analysis of BAM files uploaded directly to the cloud-based storage system after each sequencing run. The workflow used for the analysis of FURVA patient samples used the following parameters in addition to the default settings recommended by Thermofisher for the CHPv2 panel.

- VAF >0.01 and <0.90
- Down sample to 2000 reads
- Located in exonic regions

- SNVs and INDELs
- Minimum coverage ≥ 100 reads
- Minimum coverage alternate allele ≥10 reads
- Maximum strand bias 0.9
- Phred quality score ≥20 (Ewing et al. 1998)

All variants called by IR were manually reviewed in IGV. Each variant was then allocated a Tier as per the Joint Consensus Recommendation of the Association of Molecular Pathology, American Society of Clinical Oncology and College of American Pathologists (Li et al. 2017a). Databases used to gather information to assist with tier allocation included COSMIC (Tate et al. 2019), dbSNP (Sherry et al. 2001), ClinVar (Landrum et al. 2018) and CBioPortal (Gao et al. 2013). During the analysis phase an in-house list of commonly found benign variants was also created.

2.3.3

2.3.3 Droplet digital PCR (ddPCR) for detection of single nucleotide variants (SNVs) and MYC and FGFR1 copy number variations (CNVs

2.3.3.1 Background

While NGS allows efficient screening of samples for genetic variants in a wide variety of genes it is limited by sensitivity and cost. Using NGS to detect CNVs can also be challenging. Bioinformatic methods of detecting CNVs from NGS data may employ one of the following techniques; analyzing the number of reads falling in a specific genomic region, analyzing the number of reads a specific SNP site, analyzing the amount of genetic material between paired ends or looking more broadly at changes in read numbers compared to the reference genome (Liu et al. 2013). Generally, the greater the volume of data the more accurately CNVs can be called meaning that WGS or WES are preferred for accurate analysis of CNVs.

GC content can influence CNV calling as regions that are rich in GC can be more challenging to sequence and may cause artificial appearances of gain or loss within a region. This is thought to be related to the PCR amplification step, in poorer quality FFPE samples e.g. those from historical FFPE tissue samples this can be significant (Benjamini and Speed 2012). In addition, some areas of the genome are inherently more difficult to sequence than others and this can create artificially low data volumes in these regions, these DNA in these already difficult to sequence regions will be exacerbated in low quality samples such as those derived from historical FFPE samples.

There are multiple different bioinformatics approaches and techniques but many still suffer from low sensitivity (Zare et al. 2017).

Subsequent to the selection of a targeted NGS panel for this project commercially available targeted panels have incorporated CNV analysis into their bioinformatics pipelines. In this thesis the decision was made that CNVs would be investigated using ddPCR instead of using a larger NGS panel that

may have allowed the potential for CNV analysis but its size, complexity and cost would have significantly limited the number of samples that could be run.

The ddPCR technology used in this project is the Biorad QX 200 system in which DNA extracted from plasma or FFPE samples is added to a mixture of primers and probes targeting the variant of interest. The DNA, primers and probes are then encapsulated in oil-based droplets and undergo a PCR reaction. Each droplet is then read using fluorescence and droplets containing the variant of interest fluoresce more intensely than those that do not.

DdPCR can be used to detect both single nucleotide variants (SNVs) and copy number variations (CNVs), it can also be used to directly quantify DNA concentration or copies per ml amongst other uses. In breast cancer it has been used to detect ESR1 variants in patients with advanced lobular breast cancer (Desmedt et al. 2019), to screen large trial populations for ESR1 variants (Fribbens et al. 2016), to determine copy number variations in ERBB2 (Gevensleben et al. 2013) and to track variants over time attempting to predict relapse (Garcia-Murillas et al. 2015) amongst other uses. The techniques used to assess CNVs in ERBB2 can also be applied to other genes of interest in ER+ ABC (Garcia-Murillas and Turner 2018).

It is possible to multiplex small numbers of variants into the same reaction by designing primers and probes for different genetic changes and then combining them. The challenge with this method is that it becomes difficult to identify which variant is present out of those contained in the multiplex reaction. Where only two hotspots have been examined it is possible to mix the probes into the final reaction at different concentrations however in multiplexes where more than two variants are under scrutiny it is not possible to differentiate therefore one can only state that a variant in gene X is present or absent.

2.3.3.2 ddPCR materials used

Table 8 details the consumables, hardware and software required for the ddPCR SNV and CNV experiments.

DNA extraction		
QIAamp circulating nucleic acids kit	Qiagen	Cat ID 55114
Assays		
ESR1 MPX1	Bio Rad	Assay ID: dHsaMDXE91450042
ESR1 MPX2	Bio Rad	Assay ID; dHsaMDXE65719815
MYC	Bio Rad	Assay ID: dHsaCP2500322
FGFR1	Bio Rad	Assay ID: dHsaCP2500319
AGO1 (also known as EIF2C1)	Bio Rad	Assay ID: dHsaCP2500349
Reagents		
ddPCR™ Supermix for probes (no DUTP)	Bio Rad	Cat #:1863024
Droplet generation oil for probes	Bio Rad	Cat #:1863005
Hardware and Software		
QX200™ Droplet Digital™ PCR system (droplet generator and droplet reader)	Bio Rad	Cat #:1864001
PX1 PCR Plate Sealer	Bio Rad	Cat #:1814000
QuantaSoft™ Analysis Pro	Bio Rad	Version 1.0.596

Table 8 Materials required for ddPCR experiments

2.3.3.3 ddPCR methods

For all ddPCR reactions whether for SNV or CNV detection a mixture was made in each well containing 12.5µL of Supermix for probes, 1.25 µL FAM labelled probes, 1.25 µL HEX labelled probes and then up to 10µL of DNA. The aim was for each reaction to contain 10ng of DNA. If samples had a concentration >1ng/µL then the appropriate volume was added, and the total volume made to 10µL with nuclease free water.

22µL of the mixture was transferred into a droplet generator cartridge and 70µL of oil added. Droplets were generated using the QX200 droplet generator and 45µL of the droplet mixture was transferred to a 96 well plate for the PCR reaction. The outline PCR reaction is detailed in Table 9, adjustments were made to the temperature (optimised for annealing of probes to DNA target) and the number of cycles depending on the assay. To attempt to minimise the presence of 'rain' in the FFPE assays the number of PCR cycles was increased to 50 (Lee et al. 2019).

Step	Temperature	Time	Notes
1	95°C	10 minutes	
2	95°C	15 seconds	40 cycles for SNV, 50 cycles for CNV. 53.8°C for ESR1 multiplex, 55°C for SNV and 60°C for CNV
	XX°C	60 seconds	
3	98°C	10 minutes	
4	10°C	Hold	

Table 9 Thermal cycler conditions for ddPCR (Biorad QX100 thermocycler)

After the PCR reaction droplets were read using the QX200 Droplet Reader and Quantasoft Software. Final analysis was performed with the aid of the Quantasoft Analysis Pro software package (version 1.0.596).

Variants that were detected in the first cohort of samples sequenced with NGS underwent verification with ddPCR where probes were available to assess concordance between NGS and ddPCR as methods of detecting variants. Where additional samples that had not been sequenced using NGS were available then variants that had been found in the patients corresponding tissue of cfDNA samples that had been sequenced using NGS were tracked over the treatment course. The probes, along with the annealing temperatures used are shown in Table 10.

Probes	**Annealing temperature (°C)**	**Supplier and catalogue number**
ERRB2 L755S	55	Biorad (assay ID: dHsaMDS2515134)
PIK3CA multiplex 1 E545K, E542K	57	Sigma (designed in house by NHS staff)
PIK3CA multiplex 2 H1047R, H1047L	57	Sigma (designed in house by NHS staff)
AKT1 E17K	55	Biorad (assay ID: dHsaMDV2010031)
ESR1 D538G	55	Biorad (assay ID: dHsaMDS460485301)
ESR1 Y537N	55	Biorad (assay ID: dHsaMDS296069817)
TP53 R273H	55	Biorad (assay ID: dHsaMDV2010109)

Table 10 List of ddPCR probes used for variant detection and their optimum annealing temperature

Again, a minimum of 10,000 droplets with at least 300 containing target DNA was required prior to final analysis. For SNV assays positive controls identified from samples sequenced using NGS were used in each experiment. A no template control was included in each experiment to ensure there had been no sample contamination.

2.3.3.3.1 Identification of positive and negative controls for CNV assays

A positive control for *MYC* amplification was identified from in house databases. DNA was extracted from an FFPE sample which had tested positive for *MYC* amplification by FISH. No commercially available *FGFR1* controls were available and thus samples were tested blind. Once a strongly positive sample had been identified this was then used as a control for further assay batches. For *FGFR1* four FFPE samples were tested, one contained an amplification with 8 copies (ratio 3.56), this was confirmed on a repeat run of the four samples and was selected as a control for the cfDNA sample batches. The number of copies is calculated by $copies = \left(\frac{A}{B}\right) * 2$, where A=concentration of target gene, B = concentration of reference gene and 2 is the expected number of copies in the genome.

This data can also be presented as a simple ratio where $ratio = \frac{A}{B}$ with A and B being the same as described for the copies equation. In the subsequent experiments the ratio is used as the copies of the genome remains constant.

2.3.3.3.2 Sample QC: SNV detection

Samples were run in batches in 96 well plates, a maximum of 6 columns were run to reduce the risk of sample contamination. All runs included at least one positive and negative control along with a well containing only water to check for any DNA contamination. Data was analysed using Quantasoft Analysis Pro. Quadrants were set to identify the four main clusters from the positive control. Each sample was then manually inspected using the 2D Amplitude window to check that droplets had been assigned to the correct cluster. A CSV file was exported, and the following QC checks were performed for each sample:

- Total number of droplets generated >10,000
- Total number of informative droplets (those containing either mutant or WT DNA) > 300
- Sample deemed positive if ≥5 positive droplets present (equivalent to VAF of ~0.7% with a DNA input of 116 copies/µL or ~0.4ng/µL)
- No template control ('sample' was 10µL of nuclease free distilled H_2O) did not have any droplets containing DNA

Where possible positive results were confirmed using either the same sample or an alternative sample from the same patient but at a different time point if the original sample had run out. A selection of negative and borderline samples was also run in duplicate to try and assess for the occurrence of false negatives.

2.3.3.3.3 Sample QC: CNV detection

A minimum of 10,000 droplets was required to pass a sample. Samples where the number of droplets fell between 5000 and 10,000 were considered on an individual basis as if the DNA input

was satisfactory (>10ng) and sufficient amplification of the region of interest had occurred (defined as a minimum of 400 droplets containing the amplification of interest and 400 droplets containing the control gene sequence). (Davis Bell et al. 2018).

Once methodological QC had taken place samples were manually inspected using the 2D amplitude graph in Quantasoft Analysis Pro to ascertain that droplets had been assigned to the correct cluster. Where 'rain' was present then droplets not included in a definitive cluster were re-assigned. Assignment of positive droplets by either manual droplet identification or application of the thresholds applied to the control sample to other samples in the batch were compared; the results will be presented in chapter 5.

2.3.4 Statistical analysis plan

The purpose of the clinical analysis in this study is to identify potential biomarkers of interest to be studied further in future clinical trials in the setting of ER+ aromatase inhibitor resistant breast cancer. With the small numbers of samples and patients involved any significant results will be, at best, hypothesis generating. This statistical analysis plan was written prior to any knowledge of clinical outcomes and was a pre-requisite to accessing the clinical outcome data from the trial sponsor. This plan is used to pair the experimental datasets with the clinical outcome data in chapter 6. The final statistical analysis of RET expression as a predictive and prognostic biomarker will be performed in SPSS (IBM, New York, USA) and R Studio using the statistical package 'Survminer' (Kassambara 2019) by ZH.

2.3.5 Hypotheses relating to RET protein expression as measured by immunohistochemistry

Hypothesis 1: High expression of t-RET or p-RET will predict shorter progression free survival (PFS)

- PFS will be calculated from time of trial entry to disease progression or censoring
- t-RET and p-RET scores will be deemed RET high or RET low after exploration of four different cut points (see chapter 3) and after using clinical outcome data (chapter 6)
- Kaplan Meier plots will be produced to illustrate this, and the log rank test will be applied to assess for any statistically significant difference between the two groups
- Cox regression modelling will be used to calculate the hazard ratio between the two groups (RET high and RET low)

Hypothesis 2: Response to vandetanib will be enhanced in patients with high t-RET and/or p-RET expression

- Subgroup analysis will explore the relationship between RET status and treatment with fulvestrant +/- vandetanib

2.3.6 Hypotheses relating to the detection of genetic variants by next generation sequencing

Only samples from patients who had received treatment with fulvestrant and vandetanib were used for the next generation sequencing part of the project. This means there is no placebo group for comparison. All analysis in this section was carried out by ZH.

Hypothesis 3: More variants detected in tumours either in FFPE or ctDNA will result in shorter durations of response

In this section patients who have had early progression (<62 days PFS) and patients whose PFS exceeded that expected by a clinically meaningful number of months (PFS >312 days) will be examined. The frequency of tier II variants as determined by NGS will be compared amongst the two groups. All patients who had at least one sample analysed by NGS (either FFPE or cfDNA) will be included. This initial analysis will be descriptive only due to the small number of patients with variants.

2.3.7 Hypotheses relating to the detection of genetic variants by ddPCR

Data will be produced regarding the prevalence of SNVs in ESR1 along with CNV data for MYC and FGFR1 from plasma samples representative of metastatic disease. Within this set of samples there will be patients from both arms of the trial. This will enable exploration of whether response to vandetanib is dependent on the presence or absence of ESR1 variants and/or amplification of MYC or FGFR1.

Hypothesis 4: Patients with variants in ESR1 will have longer PFS than those without due to a superior response to fulvestrant as per the analysis in the SoFEA trial. The presence of a variant will have no bearing on response to vandetanib.

Hypothesis 5: Patients with copy number amplification in MYC will have a shorter PFS than those that do not, the presence of amplification will have no bearing on response to vandetanib.

Hypothesis 6: Patients with an FGFR1 amplification are likely to have a shorter PFS than those that do not (Drago et al. 2019)

- PFS will be calculated from time of trial entry to disease progression or censoring
- Methodology to determine the presence or absence of a variant or amplification will be detailed in chapter 5 (in addition to that described briefly in section 0).
- Kaplan Meier plots will be produced to illustrate the relationship between the presence of amplification and PFS, and the log rank test will be applied to assess for any statistically significant difference between the two groups
- Cox regression modelling will be used to calculate the hazard ratio between the two groups (variant/amplification present vs variant/amplification absent)

2.4 Method development

2.4.1 IHC: Development of t-RET assay

An optimised t-RET immunohistochemical assay that had been successfully used on FFPE lung cancer samples was available as a starting point in this project for assay development (as provided by AstraZeneca, P.Elvin personal communication, and subsequently published (Platt et al. 2015)). Review of the literature revealed that the majority of the limited t-RET assays reported in breast cancer had involved an assay step involving heat mediated antigen retrieval of FFPE sections to maximise antigenicity and the use of RET primary antibody concentrations ranging from 1/100 to 1/25 (Plaza-Menacho et al. 2010; Nguyen et al. 2015) alongside a sensitive immunoperoxidase-based secondary detection system.

2.4.1.1 *t-RET Assay optimisation*

After laboratory training with Dr Gee's team, the Platt assay for t-RET was evaluated by ZH using heat mediated antigen retrieval of FFPE breast cancer sections in a microwave pressure cooker using a pH9 EDTA buffer. This was followed by a step to block any endogenous peroxidases. Next the primary antibody was applied. The original antibody Epitomics 3454-1, used in the Platt assay was no longer available and was replaced with Abcam Ab134100 an IgG monoclonal anti rabbit antibody, initially at 1/1000 concentration for 2 hours and the use of a Dako DAB chromogen/substrate detection and counterstaining. All antibody incubations were performed at 23°C incubation chambers. A small panel of in house FFPE primary clinical breast cancer samples known to be positive for activity of p-RET (Gee et al. 2014) (and thus RET expressing) were used for further assay optimisation (Nottingham REC2: C202031).

Table 11 shows the various comparison assays run to determine the best overall protocol for breast cancer samples. No staining was seen with low antibody concentrations such as 1:1000 dilution;

detectable staining began to appear in the tumour epithelial cells with a primary antibody dilution of 1:250 but better clarity of staining was achieved with a 1:100 dilution. If the primary antibody was more concentrated than this e.g. 1:50 some of the stronger staining samples became oversaturated thus masking any heterogeneity or any negative cells. There was also some non-specific background staining throughout the section that in total made accurate tumour cell assessment for t-RET difficult.

Clarity of the staining was then further optimised using a serum free protein block and a hydrogen peroxide block to eliminate non-specific background and any endogenous peroxidase derived staining in FFPE breast cancer material respectively. Further assays were run to compare methodologies and also buffers of different pHs, with or without the detergent Tween 20 for the heat mediated antigen retrieval step in order to maximise RET tumour epithelial staining signal. A further assay compared two different antibody diluents with TBST 0.05% favoured as the carrier.

Protocol step	Variables tested
Antigen retrieval	Heat mediated using microwave, microwave pressure cooker, non-microwave pressure cooker and no retrieval
Antigen retrieval buffer	pH6 (sodium citrate buffer), pH9 (EDTA buffer) and pH6 with and without Tween-20
Hydrogen peroxide block	0.18% for 20minutes or 3% for 5 minutes
Dako protein block	Presence or absence of this blocking step (20-minute application)
t-RET primary antibody concentration	1:1000, 1:250, 1:100, 1:50 all made in TBST 0.05%
t-RET primary antibody incubation time	1 hour, 2 hours, 3.5 hours, overnight at 23°C
Primary antibody diluent	TBST 0.05% or Dako S0809 ready to use antibody diluent. This comparison was performed once optimum antibody dilution had been determined at 1:100.

Table 11 Variables tested for each protocol step of T-RET assay development

These optimisation steps showed superiority for (i) antigen retrieval using a pH 9 buffer, (ii) use of an 0.18% H2O2 endogenous peroxidase block and a protein block step and (iii) t-RET antibody at 1:100 in TBST 0.05% with overnight incubation. This gave a clear assessable heterogeneous cytoplasmic/plasma membrane signal in the tumour epithelial cells with little background.

t-RET Assay Validation

The optimised assay was then tested on a series of 10 FFPE breast cancer tumour samples from the Nottingham University cohort (Nottingham REC2: C202031). The series contained both ER+ and ER- tumours. The expectation was that a range of t-RET staining would be seen within the samples from negative through weak to strongly positive. The assay included samples that had been identified by previous work in the Gee lab to be positive for p-RET (Gee et al. 2014). (panels B and C) shows two

examples of positive immunostaining achieved with the final, optimised assay (see), found in the cytoplasm of tumour epithelial cells comprising positive breast cancers.

Further evaluation of the assay was undertaken in the form of an IgG isotype antibody control diluted to the same concentration as the primary antibody (Abcam Ab172730, rabbit monoclonal, concentration 1.670mg/mL). As illustrated in Panel B the application of an IgG isotype antibody solution in place of the tested primary antibody does not show any staining inferring specificity of the tested primary antibody for t-RET. The assay was also tested on FFPE sections of cell line pellets from HeLa (known to be t-RET low/negative (Platt et al. 2015)) and T47D (known to be RET positive (Gattelli et al. 2013)) cells (Panel A), with weak staining in the former and moderate-strong staining achieved in the latter cell line in accordance with the literature. Furthermore, a no primary negative control was performed (Panel C) which showed no staining indicating that there was no background staining as a result of the secondary detection system.

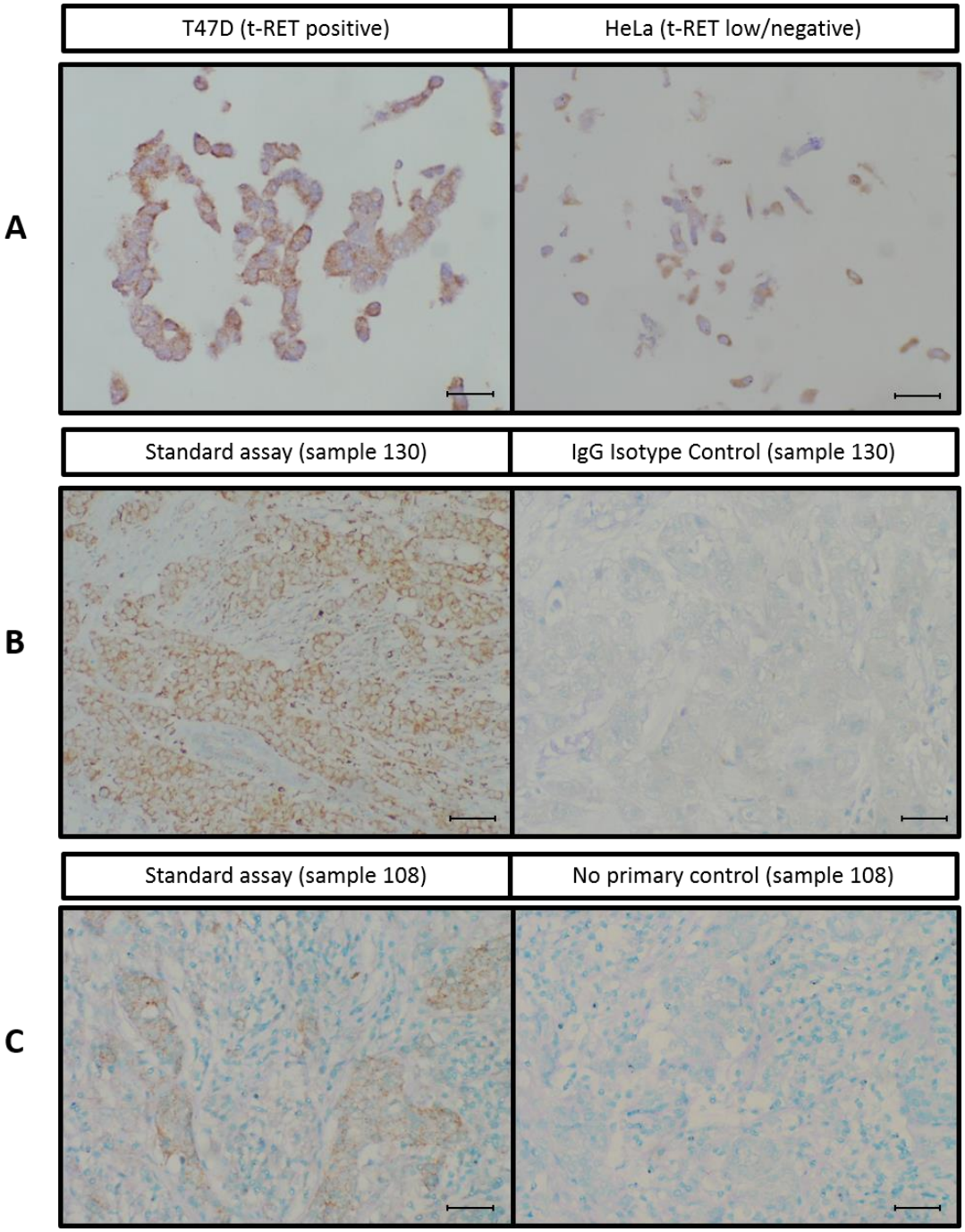


Figure 11 Use of controls to validate the t-RET IHC assay. Panel A shows two FFPE cell lines stained for t-RET; one staining strongly (T47D), the other with much weaker staining (HeLa). Panel B shows the lack of signal with IgG control antibody applied. Panel C shows that no staining is present if the primary antibody is omitted, compared to with positive staining for t-RET in breast cancer samples 108 when the standard assay is applied. All images were taken at x20 magnification. Scale bar represents 50µm.

In addition to the above work the optimised T-RET assay was also evaluated on a fuller historical series (n=93) of breast cancer sections, in this instance that had been pre-cut >10 years ago from the

primary breast cancer samples from Nottingham University as part of an MPharm student research project (co-supervised by ZH and JG[8]) (Hudson et al. 2019). The technical aspects of the project were performed by the student under supervision. Due to the considerable age of these long term stored sections the primary antibody was used at a slightly higher concentration to try and mitigate any potential compromised antigenicity in the sections (1:70 dilution; although this remained comparable to the optimal concentration range for different batches of t-RET antibodies). The EnVision secondary antibody application increased to 2hrs and DAB and methyl green application times were extended to 20 minutes. However, the key components and all other conditions of the assay remained the same.

As part of the MPharm project some repeat validation work was carried out confirming that the t-RET assay performed best with antigen retrieval using a microwave and ph9 buffer when compared to using a microwave pressure cooker or changing the pH of the buffer to pH6. Repeat validation also compared the use of TBST and S0809 as a diluent for the primary antibody, again showing that TBST produced a clearer overall signal for interpretation (Marks et al. 2018). Importantly, the t-RET assay was capable of generating biologically meaningful data even in sections that had been cut and stored for many years, showing positive correlations with one of its co-receptors (GFRα1), with increased tumour size and higher grade in its historical series (Marks et al. 2018; Hudson et al. 2019) adding further support for its application as a sensitive, potentially informative assay to assess FURVA trial samples in this thesis.

2.4.1.2 *Assay re-optimisation*

Due to the collection timeframe of specimens during the FURVA clinical trial over a year passed between receiving the first collection (115 samples) and second collection (18 samples) from the trial. During this time the original batch of Abcam Ab134100 t-RET primary antibody became no longer available. On receipt of the new t-RET antibody batch the staining pattern of the selected

[8] JG = Dr Julia Gee

positive control breast cancer slide was too weak when the new primary antibody was used at a dilution of 1:100. Thus, the primary antibody concentration was re-optimized on Nottingham positive primary breast cancer material and after testing a 1:50 and 1:75 dilution range it was determined that a dilution of approximately 1:60 for the new antibody batch resulted in a staining pattern of the positive control material comparable with that seen for the original batch of antibody at a concentration of 1:100. In this way, optimal assays were always used in FURVA samples to ensure consistent IHC results during the trial despite antibody batch changes.

2.4.2 IHC: Development of the p-RET assay

A p-RET IHC assay protocol had previously been developed and validated in-house which had shown a relationship between increased p-RET, shortened disease free interval and poorer survival on tamoxifen in an ER+ breast cancer series presented at an AACR conference (Gee et al. 2014). However, the antibody that was used in this study was no longer commercially produced for use in this thesis. An alternative p-RET antibody (anti-human, polyclonal rabbit) was thus sourced which bound to the same phosphorylation site (Ab55103 Anti RET (phospho Y1062)). There is no published literature on the use of p-RET assays in clinical breast cancer thus the final assay (see Table 5) optimised in this thesis remains entirely experimental.

The previous in-house p-RET assay had been optimised to be sensitive in FFPE material (by including heat-mediated antigen retrieval and using a 2 hour EnVision secondary detection incubation time) with low background staining (by including blocking steps) and had been used successfully on breast cancer sections, so little was changed from the original p-RET IHC protocol apart from the primary antibody. Due to the new antibody producing no detectable staining at the same dilution as the original assay (1:700) the concentration was reduced to 1:50. The duration of the primary and secondary incubation also had to be increased to ensure sensitivity. The antigen retrieval method and peroxidase/protein blocking steps remained identical.

P-RET validation

Due to the polyclonal nature of the primary antibody used no IgG isotype control was available, but an omission of primary antibody negative control was evaluated and the assay was also run on T47D (RET+) and HeLa (RET +/-) FFPE pelleted cells as per t-RET validation (section 2.1.1.1) although with no prior literature based hypothesis as to whether either would be positive for RET signalling activation. The finalised p-RET assay (Table 5) was used on the same 10 breast cancer samples as the t-RET assay had been validated with. Again, the expectation was that a variety of positive tumour epithelial staining would be seen, as had been observed with the original p-RET antibody as used by Gee et al, although at lower levels than for total RET as it is likely that not all RET in samples is activated

However, challenges were encountered when the final p-RET assay with the new primary antibody was used on patient samples with significant staining variability between assays where positive signals were not always reproducible. Detection of phosphor-specific epitopes can be problematic in such material, due to antigenicity being compromised by fixation and further sample processing. At this point therefore, the assay was further optimised by changing the diluent for the primary antibody from PBS to TBS to try and increase the robustness of positive signals, and this resulted in an improved p-RET assay with less inter-run variability. All FURVA samples that had been assayed with PBS as the primary antibody diluent were thus repeated with TBS diluent and this was retained routinely we subsequently assaying the remainder of the samples.

2.4.3 IHC: Evaluation of FURVA series samples and quality control for the t-RET and p-RET assays

Assays for the FURVA series of FFPE breast cancer samples were run manually using the optimised t-RET and p-RET IHC procedures by ZH alone in order to avoid inter-operator variability. Batches of between 4 and 14 samples cut on the same date were run alongside the same positive control taken from the in-house tissue breast cancer panel of Nottingham FFPE samples.

What could not be fully controlled for was how samples had been originally fixed at the point of surgery or biopsy at the source treating hospital. While all samples were FFPE, samples in the series were diverse as many were diagnostic and thus often several years old. Historical changes in fixation protocols may have occurred at the different hospitals. Furthermore, there are many steps during the fixation process that can cause variation in the final fixation and hence assay performance such as fixation delay, conditions of dehydration and fixative type (Engel and Moore 2011). In this project these variables had to be accepted as part of the sample collection process.

To allow correlation between t-RET expression by IHC and clinical outcome a cut point for 't-RET high' and 't-RET low' needed to be determined. In the literature two methods of determining a 't-RET high' result in breast cancer samples were identified. Gattelli et al (2013) evaluated 200 tumour cells per core biopsy in a tissue microarray (TMA) of 108 breast cancer patients with each patient represented by three cores and assigned a percentage of cells to each of three intensity scores; 0 no staining, 1, weak or moderate staining and 2, strong staining. The percentage was multiplied by the score for each intensity category and an overall score calculated. Only 89 of the samples were evaluable in the publication after the assay had been performed. It was then stated that a score of >60 was considered 'high RET expression' and a score ≤ 60 'low RET expression' (Gattelli et al. 2013).

A simplified method was used by Nguyen et al (2015) where a TMA of over 4000 fresh frozen breast cancer samples was tested. For the 2800 cores that remained evaluable after the assay an overall score was assigned to the sample of either 0, negative, 1, weak staining and 2, strong staining. With a score of 1 or 2 counting as positive (Nguyen et al. 2015).

The proportion of patient samples deemed 'RET high' varied between the two papers; in Gattelli et al 66/89 (74%) samples showed 'High RET', while in Nguyen et al 1596/2800 (57%) of patients were 'positive'. These variations could be due, for example, to assay performance (and thus signal intensity) to sample size or fixation method impacting on antigenicity. Nguyen et al used a polyclonal goat anti human antibody, while Gattelli et al used a polyclonal rabbit anti human. It is not stated by

Gattelli et al if their TMA was made with FFPE samples or with fresh frozen samples. Both TMAs featured all histological subtypes of breast cancer.

Other assays have shown significantly lower levels of RET expression. For example, Plaza-Menacho et al evaluated two TMAs of invasive breast cancer (all subtypes); the first showed RET expression in 41/161 (26%) of cases while the second, featuring primary breast cancer cases that had developed recurrent disease after tamoxifen treatment showed RET expression in 37/66 (56%) of cases. No detail is given in the paper or supplementary methods about how RET expression was determined although an H-score is mentioned (Plaza-Menacho et al. 2010).

Taking on board the range of positivity data from these studies and allowing for significant variability in the samples used and numbers of patients, it is estimated that >50% of patient samples will show some degree of RET expression. However, there is no consensus with regards to the best scoring method and where the cut-off should be drawn to identify 'positive' samples as each assay detailed here used different antibodies, sample types and scoring methods.

Thus, both methods detailed will be applied to the FURVA dataset. In addition, three cut points based on the methodology used to score HER2 positivity will be explored (Table 12). When scoring HER2 status in breast cancer samples original recommendations were that samples containing >30% cells showing intense membrane staining were considered positive. However, updated guidance in 2013 suggested that samples where >10% of cells showed intense membrane staining should be considered positive (Nitta et al. 2016). When scoring the FURVA dataset both cut-points were explored (Method A and B, Table 12)and a combination cut point was included in addition (Method C, Table 12) where allowances were made for less intense staining to be counted as positive but only if >30% of cells showed moderate staining intensity.

Method A: >10% of cells showing 3+ staining	Method B: >30% of cells showing 3+ staining	Method C: >10% of cells showing 3+ staining or >30% of cells showing 2+ staining

Table 12 Methods for calling a 'positive' or 'high' scoring sample. Based on American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines.

Furthermore, survival data will be used to statistically determined a cut point using the maxstat method (Hothorn and Zeileis 2008). In brief the maxstat method uses maximally selected rank statistics. This means that in this example the RET score is used as a continuous variable. RET scores are paired with clinical outcome data, in this case progression free survival and the cut point which demonstrates the strongest ability to demonstrate a difference in survival between two groups is selected.

In the original p-RET assay from Gee et al a cut off H-score of ≥25 was deemed 'positive'. However, only staining that was evident in the plasma membrane was counted. The score of 25 was determined from the median H-score for the dataset and a score ≥25 correlated with disease free interval and overall survival (Gee et al. 2014). In this assay the staining was predominately cytoplasmic; likely due to the different antibody that was used. Therefore, a cut off value of 25 is unlikely to be of use. Instead, pre-specified cut offs will be applied as per t-RET; firstly, methods related to the scoring of HER2 positivity and secondly using a retrospective cut point analysis; maxstat (Hothorn and Zeileis 2008).

The results of the IHC work including reproducibility between batches and applying different scoring cut-offs to determine 'positive' and 'negative' samples will be presented in Chapter 3.

2.4.4 NGS: Identification of genes of interest in ER+ primary breast cancer samples

Multiple methods of identifying frequent genetic variants were used to generate a list of genes of interest, this list was then compared with commercially available panels. The decision regarding the choice of sequencing technology needed to be made early in the project. The final decision was

made in June 2017 based on the best available data at the time. In general, the panels available at the time did not allow for the evaluation of copy number variations from single DNA samples as there were too few reference genes included in the panel to allow for bioinformatic identification of amplified genes. Therefore, at this stage of the project gene amplification by copy number gain was not included in the decision-making regarding panel selection.

Genes related to the development of endocrine resistance have been discussed in Chapter 1 and variants in these genes are key potential biomarkers to predict response to fulvestrant and vandetanib e.g. *PIK3CA, AKT1, ESR1, TP53, PTEN, CDKN2A, MYC* and those genes in the MAPK pathway such as the *EGFR* family. These genes frequently harbour pathogenic genetic variants in large datasets such as the METABRIC dataset (targeted sequencing of 173 genes in 1398 primary ER+ breast tumours) (Pereira et al. 2016) and TCGA (WES from 348 primary ER+ breast tumours) (TCGA 2012). See for more detail.

Interestingly these datasets also highlighted frequent variants in genes that were not immediately associated with endocrine resistance such as *GATA3, CDH1* and *MAP3K1*. Further genes that occurred at a frequency of >5% included *CBFB, KMT2C, RUNX1* and *TBX3*.

2.4.5 NGS: Metastatic datasets in CBioPortal

At the time of panel selection there was much less information available on the changes in variants found in patients specifically with endocrine resistant breast cancer as many datasets were a mixture of breast cancer subtypes. The closest dataset available was that from Lefebvre et al, this included the WES data from over 200 metastatic breast cancer patients. However, the majority had heavily pre-treated disease thus potentially not making them a reasonable comparator group to the patients from the FURVA trial who had received a maximum of one prior line of chemotherapy for advanced disease. In the paper by Lefebvre et al comparison was made with the mutational frequencies from the TCGA dataset to try and ascertain the changes in genomic landscape from primary to metastatic tumour. The authors identified 8 genes that contained genetic variants more

frequently in metastatic samples than in those from primary tumour; *ESR1, FSIP2, FRAS1, OSBPL3, EDC4, PALB2, IGFN1* and *AGRN*. Of these *ESR1* was the only variant to act as a potential driver mutation. The authors also noted an increase in the frequency of *TP53* variants in the metastatic setting (Lefebvre et al. 2016). From this it would seem prudent to include analysis of *ESR1* variants in any metastatic samples.

In addition to searching large datasets a second literature review focussed on smaller but more specific studies in the area of endocrine resistant breast cancer (Jansen et al. 2016; Giltnane et al. 2017). No additional genes of interest were identified.

2.4.6 NGS: Final list of genes and regions of interest in ER+ endocrine resistant breast cancer

From literature review of mechanisms of endocrine resistance and exploration of large published datasets a list of genes of interest was created. In addition, although variants in RET occurred infrequently in breast cancer it was felt important to be able to sequence the hotspot regions where variants had been known to occur in case treatment with vandetanib precipitated genetic changes in RET as a possible mechanism of resistance. The genes of interest are summarised in Table 13.

Gene	Size in amino acids	Hotspot variants in breast cancer?	Summary of role of protein encoded	Predicted variant frequency in breast cancer	References
Key genes of interest in endocrine resistant ER+ breast cancer					
PIK3CA	1069	Yes, E545X, E543X and H1047X	Codes for PI3K, key enzyme in PI3K/AKT signalling pathway	44%	See Chapter 1 Introduction section 1.2
TP53	394	No, but R175H, R248X and R273X most common.	Encodes p53; key regulator of DNA damage repair	19%	
PTEN	404	No	Key regulator of AKT	6%	
AKT1	480	Yes, E17K	Encodes key protein in PI3K/AKT pathway	5%	
ERBB2	1256	No, L755S most frequent	Increased signalling via HER2 receptor possible mechanism of endocrine resistance.	2% in primary tumour	
RB1	929	No	Encodes key protein in cell cycle regulation.	5% in metastatic samples (heavily pre-treated)	
MAP2K4	400	No, S184L most frequent	Involved in MAPK signalling	5%	
MAP3K1	1350	No	Involved in MAPK signalling	11%	
CDH1	883	No	Encodes E-cadherin, variants may increase proliferation and metastases.	13%	(Lopez-Knowles et al. 2019)
Genes commonly containing genetic variants in ER+ breast cancer but without obvious link to endocrine resistance					
GATA3	445	No, M294K most common but still infrequent	Regulator of T cell development	16%	Variant frequencies from TCGA and METABRIC datasets in CBioPortal (Gao et al. 2013)
KMT2C	4912	No	Transcriptional co-activator	9%	
CBFB	183	No, but X55 splice most frequent	Protein involved in haematopoiesis and osteopoesis	5%	
RUNX1	481	No	Transcription factor, contributes to haematopoiesis	5%	
TBX3	744	No	Transcriptional repressor	5%	
Genes that could be linked to response or resistance to fulvestrant and vandetanib. ESR1 and EGFR also key genes in endocrine resistance					
ESR1	596	Yes, Y537X and D538X	Encodes ER, key driver of growth and proliferation.	14% in metastatic samples (heavily pre-treated)	(Lefebvre et al. 2016)
RET	1115	No variants in METABRIC dataset	Target of vandetanib	Unknown	(Morandi et al. 2011)

VEGFR2 (KDR)	1357	No variants in METABRIC dataset	Target of vandetanib	Unknown	
VEGFR3 (FLT4)	1299	No variants in METABRIC dataset	Target of vandetanib	Unknown	
EGFR	1211	No	Target of vandetanib	~1% (Network 2012)	
Other genes of potential interest in endocrine resistance					
LYN	513	No variants in METABRIC dataset but is amplified in 12% of cases.	Potential regulator of PI3K and AKT1 activity	Unknown	(Schwarz et al. 2014)

Table 13 Genes of interest for investigation. X indicates that there are several alternative amino acids that can occur at this location all with potentially pathogenic consequences. Size in amino acids taken from COSMIC database https://cancer.sanger.ac.uk/cosmic. Where little is known about the gene and its role in endocrine resistance the brief description comes from the gene specific entry in https://www.genecards.org/

2.4.7 NGS: Selection of sequencing panel

Targeted sequencing panels that were commercially available at the time of panel selection (June 2017) and could be run using the technology available in the laboratory were identified. Gene coverage of panels was then compared to the list of genes and regions of interest (Table 13). At this point in the project it was estimated that 30 sets of patient samples would be available. A set would comprise a tissue sample and three plasma samples taken before entry into the clinical trial, after 8 weeks on trial and on stopping trial treatment (either due to toxicity, disease progression or patient choice).

In the investigation of potential predictive biomarkers for response to treatment with fulvestrant and vandetanib emphasis was placed on generating data from the baseline tissue sample and from the final plasma sample. This meant that approximately 60 samples would need to be sequenced in addition to validation work. On this basis panels and their associated sequencing costs also had to be considered when making a final decision.

Two panels from Qiagen were considered (GeneRead DNASeq Targeted Panels V2 Breast Cancer Panel and QiaSeq targeted DNA panels human breast cancer panel) alongside the Ion Ampliseq

panel from Thermofisher which could be customised. Ultimately the Qiagen QiaSeq panel did not work despite repeated attempts at optimisation including by company experts. The Qiagen GeneRead panel offered greater coverage of the genes of interest but at a higher sequencing cost. The final decision was to select a combination of the Thermofisher Ampliseq Cancer Hotspot Panel v2 (CHPv2) and an additional small customised Ampliseq panel (hereafter referred to as CUSTOM) to improve coverage of *RET* and to include hotspots in *ESR1, GATA3* and *LYN*. A fully customised Ampliseq panel was designed to cover all genes of interest but the amplicon coverage was significantly less than that achieved with the 'off the shelf' CHPv2 so this was not taken forward.

The Ampliseq technology also had the advantage of being well used in the literature offering some insight into the potential limits of detection; furthermore, despite it being designed for use with FFPE samples there were several studies where it had been successfully used with cfDNA. Additionally, there was local expertise and easily accessible equipment to support the use of Ion Ampliseq (Thermofisher) panels using the Ion Chef (Thermofisher) for template preparation and the Ion Proton (Thermofisher) for sequencing. Furthermore, a fully customisable bioinformatic pipeline was available through Ion Reporter meaning that in house bioinformatic pipeline development was not required.

2.4.8 NGS: Use of Ampliseq Cancer Hotspot Panel V2 (CHPv2) in the literature and validation of CHPv2 and CUSTOM Ampliseq NGS panels

Table 14 summarises references to the use of Ampliseq technology with FFPE and cfDNA samples in the literature.

Title	Reference	DNA source	Input DNA	Mean depth of coverage	Additional information
Serial next-generation sequencing of circulating cell-free DNA evaluating tumour clone response to molecularly targeted drug administration	(Frenel et al. 2015)	cfDNA from early phase trial patients	3ng	Aim: 500 Actual: 1685	Ion Ampliseq V2 Hotspot panel on Ion PGM sequencer. 10pM loading conc. LOD of 5% for de novo variants and 1% for previously identified variants. No description of how values were selected.
Mutation Analysis of Cell-Free DNA and Single Circulating Tumor Cells in Metastatic Breast Cancer Patients with High Circulating Tumor Cell Counts	(Shaw et al. 2017)	cfDNA from patients with ABC	Average 8ng (extracted from 3mls)	4537	In house designed 30 amplicon panel using Ampliseq Designer. Sequenced on Ion PGM. Downsampled to 6000 and omitted all calls with Phred quality score <25.
Cell-free DNA mutations as biomarkers in breast	(Jansen et al. 2016)	cfDNA from patients with ABC	165-573pg	Aim: 5000	Custom panel (3000 amplicons over 45 genes)

cancer patients receiving tamoxifen.		progressing on tamoxifen			on Ion PGM. Claimed 1% LOD for de novo variants.
Plasma circulating tumor DNA as an alternative to metastatic biopsies for mutational analysis in breast cancer	(Rothé et al. 2014)	cfDNA/FFPE from patients with ABC	150ng	Aim: 25000 Achieved:18000 1000 for FFPE	Ion Torrent Ampliseq hotspot. Detected down to frequency of 0.5% (not de novo)
Circulating tumor DNA as a non-invasive substitute to metastasis biopsy for tumor genotyping and personalized medicine in a prospective trial across all tumor types	(Lebofsky et al. 2015)	cfDNA from patients with metastatic tumours (all tumour types)	5ng	Aim for 95% covered by >100 reads.	Ion Ampliseq V1 and V2. Called variants at ≥1% VAF.
Targeted next-generation sequencing detects a high frequency of potentially actionable mutations in	(Muller et al. 2016)	FFPE from patients with ABC	10ng	2800	Cancer Hotspot V2 Panel. No detail on LOD or bioinformatics.

metastatic breast cancers					
Comparative genomic analysis of primary tumors and metastases in breast cancer	(Bertucci et al. 2016)	Fresh frozen tissue. 365 gene panel.	Not stated	300	min tumour content 50%
PIK3CA and TP53 gene mutations in human breast cancer tumors frequently detected by ion torrent DNA sequencing	(Bai et al. 2014)	FFPE from primary breast cancer	50ng	Average 1639 (28-4732)	Ion Ampliseq cancer Hotspot panel V1 sequenced on Ion PGM
Rapid detection of genetic mutations in individual breast cancer patients by next-generation DNA sequencing	(Liu et al. 2015)	FFPE from primary breast cancer	50ng	Mean 1639 (22-6020)	Ion Ampliseq cancer hotspot panel V1
Next Generation Sequencing of Circulating Cell-Free DNA for Evaluating Mutations and Gene Amplification in Metastatic Breast Cancer	(Page et al. 2017)	cfDNA from patients with ABC	3ng minimum extracted from 3mls plasma	Average coverage of 500x per amplicon	Custom 158 amplicon Ion AmpliSeq panel. Down sample to 2000, VAF of >1%.

Table 14 Summary of references where Ion Ampliseq technology has been used to analyse genetic variants in cfDNA and FFPE breast cancer samples. This is not comprehensive list but has selected high quality studies that offer some realm of comparison for the FURVA samples and the approach taken in this project. VAF = Variant allele frequency

Table 14 allows exploration of the potential limits of detection of the CHPv2 panel along with aims for amplicon coverage for both FFPE and cfDNA samples. For cfDNA samples quality sequencing data could be obtained with as little as 3ng of input DNA (Frenel et al. 2015; Page et al. 2017) this allowed detection of variants at ~1% VAF based on the aim of a minimum depth of coverage of 500 per amplicon. Only one paper has detected variants below 1%, in this paper variants were called at 0.5% as long as they had already been identified in paired samples at higher frequencies. This was based on very high DNA input – up to 150ng which will not be possible with the FURVA cfDNA samples (Rothé et al. 2014).

Validation of the Ion Ampliseq panels involved a stepwise approach; initially testing the panel with reference standards designed to mimic cfDNA and then with patient samples containing known variants. This strategy enabled testing of the library preparation, sequencing and bioinformatic pipeline. Although it was not possible to use cfDNA from breast cancer patients prior to the first sequencing run using FURVA patient samples it was felt that this approach was pragmatic given restrictions imposed by the cost of each sequencing run and availability of patient samples for validation work.

2.4.8.1 *Experiment 1: Limit of detection of CHPv2 panel*

A library was prepared according to manufacturer's instructions using cfDNA from reference standard HD780 (Horizon Discovery, Cambridge, UK). 9 samples were prepared at varying DNA concentrations with varying mutational frequencies. The experiment aimed to assess the minimum DNA input requirements and the lowest variant frequency detectable. The reference standard included variants in *EGFR, KRAS, NRAS* and *PIK3CA*. Based on the limits of detection used in the

literature as detailed in Table 15 samples with variants at a variant allele frequency (VAF) of between 5% and 0.5% were tested with DNA input down to 5ng.

Sample	DNA input (ng)	Approximate VAF (%)
1	20	5
2	10	5
3	5	5
4	20	1
5	10	1
6	5	1
7	20	0.5
8	10	0.5
9	5	0.5

Table 15 Samples run in first limit of detection experiment CHPv2

All final libraries were quantified using a Qubit Flurometer (Life Technologies, Carlsbad, CA, USA) and selected samples were evaluated using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Qubit concentrations were expected to be between 300ng and 1400ng/mL; the range for the nine samples was 242-848ng. With only sample 8 and 9 falling below the 300ng value.

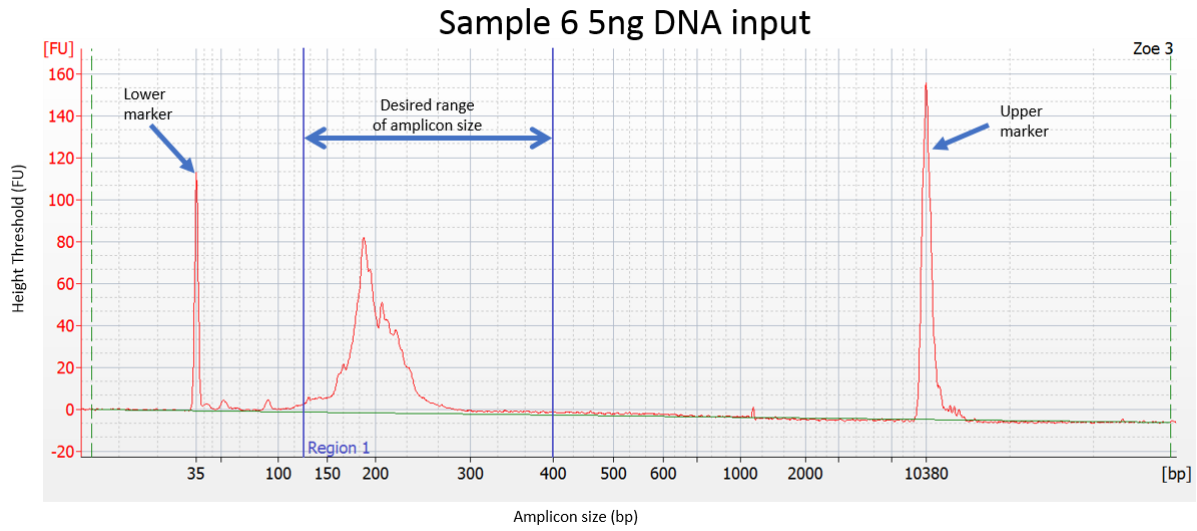


Figure 12 Annotated Bioanalyzer trace for sample 6 showing peaks in the expected range of amplicon size

3 samples were selected for analysis using a Bioanalyzer. This allows visualisation of final fragment size of the library (see example in). For all three samples tested, traces showed in the range expected in the protocol (120-400bp). The Bioanalyzer also allows calculation of the final concentration of the samples. The predicted concentrations as per the manufacturer's instructions were 200-10,000pM and the concentrations of the samples fell within this range. Samples were pooled to give equal coverage at a concentration of 1000pM, this pool was then further diluted to 100pM and finally 75pM for template preparation using Ion PI sequencing chips on the Ion Chef (Thermofisher, Waltham, MA, USA). Following template preparation chips were sequenced using the Ion Torrent Proton Sequencer (Thermofisher, Waltham, MA, USA). Torrent Suite software Version 5.8.0 (Thermofisher, Waltham, MA, USA) created FASTQ, BAM and BAI files for each barcoded sample which could then be downloaded and reviewed.

Each BAM file was manually reviewed using Integrative Genomics Viewer (IGV) to ascertain whether the variants present in the reference standard had been sequenced correctly. Subsequently, each sample was analysed using Ion Reporter (Thermofisher, Waltham, MA, USA) using a customised workflow to the appropriate expected VAF If variants were not detected by the automated bioinformatic pipeline but were present in the BAM file on IGV then the sample was re-analysed with a lower VAF cut off e.g. where the expected allele frequency was 5% all variants above 1% were reviewed. If the VAF was 1% then all calls above 0.5% were reviewed. The customised workflow allows for downsampling to a depth of 2000 reads. This means that for samples where coverage is very high downsampling can mean variants appear at lower frequencies than expected. Increasing the downsampling significantly would reduce the risk of this but at the expense of a much longer analysis time and for many samples may not increase the quality of variant calls.

			Sample 1 20ng 5%		Sample 2 10ng 5%		Sample 3 5ng 5%		Sample 4 20ng 1%		Sample 5 10ng 1%		Sample 6 5ng 1%		Sample 7 20ng 0.5%		Sample 8 10ng 0.5%		Sample 9 5ng 0.5%	
Gene	AA change	Base change	IGV	IR (1%)	IGV	IR (1%)	IGV	IR (1%)	IGV	IR (0.5 %)	IGV	IR (0.5 %)	IGV	IR (0.5 %)	IGV	IR (0.5 %)	IGV	IR (0.1 %)	IGV	IR (0.1 %)
EGFR	L858R missense	c.2573T>G	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	N*	N	N*	N
EGFR	E746 DEL	c.2235_2249del 15	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
EGFR	T790M missense	c.2369C>T	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N	Y	Y
EGFR	V769 in frame INS	c.2308_2309ins CCAGCGTGG	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y**	N	Y	N	N
KRAS	G12D missense	c.35G>A	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
NRAS	Q61K missense	c.181C>A	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
NRAS	A59T missense	c.175G>A	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N*	N
PIK3CA	E545K missense	c.1633G>A	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

*Table 16 Comparing variants identified manually or via semi-automated bioinformatic pipelines for samples of varying VAF and DNA input. BAM files from each sample were reviewed manually in Integrated Genome Viewer (IGV) to ascertain whether variant was manually detectable. BAM files were then run through customised workflows in Ion Reporter V5.10 (IR). The cut off VAF frequency was adjusted in each workflow in order to allow calls within the expected range of VAF in the sample e.g. a sample with an expected VAF of 0.5% was run on a workflow that allowed review of all variants at a VAF >0.1%. The cut off is indicated in brackets at the top of each IR column. *denotes variants that were present on manual review but with less than 10 reads for the alternate allele. **denotes a variant that required the VAF filter level to be lowered to 0.1%.*

Table 16 shows the nine samples run using the CHPv2 panel. The BAM file for each sample was reviewed manually in IGV to ascertain whether the expected variants could be detected. The BAM file was then run through a customised workflow designed using Ion Reporter V5.10 (IR). The workflow could be further customised by applying filters to adjust the cut off VAF for calls. Table 16 shows that variants present at ~5% can be detected with DNA input as low as 5ng. Variants with a VAF of ~1% can also be detected as long as the VAF cut off is adjusted to 0.5%. Two variants in sample 4 were not detected this was due the failure in the sample preparation; in both locations the Phred Quality score was <6 indicating poor sequencing. Below a VAF of 1% it is still possible to detect variants, particularly if the original DNA input was high (e.g. Sample 7) however, pragmatically this is not useful for de novo variant detection as it would require the manual review of 2646 variants in this example.

This data is limited by the lack of duplicate experiments and the lack of samples with VAF between 1% and 5% however it allows a confident limit of detection of a VAF of 5% and suggests that a workflow using a cut off of 1% allows balance between number of variants called while minimising the risk of missing variants with a VAF of between 1% and 5%. An additional factor to consider is that these samples were reference standards. Although attempts have been made to create standards representative of cfDNA e.g. short fragments of DNA it is likely that patient samples will be less pure and therefore the actual limit of detection may vary sample to sample. Practically the limit of detection for the CHPv2 panel probably lies around 2% VAF if at least 5ng of DNA is used as a starting material. Below 5ng the sensitivity of the panel to detect low level somatic variants will be reduced.

Furthermore, no samples with a total DNA input less than 5ng were tested. At this point it was not known that a significant number of the FURVA patient samples would have a total DNA input of <5ng.

2.4.8.2 *Experiment 2: second validation run testing both CHPv2 and CUSTOM panel using a range of reference standards and patient samples*

Before using either panel on the samples from participants in the FURVA trial it was important to test the panels using DNA from patient samples and reference standards including genes of significant interest in breast cancer. In this second experiment 10 samples were prepared for sequencing using the Ion Ampliseq Library Kit 2.0 (Thermofisher, Waltham, MA, USA) as per manufacturer's instructions (see Table 17) using both the CHPv2 panel and the CUSTOM panel. The 10 samples comprised 4 reference standard samples (3 at 10ng DNA input and one at 5ng, all representing cfDNA), 4 patient samples (all at 10ng DNA, all representing FFPE extracted DNA) and two further reference standards known wild type for certain mutations (one from cell line DNA and one representing cfDNA). No patient derived cfDNA samples with known variants were available for validation. The lower VAF frequencies of *AKT1* and *PIK3CA* were made by mixing DNA that was known to be WT for AKT1 with the reference standard containing known variants at known VAF. No VAF frequency was available for the FFPE patient samples (sample 1-4) as the variants had been identified using Sanger sequencing

Sample	Details	DNA source	Input DNA	Expected mutation and VAF (if available)	Results
1	Patient 1	FFPE	10ng	*TP53* C135Y	Detected by IR 56%
2	Patient 2	FFPE	10ng	*TP53* K132N	Detected by IR 18%
3	Patient 3	FFPE	10ng	*TP53* S127Y	Detected by IR 49%
4	Patient 4	FFPE	10ng	*TP53* Q103*	Detected by IR 21%
5	HD786 multiplex RS	fragmented cell line	10ng	*AKT1* E17K 5%, *PIK3CA* E545K 5.6%	Detected by IR at 6.3% and 5.1% respectively
6	HD786 multiplex RS	fragmented cell line	10ng	*AKT1* E17K 2.5%, *PIK3CA* E545K 2.8%	Detected by IR at 4.1% and 3.5% respectively
7	HD786 multiplex RS	fragmented cell line	10ng	*AKT1* E17K 1.25%, *PIK3CA* E545K 1.4%	*PIK3CA* variant detected by IR at 2.75%, *AKT1* at 0.35% only detected by manual review of filtered out variants
8	HD786 multiplex RS	fragmented cell line	5ng	*AKT1* E17K 5%, *PIK3CA* E545K 5.6%	Detected by IR at 5.4% and 5.6% respectively
9	HD 659 AKT1 WT RS	cell line	10ng	NIL in *AKT1*	No variants called in *AKT1* in IR
10	HD 776 WT RS	fragmented cell line	10ng	NIL in variant list for HD776	No variants detailed in HD776 called

*Table 17 Comparing different DNA sources and variants analysed using IR. All reference standards are identified by the prefix HD (Horizon Diagnostics) followed by their catalogue number. RS = reference standard. * = termination (STOP codon).*

This second validation experiment demonstrated that the library preparation, sequencing and bioinformatic workflow allowed detection of variants in patient derived FFPE samples and gave examples of variants from reference standards representative of cfDNA showing that it is possible to detect variants between 1% and 5% VAF (sample 6 and 7). In sample 6 both variants were detected by the standard bioinformatic pipeline in Ion Reporter whereas for sample 7 only the variant with a VAF >1% was detected using IR.

2.4.8.3 *Experiment 3: Validation of the CUSTOM Ion Ampliseq panel*

The CUSTOM Ampliseq panel covers variants in *ESR1, GATA3, RET* and *LYN*. At the time of the validation no reference standards were commercially available for variants in these genes. Search of in-house databases for samples containing variants in these genes did not identify any suitable

samples. Therefore, it was not possible to validate that the panel could correctly identify variants, but it was possible to check that the chemistry of the panel design covered the ROI.

Libraries were prepared using a variety of DNA sources (see Table 17). After sequencing, the CoverageAnalysis plugin in Torrent Suite version 5.8.0 (Thermofisher) was used to calculate coverage per amplicon. A minimum coverage of 1000 reads was set for each amplicon with the hope of being able to detect low frequency (<5% VAF) in low DNA input samples. Table 18 shows that in 7/10 samples depth of coverage was >1000 for all amplicons. The three samples where coverage is less than 100% had very low coverage overall compared to other samples. This could either be due to poor quality DNA input, technical issues in library preparation or uneven pooling of final libraries. As all samples that had good overall coverage had full coverage of the amplicons of interest it would be likely that the problem was with the samples rather than the panel.

Sample information				Percentage of amplicons with ≥1000 coverage				
Sample	Detail	Source	DNA input	ESR1 n=2	RET n=20	GATA3 n=11	LYN n=8	All amplicons n=41
S1	Patient 1	FFPE	10ng	100%	100%	100%	100%	100%
S2	Patient 2	FFPE	10ng	100%	100%	100%	100%	100%
S3	Patient 3	FFPE	10ng	100%	100%	100%	100%	100%
S4	Patient 4	FFPE	10ng	100%	80%	73%	75%	85%
S5	Patient 5	FFPE	10ng	100%	100%	100%	100%	100%
S6	HD786 multiplex RS	fragmented cell line	10ng	100%	55%	75%	63%	68%
S7	HD786 multiplex RS	fragmented cell line	10ng	0%	50%	27%	50%	41%
S8	HD786 multiplex RS	fragmented cell line	10ng	100%	100%	100%	100%	100%
S9	HD786 multiplex RS	fragmented cell line	5ng	100%	100%	100%	100%	100%
S10	HD 659 AKT1 WT RS	cell line	10ng	100%	100%	100%	100%	100%

Table 18 Ten samples run using the CUSTOM Ampliseq Panel. Samples were then analysed using the CoverageAnalysis plugin in Torrent Suite version 5.8.0 to assess coverage of the ROI in each gene.

2.4.9 NGS: Summary of limit of detection (LOD) experiments and final bioinformatic pipeline for use with FURVA patient samples

The three validation experiments performed showed that variants could be detected from FFPE patient samples and cfDNA reference standards. Variants could be detected manually using IGV as low as 0.5% VAF, however automated variant detection at these low levels relied on 20ng of DNA input and would not be suitable for de novo variant detection as over 2000 variants would need to be manually reviewed. More realistically the LOD lies between 1 and 5% depending on the quality and quantity of the DNA used for library preparation. For samples with low DNA input (≤5ng) the confident LOD is 5%. It is possible to detect variants between 1 and 5% even in low DNA input samples but as these samples were reference standards it may be that variants at <5% need to be interpreted with caution, particularly in low quality samples or those where there is no additional sample present to cross reference.

2.4.10 DdPCR: Assay specifics and validation

A limitation of the NGS work in this project was that the NGS panel selected was unable to analyse copy number changes in samples. Copy number changes are reported frequently in ER+ breast cancer with the most common as per the Razavi et al dataset in CBioPortal limited to ER+/HER2- metastatic samples (n=687) detailed in Table 19.

Gene	% of patients with CNA	Gene role	Reference
CCND1	25.5	Encodes Cyclin D1 which in turn regulates CDKs	(VanArsdale et al. 2015)
FGF19	23.7	Hormone like growth factor	(Perez-Garcia et al. 2018)
FGF4	23.0	Paracrine or autocrine like growth factor	
FGF3	22.6	Paracrine or autocrine like growth factor	
FGFR1	15.0	Growth factor receptor	
PAK1	13.5	Kinase involved in cell motility. Potentially acts within the PI3K pathway.	(Thillai et al. 2017)
MYC	9.3	Oncogene involved in cell cycle progression	(Green et al. 2016)

Table 19 Genes commonly showing copy number alteration (CNA) in ER+/HER2- metastatic breast cancer as determined by Razavi et al (2018) using FFPE tissue and next generation sequencing

Two of the commonly amplified genes were selected for investigation. Firstly, *MYC* and secondly *FGFR1. MYC* was selected due to its key role in the cell cycle and its documented role in endocrine resistance; if *MYC* was significantly amplified in a tumour it would be likely that it was the key driving variant of that tumour. One could hypothesise that a CNA in *MYC* in the primary tumour may result in primary endocrine resistance and amplification in the metastatic setting could be a potential mechanism of resistance to endocrine therapies. *FGFR1* was selected over the associated growth factor encoding genes as it is targetable with emerging therapies in the clinic, it also has a stronger evidence base of pathogenesis in endocrine resistant breast cancer. Analysing *PAK1* and/or *CCND1* would also be interesting but more than two targets were beyond the sample availability and scope of this section of the project. One could argue that investigation of *CCND1* amplification is of more interest where CDK4/6 inhibitors are in use while *PAK1* is of interest but lacks concrete evidence of association with endocrine resistance.

Using cfDNA to examine copy number is a technique that has potential for many clinical applications and in this respect analysing copy number by ddPCR rather than NGS has advantages such as speed and cost. CNV analysis by ddPCR is commonly investigated using HER2 as a target due to its clinical significance and the range of targeted therapies available for patients whose tumours are HER2 amplified.

MYC CNA has, to my knowledge, been looked at only in colorectal cancer using ddPCR (Lee et al. 2019). It has been studied in breast cancer using FISH in a number of settings including neo-adjuvant where use of the Shannon Index showed that patients with high *MYC* has a worse disease free survival particularly in ER+ patients (Chung et al. 2018). In a 2017 meta-analysis of *MYC* expression in breast cancer FISH was the most commonly used detection method (48%) while other methods included qPCR and IHC; no distinction was made between protein and gene expression. The meta-analysis showed that *MYC* expression was associated with worse RFS/DFS and OS, more so in patients with ER negative disease (Qu et al. 2017).

2.4.10.1 *ESR1 multiplex assays*

Pre-designed multiplexed probes were purchased from Biorad (Cat no: 12003910 and 12004118 – see Table 20 for list of variants). *ESR1* variants that had been identified using NGS were used to validate the assays for the detection of D538G and Y537N variants. Validation was repeated using gBlock synthetic double stranded DNA fragments (Integrated DNA technologies, Coralville, IO, USA) for the same variants. Samples that did not contain ESR1 variants as determined by NGS were used as negative controls and to assess whether false positive droplets occurred.

Multiplex 1 (MPX1)	**Multiplex 2 (MPX2)**
E380Q	S463P
Y537C	Y537S
D538G	Y537N
L536R	

Table 20 Variants covered by each multiplex

shows four ddPCR reactions demonstrating a positive control (g-block) and a negative control (patient FFPE sample) for each multiplex assay. In panel A and B blue dots indicate the presence of the variant and the green dots represent wild type DNA. While in panel C and D the green dots indicate wild type DNA present for the regions of interest.

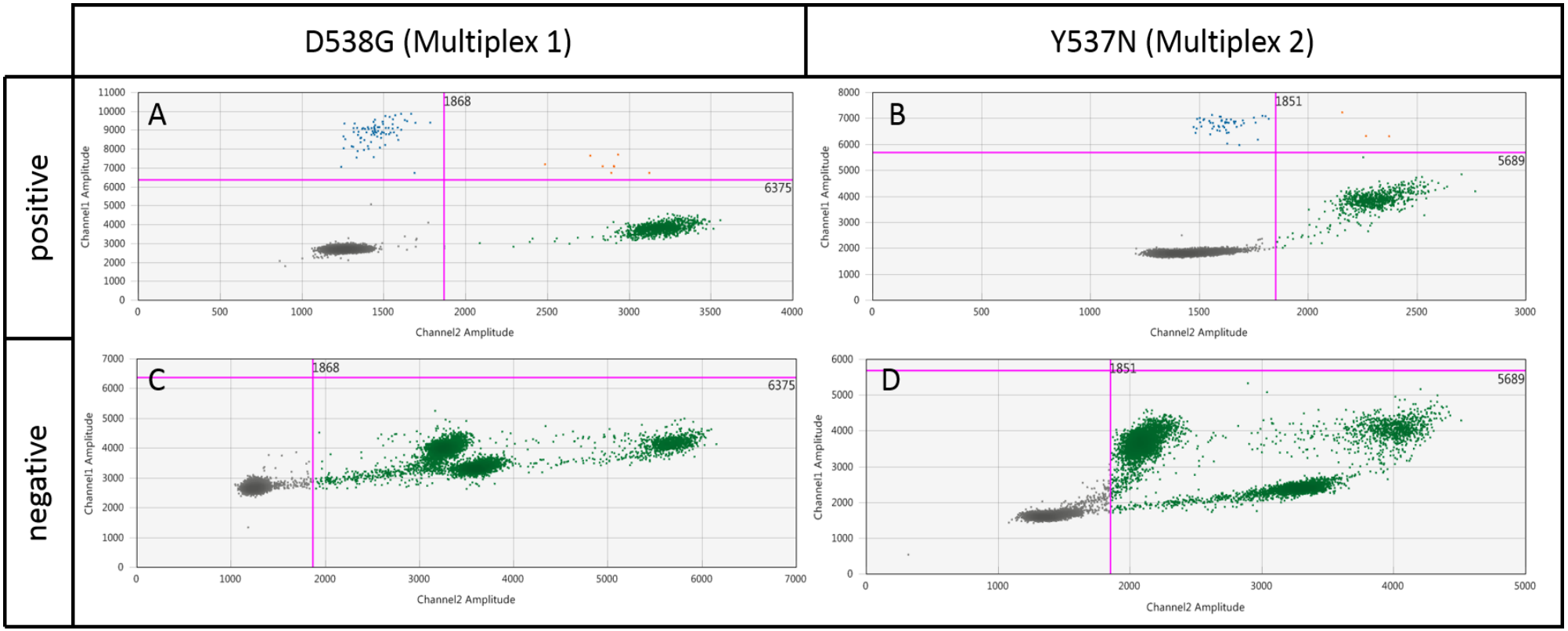


Figure 13 ESR1 multiplex controls. In both A and B blue dots in the top left of the graph illustrate droplets containing the variant DNA sequence. Orange dots illustrate droplets containing both WT and variant DNA. Grey dots illustrate droplets that contain neither WT nor variant droplets while the green dots represent droplets containing WT DNA. Both multiplex assays contain probes for three or four variants and thus there are multiple groups of WT droplets noted. In A the positive droplets number 89 which relates to a VAF of 7%. In B the positive droplets number 55 relating to a VAF of 4%. WT = wild type

Previous studies have explored the frequency of *ESR1* variants in patients with ER+ ABC. The limit of detections in these studies ranged from 0.1% (no details given on how LOD was established) (Najim et al. 2019) to '2 positive droplets' (Fribbens et al. 2016). The two positive droplet cut off was extrapolated from the methodology detailed in (Garcia-Murillas et al. 2015; Schiavon et al. 2015) which the authors suggest is equivalent to 3 copies or 0.00024%. This limit of detection was calculated by extracting gDNA totalling 15,000 copies from a cell line and spiking it with 3 copies of DNA containing a D538G variant. Others even accepted a single positive droplet as a 'positive' sample (Chandarlapaty et al. 2016). In another study the limit of detection was around 0.02% with 20ng DNA input, no detail is given on how the authors arrived at this figure (Spoerke et al. 2016).

The probes used here were the same as those used by Fribbens et al. In their study low DNA input samples (not quantified) a minimum of 300 WT droplets were required; this equated to a 95.1% probability of detecting variants at a VAF of 1% (Fribbens et al. 2016). This cut off has been applied to the FURVA samples when the estimated DNA input was <10ng as per Qubit concentration.

For many cfDNA samples from the FURVA study the total DNA input into the reaction was <5ng. Experiments were designed to explore the limits of detection of the assay both in terms of VAF and input DNA. Input DNA was calculated as per copies/µL as per the absolute quantification by ddPCR. In the experiments detailed in Table 21 synthetic DNA fragments containing the variant of interest (gBlock) were mixed with wild type synthetic DNA fragments to create samples with varying DNA inputs and variant allele frequencies (VAF). The aim of the experiment was to create samples with decreasing DNA concentration but similar VAF (experiment A) and with decreasing VAF but similar DNA concentration (experiment B). However, as seen in this was difficult to achieve with the gBlock DNA fragments. Even when sample concentration had been quantified using ddPCR in prior experiments attempts to mix DNA containing the variant of interest with wild type DNA did not manage to produce consistent VAF or DNA depending on the variable that was supposed to be constant.

gBlock gene fragments are artificially made double stranded DNA fragments usually measuring between 125 and 3000bp. They allow creation of DNA sequences containing specific variants and in this thesis are used as controls where patient samples were not available. The serial dilutions required are challenging to create low DNA concentration reference samples such as those needed in this thesis. While all efforts were made to quantify DNA at each dilution and ensure thorough vortexing of samples it was not always possible to create a final solution of the desired concentration.

Furthermore, when exploring very low level concentrations it was not possible to check concentrations with Qubit analysis prior to use of samples in the ddPCR assay.

	DNA concentration (D538G/WT) (copies/µL)	**VAF with upper and lower CI from Poisson distribution**	**D538G droplets**	**WT droplets**	**Equivalent DNA input**
	A: Exploring minimum DNA input, VAF should be around 8% for all samples				
S1	17/193	8.12 (9.22-7.01)	193	2030	~0.7ng/µL
S2	0.637/7.88	7.48 (12.3-2.68)	9	111	~0.03ng/µL
S3	0.24/1.53	13.8 (26.9-9.694)	4	25	~0.006ng/µL
S4	0.194/0.84	18.7 (39.1-0)	3	13	~0.004ng/µL
	B: Exploring VAF detectable with varying low DNA concentration samples				
S5	1.19/36.7	3.13 (1.65-4.61)	17	519	~0.13ng/µL
S6	0.255/55.2	0.459 (1.01-0)	3	635	~0.2ng/µL
S7	0.717/6.47	9.98 (3.27-16.7)	8	72	~0.025ng/µL
S8	0.1/110	0.09 (0.305-0)	1	1053	~0.4ng/µL

Table 21 Experiments run using G-block DNA fragments for ESR1 D58G and ESR1 WT to attempt to establish limits of detection for low DNA input samples

The data in Table 21 shows that in experiment A samples with a VAF of ~8% can be detected with a DNA input as low as 0.006ng/µL albeit with large confidence intervals. In experiment B the best conclusion that can be drawn is that low DNA input samples e.g. 0.13ng/µL can detected variants at 3%. For variants around 0.5% VAF, more DNA is needed than 0.2ng/µL. This means that some of the FURVA samples will not contain enough DNA to detect variants with a VAF of <1%. In experiment B it has also been demonstrated that even with reasonable DNA input very low-level variants e.g. 0.09 VAF cannot be confidently detected.

Using G-block containing the variant Y537N similar experiments were performed using the MPX2 probes to try and establish the frequency of variant that could be detected from low DNA input samples.

	DNA concentration (Y537N/WT) (copies/µL)	VAF (fractional abundance %)	Y537N droplets	WT droplets	Equivalent DNA input
	A: DNA input higher				
S1	0.406/11.5	3.41 (6.46-0.361)	5	141	0.045ng/µL
S2	0.234/23.2	0.997 (2.19-0)	3	295	0.08ng/µL
S3	0.155/24	0.641 (1.64-0)	2	307	0.085ng/µL
S4	0.132/19.2	0.684 (1.72-0)	2	288	0.075ng/µL
	B: DNA input lower				
S5	0.383/4.53	7.8 (14.6-0.994)	5	59	0.015ng/µL
S6	0.268/13.8	1.9 (3.84-0)	4	205	0.05ng/µL
S7	0.143/10.3	1.37 (3.45-0)	2	143	0.035ng/µL
S8	0.07/11.1	0.064(2.17-0)	1	153	0.04ng/µL

Table 22 Samples containing G-block DNA at similar variant frequencies with low and very low total samples DNA concentrations. The VAF shows the estimate VAF and the 95% confidence interval in brackets. The equivalent DNA input was calculated using the online calculator available at https://www.thermofisher.com/uk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html to convert copies/µL to ng/µL.

gain, working with g-block was challenging. The aim of the experiment detailed in Table 22 had been to create samples with decreasing variant frequency and then test those variant frequencies at low and very low overall sample concentrations with the aim of being representative of those samples which were either very low in cfDNA concentration at the point of extraction or were diluted to allow testing as insufficient material remained. Despite using dilutions that had been directly quantified by ddPCR as the starting sample the samples used in Table 22 had lower overall DNA concentrations than I would have liked.

The conclusion that can be drawn from this experiment is that only variants above 3% can confidently be identified from samples with very low DNA concentrations, the concentrations can be as low as 0.015ng/µL (S5 in Table 22). This expands on the findings from Table 21 where it has now been shown that samples with a VAF ~3% can be detected with low DNA input (S1 in Table 22).

In summary, very low concentration samples (cfDNA concentration ≥ 0.015ng/µL) can be used to detect ESR1 variants using the multiplex probes if the VAF is >3%. These experiments do not answer the question of the lowest VAF detectable using samples with good DNA concentrations. This will be retrospectively interrogated with the results from the patient samples (see Chapter 6).

2.4.10.2 *MYC and FGFR1 copy number variation (CNV) assays*

DdPCR can be used to evaluate copy number by designing probes that contain a sequence unique to the gene of interest and pairing them with a DNA sequence in a reference gene that is not known to amplify. The number of copies of the sequence of interest can act as a surrogate marker for the number of copies of that gene contained in the sample. This has successfully been used to evaluate a number of targets, for example HER2 in breast cancer (Gevensleben et al. 2013). The use of ddPCR in this project has allowed data on CNV in two key genes in ER+ breast cancer to be collected from patients in both arms of the trial.

2.4.10.2.1 Defining amplified samples

2.5.4.2.3.2 FFPE

Much of the published work to date has focussed on using ddPCR to assess *ERBB2* CNV. This is an obvious target as there are established gold standard methods to identify amplified samples (IHC and FISH). In FFPE samples where the percentage of DNA representing tumour is known one can make a good estimate of the ratio of copies of gene of interest, that will suggest that amplification is present. An example is shown, taken from Otsuji et al in (Otsuji et al. 2017). From this one can confidently call any ratio >2 as amplified in an FFPE sample. Additionally, for samples with lower tumour content the ratio threshold for a positive sample can lie between 1 and 2. The threshold for an individual sample can be calculated using the question R = x+1 where R is the ratio above which a sample is amplified and x is the tumour percentage expressed as a decimal.

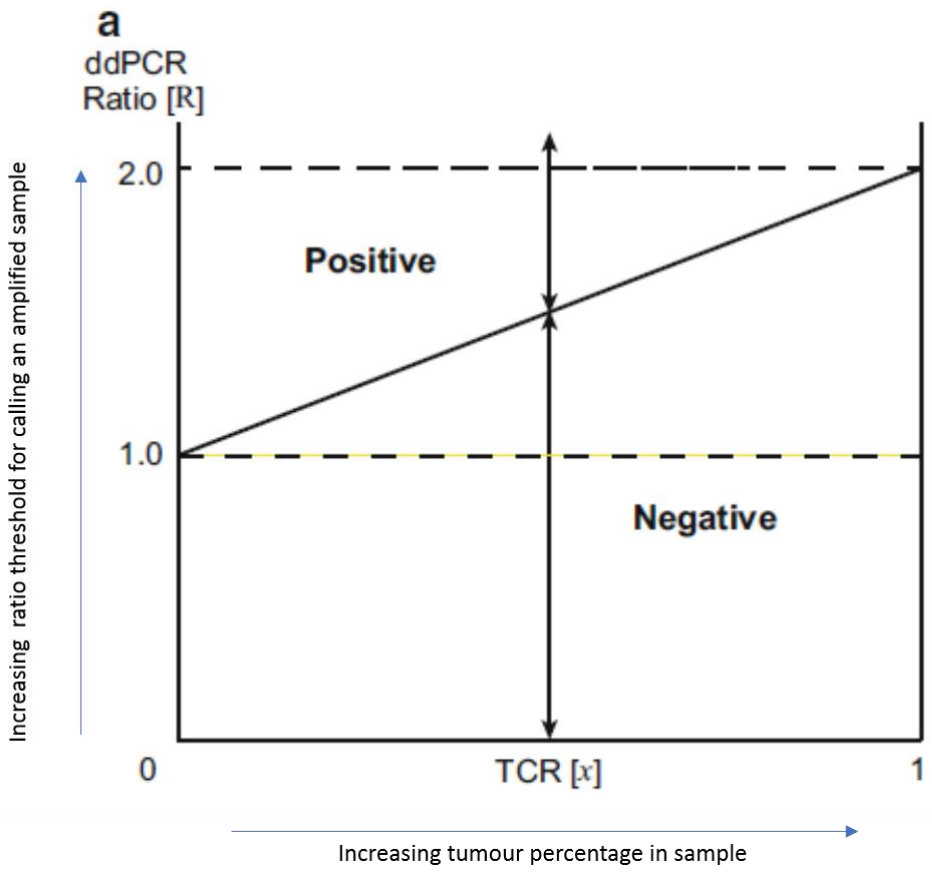


Figure 14 This graph taken from Otsuji et al shows the broad relationship between percentage of tumour present in FFPE samples and the ratio of gene of interest : reference gene required to call an amplified result. Assessment of tumour percentage (TCR) is usually by circling the area of highest tumour percentage on an H&E stained slide and always has a degree of subjectivity.

In interpretation of the results from the copy number experiments using DNA derived from FFPE samples both a strict cut off of a ratio ≥2 and the more lenient cut off as per the methodology in were explored. However, we must bear in mind that a sample that is mathematically amplified may not be clinically significant The results of these are presented in Chapter 5 and again in Chapter 6 when clinical outcome data allows more nuanced thresholds to be explored.

2.5.4.2.3.2 cfDNA

Defining the cut off for an amplified cfDNA samples is more challenging as the percentage of the DNA going into the test is a mixture of tumour and non-tumour DNA (as shown in), thus the tumour DNA is likely to be at a much lower percentage than in FFPE samples and is unknown.

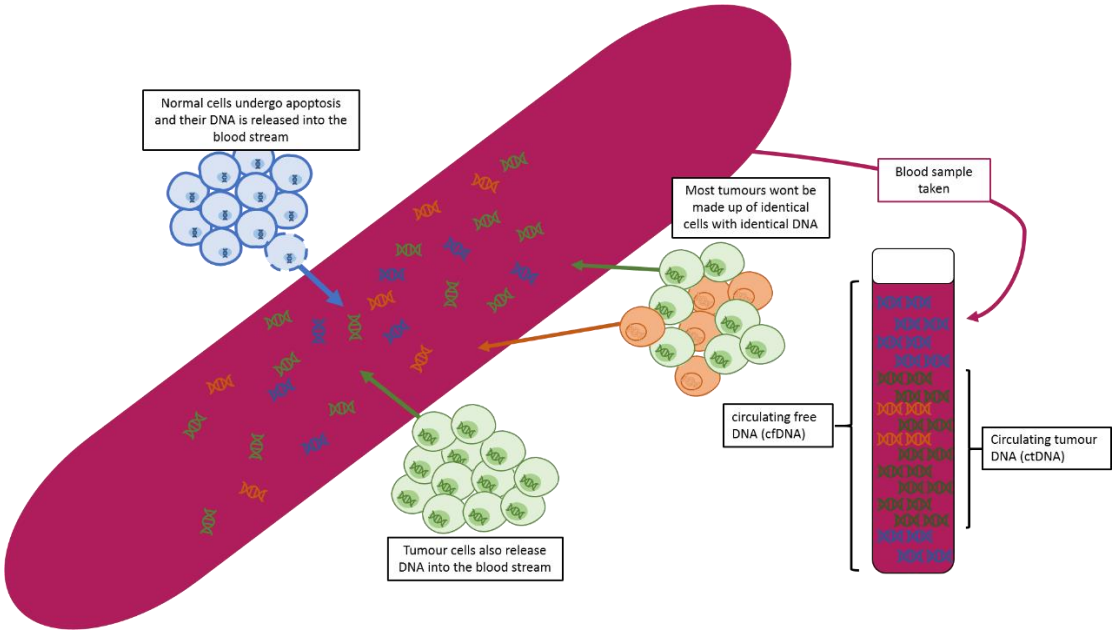


Figure 15 Infographic showing ctDNA as a proportion of cfDNA. DNA from both normal (blue) and tumour cells (green and orange) is shed into the blood stream via apoptosis, necrosis and potentially by direct secretion(Wan et al. 2017). This mixed collection of DNA forms cfDNA, of which a percentage will be DNA from tumour cells (ctDNA).

The amount of tumour DNA will also vary between samples and in a significant minority will be zero. This makes it very difficult to apply a single cut off value to a set of samples. The best worked example of this challenge I have identified is the work by Gevensleben et al. Here, upper and lower limits were defined where any values above the upper limit were highly likely to be amplified and those below the lower limit were not. The thresholds were determined by a 95% CI of a threshold of 1.25 which was the optimum cut point as determined by ROC curve analysis of 44 cfDNA breast cancer samples where the HER2 status was known from paired FFPE samples tested with IHC and FISH if necessary (Gevensleben et al. 2013).

However, in this thesis no prior knowledge of *MYC* or *FGFR1* copy numbers was available making the methodology used by Gevensleben inappropriate due to too many unknown factors. Instead the ratios of the gene of interest to reference gene will be paired with clinical outcome data and retrospective statistical analysis will be used to determine whether an optimum cut-point can be determined to identify prognostic groups using the maxstat method (Hothorn and Zeileis 2008)

which incorporates the ratio of the gene of interest to the reference gene and a survival variable (in this thesis progression free survival (PFS)) to identify the cut point most able to identify a meaningful difference between two groups.

In samples that were identified as amplified, where possible, another sample from the same patient, either cfDNA or FFPE was tested to compare the amplification status between time points.

2.5 Chapter discussion

The materials available for this project in terms of tissue blocks and plasma samples have been limited. Both in terms of the number of patients with complete sets of tissue and plasma samples representing trial entry, 8 weeks and end of treatment and physically limited in the number of slides available from each block and the volume of plasma available for DNA extraction.

Within the treatment arm of the trial only seven patients out of a possible 80 had a full set of samples available. As the focus of the project was biomarkers of response to vandetanib it would have not been logical to seek an alternate source of samples. However, the lack of sets of samples meant that the final data is not as uniform as I would have liked. For example, much of the cfDNA work has been performed on a heterogeneous mix of cfDNA samples with some coming from trial entry and others having been exposed to vandetanib or fulvestrant at either 8 weeks or end of trial treatment, particularly the latter ddPCR work (*MYC* and *FGFR1* copy number) where the lack of uniformity in drug exposure could introduce bias and act as a confounding factor.

The potential risks of using cfDNA samples from a mixture of time points are twofold: if a baseline sample has been used then any variants that may have either appeared as a result of treatment or become detectable due to sub-clonal proliferation will not have been detected; where the 8-week sample has been used there is a risk that if a resistance variant has developed it may have not yet become detectable. If the patient is responding well to treatment even clonal variants may not be detectable. With all cfDNA samples there is a risk that while there are variants in the tumour tissue,

DNA containing these variants is not being shed into the blood. This is particularly true for patients with luminal A and B breast cancers where the rate of detectable variants has been shown to be significantly lower than for HER2+ or TNBC (Zhou et al. 2019). This study predominately used primary breast cancer samples but it is likely that the same trend would be seen in metastatic samples. There are many pre-analytical variables prior to the use of cfDNA to detect cancer variants in ctDNA such as sample collection, sample storage and DNA extraction all of which can have significant influence on the final quality and quantity of DNA going into the final test (Markus et al. 2018). Thus, the absence of ctDNA can be due to either clinical factors, pre-analytical factors or a combination of both.

Where possible the EOT sample has been used as it was hypothesised that these samples would contain the highest quantity of ctDNA due to the progression and later stage of the disease. In addition, a further hypothesis was that patients may develop variants that conferred resistance to treatment during the course of treatment and these would only be detectable in the EOT sample. However, the EOT samples were a finite resource and thus, where necessary, were replaced by either 8W or BASE plasma samples.

2.5.1 Immunohistochemistry

Immunohistochemistry is a well-established, if partially subjective, technique for examining protein expression in FFPE tissue. Its advantages over other antibody-based techniques such as western blotting or enzyme linked immunosorbent assay (ELISA) include the ability to quantify and investigate the staining pattern of the protein of interest, for example where the staining occurs in the cell – membrane, cytoplasm or nucleus. By using whole sections rather than creating a TMA further detail can be gained about the expression of RET in any normal structures contained in the sample, it also allows visualisation of any heterogeneity of staining in different regions of the sample.

115 of the samples available for IHC were primary tumour sample. As it has been shown that RET expression increases as endocrine resistance develops it would have been interesting and potentially more accurate to use tissue taken from a biopsy at the point of endocrine resistance to try and ascertain whether RET protein expression could be a predictive biomarker for response to vandetanib and fulvestrant. However, this was not mandated in the study protocol and is often impractical in a clinical setting.

Furthermore, the p-RET antibody selected resulted in significant background staining despite optimisation. This is likely due its polyclonal nature. Had a monoclonal antibody been available this would have been selected. In addition to this it would have been ideal if the antibody used had already been used in other studies in breast cancer. However, the antibodies used in the publications available at the time of antibody selection were no longer in production.

2.5.2 Next generation sequencing

Next generation sequencing has been used extensively in breast cancer research to identify genes containing variants that could act as potential biomarkers. Common variants have been identified and characterised but aside from *TP53* variants are often not prognostic biomarkers in isolation. The purpose of the next generation sequencing element of this project was to search for variants at multiple time points during treatment with vandetanib and fulvestrant. It was hypothesised that new variants might emerge after treatment with vandetanib and fulvestrant. Thus, the technology selected for NGS needed to cover a good variety of genes of interest in ER+ breast cancer, along with detailed coverage of *RET* as it was thought that cells resistant to RET inhibition may contain variants in *RET*. The technology also needed to be compatible with DNA from FFPE samples and cfDNA extracted from plasma. At the time of selection (June 2017) the Ampliseq CHPv2 panel and an additional custom designed panel covering hotspots in *RET, LYN, GATA3* and *ESR1* offered a reasonable compromise between size and design of panel, DNA input required, DNA input type and expected coverage using the technology available (Ion Proton, Thermofisher, Waltham, MA, USA).

Subsequent to panel selection, technology has continued to improve, and panels are now often significantly larger. With the advent of unique molecular barcoding the limits of detection have also been improved. However, the absolute quantity of cfDNA that it is possible to extract from 2mls of plasma from patients with ER+ breast cancer has not increased, and this continues to be a limiting factor in the use of large sequencing panels with low concentration cfDNA samples.

It was challenging to design experiments to accurately assess the limit of detection of the selected NGS panel. It is not possible to validate every possible variant in the panel therefore validation is limited to common variants. Validating with commercially produced reference standards allows the most accurate estimation of input DNA and expected VAF but is also the purest form of DNA it is possible use and is not representative of patient samples. Validating with patient samples relies on availability of samples with a specific variant and then contains many variables that it is not possible to keep the same across all samples such as the way the sample was collected and stored or the way the DNA was extracted. The validation performed here used reference standards specifically designed to mimic cfDNA and patient samples for FFPE.

Ultimately each clinical sample has variables that cannot be fully controlled for such as DNA quantity and quality which can both be affected by the processing and storage of that sample. The most accurate conclusion from is that with DNA from a commercially produced reference standard and adapted bioinformatic pipeline the technology could detect variants as low as 0.5% VAF providing at least 5ng of DNA had been used. However, when analysing clinical samples where variants are unknown this level is impractical as the number of variants that would have needed manual review would be in the thousands for each sample. With clinical samples the practical limit of detection is around 5% VAF. For samples with good DNA quality and >5ng DNA input it is likely to be lower therefore all variants called with a VAF of >1% were manually reviewed.

There are ways in which DNA can be more accurately quantified such as using ddPCR for direct quantification. In addition, there are also methods available to assess the quality of DNA such as

using an electrophoresis-based method like the Agilent Bioanalyzer. In this project, the number of samples was small and finite and, even if armed with more information, poor quality and low DNA concentration samples would still be run in the hope that usable data could be generated. Thus, these additional methods of interrogating samples prior to library preparation and sequencing were not used. The use of vigorous post sequencing QC thresholds for individual variant calls aimed to ensure that false calls were not made.

2.5.3 Droplet digital PCR

DdPCR is complementary to the use of NGS in this project allowing data to be generated about specific variants in larger groups of patients and has been the only way copy number variation has been explored. An alternative to ddPCR to explore CNV in cfDNA would have been to use qPCR but the increased sensitivity of ddPCR for samples with low DNA input meant that ddPCR was selected as the preferred method. The gold standard test for CNV is fluorescence in situ hybridisation (FISH) but this can only be used on FFPE sections. Alternatively, techniques such as array comparative genomic hybridisation (aCGH) can be used, these can be used with DNA extracted from plasma but are only suitable for detecting large region wide CNVs e.g. loss or gain of segments of DNA measuring >1Mb.

The hypothesis that amplification of certain genes may increase over time and contribute to resistance to endocrine therapies meant that cfDNA samples were the primary source of DNA for the CNV evaluation. This came with significant challenges for interpretation of the resulting data as it was not possible to know whether the thresholds set (based on HER2 validation by Gevensleben et al) to determine whether a sample was amplified or not were applicable to the genes of interest. Effort has been made to explore ways to accurately identify amplified samples, but the methodology has limitations and if a signal was identified would need further validation before use on large groups of clinical samples, ideally by identifying a cohort of paired tissue and plasma samples.

A major limitation to using cfDNA to assess CNV is that the percentage of cfDNA that is ctDNA is unknown.

When it comes to assessing SNVs ddPCR is a much more established method and the use of cfDNA as an input material could be argued to be preferred to FFPE as it offers a more up to date 'snapshot' of the disease and the improved DNA quality can lead to more easily interpretable results. Here variants in *ESR1* were looked for across both arms of the trial using multiplexed reactions. Arguments could also have been made for looking for variants in *PIK3CA* and *AKT1*, however, due to the limited number of samples available the decision was made to only look at one of the three. As *ESR1* had been the only gene where variants had shown potential as a predictive biomarker to one of the two active drugs being investigated it was decided to prioritise this over *PIK3CA* or *AKT1*. If samples had been unlimited then all three targets could have been evaluated. If funds had been unlimited then all samples could have been assessed by NGS and ddPCR only used for CNV analysis. Using multiplex probes for *ESR1* meant that more variants could be tested for in 10µL of sample than if single probes had been used. The disadvantage of multiplexed probes is that no definite conclusions can be drawn regarding which variants are present and at what VAF.

2.5.4 Methods not used in this project

When considering methods of biomarker discovery in patients treated with vandetanib and fulvestrant it must be stated that the methods selected here are just a small number of those available. There has been no attempt to investigate gene expression by mRNA analysis, insufficient sequencing data has been generated to look at genetic signatures or tumour mutational burden (TMB) and evaluation of the regulatory mechanisms of gene expression such as methylation changes has not been possible. This is partly due to attempting to match the methods to the samples available; for example, mRNA analysis would be best used with tissue samples from metastatic lesions ideally comparing time points before and after treatment. Analysis of gene signatures and TMB require significantly larger amounts of sequencing data than that generated from the CHPv2 panel.

2.6 Chapter summary

This chapter outlines the three key methods used to investigate potential biomarkers within the FURVA study participants. Immunohistochemical analysis of protein expression, next generation sequencing of DNA using a targeted Ampliseq panel and ddPCR for both variant detection and copy number variation using cfDNA samples are considered in turn.

Selection of techniques was based on the samples that would be available and the in-house expertise and experience that could be drawn from. Particular attention was paid to the choice of technology for next generation sequencing and the final panel design. Compromise was needed in some areas to ensure that after consideration of time, cost and expertise available the technique most suited to the samples available was selected. This is particularly true of the use of NGS where with an unlimited budget and sample access different decisions would have been made.

Validation of techniques using samples as close to those from the FURVA trial participants; both FFPE and cfDNA extracted from plasma has been performed.

3 Analysis of total RET (t-RET) and phosphorylated RET (p-RET) expression in FFPE samples from participants in the FURVA clinical trial using immunohistochemistry (IHC)

3.1 Chapter Overview

This chapter presents the results of IHC analysis of t-RET and p-RET protein expression using assays developed specifically for clinical breast cancer samples used in this project. The development and validation of the assays was discussed in Chapter 2. The analysis of the t-RET assay and p-RET assay are presented in turn along with discussion about the reproducibility of the assays and the challenges of working with archived clinical samples.

3.1.1 Chapter Aim

This chapter aims to determine the level of t-RET and p-RET protein expression in FFPE clinical breast cancer samples from participants in the FURVA trial using immunohistochemistry.

3.1.2 Chapter Objectives

- To determine the range of protein expression using IHC assays for both t-RET and p-RET in FFPE primary tumour samples from patients who have gone on to develop endocrine resistant breast cancer
- To assess reproducibility of the assays over large clinical series
- To explore different staining cut points to determine whether samples overexpress t-RET or p-RET
- To discuss limitations and challenges of working with archival FFPE specimens in the context of the FURVA clinical trial

3.1.3 Materials

165 patients participated in the FURVA trial, FFPE tissue blocks were provided from 136 participants. In total 128 samples were eligible for analysis; 115 primary tumour samples and 13 metastatic

samples. 8 sample blocks were not eligible as they either did not contain any tissue (n=3), did not contain any tumour epithelial cells (n=1), did not contain ≥100 tumour epithelial cells (n=2) or came from a cytology sample (n=2). This is summarised in Figure 16.

Of the 13 metastatic samples 6 were from lymph nodes, 4 were from bone and single samples each from lung, liver and skin were also identified.

Of the samples suitable for the main analysis (untreated primary tumour) 38 were core biopsy FFPE samples, 69 were full FFPE sections >1cm^2, two were from 'mega blocks' where each section was >5cm^2 and 6 were from full sections <1cm^2.

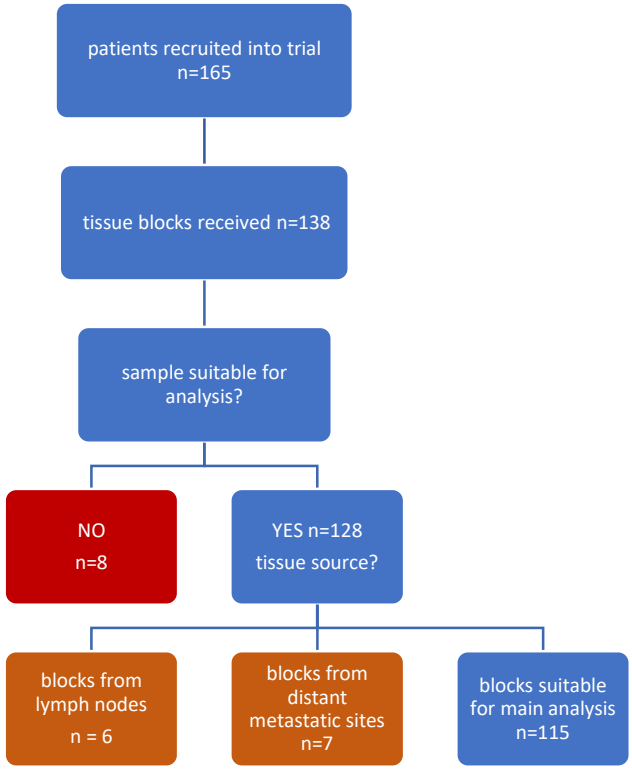


Figure 16 Flow diagram of available clinical samples for analysis in the FURVA study. Blue shading indicates flow path for primary breast cancer samples included in main IHC analysis. Red shading indicates samples excluded from main analysis. Orange shading indicates metastatic samples included in a secondary analysis.

3.2 Total (t-RET) main analysis – primary tumour samples only n=115

3.2.1 Assay characteristics

The assay used to assess t-RET expression in this project used a primary antibody that had not previously been used on breast cancer patient samples. The monoclonal antibody selected; Ab134100 (Abcam) produced immunostaining that showed largely cytoplasmic staining where RET was present in the clinical primary breast cancer samples tumour epithelial cells. Staining intensity varied between samples and on occasion varied between epithelial cells in individual sections. All samples showed some degree of RET staining (H-score >0). Examples of 1+ (weak), 2+ (moderate) and 3+ (strong) staining are shown in Figure 17. Only tumour epithelial cells were scored. For more detail on the methodology please refer to Chapter 2. Figure 17 demonstrate examples of staining representing weak, moderate and strong expression of t-RET.

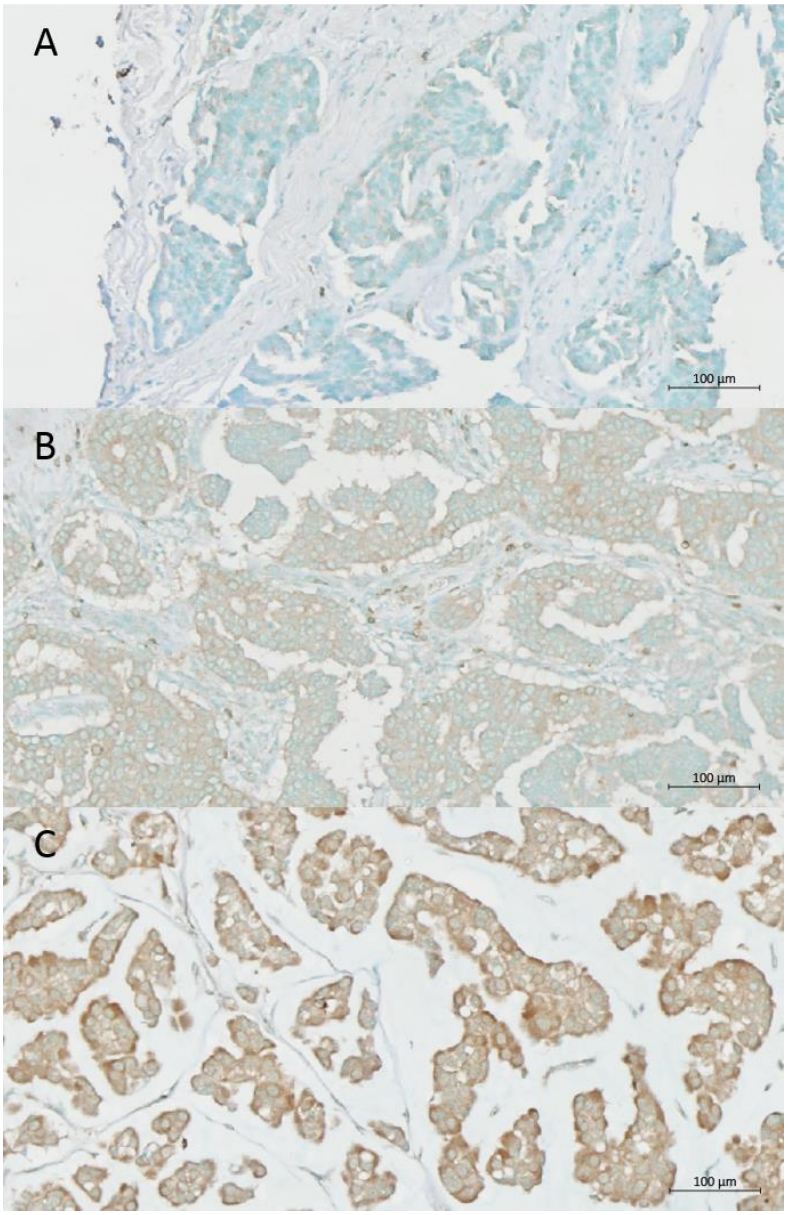


Figure 17 Examples of t-RET staining patterns in FURVA primary breast cancer sections. A = sample 242008 showing 1+ staining. Overall H-score 85. B= sample 86012 showing 2+ staining. Overall H-score 170. C= sample 86003 showing 3+ staining. Overall H score 285. Original magnification x 10, scale bar = 100µm.

3.2.2 Assay performance

Samples were assayed in fifteen separate batches for the trial due to sample availability, each including a positive control breast cancer slide. Control slides were scored for t-RET, and scores compared across batches. The median H-score for the control sample in the t-RET assay was 100 (range 50-140). Figure 18 shows mean H-score per batch alongside that of the control sample run

concurrently. Batch 11 (n=8 patient samples) had a lower than normal control H-score but an average sample H-score. On manual review it was felt that this control score was low due to technical variation but that the patient samples showed the expected heterogeneity of staining between samples and an appropriate range of overall scores, thus the results from this batch were included in the final analysis. The variation could have been due to a poor quality section cut for the control or a change in thickness of the section. Batches 12-15 were run more than a year after batches 1-11 due to sample availability. By this time the original antibody batch used was no longer available and the assay was performed with the same antibody but at a re-titrated concentration for the new batch to achieve similar control sample scores. Batch 12 (n= 3 patient samples) and batch 13 (n=5 patient samples) had slightly higher control sample h-scores (125 and 140 respectively), however the average score for samples included in these batches was not higher than expected so it was not felt that the assay had resulted in artificially high sample H-scores. Ideally samples would have been run over a short time frame with an equal number of samples per batch, however, within the timeframe of this project this was not logistically possible.

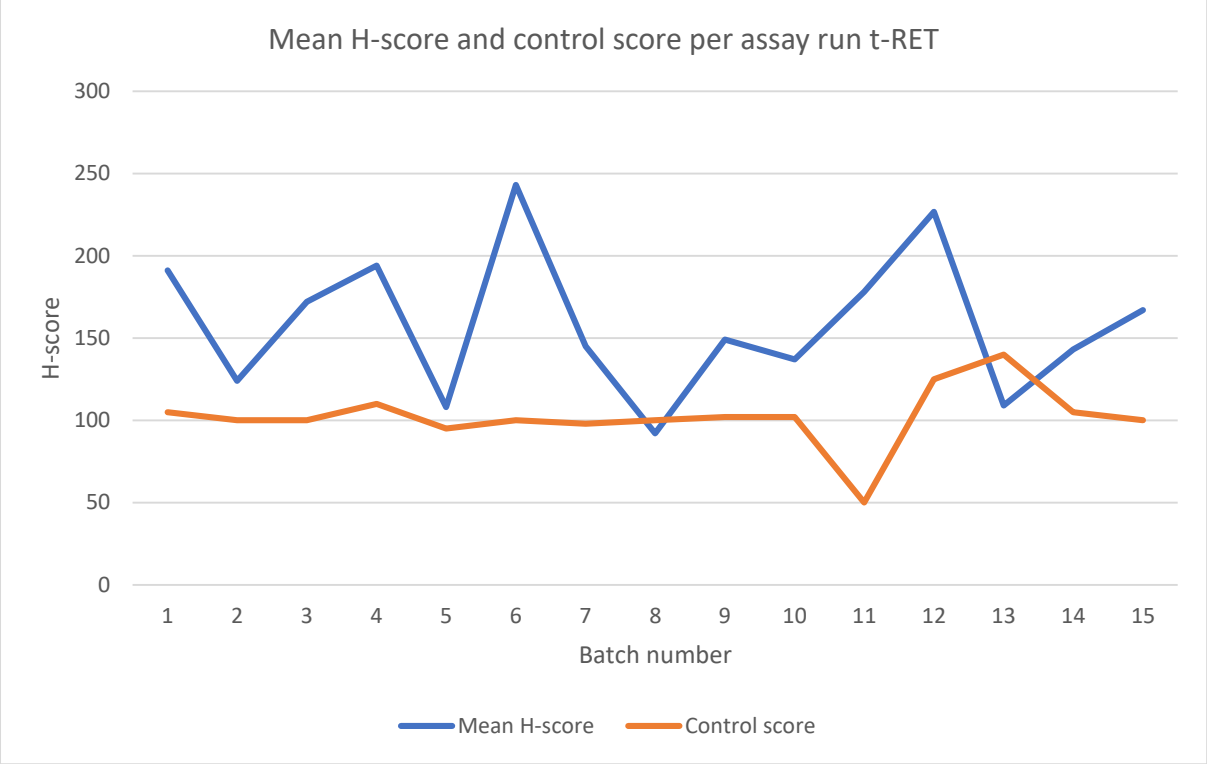


Figure 18 Chart showing mean t-RET H-score per FURVA sample assay (blue line) and for the positive control breast cancer sample H-score (orange line) for each of the 15 separate sample batches.

3.2.3 Scoring

Each assay was scored first by Zoe Hudson (ZH) (PhD candidate) and then by Dr Fouad Alchami (FA) (Consultant Pathologist). FA scored the samples 'live' whereas ZH had reviewed each slide prior to the scoring session. Where discrepancies in scoring occurred consensus was reached while both ZH and FA reviewed the stained sample resulting in an agreed score (shown as ZH/FA in Figure 19 graph A).

Prior to this project ZH had not received advanced training in histopathology. Basic training in identifying tumour epithelial cells in FFPE clinical breast cancer samples and H-scoring such samples was delivered by skilled technical staff from the Gee lab so that ZH was able to initially assess virtually all samples. Where there were technical challenges in scoring, for example ZH was unable to identify any tumour epithelial cells within the sample, no initial attempt at scoring was made by ZH

(n=3). Assessment of the trial samples by consensus with the pathologist was thus important to ensure the H-score data for the trial were as robust and comprehensive as possible. Overall, correlation between the two methods of scoring was achieved with a correlation coefficient of 0.72 (95% CI 0.62-0.80) (Figure 19 Graph A).

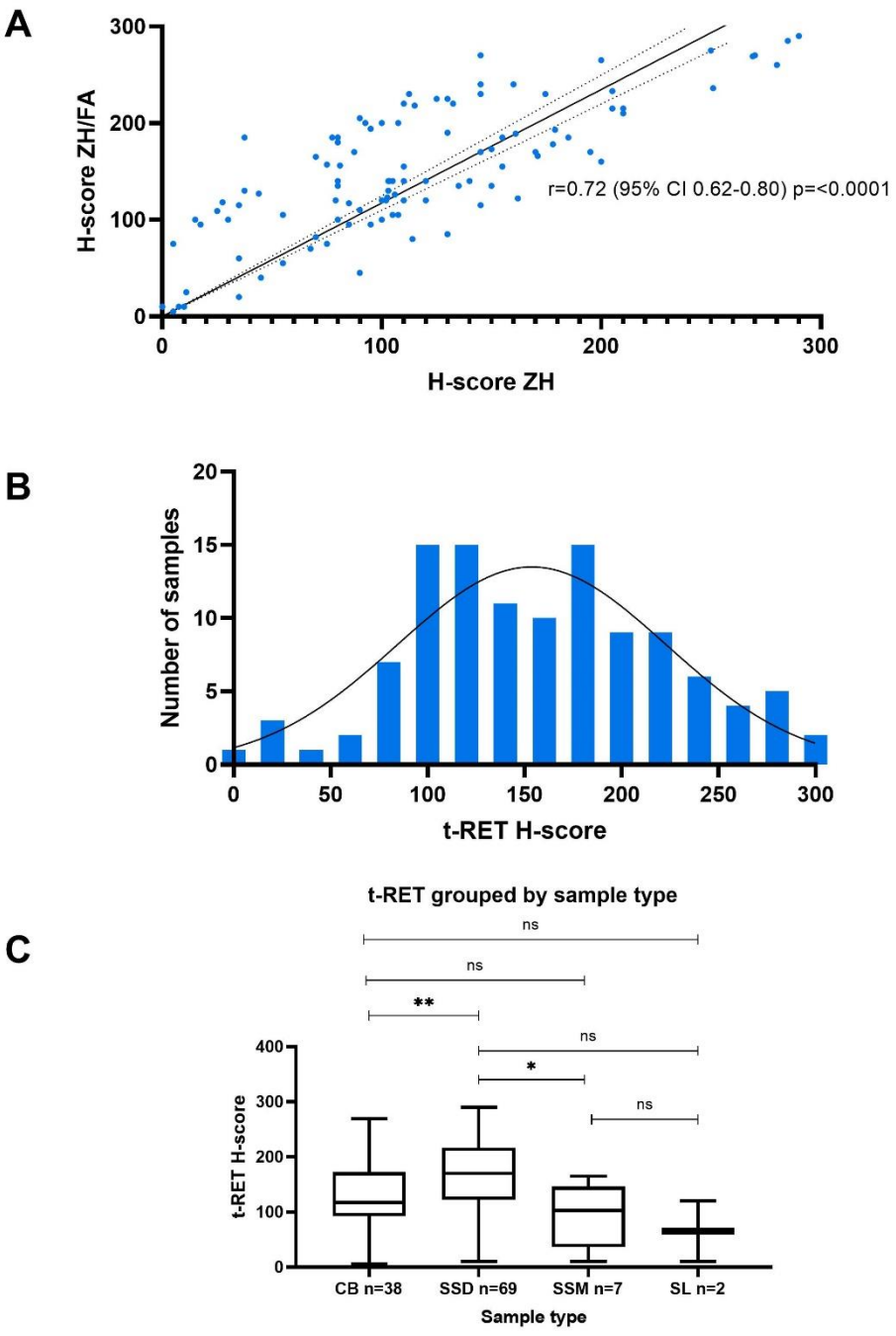


Figure 19 ***A)*** *Scatterplot showing correlation between ZH H-score and ZH/FA consensus H-score for the FURVA clinical samples stained for t-RET. r=Spearman's rank correlation coefficient. p value is two sided with an alpha significance value of 0.05. Dotted lines represent upper and lower 95% confidence intervals* ***B)*** *Histogram and Gaussian distribution of the final H-scores across the series of primary tumour (PT) samples stained for t-RET.* ***C)*** *Box and whisker plot comparing range of scores by sample type; CB = core biopsy, SSD = standard section >1cm^2, SSM = small section <1cm^2 and SL = large section >5cm^2. P-values from one-way ANOVA test. ns = >0.05, * = 0.01 to 0.05, ** = <0.01*

All samples showed staining for t-RET (H-score >0). The pattern of scores showed a normal distribution (Figure 19 Graph B). Figure 19 Graph C explores whether scores altered based on the sample type. Most samples were either core biopsies, 38/115 (30%) or standard sections measuring >1cm^2, 69/115 (60%). The median H-score for standard sections was higher than that for core biopsies as was the interquartile range reaching statistical significance, however the overall range was similar.

In previously published t-RET assays the assays have been performed on tissue microarrays (TMAs) (Gattelli et al. 2013; Mechera et al. 2019). These are created by taking a 1-2mm core from each tumour block, ideally from an area with high tumour content and then embedding these cores into another paraffin wax block. Sections of this block containing multiple (typically up to 100) samples are then stained. This technique allows many more samples to be tested at the same time thus mitigating for natural variability between batched runs. However, unless multiple sampling across a tumour has been performed it does not allow investigation of the assay staining across larger tumour sections. Heterogeneity of staining was noted in some of the FURVA samples tested in this project and this would not have been detectable had a TMA been created.

3.2.4 Determining cut off values for 'RET overexpression' using the t-RET scoring system

Two published methods to define cut-offs using t-RET assays on breast cancer FFPE samples were identified from literature review and applied to the data generated from the FURVA samples; one using H-scoring and the other simply defining the staining on a 0-2 scale. The two methods are detailed in chapter 2. The two methods were applied to the t-RET data and the results shown in Table 23 and Table 24.

	FURVA n=115	Gattelli n=89
'RET high' total H-score >60	102/115 (89%)	66/89 (74%)
'RET low' total H-score ≤60	13/115 (11%)	23/89 (26%)

Table 23 Applying the Gattelli et al scoring method to the FURVA data using an >60 H-score as a cut-off for positivity

	FURVA n=115	Ngyuen n=2800
'RET Positive'	96/115 (83%)	1596/2800 (57%)
'RET negative'	19/115 (17%)	1204/2800 (43%)

Table 24 Applying the Nguyen et al method to the FURVA dataset, where an overall score was assigned to the sample of either 0, negative, 1, weak staining or 2, strong staining with a score of 1 or 2 counting as positive.

Both published methods used tissue microarrays for their analysis and both papers used different primary antibodies to the particular t-RET primary antibody used in the FURVA assay. This means their scoring systems are not directly comparable to the current study, but they offer a reasonable starting point to explore possible definitions of t-RET 'positive' and 'negative' samples. However, overall, when both these scoring systems were applied to the t-RET assay used to evaluate the FURVA sample series a much larger proportion of samples (>80%) scored positive which precluded a meaningful subgroup analysis.

A further receptor tyrosine kinase IHC assay in use in breast cancer clinical histopathology is the HER2 assay. Here a sample is considered positive for HER2 protein overexpression if >10% of tumour cells in the sample show 3+ staining. When this was applied to the FURVA dataset then 46/115 (40%) of samples are t-RET 'positive' (with the remaining samples showing 1+ or 2+ staining). During the development of standardised guidelines for HER2 scoring a cut off >30% of cells showing high intensity staining was also applied for protein overexpression. If the 30% cut off is applied 26/115 (23%) of samples are 'positive'. These methods are explored in Table 25.

Using 3+ scoring alone excludes many samples which exhibit significant areas of 2+ staining in their tumour from being 'positive'. By allowing positive samples to include samples where >10% of cells exhibit 3+ staining or >30% of cells exhibit 2+ staining takes the number of 'positive' samples to 58/115 (50%). This third category ('C' in Table 25) is more pragmatic as it allows for significant 2+ staining to count as 'positive'. This would also allow some flexibility in defining a 'positive' sample if any batches had resulted in lower than average staining intensity. All three categories (summarised for FURVA in Table 25) will thus be explored with clinical outcome data for FURVA to see if a subgroup who respond better to RET inhibition can be identified based on t-RET staining.

	A: >10% of cells showing 3+ staining	B: >30% of cells showing 3+ staining	C: >10% of cells showing 3+ staining or >30% of cells showing 2+ staining
t-RET 'positive'	46/115 (40%)	26/115 (23%)	58/115 (50%)
t-RET 'negative'	69/115 (60%)	89/115 (77%)	57/115 (50%)

Table 25 Three proposed systems for defining t-RET 'positive' and 'negative' samples. All three will be applied to the FURVA dataset in this project. The cut off that best identifies a clinical subgroup will be recommended for further studies. The clinical correlation will be presented in Chapter 6.

3.3 Phosphorylated (p-RET) main analysis – primary tumour only n=115

3.3.1 Assay characteristics

P-RET has not been previously investigated in published breast cancer series (apart from briefly in occasional conference proceedings). The assay developed for this project uses a polyclonal antibody against a RET phosphorylation site Y1062. No monoclonal antibodies to p-RET were available at the time of the assay optimisation.

Figure 20 shows example of 1+ (weak), 2+ (moderate) and 3+ (strong) p-RET staining in primary breast tumour tissue samples from participants in the FURVA trial. Image A illustrates one of the major challenges in scoring the samples; at first glance the sample appears to contain strong staining but on closer inspection the strong staining is in the connective tissue whereas the tumour cells (nuclei stained blue/green) do not exhibit significant staining in their cytoplasm. Heterogeneous tumour epithelial staining can be seen in image B where some cells show 2+ staining while others are showing 1+ staining, while much stronger, more homogenous cytoplasmic staining is apparent in image C (again along with a connective tissue background signal).

It should be noted that despite significant optimisation there was a degree of cross reactivity leading to some background staining of connective tissue within samples (Figure 21 image 1). Staining was seen in the cytoplasm of tumour cells alongside staining in lymphocytes (Figure 21 image 2), axons (Figure 21 image 3) and keratinocytes (Figure 21 image 4) where these normal structures were included in the tissue sample. Where staining was present in tumour epithelial cells it showed a granular appearance, which did not stain in the nucleus. Both image 2 and 4 (Figure 21) show cells exhibiting 3+ staining. Although the staining is stronger in some cells in image 2 rather than add a fourth scoring category all cells exhibiting staining in keeping with the intensity shown in image 4 or greater would be scored as maximum of 3+.

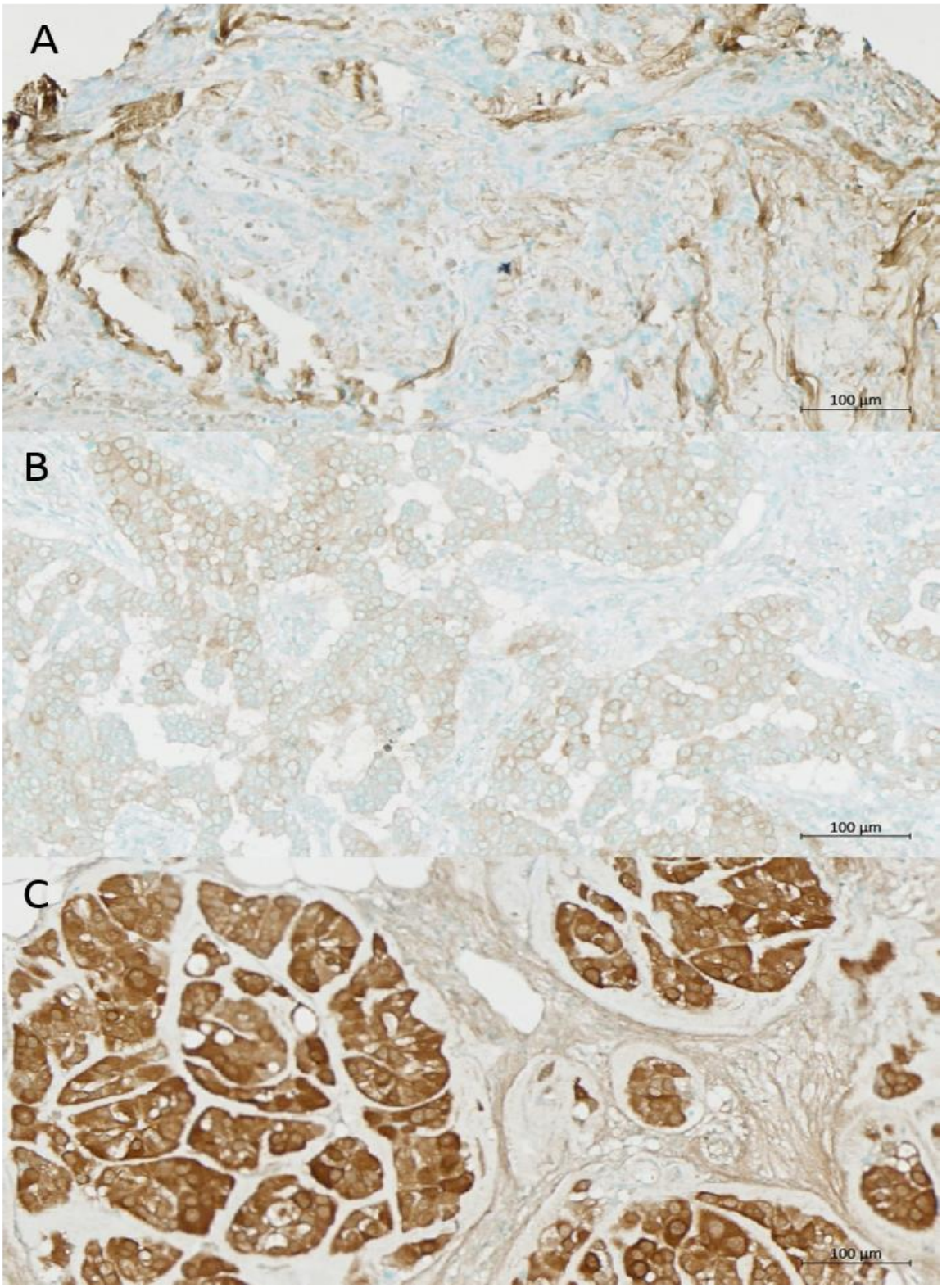


Figure 20 Examples of p-RET scoring in FURVA clinical breast cancer samples. A = sample 184002 showing low intensity tumour epithelial staining (1+). Overall H-score 25. B = sample 86012 showing moderate intensity tumour epithelial staining (2+). Overall H-score 125. C = sample 86003 showing high intensity tumour epithelial staining (3+). Overall H-score 280. Original magnification x 10. Scale bar = 100µm.

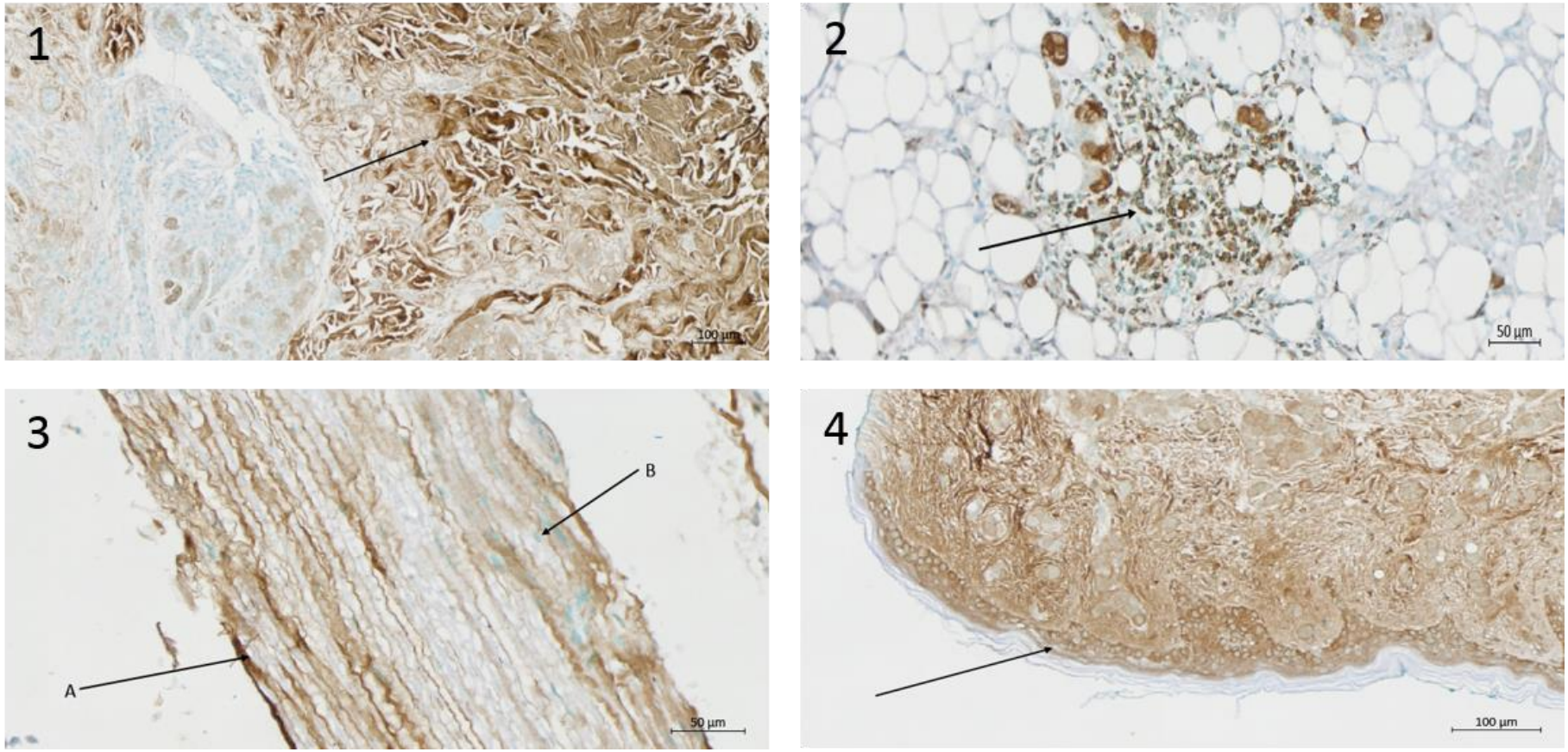


Figure 21 Images showing staining of non-cancerous structures by p-RET assay. In image 1 glycogen bundles indicated by arrow are showing high staining intensity. In image 2 lymphocytes are showing 3+ staining. In image 3 a nerve was identified in the sample and the axons (A) stain with moderate intensity, in addition image 3 shows cancer cells infiltrating the nerve (B). In image 4 the sample included skin tissue and keratinocytes, indicated with the arrow showing 3+ staining. Original magnification x 10 for panel 1 and 4 with scale bar = 100µm and x20 for panel 2 and 3, where scale bar = 50µm. Images were produced from scanned slides at x10 magnification with additional digital zoom applied to illustrate the area of interest.

3.3.2 Assay performance

For p-RET IHC samples were, again, assayed in 15 batches of varying slide numbers depending on sample availability. Each assay included a positive control breast cancer slide. Control slides were scored along with each assay batch and also evaluated against each other. Mean H-score per batch of FURVA sample was also calculated. The control and batch sample means are plotted for each batch of samples run in Figure 22. The control sample selected for the p-RET assay was a large sample which may account for the inter-run variability seen in some of the batches which was barely present in the t-RET assay. Most obviously, the control score was lower for batch 5 (n=6) but the overall mean score of the FURVA samples in this batch was within the standard range and so as deemed acceptable. It is notable that the later FURVA sample batches have lower overall average H-scores, but the control sample maintains good reproducibility. In explanation, batch 11 (n=3) and 13 (n=4) were both small batches and it is possible that it was chance alone that accounted for lower scoring FURVA samples. Batches 12 and 14 both contained 10 samples. While the average H-score of both runs was lower than expected, the control slide scored comparably to other batch runs. On the basis of the control sample working these runs will also be included in the final analysis.

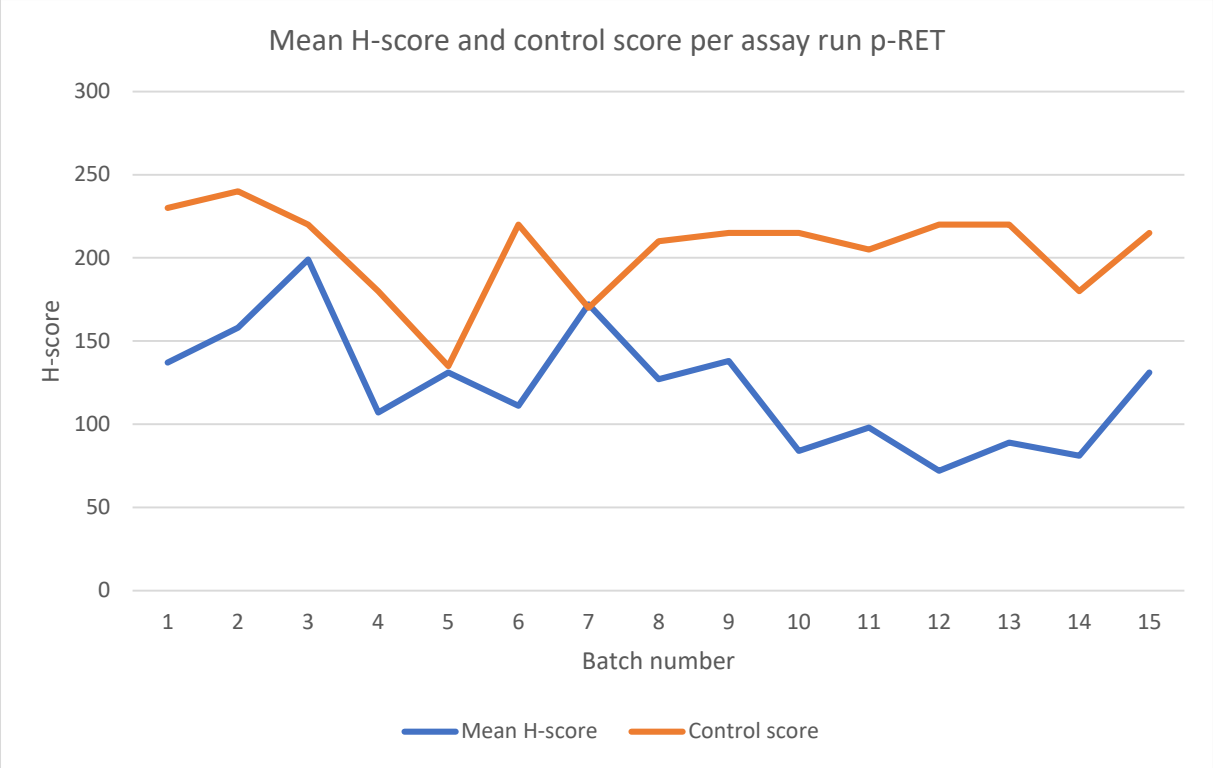


Figure 22 Chart showing mean p-RET H-score per FURVA sample assay run (blue line) and for the positive control breast cancer sample H-score (orange line) for each of 15 separate sample batches.

3.3.3 Scoring of the sample immunostaining

The p-RET assay was scored in the same manner as for t-RET (see section 3.2.3) by using the H-score method (which was also used by Gee et al in a previous meeting presentation). Correlation between ZH and ZH/FA assessment scores for p-RET were improved compared to the t-RET assay (Graph A Figure 23). The p-RET assay gave a clearer distinction between no staining and staining present in the tumour cell potentially making it easier to come to a consensus. For example; it is easier to agree whether a cell exhibits staining or not than whether the staining present constitutes 1+, 2+ or 3+, staining.

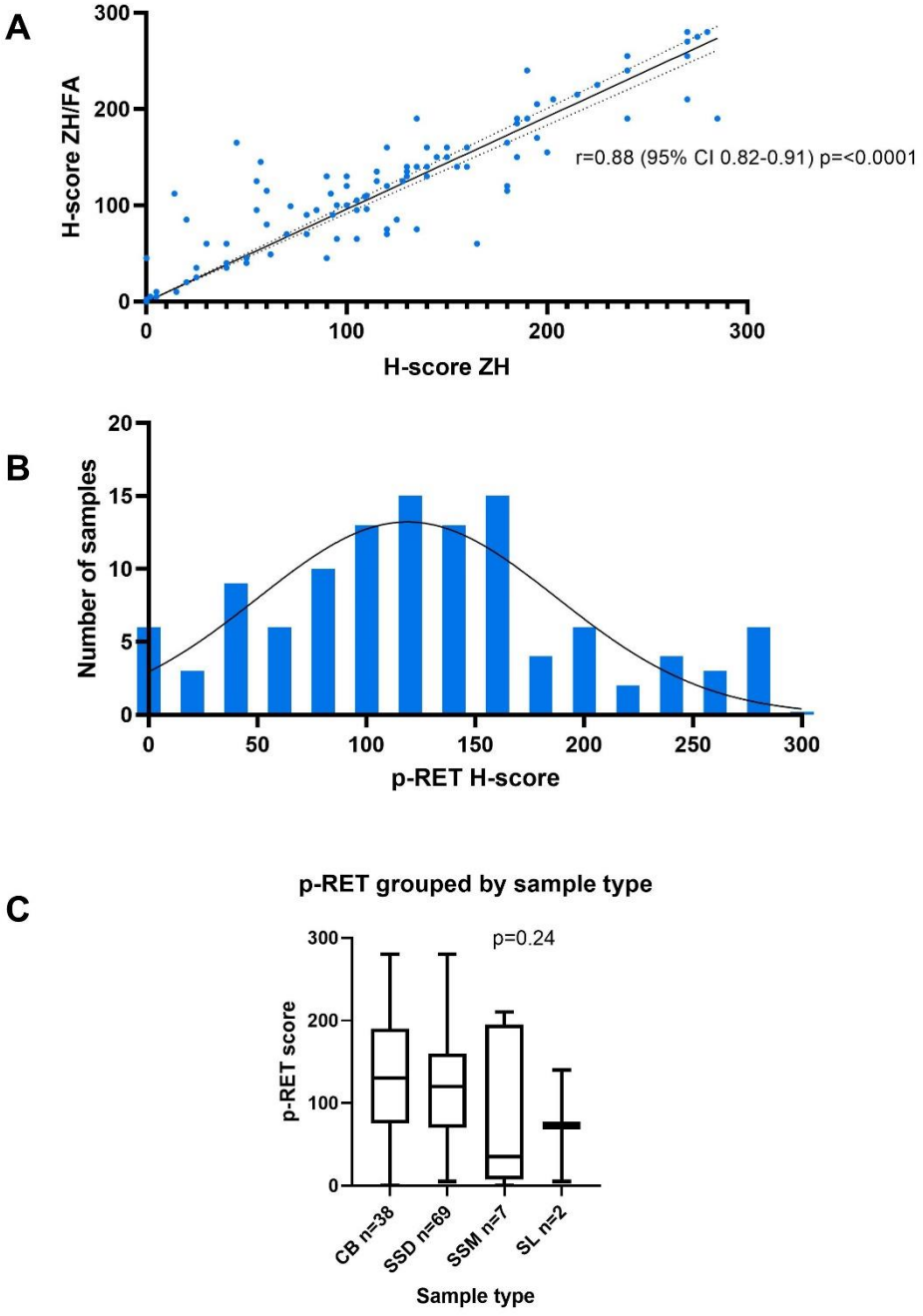


Figure 23 A) Scatterplot showing correlation between ZH H-score and ZH/FA consensus H-score for the FURVA clinical samples stained for p-RET. r=Spearman's rank correlation coefficient. p value is two sided with an alpha significance value of 0.05. B) Histogram and Gaussian distribution showing the final H-scores across the series of primary tumour (PT) samples stained for p-RET. ***C)*** *Box and whisker plot comparing range of scores by sample type; CB = core biopsy, SSD = standard section ≥1cm² and ≤5cm², SSM = small section <1cm² and SL = large section >5cm². P value from overall one-way ANOVA analysis. No significant difference detected in h-score between different sample types.*

The pattern of scoring through the cohort did not follow a normal distribution, with the median H-score being 115 (range 0-280). In the p-RET cohort 47/115 (41%) of samples had a H-score of ≤100 and 81/115 (70%) scored ≤150. Two samples did not show any staining and thus their H-score was 0. In the p-RET immunostaining the median H-score was slightly higher in core biopsy samples than in standard sections but the range was almost identical. The difference did not reach statistical significance (Figure 23 graph C).

3.3.4 Determining cut off values for 'RET overexpression' using p-RET assay

There are no published works referring to the use of a p-RET assay in breast cancer samples thus no available scoring systems for comparison. Gee et al (meeting abstract) used an H-score cut point of ≥25, but the study did not use the present antibody. The methods used to score explore cut off values for t-RET 'positive' and 'negative' samples was examined in the p-RET dataset (Table 26). With a 10% cut off for positivity this resulted in 51/115 (44%) of samples being categorised as 'positive'. If the 30% cut off was applied, then 20/115 (17%) of samples are positive. If the scoring expanded to include samples where >30% 2+ staining also counts as 'positive' then the total number of p-RET 'positive' samples rose to 82/115 (71%).

	A: >10% of cells showing 3+ staining	B: >30% of cells showing 3+ staining	C: >10% of cells showing 3+ or >30% of cells showing 2+ staining
RET "positive"	51/115 (44%)	20/115 (17%)	82/115 (71%)
RET "negative"	64/115 (56%)	95/115 (83%)	33/115 (29%)

Table 26 Three proposed systems for defining p-RET positive and negative samples. All three will be applied to the dataset for the FURVA clinical analysis and the cut off that best identifies a clinical subgroup will be recommended for further studies.

3.4 Comparing t-RET and p-RET scores in primary tumour samples

When the H-scores of t-RET and p-RET were compared for each sample; there was no significant correlation, although a weak trend was apparent suggesting a weak association (p=0.11) (Figure 24). When scores were categorised into RET 'positive' and RET 'negative' using a cut off of >10% of tumour epithelial cells showing 3+ staining 52% of samples were either both positive or both negative for t-RET and p-RET i.e. were concordant. When the cut off was determined by >30% of cells showing 3+ staining the concordance increased to 65%. It is feasible that not all samples that show high expression of t-RET should also have high expression of p-RET as not all RET present in the cell will be activated/phosphorylated so a strong correlation would not be expected. Phospho-specific epitopes can also be more sensitive to damage during fixation and processing influencing antibody performance (Pinhel et al. 2010).

Of the 27 samples where neither strategy resulted in concordance between t-RET and p-RET status, 15 samples were positive for t-RET but not p-RET and 12 vice versa. The lack of p-RET expression in t-RET 'positive' samples is explainable by not all t-RET protein being activated. However, this does not explain why samples would be 'positive' for p-RET but not for t-RET. In this instance, this may simply reflect the different affinities of each primary antibody for detecting their respective epitopes in the antigen retrieved FFPE samples, or even damage of the t-RET epitope in some material. However, in the 12 samples where p-RET was 'positive' but t-RET was not, all samples had >50% 3+ staining present in the p-RET assay. In the corresponding t-RET assays three showed very low staining, total H-score <100 while the others scored mainly 2+. The staining in the t-RET assay was thus overall subtler and it may thus be that in some cases a 2+ t-RET stained cell in the t-RET assay is similar to a 3+ stained cell in the p-RET assay but the 2+ staining did not reach the level required to be called 'positive'. The non-concordant samples came from a variety of different batches, so it is unlikely that the disparity can be explained by any technical problems with either assay.

Conversely the samples where the t-RET assay was positive and the p-RET was negative there was a clearer split; the T-RET samples were positive while the majority of the p-RET samples showed very low staining (total H-score <100), this is likely to be representative of true biological discordance where t-RET is present, but not activated or at best only minimally active.

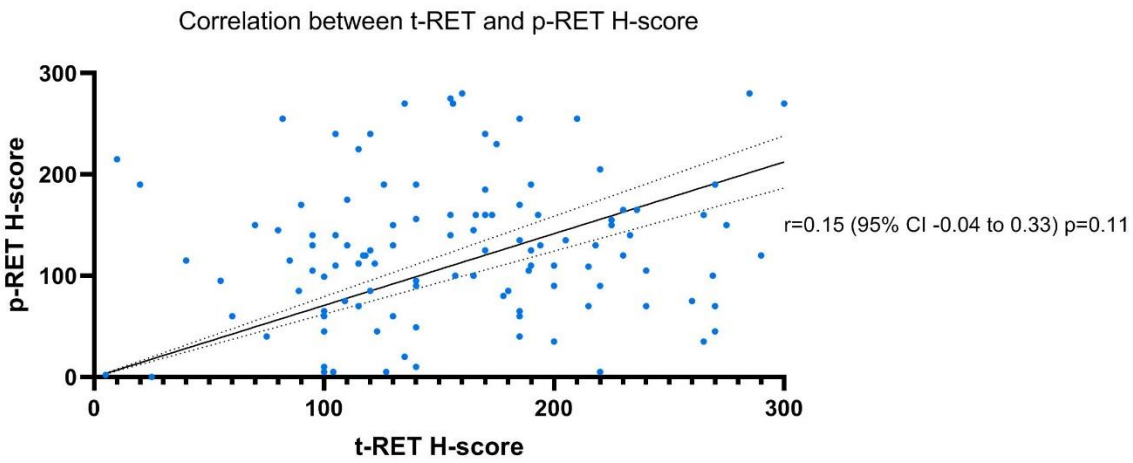


Figure 24 Correlation between t-RET and p-RET H-score. r=Spearman's rank correlation coefficient. p value is two sided with an alpha significance value of 0.05. Dotted lines represent 95% confidence interval.

3.5 t-RET and p-RET expression in metastatic samples

For 13 patients in the FURVA clinical trial the tissue block received represented metastatic disease. 6 were from lymph node samples, 4 from bone and one each from skin, lung and liver metastases. The analysis of these samples was performed separately from the primary tumour sample as the assay had not been optimised for use in these tissues and thus all results should be interpreted with caution.

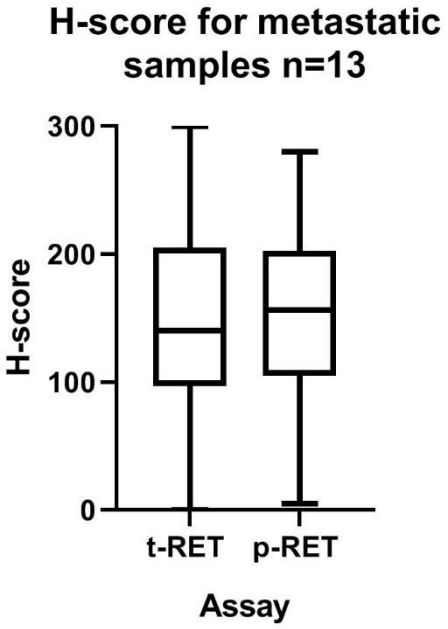


Figure 25 Box and whisker plot showing H-score for both t-RET and p-RET assays in metastatic FFPE samples

Overall, median t-RET scores were lower in metastatic samples than in primary tumours but the inverse was true for p-RET with the median scores being 140 for t-RET (155 in primary tumour) and 156 for p-RET (120 in primary tumour) (Figure 25). This may reflect prominence of such signalling in an adverse, AI resistant metastatic cohort. However, due to the very small number of samples it is difficult to conclude that such metastatic samples conclusively express more t-RET or p-RET than primary tumour samples. Moreover, there were no matched samples from the same patients available for direct staining comparisons because the specimen collection was not designed to assess change in RET expression over time. Feasibly, the change in median scores may also be due to the assay performing differently on non-primary breast tumour FFPE materials. Due to the very small numbers of samples from metastatic lesions the assay was not optimised for the different types of tissue in which the metastatic deposits were found. In future experiments it would be interesting to compare RET expression at different points during patient's disease course.

3.6 Chapter Discussion

This chapter has described the characteristics and performance of two immunohistochemical assays in FURVA clinical trial samples that had been developed and validated by ZH for this thesis; one to assess t-RET protein expression and one to detect p-RET. Both have been applied to a series of 115 breast primary tumour samples and 13 metastatic samples from participants in the FURVA clinical trial. While the FFPE blocks that sections have been cut from may be up to 20 years old and so storage may feasibly influence the integrity of the epitopes for the antibodies, the sections were cut and assayed within a month to minimise the ageing of the cut sections which adversely affect the staining intensity (Mirlacher et al. 2004).

Both IHC assays allowed assessment of t-RET and p-RET protein expression levels in FFPE samples. The staining intensity varied between the patient samples and intratumoural heterogeneity was noticed too. It was noted that in both the t-RET and the p-RET assay the predominant location of staining was in the cytoplasm. For the two previously published t-RET assays in breast cancer tissue where images were included in the publication the staining exhibited was also cytoplasmic (Gattelli et al. 2013; Nguyen et al. 2015). RET is a transmembrane protein with a large intracellular domain (Morandi et al. 2011) however, it has been noted that during an IHC assay the target epitope can move location within the cell due to the breakdown of some cellular components either during initial fixation or during the assay itself.

Two published scoring systems for the detection of t-RET using immunohistochemistry were applied to the data generated from the FURVA clinical trial breast cancer samples. When either system was applied over 80% of samples were ‘positive’ by the Gattelli method (Gattelli et al. 2013) and over 50% by the Nguyen method (Nguyen et al. 2015). Both datasets in the published literature used samples taken from unselected primary breast cancers. All samples from the FURVA dataset were from patients who had ER+ disease and had gone on to developed endocrine resistance hence they

were a group who were likely to have more aggressive disease and thus perhaps higher levels of t-RET expression detectable by IHC.

Three approaches for categorising samples into RET 'positive' and RET 'negative' for each assay were examined. These categories, along with retrospective cut point analysis using the t-RET and p-RET scores as a continuous variable will be correlated with clinical outcome in Chapter 6. The cut offs for the groups were selected to try and identify patients that may respond better to treatment with the RET inhibitor vandetanib; i.e. those who are the strongest expressers of t-RET or with more substantial p-RET signalling activity. One might hypothesise that p-RET positivity will be a better predictor of response to RET inhibition than t-RET (although ultimately this is dependent on the assay performance which can be more difficult for phosphor-specific epitopes in FFPE material (Pinhel et al. 2010). This will be examined in Chapter 6.

The prolonged timeframe needed so all of the trial samples collected during the course of the clinical trial meant that creating a TMA or running all samples with the same batch of primary antibody was not possible for this thesis work. Two techniques were used to try to assess and minimise batch variability.

Firstly, a positive control breast cancer slide was run with each assay. In both the t-RET and the p-RET assays the control slide was then scored alongside the FURVA samples in each batch and the scores compared. The positive control slide for the t-RET assay showed greater reproducibility than that for the p-RET assay. This is likely due to the size of the control slide sample (~1cm^2) and the fact that it stained homogeneously. The positive control sample selected for the p-RET assay was much larger (~4cm^2) and included a variety of different cells including areas of ductal carcinoma in situ (DCIS) which tended to stain very strongly, this meant that scoring it was more difficult due to the heterogeneity of both tissue types and staining patterns. However, despite the challenges the p-RET positive control sample posed the mean score for the control slides over 15 batches was 205 and only one control slide scored more than 50 points lower than this.

Secondly, mean H-score per FURVA sample batch was calculated. This was a cruder method as the batch sizes were not equal due to sample availability but did act as a useful second comparator if the positive control score was a little higher or lower than expected, as occurred in one batch of both the t-RET and p-RET assays. On balance, the assay performance was adequate for both the t-RET and p-RET assays, considering both the control and sample batch performance. With the benefit of hindsight, I would have selected a smaller, more homogeneously staining positive control breast cancer sample for the p-RET assay.

Both assays were developed and tested on freshly cut but historical FFPE breast cancer samples collected as part of the FURVA clinical trial. The process of tissue fixation is known to cause challenges requiring stringent unmasking of the antigen of interest from historical samples. Multiple factors during the initial collection, fixation and long term storage of samples can adversely affect the ability to detect the antigen of interest (Xie et al. 2011). The samples collected as part of the FURVA trial were also collected in a variety of centres and at different time points over the last 20 years. It is thus likely that beyond the variability of each assay, that attempts have been made to control here, there are many more variables that occurred prior to the receipt of the samples that may be a caveat when interpreting these IHC assay results. However, there was no major difference in core biopsy versus standard sections for p-RET and t-RET staining so this is unlikely to confound the findings. This is important to note as discrepancies in immunostaining have been noted previously in different sample types for example HER2 status in core biopsies and resection tissue specimens (Wojnar et al. 2013).

Like all immunohistochemistry assays there is subjectivity in the scoring of samples. By having two clinicians review the samples separately and then come to a consensus agreement attempts have been made to minimise this. An alternative approach would have been for both scorers to have formally scored the samples blindly and then met to review cases. This was not practical given the time that was donated by FA was not funded as part of the FURVA trial. Correlation between the

scores generated by ZH and those finally agreed by ZH and FA was significant, slightly more so for p-RET than t-RET (r=0.88 and r=0.72 respectively) suggesting the assessment strategy employed was appropriate to the size and scope of the project. Assessment by consensus should ensure that the immunostaining data for the two markers is as robust as possible for the FURVA series.

3.7 Chapter Summary

Both the t-RET and p-RET assays detected staining of their target antigen in the majority of the FURVA clinical samples and staining was heterogeneous between and within patients, suggesting RET signalling is present (to a greater or lesser extent) in the primary tumours of many of those patients who developed endocrine resistance after treatment with an aromatase inhibitor. There was variation in staining patterns and H-scores between FURVA sample batches but by categorising scores into t-RET and p-RET 'positive' and 'negative' cohorts some of the potential for false positives or negatives will be mitigated. These broader categories should allow for pragmatic investigation of whether strong RET expression (either t-RET or p-RET) could act as a predictive biomarker for clinical response which will be investigated in Chapter 6.

4 Genetic variants in patients with ER+ breast cancers treated with fulvestrant and vandetanib within the FURVA clinical trial

4.1 Overview

This chapter focusses on the detection of genetic variants using next generation sequencing (NGS) with the Ion Ampliseq Cancer Hotspot Panel V2 (Thermofisher, Waltham, MA, USA) which uses 207 amplicons to cover hotspots in 50 cancer related genes and a custom designed hotspot panel focussing on common variants in *ESR1, GATA3, RET* and *LYN*. These panels will hereafter be referred to as CHPv2 and CUSTOM.

Both FFPE and cfDNA samples were sequenced with the CHPv2 while only cfDNA samples were sequenced with both the CHPv2 and the CUSTOM panel.

4.1.1 Materials

The samples used were collected as part of the FURVA trial. DNA was extracted from 1) FFPE tissue samples and 2) plasma samples taken after the development of endocrine resistant metastatic disease. While there is significant overlap between the datasets with the majority of patients having both a FFPE tissue block and at least one plasma samples suitable for cfDNA extraction not all patients have both samples available.

Figure 26 shows the time course of disease and the maximum of four possible samples that were collected as part of the FURVA clinical trial. While Figure 27 shows a flow chart of the samples used in this chapter. The samples available and variants detected are also detailed in Appendix 3.

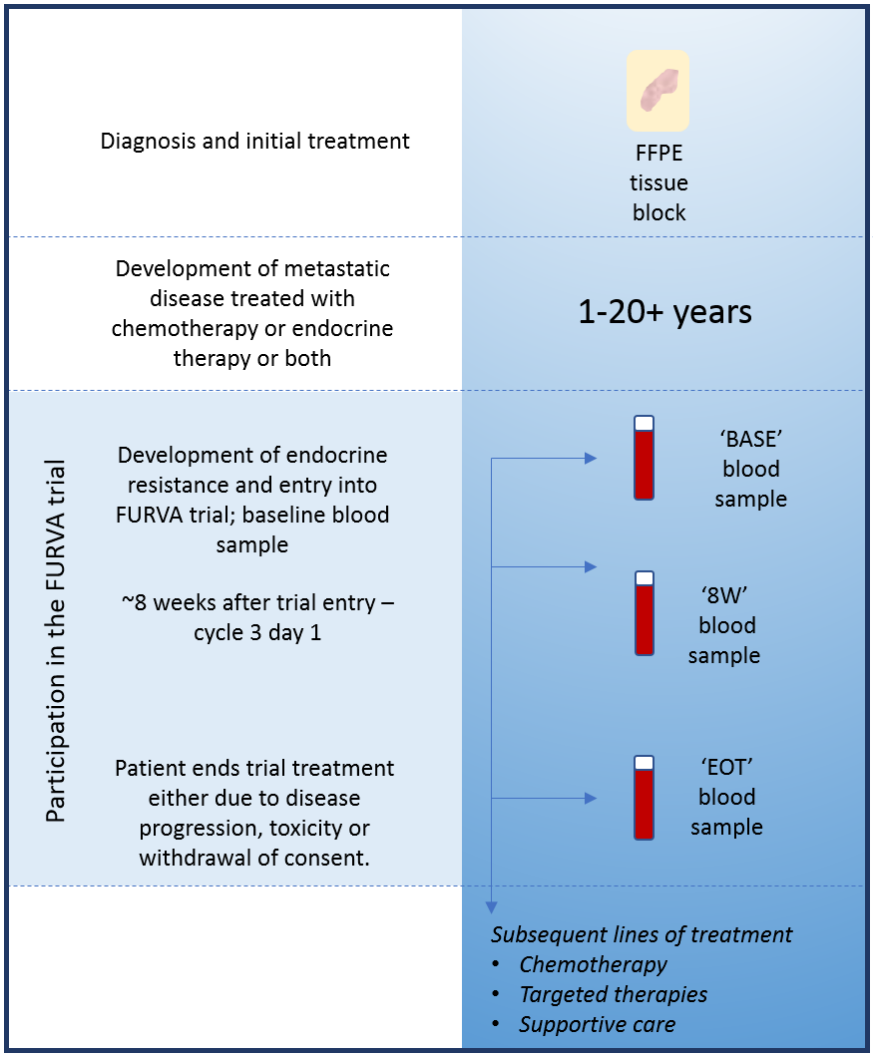


Figure 26 Infographic depicting the timeline of disease in patients recruited to the FURVA trial. There are four possible samples from each patient; one from their tissue block and three blood samples taken before, during and after treatment with fulvestrant and vandetanib.

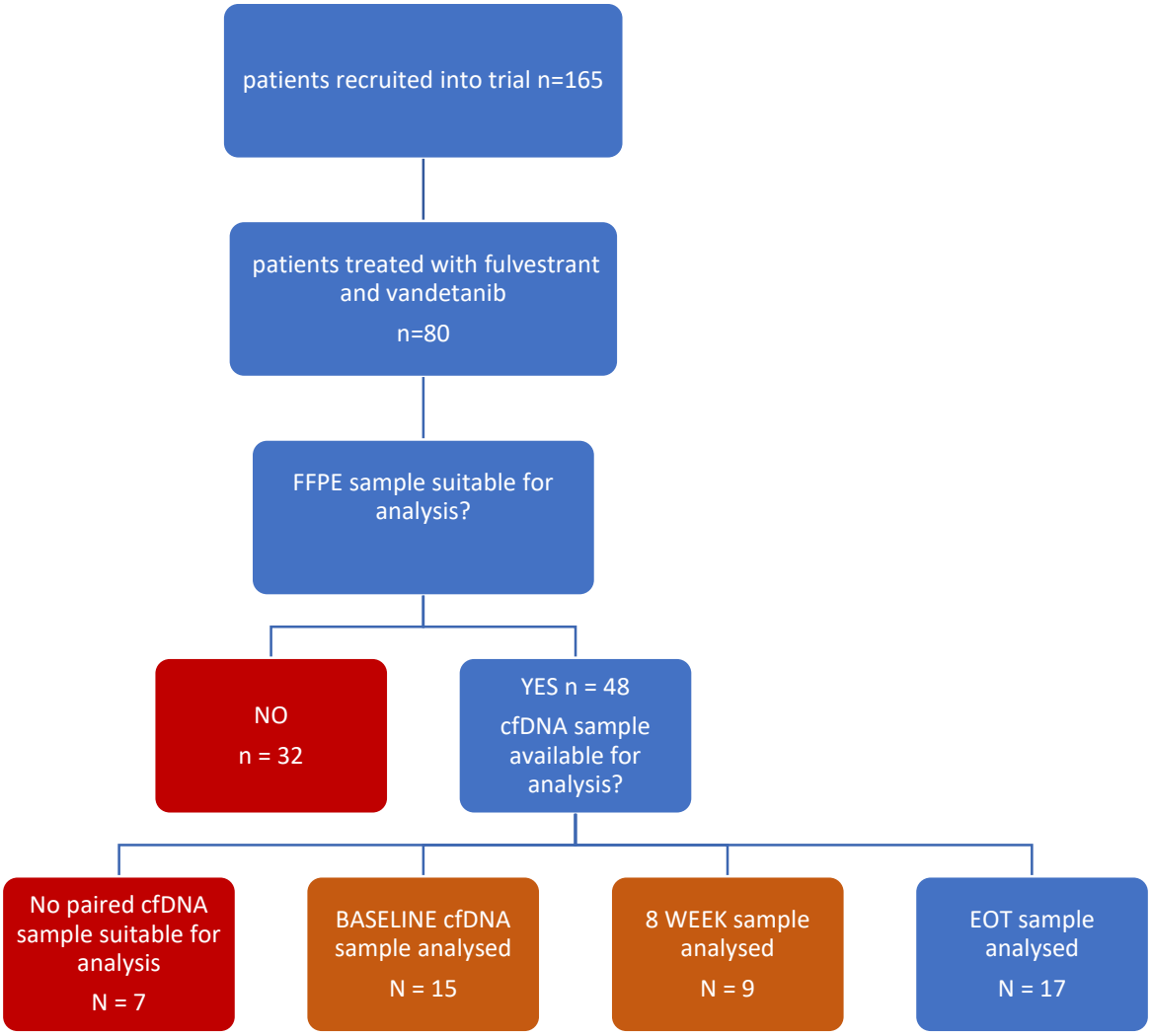


Figure 27 Flow chart showing samples used in this chapter. FFPE samples were run and then corresponding cfDNA samples were run if available. The aim had been to sequence cfDNA samples from the same time point but this was not possible due to sample availability.

4.1.2 Key methodology

Variants have been categorised according to the American consensus recommendation guidelines.

Variants are assigned a tier depending on their potential pathogenicity (Li et al. 2017a): The

summary table is shown in Figure 28.

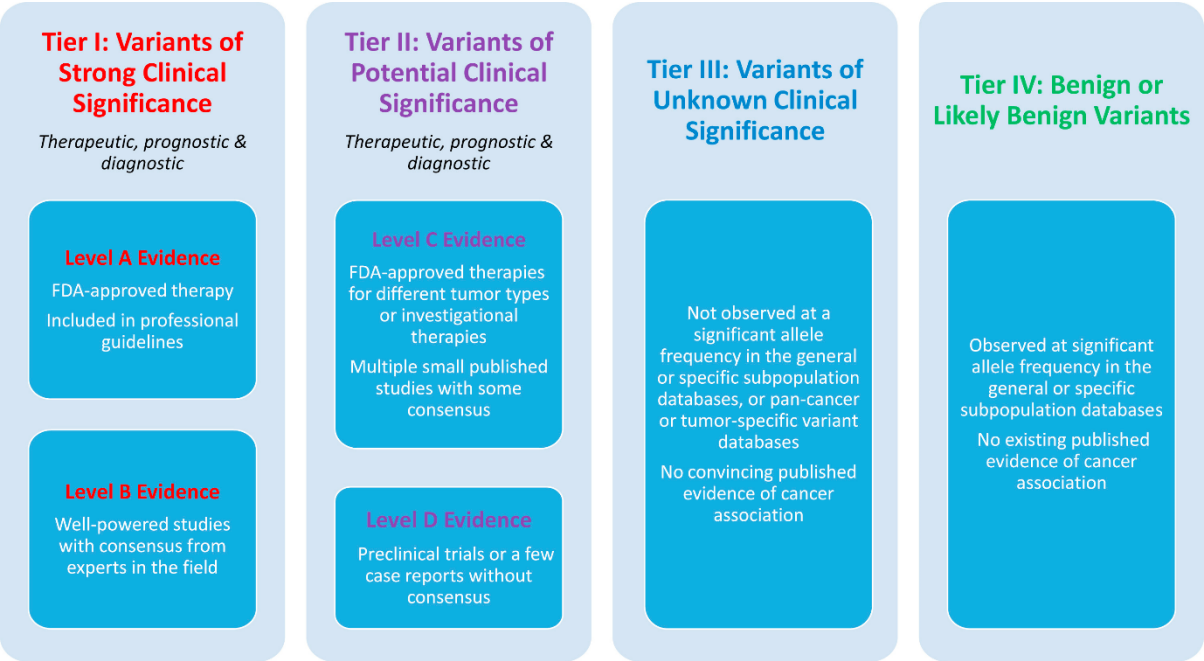


Figure 28 Classification of variants. Reproduced from Li et al, Standards and guidelines for the interpretation and reporting of sequence variants in cancer; A joint consensus recommendation of the Association for Molecular Pathology, American Society of Clinical Oncology, and College of American Pathologists (Li et al. 2017a)

Data will be presented on all tier I-III variants for both FFPE and cfDNA samples.

Key resources used and frequently referred to in this chapter include:

Catalogue of somatic mutations in cancer (COSMIC) http://cancer.sanger.ac.uk (Tate et al. 2019)

Over 4 million coding mutations curated from over 1 million tumour samples. Focus on somatic variants but also includes data on common SNPs

dbSNP http://www.ncbi.nih.gov/SNP (Sherry et al. 2001)

International collection of single nucleotide polymorphisms. For many SNPs the database contains population frequencies taken from large databases such as GnomAD.

ClinVar https://www.ncbi.nih.gov/clinvar (Landrum et al. 2018)

Publicly available database of human genetic variants containing curated clinical significance information.

CBioPortal http://cbioportal.org (Gao et al. 2013)

Open access resource allowing exploration of human cancer datasets offering access to a wealth of data from breast cancer specific studies including variant frequencies and clinicopathological variables including survival data. The main dataset used for reference in this chapter is the 'Breast Cancer (MSK, Cancer Cell 2018)' (Razavi et al. 2018). Other datasets used include the METABRIC breast cancer dataset (Pereira et al. 2016) and the TCGA dataset (TCGA 2012)

Mutation mapper, part of the CBioPortal toolkit, enables visualisation of variants within genes along with computational predictions of pathogenicity from SIFT and Polyphen-2. This has been particularly helpful when investigating potential pathogenicity of tier III variants. The protein locations come from Pfams latest database entries (http://pfam.xfam.org/ (El-Gebali et al. 2019)).

Two large genomic datasets have been used to draw comparisons to the data generated in this study. Both were accessed using CBioPortal. The first is the MSK-IMPAKT breast cancer dataset (Razavi et al. 2018) which consists mainly of patients with ER+/HER2- disease. Where subgroups of the dataset have been used this is clearly stated. Often it has been limited to patients documented to have ER+/HER2- disease. The other dataset used less frequently is the TCGA dataset as this contains predominately data from primary tumours and as such may not offer a robust comparison with the data from the cohort of patients in this project where all patients had developed endocrine resistant metastatic disease (Network 2012).

At the time of the analysis presented in this chapter there were no true tier I variants in breast cancer. Subsequent to the analysis presented here the results of the SOLAR-1 trial using the PIK3CA inhibitor alpelisib showed a benefit for patients specifically with ER+/HER2 negative *PIK3CA* variant

positive breast cancer making *PIK3CA* variants true tier I variants (André et al. 2019). The role of HER2 overexpression as assessed by immunohistochemistry or FISH has long been shown to predict benefit from HER2 directed therapies. However, the role of variants in *ERBB2* in patients whose tumours are HER2 negative by traditional methods remains under investigation. It may be that some variants result in resistance to certain HER2 directed therapies but sensitivity to others depending on the downstream effect of the variant (Gaibar et al. 2020); throughout this chapter *ERBB2* variants will be deemed tier II variants.

4.1.3 Chapter Aim

To establish the genetic variant profile of FFPE primary tumour samples and cfDNA samples representing metastatic endocrine resistant disease of breast cancers treated with fulvestrant and vandetanib using next generation sequencing.

4.1.4 Chapter Objectives

To establish the genetic variant profile of primary breast cancer tumours from patients who have gone on to develop metastatic endocrine resistant disease using NGS analysis of DNA extracted from FFPE tumour samples using the CHPv2.

To determine the genetic variant profile of breast cancers in patients who have developed endocrine resistant metastatic disease by NGS analysis of cfDNA extracted from plasma samples using the CHPv2 and CUSTOM panels.

Within the limits of sample type and availability, to compare the genetic variants present in primary and metastatic tumour.

To investigate the potential pathogenicity of tier III variants discovered using the techniques above and to establish if any tier III variants are of potential interest for further investigation in the context of the FURVA clinical trial

4.2 Investigating the genetic variant profile of primary breast cancer tumours in patients who have gone on to develop endocrine resistance; analysis of primary tumour FFPE samples

4.2.1 Samples and DNA extraction

FFPE samples were collected as part of the FURVA clinical trial. Thus, this was a group of patients who had developed metastatic disease that had become resistant to endocrine therapy. Some FFPE blocks contained tumour resection samples while others contained core biopsies. Blocks may have been collected up to 20 years previously.

The samples used in this chapter all came from patients who had received treatment with fulvestrant and vandetanib during the FURVA clinical trial. These samples were prioritised over samples from the fulvestrant and placebo arm as they would give greater insight into biomarkers of response to vandetanib should the trial return a positive result. The results of the trial were not known at the time of the analysis reported in this chapter.

51 primary tumour FFPE samples were identified for DNA extraction. Review by a pathologist allowed an estimate of tumour percentage (TP) and the area of the section with highest tumour content was macro-dissected. Two samples had tumour content <20%. Due to the small number of samples available no cut off for tumour percentage was set and all samples proceeded to DNA extraction. A single sample failed extraction with an extracted DNA concentration of 0.09ng/µL. All other samples proceeded to library preparation (n=50). Two samples failed library preparation due to undetectable final library concentration; both had low extracted DNA concentrations (0.13 and 0.15 ng/µL). This left 48 samples suitable for next generation sequencing. Of the 48 sequenced samples median tumour content was 60% (range 8%-90%). The median concentration of DNA extracted was 6.2ng/µL (range 0.25-60ng/µL). Figure 29 shows this data in graphical form along with data regarding final library concentration and number of variants detected per sample.

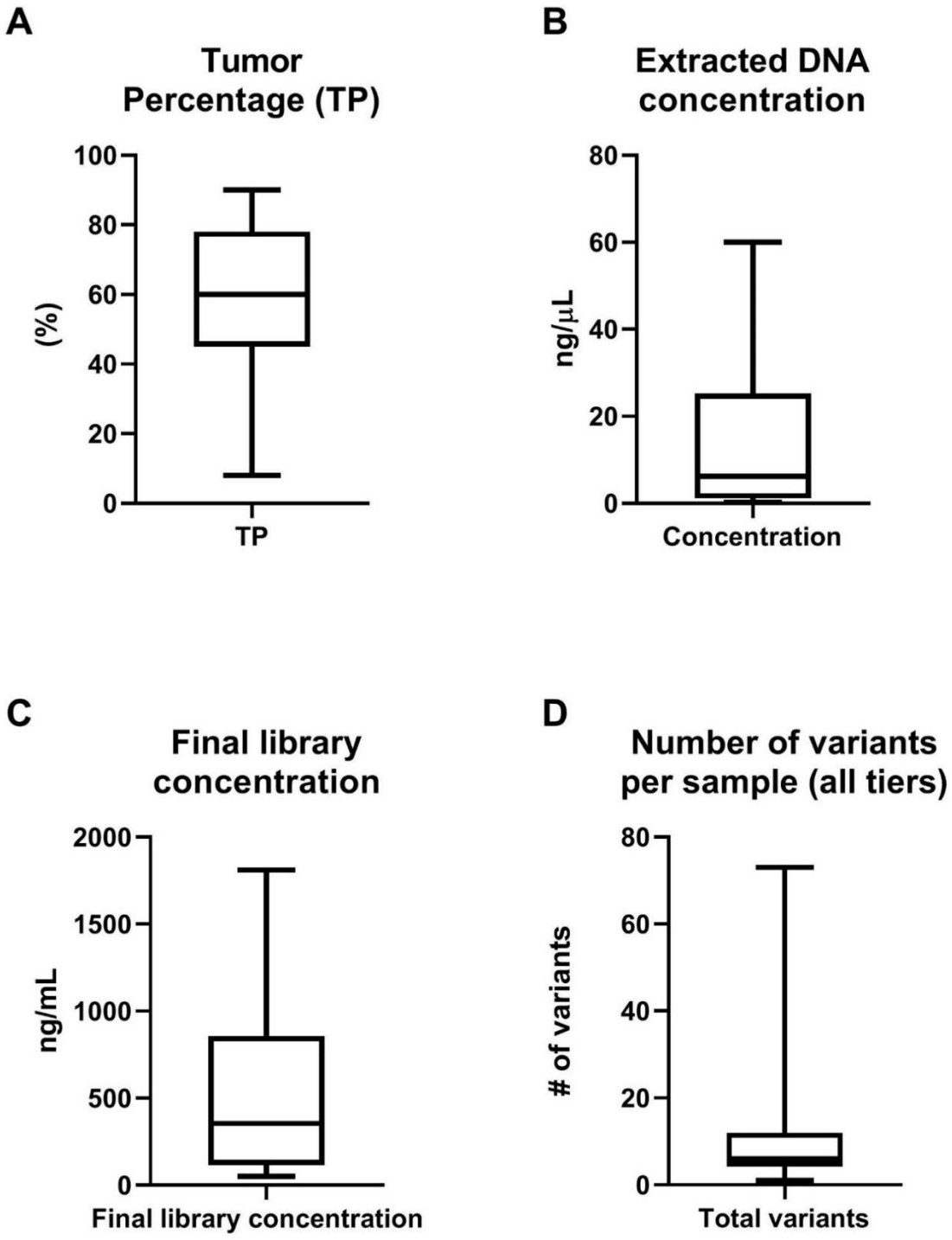


Figure 29 Four box and whisker plots demonstrating data regarding sequenced FFPE primary tumour samples n=48. The box shows the interquartile range and is bisected by a line representing the median value. The whiskers demonstrate the upper and lower limits of the variable shown. Plot A shows tumour percentage. Plot B shows extracted DNA concentration. Plot C shows final library concentration. Plot D shows number of variants per sample (all tiers I-IV). All graphs created using GraphPad Prism 8 v.8.1.1.

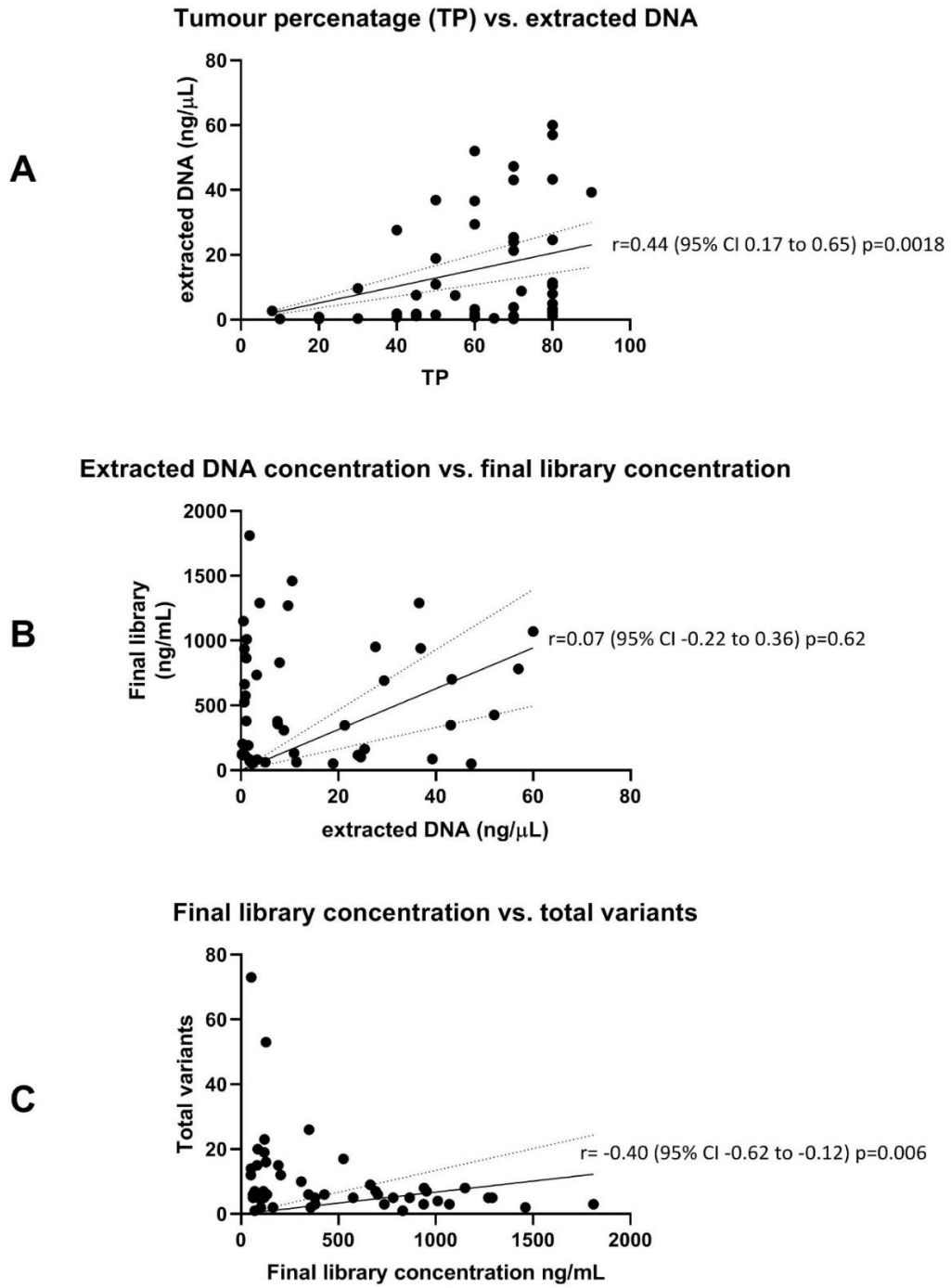


Figure 30 Three graphs exploring correlations between tumour percentage, extracted DNA concentration, final library concentration and number of variants detected per sample. Graph A shows a positive correlation between tumour percentage and extracted DNA concentration. Graph B shows no correlation between extracted DNA concentration and final library preparation. Graph C shows a negative correlation between final library concentration and total number of all tier non-synonymous variants detected per sample. r = Spearman's Correlation coefficient. P values are given based on two-tailed analysis with an α value of 0.05. All graphs produced using Graphpad Prism 8 v.8.1.1.

Figure 30 explores the relationships between the original FFPE sample and the final number of all tier non-synonymous variants detected. Graph A shows a positive correlation between tumour percentage and extracted DNA concentration. Higher tumour percentage samples generally result in higher extracted DNA concentrations. This would seem intuitive although there is always a margin of error when determining tumour percentage and it can be subjective. Graph B however shows no correlation between the extracted DNA concentration and the final library concentration. The library preparation process is designed to ensure only high-quality DNA covering the regions of interest remains for sequencing. The lack of correlation between extracted DNA concentration and final library preparation would suggest that extracted DNA concentration is not a surrogate for the quality of DNA available in the sample. Furthermore, there may have been technical variation between library preparations such as residual ethanol inhibiting PCR processes or over drying of beads resulting in difficulties returning selected DNA fragments to solution. This is further explored in graph C where there is a negative correlation between final library concentration and number of variants called. The lower the final library concentration the higher the number of variants called. This is explored further in section 4.2.2.

4.2.2 Sequencing, bioinformatic analysis and overview of variant detection in primary tumour FFPE samples

Sequencing data was generated from 48 samples using the Ampliseq CHPv2 NGS panel. Bioinformatic analysis was automated using predefined and validated workflows allowing the manual interpretation of variants to begin at the point of generation of annotated variant call files which were viewed in Ion Reporter version 5.10 (Life Technologies), a cloud based graphical user interface. Limits incorporated into the bioinformatic pathway included down sampling of 2000 reads, variant allele frequency (VAF) of ≥1% and <95% for FFPE samples Phred Quality score ≥20 (Ewing et al. 1998). All non-synonymous (i.e. those that result in a change of amino acid) variants were manually reviewed using Integrated Genome Viewer (IGV), a genome browser (Robinson et al. 2017). All analysis was performed using GRCh37 (hg19) as the reference genome.

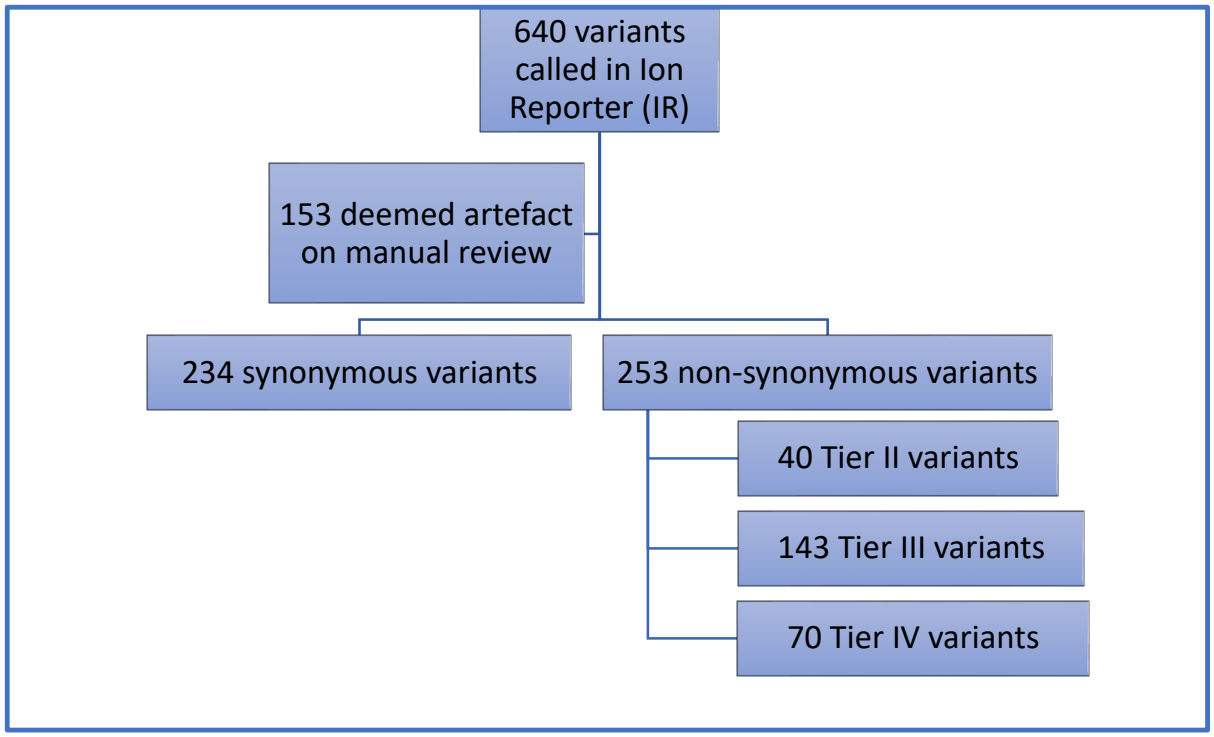


Figure 31 Flow chart showing breakdown of variants detected in FFPE primary tissue samples n=48.

Figure 31 shows the breakdown of variants detected by the automated bioinformatic pipeline in IR. Of the 640 variants called by the pipeline 153 (24%) were deemed to be artefact on manual review. Of those remaining there were 234 synonymous variants (i.e. variants not resulting in an amino acid change) and 253 non-synonymous variants. The non-synonymous variants were further categorised into tiers of clinical relevance as detailed in Figure 28. The mean number of all tier variants per sample was 10 (range 1-73, median 6) as shown in plot D Figure 29. Two outlier samples contained 73 and 53 all tier non-synonymous variants, both had low final library concentrations; 53ng/mL and 128ng/mL respectively. For these samples most variants were tier III variants with a variant allele frequency of <5%.

Overall, samples with lower final library concentrations had higher all tier non-synonymous variants. For example, the median number of variants called in samples with a final library concentration of <300ng/mL (n=21) was 5 compared to only 2 in samples with a concentration ≥300ng/mL (n=27). However, of the 13 samples with a final library concentration of <300ng/mL where a tier II variant

was detected the median VAF was 27% suggesting that despite suboptimal libraries and potentially poor-quality DNA input, significant variants can still be identified when present. Based on these findings it is reasonable to sequence low concentration libraries and interpret their results using the same bioinformatic pipeline as other samples albeit having to manually review more low-quality variant calls.

4.2.3 Primary tumour FFPE samples tier II variants detected

Tier II variants are defined as those that are variants of potential clinical significance. This means that there is level C (FDA-approved therapies for different tumour types or investigational therapies or multiple small published studies with some consensus) or level D (pre-clinical trials or a few case reports without consensus agreement) evidence of their pathogenicity. In breast cancer *ERBB2* amplification is the closest to a tier I variant although this is usually determined by FISH or IHC rather than using NGS based copy number variation analysis. All other common variants are tier II. There is current discussion that *PIK3CA* variant status may become a tier I variant with regards to response to Alpelisib on the basis of the results of the SOLAR-1 trial (André et al. 2019).

Tier II variants that are common in breast cancer were discussed in Chapter 1 and will be re-referred to here.

31/48 (65%) of samples contained at least one tier II variant. 7/48 (15%) samples contained more than one tier II variant (detailed in Table 27). Of those 4/48 (8.5%) samples contained two variants while 3/48 (6.5%) contained three.

	Variant 1	VAF (%)	Variant 2	VAF (%)	Variant 3	VAF (%)
Patient 45	*PIK3CA*	39	*PIK3CA*	36	*PIK3CA*	37
Patient 19	*PIK3CA*	14	*ERBB2*	15	*GNAS*	4
Patient 80	*PIK3CA*	18	*AKT1*	8		
Patient 61	*TP53*	22	*PIK3CA*	22		
Patient 34	*TP53*	62	*APC*	3		
Patient 51	*TP53*	50	*AKT1*	37		
Patient 55	*RET*	3	*PTPN11*	3	*CDKN2A*	4

	TP53
	PIK3CA
	AKT1
	other

Table 27 Samples containing multiple tier II variants. With potential driver variants listed as variant 1. Note low VAF for all variants in the sample from Patient 55 meaning it is unlikely that any of these represent a driver variant.

The gene most frequently containing a tier II variant was *PIK3CA* (35% of samples), followed by *TP53* (17%), *AKT1* (15%), and *CDKN2A, RET, PTPN11, GNAS, ERBB2* and *APC* (all 2% of samples). Where multiple variants were present in a sample the 'driver' mutation (here determined by the variant with the highest frequency) was *PIK3CA* in 3/7 samples and *TP53* in 3/7 samples. In one sample

(Patient 55) three low VAF variants were present (*RET, PTPN11* and *CDKN2A*) making it unlikely that any were driver variants, and raising the possibility that all were artefact as the confident limit of detection for the panel was around 5%. This is summarised in Figure 32.

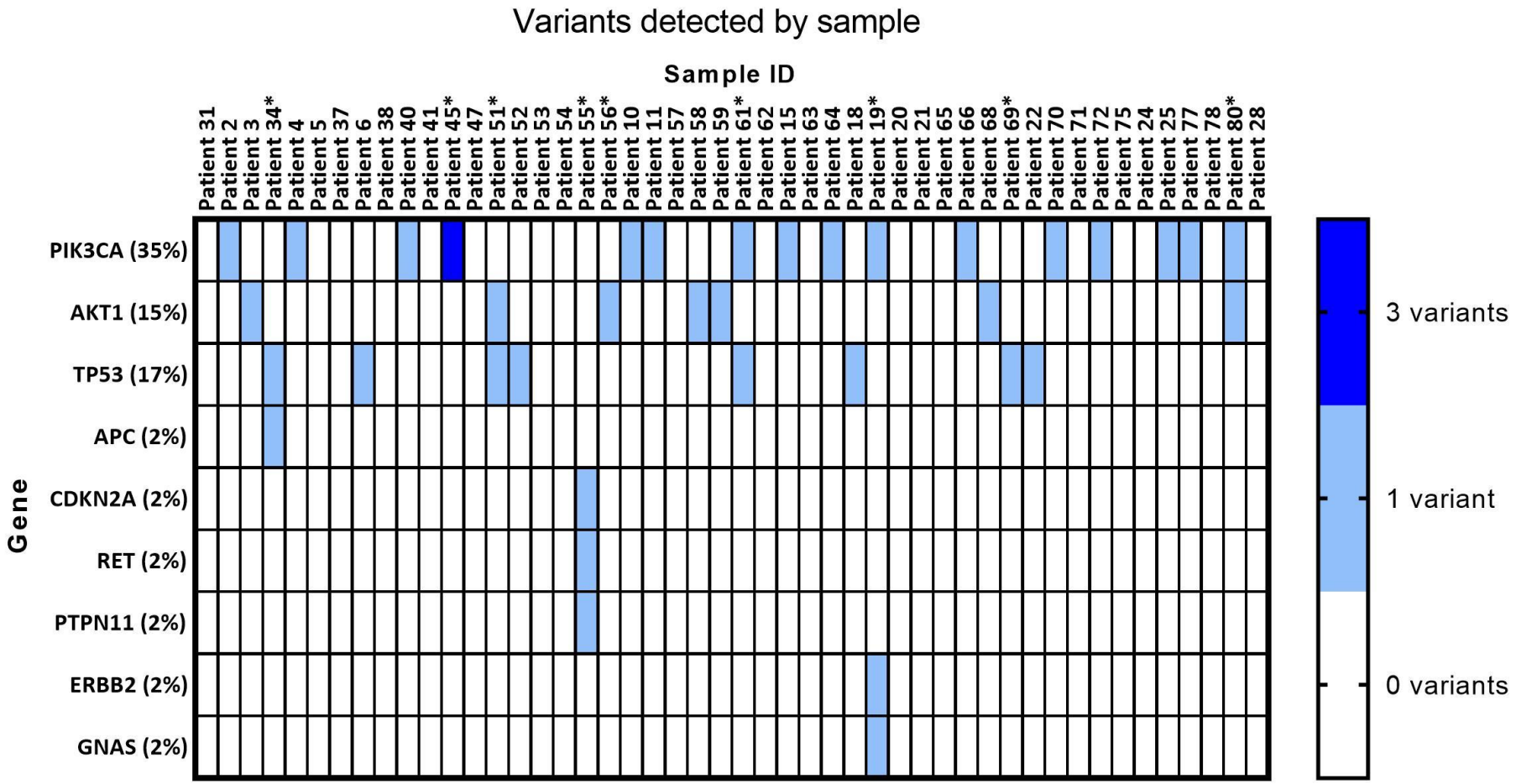


Figure 32 Primary tumour FFPE patient sample sequenced (n=48) along with tier II variants detected. Percentage by gene refers to the overall frequency of variants in that gene in this dataset.

*Sample ID's denoted with * are those with >1 variant detected n=8. A single sample had multiple variants in one gene (Patient 45 in PIK3CA). For variant details please see Table 28.*

4.2.4 Focus on specific variants

The targeted sequencing panel used in this project focuses on hotspot variants i.e. variants that have been noted to occur frequently in samples from cancer tissues. Generally, tier II variants are likely to be hotspot variants as they are well described and investigated. This can be seen in Table 28 and Figure 33 where all variants detected in *AKT1* were the commonly identified E17K variant resulting in an amino acid change from glutamic acid to lysine. Of the 19 variants detected in *PIK3CA* 15 were in the classic 'Exon 9' and 'Exon 20' hotspots. In this series only one patient had a variant in both *AKT1* (E17K) and *PIK3CA* (H1047R) (Patient 80), this is in keeping with other ER+ breast cancer datasets where variants in *AKT1* and *PIK3CA* are often mutually exclusive[10]. In *TP53* variants are less likely to cluster so variants tend to spread over the large DNA (193 amino acid) binding domain as well as throughout the rest of the gene. This can be visualised using lollipop plots (see Figure 33)

[10] When the MSK-IMPAKT breast cancer dataset was limited to patients with ER+/HER2- disease in CBioPortal and an analysis for mutual exclusivity between *PIK3CA* and *AKT1* was performed 276/621 patient samples contained a *PIK3CA* variant while 43/621 contained an *AKT1* variant. Only 7 contained both (Log2 Odds ratio -2.102 $p<0.001$).

Gene	Variant	Detected in *n* samples
PIK3CA	E542K	2
	N345K	1
	D1029H	1
	C420R	1
	E453Q	1
	E545K	4
	H1047R	8
	H419delR	1
TP53	R248Q	1
	P278L	1
	R158L	1
	C182*	1
	R337C	1
	L194R	1
	E285L	1
	C238Y	1
AKT1	E17K	7
APC	**G1120E**	1
CDKN2A	H83Y	1
ERBB2	L755S	1
GNAS	R201C	1
PTPN11	**S502L**	1
RET	**A883T**	1

*Table 28 List of specific variants organised by gene alongside their frequency of occurrence. Bold indicates variants that were detected at ≤3%. These are unlikely to be representative of driver variants. Amino acids are indicated by their single letter abbreviation. * refers to a nonsense variant resulting in termination of the protein.*

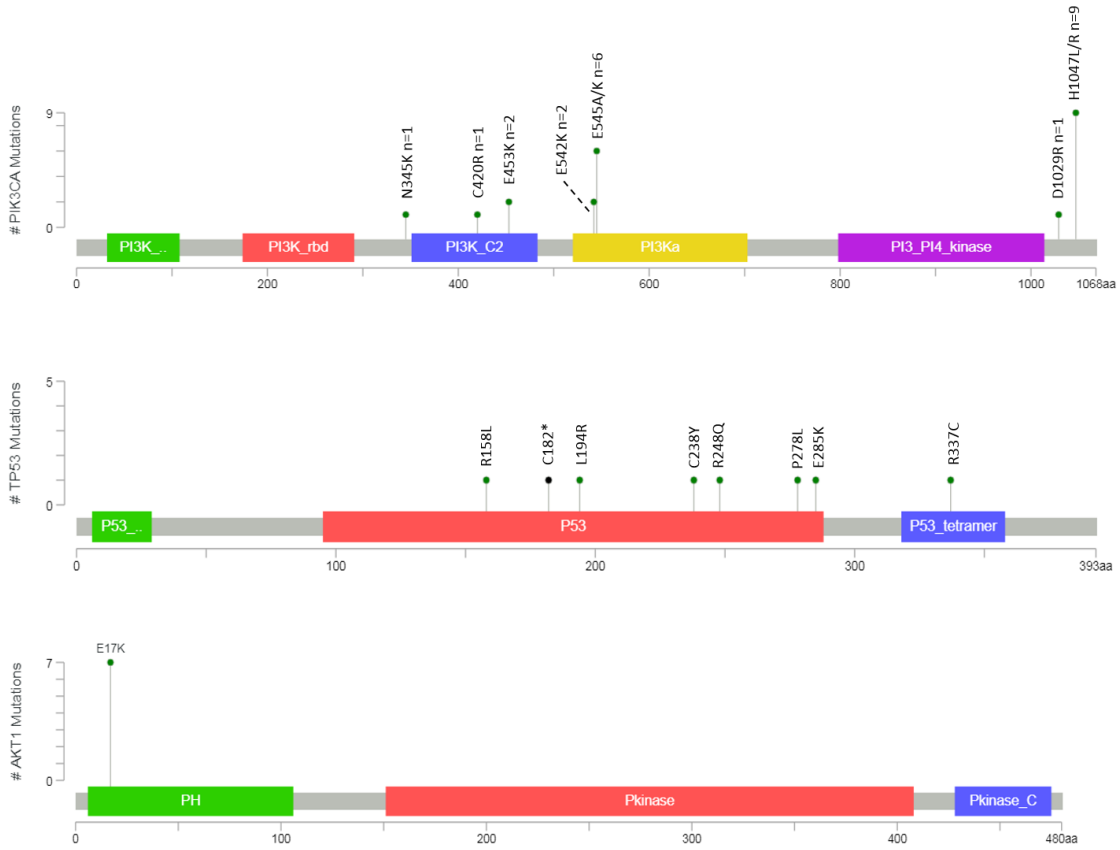


Figure 33 Lollipop plots showing position of detected tier II variants by gene. Each variant is denoted by a green (missense) or black (nonsense) dot. The length of the stalk relates to how frequently the variant occurred. This demonstrates the 'hotspot' nature of variants in PIK3CA (E545A/K and H1047l/R) and AKT1 (E17K). In comparison the eight variants detected in TP53 are spread throughout domains. Created using mutation mapper part of the CBioPortal online toolkit.

4.2.5 Comparing findings to current literature

Large scale sequencing projects have made data publicly available and searchable such as that contained in CBioPortal. The MSK-IMPACT breast cancer cohort (Razavi et al. 2018) can be interrogated to form a dataset which acts as a good comparator to the FFPE primary tumour samples used here. Using CBioPortal the original MSK-IMPACT dataset (n=1918) was limited to patients who were peri or post-menopausal and had been diagnosed with ER+/HER2- breast cancer and had gone on to develop metastatic disease (n=104). The MSK-IMPACT pipeline examines 410 genes in their entirety (compared to hotspots only in the panel used for the FURVA samples) and the

final dataset included all variants at a VAF >1% which are not known benign changes (i.e. all tier I-III variants). The dataset examines single nucleotide variants (SNVs), insertions, deletions and copy number variations (CNVs).

The data from the 104 primary tumours from the MSK dataset was compared to the data from the Ampliseq CHPv2 used in this project. Genes that contained variants in the MSK-IMPACT dataset at ≥3% VAF but were not included in the 50 gene cancer hotspot panel were not included in the comparison (*FAT1, NCOR1, ARID1A, TBX3, RUNX1, MAP2K4, KMT2C, MAP3K1, GATA3*).

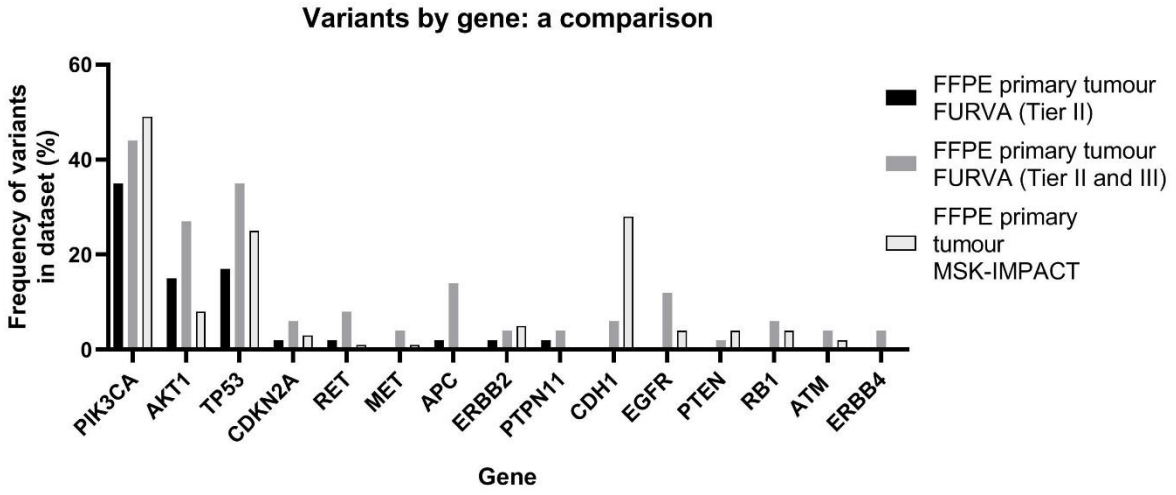


Figure 34 Comparing the frequency of variants by gene between the FURVA primary tumour dataset and a matched dataset from the MSK-IMPACT breast cancer cohort. The bioinformatic pathway for the MSK-IMPACT dataset is less stringent than that applied to the FURVA dataset so combining tier II and tier III variants in the FURVA dataset may offer a better comparison in some genes.

Figure 34 shows the frequency of variants by gene, firstly in the tier II variants in the FURVA dataset, then by tier II and III variants combined and finally by the MSK-IMPACT breast cancer dataset. The bioinformatic pathway in the MSK-IMPACT dataset was more lenient than that which was applied to determine tier II variants in the FURVA dataset in particular with regards to known hotspot variants; the MSK pipeline permitted inclusion of all non-synonymous variants that were not known to be benign to be included in the final reports (Cheng et al. 2015).

This is demonstrated in *PIK3CA* where around 35% of samples in the FURVA dataset contained a tier II variant. Whereas the frequency in the MSK dataset was 49%. However, if both tier II and III variants in the FURVA dataset are included then PIK3CA variants are present in 44% of the FURVA samples. In *TP53* tier II variants were found in 15% percent of samples whereas if tier III variants are included the frequency increased to 35%. The rate of *TP53* variants in the MSK dataset was 25%. With the FURVA combined results being higher than those in the MSK-IMPACT dataset there is a possibility that the interpretation of some of the low VAF tier III *TP53* variants in the FURVA dataset was too lenient.

There are several genes where the frequency of variants is notably different between the FURVA and MSK datasets. Firstly, in *AKT1* even without combining tier II and III variants the rate of *AKT1* variants in the FURVA dataset was 15% compared with 8% in the MSK-IMPAKT dataset. The VAF of the variants was above 20% for 5/7 variants while the other samples had VAF of 4 and 8% respectively. Although the region is only covered by a single amplicon in the Ampliseq CHPv2 it sequences cleanly.

The level of *CDH1* variants in the MSK-IMPACT dataset was high with 28% of patient samples containing a variant making it the second most frequently mutated gene. This is at least partially explained by the limited coverage of *CDH1* in the Ampliseq CHPv2 panel where only 7 hotspots were covered by 3 amplicons (12% of gene covered). In the MSK-IMPAKT dataset variants in *CDH1* were spread throughout the gene.

Given the significant role that the PI3K pathway plays in ER+ breast cancer one might expect a higher frequency of variants in *PTEN*, one of AKTs key regulators. However, in both datasets the rate of variants in *PTEN* was below 5%. Even in expanded datasets encompassing all stages and histological diagnoses of breast cancer the rate only increases to around 7%. *PTEN* is reasonably well covered in the Ampliseq CHPv2 with amplicons covering 70% of the gene, thus it might have been reasonable to expect one or two *PTEN* variants in the cohort of 48. *PTEN* has been noted to be a difficult gene to sequence due to the presence of a pseudogene {Claes, 2014 #363}.

4.3 Investigating the genetic variant profile of metastatic ER+ breast cancer in patients treated with fulvestrant and vandetanib using cfDNA

4.3.1 Samples and DNA extraction

For each patient who had already had a tissue sample sequenced DNA was extracted from the last available corresponding plasma sample if available. 41/48 patients who had had an FFPE tumour available for sampling has a cfDNA sample suitable for sequencing; 17 'end of treatment' (EOT) samples, 9 '8 week' samples and 16 'baseline' (BASE) samples. In the time course of the patients' disease the BASE samples represent a snapshot of the disease after the initial diagnosis, adjuvant endocrine therapy and up to one line of chemotherapy. Then, depending on where in the trial treatment they were taken may also represent the disease during (8 week) or after treatment with fulvestrant and vandetanib (EOT). Ideally the same time point for each patient would have been sequenced but due to sample availability this was not possible. Samples were taken at sites around the country and sent to the All Wales Genetics Laboratory in Cardiff for processing. Samples that arrived after the 96-hour cut off were not processed. Other trials, particularly those based in a single centre have had much stricter processing times with many samples processed within hours rather than days.

All plasma samples apart from the 8-week sample would have been taken when the patients' disease was progressing on its current treatment. At baseline this would have likely been hormone therapy, while at EOT this would have been fulvestrant and vandetanib. To be eligible for entry to the FURVA trial patient's disease needed to be progressing but without evidence of visceral crisis which would necessitate treatment with chemotherapy. This means that patients who had a higher disease burden or potentially had more aggressive disease may not have been included in the trial.

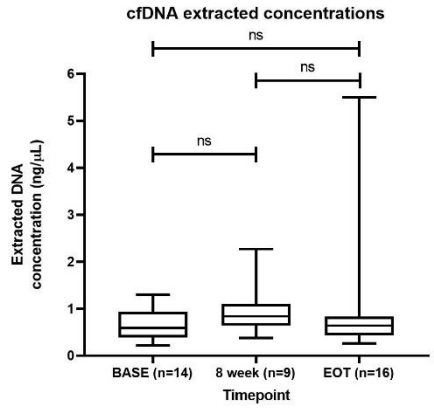
Extracted DNA concentrations varied widely between samples. Where possible DNA was extracted from 2mls of plasma (n=35), alternatively 3mls were used for two patients who had >4 vials of plasma available while only 1ml was available for four patients. In Figure 35 the two cfDNA samples

extracted from 3mls of plasma were removed from the analysis as they were significant outliers with extracted DNA concentrations of 56ng/µL and 45.6ng/µL.

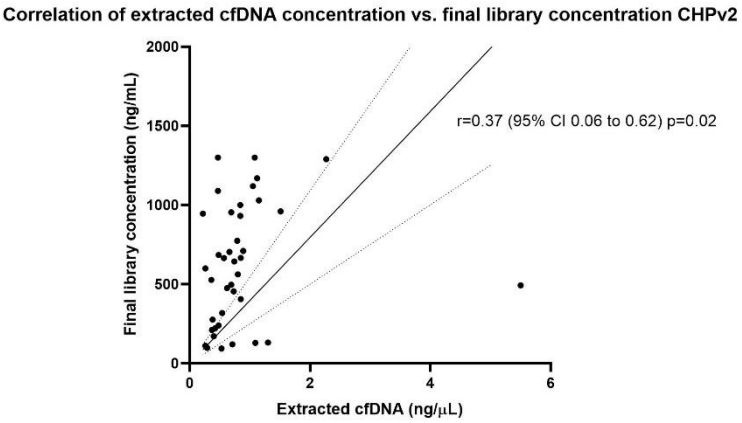
Figure 35 graph A shows cfDNA extracted from plasma taken at baseline (entry into the FURVA trial) had a lower extracted concentration than that taken at 8 weeks or EOT. Median baseline concentration was 0.70ng/µL compared to 0.84 ng/µL and 0.62 ng/µL for 8 week and EOT respectively. The range of concentrations increased as time progressed with the range at baseline being 0.22-1.3ng/µL, while 8 week was 0.38-2.27ng/µL and EOT greater still at 0.26-5.5ng/µL. Analysis here is complicated by the fact that some or all of the 8 week samples may actually represent EOT samples that have been incorrectly labelled, if even a couple are actually EOT samples then these patients are very poor responders and this may account for why the median extracted cfDNA concentration is highest in the 8 week group. The range of the EOT concentrations is likely to account for why the median values across time points are lower in EOT than baseline samples (0.62 vs 0.70ng/µL).

In Figure 35 extracted cfDNA concentration is compared to the final library concentration for the CHPv2 panel (graph B) and the CUTSOM panel (graph C). In both cases the higher the DNA input amount in nanograms the higher the final library concentration, more markedly for the CUSTOM panel. This may be due to the higher number of PCR cycles used in the custom panel. The correlation between extracted DNA concentration and final library concentration is stronger for cfDNA (r=0.37) than DNA extracted from FFPE primary tumour tissue (r=0.07). This is likely to be due to the better quality and sample storage and collection of cfDNA samples and potentially more uniform sample collection, processing and storage prior to DNA extraction.

A



B



C

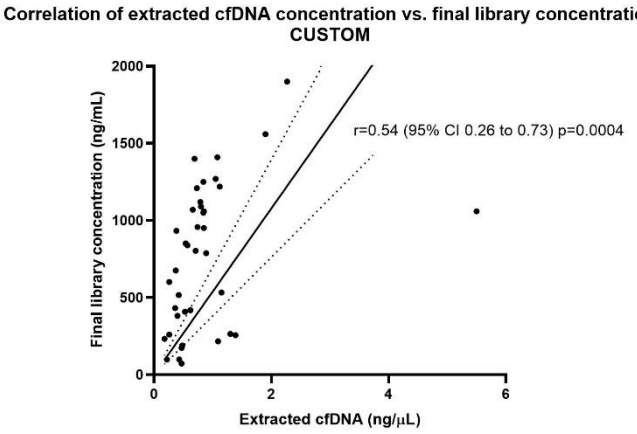


Figure 35 Three graphs exploring extracted cfDNA concentration (Graph A) and final library preparation concentrations for CHPv2 (Graph B) and CUSTOM (Graph C). Even after removal of two major outliers in the dataset the range of extracted cfDNA concentrations is still greater in EOT samples compared to those taken at baseline or 8 weeks. In both panels (CHPv2 and CUSTOM) the DNA input correlates significantly with the final library concentration. P values are given based on two-tailed analysis with an α value of 0.05.. r=Spearman's correlation coefficient. ns = not significant. Comparisons of cfDNA concentrations (Graph A) performed using one-way ANOVA test. Graphs created using GraphPad Prism 8 v.8.1.1

4.3.2 Sequencing and bioinformatic analysis

Bioinformatic analysis was automated as per methodology used for the FFPE samples (see 0). Variants were called if the VAF, as determined by down sampling to 2000 reads, was ≥1%. Data from the CHPv2 and CUSTOM panel were analysed using separate bioinformatic pipelines due to the need for different reference files. Once variants had been called by Ion Reporter, they were pooled to create a single dataset of variants for each patient.

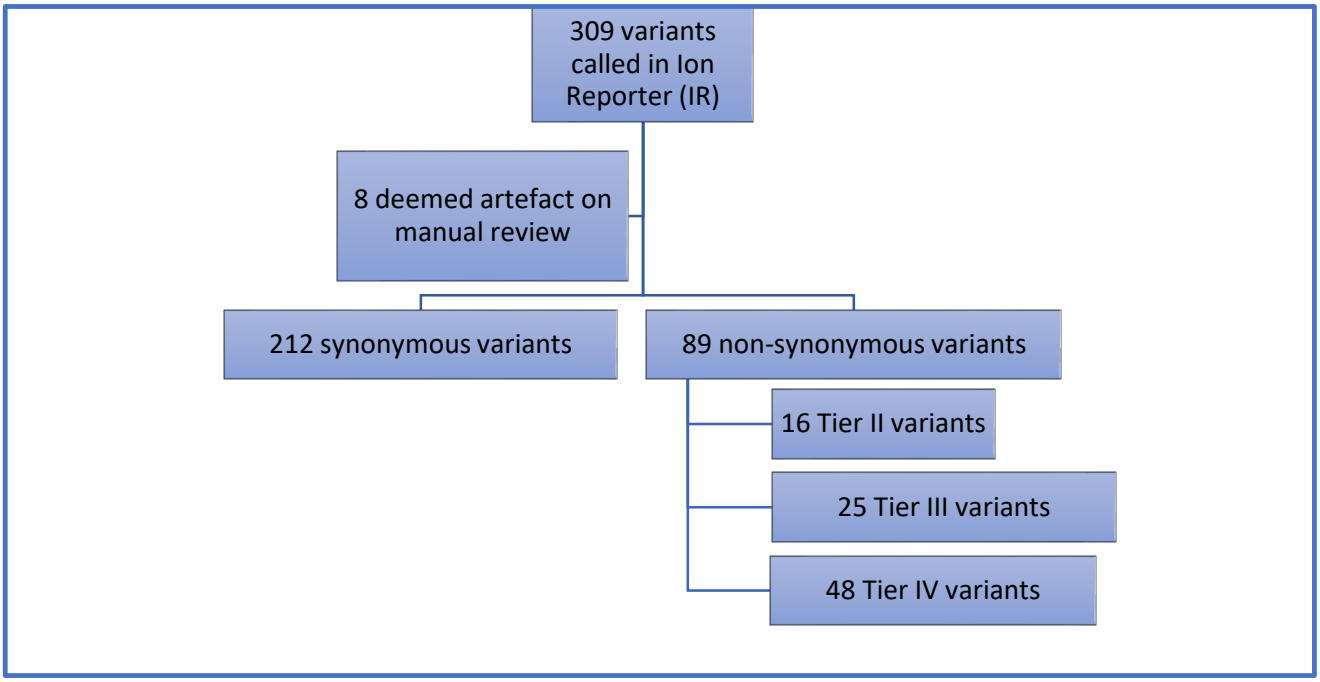


Figure 36 Flow chart showing breakdown of variants detected in cfDNA in the setting of endocrine resistant metastatic disease n=41

Very few artefact variants were called by Ion Reporter in cfDNA samples (as shown in Figure 36). Over half of all variants called were synonymous variants. Of the 89 non-synonymous variants 16/89 (18%) were tier II variants. The number of variants called overall was less than in the FFPE primary tumour samples across all tiers. The data reported in section 4.3.3 refers to variants detected by the automated bioinformatic pathway and manually checked using IGV, furthermore results were then cross checked with databases such as COSMIC and ClinVar to assign variants to the correct tier.

4.3.3 Metastatic endocrine resistant cfDNA tier II variants detected

The median number of all tier variants per sample was 2 (range 0-7). Overall, a tier II variant was detected in 11/41 (24%) of samples. Seven patient samples contained a single tier II variant, three contained two and a single sample contained three tier II variants as detailed in Table 29. In Patient 12 and 60 the variants were present at levels of 4-8%, that is to say that of all the DNA sequenced (both tumour and normal DNA make up the cfDNA) X% contains the variant. In comparison the variants in the sample from Patient 51 were present at much higher VAF suggesting that the cancer in this patient was shedding large volumes of DNA into the bloodstream. The VAF could also indicate that this was a tumour where the majority clone was one harbouring a variant causing significant impact on *TP53* function.

	Variant 1	VAF (%)	Variant 2	VAF (%)	Variant 3	VAF (%)
Patient 51	*TP53*	72	*AKT1*	49	*ESR1*	42
Patient 12	*ERBB2*	8	*PIK3CA*	4		
Patient 60	*PIK3CA*	4	*ESR1*	4		
Patient 22	*ESR1*	22	*GATA3*	50		

Table 29 Four samples containing multiple tier II variants

The genes containing variants were similar to those in the FFPE tumour samples, albeit with less variants detected overall. *ESR1* variants were not looked for in primary tumour samples as they are rarely found in primary tumours. Variant tracking work with ddPCR will determine at what point in the patient's disease course they became detectable. This will be covered in section 4.5. Of the four *ESR1* variants detected three occurred in samples where multiple variants were present. This may be due to the corresponding tumours shedding more cfDNA than others making any variant detection easier or one could hypothesise that the presence of another variant increases the change of an *ESR1* variant occurring or vice versa. Figure 37 summaries is the genes where variants were detected in cfDNA samples.

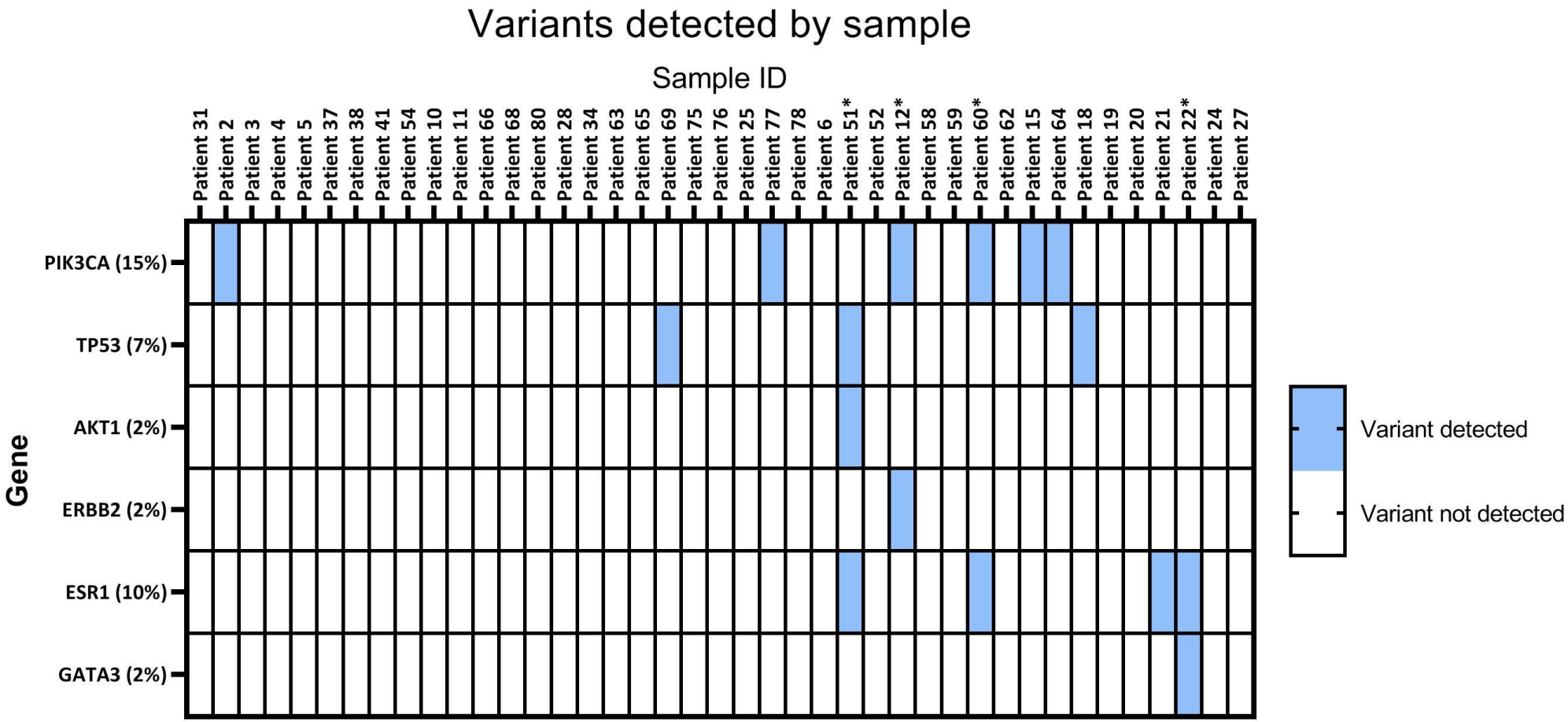


*Figure 37 Variants by cfDNA sample. Sample IDs marked with * indicate samples with variants present in multiple genes. The percentage of samples containing a variant in gene X is also shown.*

4.3.4 Focus on specific variants

Table 30 details the specific tier II variants detected in cfDNA samples.

Gene	Variant	Detected in *n* samples
TP53	p.Glu285Lys	1
	p.Arg158Leu	1
	p.Leu194Arg	1
PIK3CA	p.Glu542Lys	1
	p.Glu545Ala	1
	p.Glu545Lys	2
	p.His1047Arg	2
ESR1	p.Asp538Gly	2
	p.Tyr537Asn	2
ERBB2	p.Leu755Ser	1
GATA3	p.Arg367Ter	1
AKT1	p.Glu17Lys	1

Table 30 Variants by gene cfDNA tier II variants

Where variants were detected in cfDNA samples they were frequently the most well-known variants in key breast cancer genes. For example, in *PIK3CA* unlike in FFPE primary tumour samples only common variants in exon 9 and 20 were detected from cfDNA samples. The three *TP53* variants are also amongst the most common *TP53* variants detected in breast cancer.

The four *ESR1* variants detected are those that are most frequently detected in ER+ metastatic breast cancer, clustering around amino acid 537 (tyrosine) and 538 (aspartic acid). The *ERBB2* variant detected is the frequently occurring L755S variant. The *GATA3* variant is unusual in that it occurs at 50% VAF in cfDNA suggesting it could be a germline variant. This is corroborated by its presence in the primary tumour tissue sample with a VAF of 54%. Usually in these circumstances these variants tend to be benign unless the patient is known to have an inherited cancer predisposition disorder. In this case the variant is listed as pathogenic in ClinVar (rs104894164) with links to Barakat syndrome. Barakat syndrome is a vanishingly rare autosomal dominant inherited condition which usually presents with a triad of hypoparathyroidism, sensorineural deafness and renal disease. It is not known to increase risk of breast cancer (Barakat et al. 2018).

4.3.5 Comparing findings to current literature

While large databases of sequenced ER+ metastatic samples are available the majority have used FFPE biopsy samples for their genetic sequencing making comparisons of variant frequency with the data here difficult. With cfDNA samples one cannot equate absence of variant in cfDNA with absence of variant in tumour; it may just not be detectable either due to lack of ctDNA within cfDNA or ctDNA being present but the variant only being present in a sub-clonal population and thus not detectable when a limit of detection of ~1% is applied.

The CHPv2 Ion Ampliseq panel has been frequently used with FFPE samples proving reliable and effective at detecting variants. In some laboratories it is used for clinical diagnostics with a limit of detection of 3% (Rathi et al. 2017) providing at least 10ng of DNA input and a final library concentration of >100ng/ml. In other studies it has been used in a research setting to detect variants in a clinical breast cancer series (Bai et al. 2014) with a minimum DNA input of 50ng.

However, less has been published about its use with cfDNA samples. In the FURVA samples the median DNA input was 8.52ng (range 2.64-27.40ng) for the CHPv2 and 4.38ng (range 1.32-20ng) for CUSTOM; less than the recommended DNA input despite adding the maximum permissible volume to the library preparation kits.

Ion Torrent technology has been used to successfully sequence cfDNA samples from metastatic breast cancer patients but not in large series. For example, Nakauchi et al sequenced the exonic regions of *TP53* and *PIK3CA* at varying stages of disease treatment for 17 patients. Comparing the results from cfDNA to those obtained from sequencing tumour DNA. 8/17 tumour samples contained a variant in *TP53, PIK3CA* or both. Of these variants 7 were detected in cfDNA (Nakauchi et al. 2016). Frenel et al used the CHPv2 panel to monitor changes in variants over time in 39 patients with solid tumours who were participating in a phase I clinical trial. Amongst the 7 patients with breast cancer all had a detectable variant in tissue, concordance occurred in 6 patients. Five patients had the same variant detected in tissue and plasma (4 x *TP53*, 1 x combination *PIK3CA* and

KRAS). One patient had three variants detected in tissue but only one of these was detectable in plasma after treatment failure. The recruitment of patients from the phase I trial unit who had no available active cancer treatment options is likely to account for the high rate of variants detected (Frenel et al. 2015). These studies, while offering comment on the use of Ion Torrent sequencing technology, do not act as reasonable comparators to the samples sequenced here due to the different panels used, small numbers and significantly different stage of disease. Thus there is minimal published data to act as a comparator for cfDNA samples sequenced using the CHPv2 NGS panel.

4.3.6 Comparing tier II variants in primary tumour FFPE and cfDNA metastatic samples

The overall frequency of variants detected in the cfDNA samples was lower than the FFPE samples (see Figure 38). For example, variants in *PIK3CA* were detected in 35% of primary tumour FFPE samples but only 15% of metastatic cfDNA samples (20% if manually detected variants included). Manual detection of variants refers to specifically looking for a variant in the aligned read files (BAM files) using IGV genome browser to identify if a variant present in tissue was also present in cfDNA. In most cases these variants were not called by the bioinformatic automated pipeline because their frequency was ~1%. In *AKT1* the detection rate fell from 15% in FFPE samples to 2% in cfDNA samples. Again, in *TP53* variants were detected in 17% of FFPE primary tumour samples but only 7% of cfDNA samples (10% if manually detected variants included). *ESR1* variants were detected in cfDNA metastatic samples and are unlikely to have been present in the primary tumour samples due to what we know from the literature around the development of *ESR1* variants as a response to prolonged endocrine treatment (see Chapter 1). However, it is important to note they were not tested for in the primary tumour.

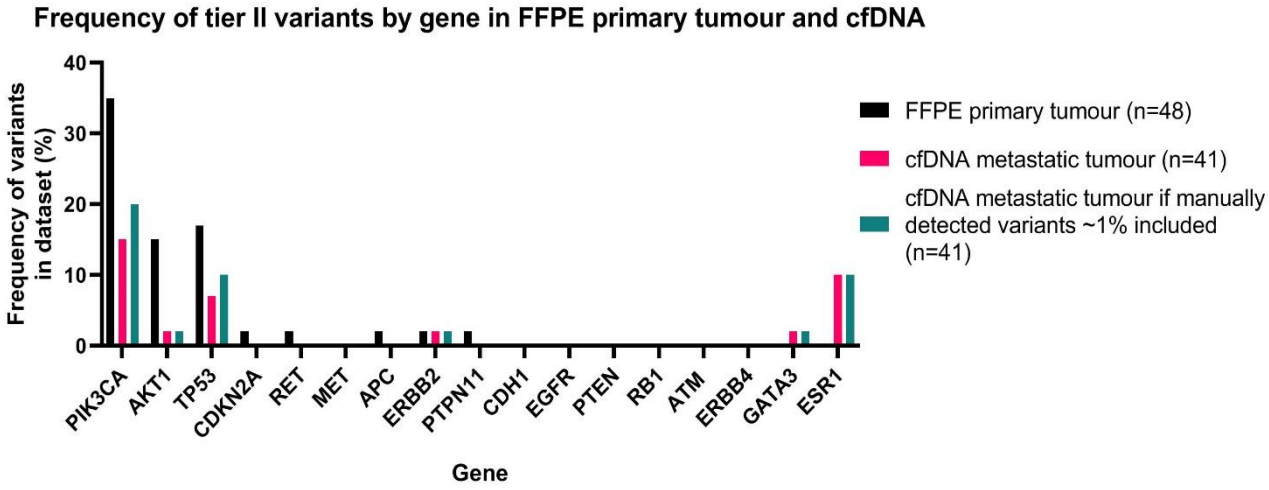


Figure 38 Frequency of variants by gene showing comparison in frequency of variant between FFPE primary tumour samples (black bars) and cfDNA metastatic tumour samples both with (green bars) and without (pink bars) the addition of manually detected variants ~1%

These differences warrant exploration. It is accepted that there is not 100% concordance between FFPE and cfDNA samples in the literature. For example, the MOSCATO trial recruited 283 patients with advanced solid tumours including 41 patients with breast cancer. The sensitivity of cfDNA analysis to detect variants using the CHPv2 panel identified in tumour was 49.9% (95% CI 44.6-55.1%) in variants with a VAF >1%. It was noted that the sensitivity increased with a greater number of metastatic sites (p=0.0006), decreased albumin level (p=0.0007) and number of previous treatment lines (p=0.047). (Jovelet et al. 2016). As the samples used in this study were from patients relatively early in their disease course this may partially explain why the concordance was low. Table 31 shows the concordance between variants detected in primary tumour and cfDNA.

	FFPE primary tumour			cfDNA plasma sample			
Patient	**Gene**	**Variant**	**VAF**	**Gene**	**Variant**	**VAF**	**Concordance**
Patient 2	*PIK3CA*	E542K	27	*PIK3CA*	E542K	6	100%
Patient 51	*TP53*	R158L	50	*TP53*	R158L	72	100%
	AKT1	E17K	37	*AKT1*	E17K	49	100%
Patient 52	*TP53*	C182Ter	56	***TP53***	**C182Ter**	**2**	100%
Patient 15	*PIK3CA*	E545K	27	*PIK3CA*	E545K	5	100%
Patient 64	*PIK3CA*	H1047R	69	*PIK3CA*	H1047R	75	100%
Patient 10	*PIK3CA*	H1047R	30	***PIK3CA***	**H1047R**	**1**	100%
Patient 11	*PIK3CA*	E542K	34	***PIK3CA***	**E542K**	**1**	100%
Patient 18	*TP53*	L194R	49	*TP53*	L194R	16	100%
Patient 69	*TP53*	E285K	32	*TP53*	E285K	33	100%
Patient 75	*PIK3CA*	E545K	23	***PIK3CA***	**E545K**	**1**	100%
Patient 25	*PIK3CA*	H1047R	32	***PIK3CA***	**H1047R**	**1**	100%
Patient 77	*PIK3CA*	H1047R	15	*PIK3CA*	H1047R	20	100%
Patient 6	*TP53*	P278L	2	ND			0%
Patient 61	*PIK3CA*	N345K	22	ND			0%**
	TP53	R337C	22	*TP53*	*R337C*	*0.07*	
Patient 19	*PIK3CA*	C420R	14	ND			0%**
	ERBB2	L755S	15	*ERBB2*	*L755S*	*0.1*	
	GNAS	R201C	4	*GNAS*	*R201C*	*0.1*	
Patient 54	*CDKN2A*	H83Y	5	*CDKN2A*	*H83Y*	*0.18*	0%*
Patient 3	*AKT1*	E17K	4	*AKT1*	*E17K*	*0.14*	0%*
Patient 4	*PIK3CA*	E545K	33	*PIK3CA*	*E545K*	*0.45*	0%*
Patient 58	*AKT1*	E17K	52	*AKT1*	*E17K*	*0.13*	0%*
Patient 59	*AKT1*	E17K	63	*AKT1*	*E17K*	*0.23*	0%*
Patient 66	*PIK3CA*	H1047R	7	*PIK3CA*	*H1047R*	*0.28*	0%*
Patient 68	*AKT1*	E17K	22	*AKT1*	*E17K*	*0.17*	0%*
Patient 22	*TP53*	C238Y	3	*TP53*	*C238Y*	*0.07*	0%*
Patient 80	*PIK3CA*	H1047R	18	*PIK3CA*	*H1047R*	*0.21*	0%*
	AKT1	E17K	8	AKT1	E17K	0.24	
Patient 34	*TP53*	R248Q	62	NA			
	APC	G1120E	3				
Patient 70	*PIK3CA*	H419delR	17	NA			
Patient 72	*PIK3CA*	H1047R	33	NA			
Patient 40	*PIK3CA*	E545K	43	NA			
Patient 45	*PIK3CA*	H1047R	39	NA			
	PIK3CA	E453Q	36				
	PIK3CA	D1029H	37				
Patient 55	*RET*	A833T	3	NA			
	PTPN11	S502L	3				
Patient 56	*AKT1*	E17K	37	NA			

*Table 31 Tier II variants detected in FFPE samples and their presence or absence in corresponding cfDNA samples. Bold type indicates variants that were only detected on manual review of data in IGV. *indicates variants that were present on manual review but at levels so low that automated detection is not practical. ** indicates a sample where some variants*

*detected in cfDNA were absent in cfDNA while others were present at very low levels. Overall, concordance for samples marked with * was deemed 0% due to the very low VAF of the variant called. C182Ter refers to a variant where the change in base has resulted in a stop codon.*

In this dataset 31/48 FFPE primary tumour samples contained tier II variant. Of these 24/31 had a cfDNA sample for comparison. 7/24 (29%) variants were detected in both cfDNA and FFPE using the standard bioinformatic pipeline. Due to the limit of detection for the bioinformatic pipeline being set at 1% it was hypothesised that some variants may be present in cfDNA but had not been detected by the bioinformatic pipeline. This was true for an additional 5 cases where on manual review the variant that was present in FFPE primary tissue was present at ~1% in cfDNA but may have been called at <1% due to the downsampling effect where the bioinformatic pipeline only reviewed 2000 randomly selected reads at a single location. If these variants were included concordance would improve to 12/24 (50%). A further 11 patients had variants that were detectable on manual review but at VAF of <0.5%. Given their presence in the FFPE sample one would like to believe that they were not artefact, but they should not be counted unless confirmed with an alternative method such as ddPCR. In 1/24 patients, even with manual review the variant was not present in cfDNA and thus may have represented in a subclone in the primary tumour that either remains as such or has been removed by treatment between the primary tumour and the development of metastatic disease.

4.4 Tier III variants in primary tumour FFPE and cfDNA metastatic samples in genes of interest in breast cancer

Tier III variants were present in both FFPE and cfDNA samples. Each tier III variant frequency of ≥5% was manually reviewed and a literature search performed to try and establish whether it was a potential variant of interest in the setting of ER+ metastatic breast cancer treated with fulvestrant and vandetanib. Table 32 shows the tier III variants in genes of interest in this project. For the 8 tier III variants literature review and cross-referencing with other samples from the same patient has attempted to establish whether these variants are potentially pathogenic and could represent targets for wet lab validation and exploration of their effects on gene function.

GENE	AA	Effect	COSMIC ID	dbSNP ID	Source	VAF (%)	Location within gene
ATM	p.Val3005Ile	Missense (G>A)	NA	rs1555151745	FFPE	5	intradomain
ERBB4	p.Ser303Phe	Missense (C>T)	COSM1015992	NA	FFPE	34	Furin like cysteine rich domain
ESR1	p.Val533Leu	Missense (G>A)		rs778116774	cfDNA (EOT)	48	intradomain
PIK3CA	p.Ala694Val	Missense (C>T)	NA	rs754404652		5	PI3K accessory domain
RET	p.Val899Ile	Missense (G>A)	COSM5991507	NA		6	Protein tyrosine kinase
RET	p.Asp322fs	FS deletion	NA	NA	cfDNA	8-10	intradomain
TP53	p.Thr102Ile	Missense (C>T)	COSM43678	rs786202717	FFPE	37	DNA binding domain
GATA3	p.Gly279_Thr280insLysAla	FS insertion	NA	NA	cfDNA (EOT)	10	Zinc finger

Table 32 Tier III variants in genes of interest in this project at VAF >=5%. Location within gene has been determined suing mutation mapper part of the CBioPortal toolkit (accessed 17/5/19)

Each gene/ variant is considered in turn.

4.1.1 *ATM* V3005I/rs1555151745

This variant occurred at a VAF of 5% in a FFPE sample. No corresponding plasma samples were available. *ATM* is a key gene in the DNA damage response, particularly the repair of a subset of double strand breaks not repaired by non-homologous end joining. *ATM* is a large gene (3056 amino acids) (Choi et al. 2016). V3005I occurs in between the kinase and FAT C-terminal domains, it is predicted as deleterious by SIFT and possibly damaging by Polyphen-2 as modelled using mutation mapper. However, no references concerning this variant could be identified in the literature. Thus, it remains a true variant of uncertain significance. In this study it is low priority for further investigation given its low VAF and non-domain location. This variant has not been reported in population databases.

4.4.1 *ERBB4* S303F/COSM1015992

ERBB4 S303F was identified in a single patient (patient 28) FFPE sample at a VAF of 33%. This variant has not been reported in population databases. The variant was present in the baseline cfDNA sample, only on manual review, at a frequency of 0.67%. The EOT sample failed library preparation, likely due to low extracted DNA concentration (0.18ng/µL). This patient stopped taking trial treatment after 81 days. The S303F variant was the only potential driver variant identified in this sample.

ERBB4 is part of the epidermal growth factor receptor subgroup of tyrosine kinase receptors which activate both the PI3K and MAPK pathway downstream controlling cellular growth and proliferation. Pathogenic somatic variants in the HER family are of interest as a potential mechanism for resistance to HER2 directed therapies (Canfield et al. 2015). S303F is seen in breast cancers and was identified in an ER+/HER2- liver metastasis sample in the MSK-IMPAKT breast cancer dataset but not in either the METABRIC or TCGA datasets.

S303F occurs in the Furin like domain and S303F variant containing cells exhibit altered signalling in the ERBB4 receptor and the PI3K and MAPK pathways. Although signalling can be demonstrated to be abnormal in HER2+ cell lines no subsequent increase in migration or invasion was demonstrated in a cell line-based study. However, the presence of an S303F variant predicted for increased sensitivity to PI3K inhibition adding weight to the hypothesis that an S303F variant in *ERBB4* causes upregulation of the PI3K pathway. (Elster et al. 2018).

No literature could be identified showing the effect of the variant S303F in ER+/HER2- breast cancers. Furthermore, the impact of the variant on response to fulvestrant has not been tested. Although vandetanib is not reported to inhibit ERBB4 other EGFR family targeted drugs such as lapatinib often have pan EGFR effects (Qiu et al. 2008). Therefore, it is not beyond possibility that vandetanib may have inhibitory effects on ERBB4 alongside EGFR and RET. If this were the case, then a gain of function variant such as S303F could influence the response to vandetanib.

4.4.2 *ESR1* V533L/ rs778116774

This variant occurred in a single cfDNA sample (patient 5) at a VAF of 48%. This would suggest either that it is a benign germline variant, or it is present in a significant number of cells in an aggressive cancer. This patient has a paired FFPE primary tumour sample where the variant was present at 25%. It has been noted in population databases albeit at very low frequencies e.g. 0.00004% in GnomAD. Its location within *ESR1* falls between the ligand binding domain and the C-terminal. It is not reported in over 8000 breast cancer samples in CBioPortal. Overall, the presence in primary tumour and the intra-domain location make this unlikely to be a key variant in treatment resistance, the fact that it also occurs at low frequencies in population databases means that it is likely to show low or no pathogenicity.

4.4.3 *PIK3CA* A694V/rs754404652

This variant has been reported in dbSNP with population frequencies <0.0001%. It is not reported in COSMIC. It occurs in the PI3Ka domain. While many papers have examined the functional effects of

the common *PIK3CA* variants in exons 9 and 20 there is no published literature on the functional impact of this variant. This variant occurred at VAF of 5% in a single FFPE sample (patient 22), it was not detectable in matched cfDNA samples. This sample also contained an *ESR1* variant which was detectable in matched cfDNA samples. This would suggest that even if the variant was pathogenic it was not the key driving variant in this case or was representative of a subclone in the primary tumour.

4.4.4 RET

4.4.4.1 RET V899I/COSM5995107

RET p.Val899Ile was found in a single sample (patient 55) at a VAF of 6%. The sample also harboured 5 SNPs but no obvious driver mutation from the genes sequenced. This patient has potential to be an exceptional responder but unfortunately no plasma samples have been collected. Total and phosphorylated expression scores were moderate (h-score 155 and 140 respectively) in the IHC analysis of this patient sample.

Somatic variants in *RET* are rare in breast cancer. A recent study looked specifically at *RET* gene alterations in 9693 breast cancers. The authors showed an overall frequency of gene alterations (including fusions, amplifications and SNVs) of 1.2% (121/9693). Of these 25/9693 were missense variants. P.Val899Ile was not detected in this series and also has not been reported in breast cancer samples in COSMIC or CBioPortal (Paratala et al. 2018).

V899I sits within the large tyrosine kinase domain along with common variants such as M918T (the most common *RET* variant in medullary thyroid cancer) and S904F (also common in medullary thyroid cancer). In silico and in vitro work has established that these variants result in functional changes and a more aggressive phenotype (Cosci et al. 2011). While it cannot be assumed that changes in a similar region will have the same effect it does mark this an interesting variant for further investigation.

4.4.4.2 RETp.Asp322fs

This variant occurred in three samples; all cfDNA samples. The VAF ranged from 8-10%. It results as a deletion of two bases. It was only detected using the CUSTOM panel, so it is unknown if it is also present in FFPE samples as these were not tested with the CUSTOM panel. It occurs reasonably early in the protein (AA 322 out of 1114) but is in a region between the cadherin and protein kinase domain. The corresponding h-scores from the IHC work for these patients are shown in Table 33.

	Total-RET IHC score	Phosphorylated RET IHC score
Patient 51	140	0
Patient 77	117	120
Patient 64	140	10

Table 33 Three patients with RET D322fs variant and their corresponding RET IHC scores, low score indicates absence of protein. The maximum H-score possible is 300 where 100% of tumour cells within the sample exhibit strong staining suggestive of high protein expression. Phosphorylated scores relate to the protein being phosphorylated or 'active'.

In two of the three patients there was an absence of phosphorylated or active RET in the samples where the cfDNA sample contained the variant. Potentially, this could act as hypothesis generating data that the variant results in a loss of function however these are very small numbers and could have easily occurred by chance.

4.4.5 *TP53* T102I/rs786202717/COSM43678

This variant occurred at 37% VAF. It has not been reported in population databases. No plasma samples were available for this patient. It occurs in the DNA binding domain, within exon 4, but outside the L2 and L3 loops and the LSH motif where variants have been shown to be stronger predictors of poor prognosis than those outside of these areas (Olivier et al. 2006). However, a significant limitation of the work by Olivier et al is that only 651/1794 patients included in the study had all exons sequenced. Thus, the analysis focuses on variants in exons 5-9 and is likely underpowered to predict the pathogenicity of variants in other exons.

4.4.6 *GATA3* p.Gly279_Thr280insLysAla

This frameshift insertion occurs in the proximal zinc finger like domain of *GATA3*. It was detected in a cfDNA sample at 10% VAF. It is not present in the associated FFPE sample. *GATA3* is a transcription factor which plays a role in cell differentiation, proliferation and movement. *GATA3* is linked to the ER pathway in mRNA analyses and as such variants within the gene may influence clinical outcomes in ER+ breast cancer along with response or resistance to ER directed therapies such as fulvestrant (Asch-Kendrick and Cimino-Mathews 2016). Recent research has focussed on variants in zinc finger binding domain 2 where the majority of *GATA3* variants in breast cancer occur (Takaku et al. 2018). Hypothetically, a significant change in amino acid sequence in the proximal would also result in a change in ability to bind zinc resulting in functional change. At this point in time this is a true variant of uncertain significance.

4.4.7 Summary

Overall variants that occur outside major functional domains e.g. those in *ATM* and *ESR1* are unlikely to be key drivers in this setting are and likely to remain variants of uncertain significance. In addition, *PIK3CA* A694V occurs at low frequencies in FFPE and is not detectable in cfDNA making it unlikely to be a key driver variant and therefore not recommended for further investigation. The variant found in *GATA3* could result in functional change and potentially influence response to fulvestrant via the ER pathway; the patient in which this variant was found had a PFS of less than 3 months therefore this variant may have contributed to a poor response to fulvestrant. The S303F variant in *ERBB4* is of potential interest as hypothetically it could influence the receptor and pathways involved with treatment with vandetanib. In addition, the variants reported in *RET* could have influence over response to treatment with vandetanib if they resulted in upregulation of downstream signalling pathways. The frameshift variant although occurring in an intra-domain region could be a cause of the absence of active RET in these patients and thus combined with the immunohistochemistry data marks it as a variant for potential further study. The *TP53* variant is truly a variant of uncertain significance.

4.5 Confirmation of variants detected by NGS using ddPCR

Where probes and samples were available variants detected using NGS were confirmed using ddPCR for all *AKT1* variants and variants in *PIK3CA, ESR1, TP53* and *ERBB2* in the initial cohort. All *AKT1* variant samples were tested due to the higher than expected frequency of *AKT1* E17K variants in tissue samples. Table 34 shows the concordance between variants detected using NGS and ddPCR. Here we can see that every variant detected in FFPE that was subsequently tested for using ddPCR was confirmed, often with similar VAF. There was an exception to this in patient 344002 where the variant was called at 8% in tissue by NGS but 35% by ddPCR. This is likely to be due to low DNA input into the ddPCR assay, strictly speaking this assay did not meet minimum QC as only 108 WT droplets were formed. However, the presence of 59 *AKT1* E17K containing droplets suggests that this is a true positive sample.

Elsewhere in the table the challenges of sample availability are evident with only a few patients having samples at a range of time points to allow tracking of variants. While this shows that it is possible to track variants it is impossible to draw any meaningful conclusions about the change in VAF over time from such small numbers of samples.

		TISSUE		BASELINE cfDNA		8 WEEK cfDNA		EOT cfDNA	
		NGS	ddPCR	NGS	ddPCR	NGS	ddPCR	NGS	ddPCR
AKT1 E17K	1006	NA	NA	6.91	6.29 (9.79-2.79)	NA	35.2 (38.2-32.1)	7	1.09 (1.91-0.264)
AKT1 E17K	67001	48.9	48.3 (46-50.6)	NA	NA	NA	NA	49	45.6 (44.7-46.5)
AKT1 E17K	86007	37	27.7 (25.4-30)	NA	1.55 (0.28-2.83)	NA	NA	NA	NA
AKT1 E17K	185001	52	45.3 (41.4-49.2)	NA	NA	NA	NA	**ND**	NA
AKT1 E17K	185003	63	54 (52.1-55.9)	NA	NA	NA	NA	NA	NA
AKT1 E17K	185022	**ND**	***0.395 (0.87-0)***	7	3.19 (4.92-1.45)	NA	***0.702 (1.55-0)***	**ND**	**ND**
AKT1 E17K	207012	22	18.5 (16.1-20.9)	1	***0.187 (0.629-0)***	NA	NA	NA	NA
AKT1 E17K	344002	8	35.3 (28-42.6)*	**ND**	**ND**	NA	NA	NA	NA
ERBB2 L755S	185022	15	16.5 (18.3-14.6)	**ND**	**ND**	NA	**ND**	**ND**	**ND**
ESR1 Y537N	207002	NA	***0.0529 (0.178-0)***	NA	5.86 (7.02-4.71)	NA	NA	13	16.9 (19.16-14.7)
ESR1 D538G	207014	NA	**ND**	NA	15.3 (19-11.7)	NA	NA	28	22 (24.3-19.6)
PIK3CA E545K	16007	33	32.7 (34.6-30.9)	**ND**	***0.393 (0.867-0)***	NA	NA	NA	NA
PIK3CA E542K	163002	34	30.9 (33.1-28.6)	1	***0.667 (2.24-0)***	NA	***0.27 (0.908-0)***	5	***1.57 (3.18-0)***
PIK3CA E545K	185015	27	25.3 (26.5-24.0)	4.6	4.34 (5.3-3.39)	NA	3.76 (3.03-2.3)	3.75	4.09 (5.82-2.36)

*Table 34 Confirmation and tracking of variants detected by NGS using ddPCR. Showing samples where a variant is present by both NGS and ddPCR (dark green). Samples where only one modality was available (light green). NA indicates a sample was not available for testing. ND indicates a sample that was tested but no variant was detected. Bold type indicates samples where the VAF was so low that the 95% Poisson Confidence Intervals include 0 and thus this result should be interpreted with caution. *shows a single sample where there was low DNA input into the ddPCR assay; the assay detected the variant as it was at high frequency but the VAF may be falsely high due to low DNA input.*

4.6 Chapter discussion

4.6.1 Sample availability and quality

Both FFPE samples and cfDNA samples were collected as part of the FURVA clinical trial. Neither were mandatory to trial participation. This meant that not all trial participants had samples available for use. It also meant that, unlike other studies, no cut offs were put in place for tumour percentage or extracted DNA concentration as if there was a possibility meaningful data could be generated from the sample then it was deemed worth running. Overall FFPE primary tumour tissue samples were collected from 51/80 trial participants who had been treated with fulvestrant and vandetanib.

The quality of the DNA extracted from FFPE samples remains an issue in the routine use of next generation sequencing for variant identification in cancers. FFPE samples can have been stored for many years before being used for NGS. During the process of collection, fixation and storage there are many variables that can lead to breakdown in DNA and subsequent poor quality sequencing data (Ascierto et al. 2019). In particular, the act of formalin fixing samples can cause cross linking of DNA and thus significant DNA fragmentation when samples and dewaxed and DNA extracted (Do and Dobrovic 2015). Furthermore, different protocols for fixing tissue can have adverse effects on DNA quality, in particular the concentration and the duration of formalin exposure (Einaga et al. 2017). It is likely that many different protocols were used for the samples in this project as they were collected from sites all over the UK. There is evidence to suggest that the DNA cross linking that occurs during formalin fixation can cause false pick up of C>T changes in FFPE samples (Bhagwate et al. 2019); this means that further caution should be applied to a number of the tier III variants identified where the change detected was from C>T as this could have occurred due to sequencing error. Fresh frozen tissue has been shown to give superior sequencing accuracy when compared to FFPE samples (Bhagwate et al. 2019) but has practical disadvantages. Reassuringly though there is good evidence that acceptable sequencing data can be gained from FFPE samples stored for a number of years (Carrick et al. 2015).

Collection of plasma samples for cfDNA extraction was sporadic. Initially it was planned to only analyse end of treatment samples as they were hypothesised to contain the most cfDNA thus increasing the chance of detecting variants of interest. However, only 17 EOT samples were available for patients where an FFPE block had been submitted so the analysis extended to include a paired cfDNA sample taken at any time point for each patient; with order of preference being EOT, 8 week and finally baseline if no other samples were available. This means that samples have been sequenced at varying points in the treatment course with potential for exposure to treatment to change the quantity of ctDNA within the sample.

4.6.2 Panel and technology selection

Since the selection of the CHPv2 and CUSTOM panel for this project (June 2017) technology has made significant progress in the design, scope and limits of detection of sequencing panels. For example, most large-scale sequencing projects now use whole gene targeted panels as opposed to hotspot panels and most of these panels cover 100+ genes, often closer to 500. There has also been the advent of unique molecular barcoding technology to push the limits of detection regarding cfDNA samples for example Oncomine technology (Thermofisher). It has even been possible to perform whole exome sequencing from cfDNA in patients with metastatic breast cancer (Adalsteinsson et al. 2017).

Due to the small size of the panel selected for this project it has not been possible to investigate copy number variation, fusion proteins, tumour mutational burden or gene signatures. These are all potential biomarkers and their absence is a notable limitation of the technology selected.

The selection of the CHPv2 and CUSTOM panel was the best scientific and practical choice at the time, but it must be acknowledged that this technology is now being superseded by more sensitive and comprehensive (although not necessarily cheaper) technologies. It is also important to note that the selection of samples to sequence had to be made prior to clinical outcomes being available. If

data distinguishing good from poor responders was available, then more detailed sequencing on less samples may have been appropriate.

Furthermore, some newer panels enable the investigation of genetic variants in DNA and RNA simultaneously e.g. Trusight 170 (Illumina, San Diego, USA) although this is only possible from FFPE samples. Given that the time points of most interest in these patients is their genetic landscape immediately before and after treatment with fulvestrant and vandetanib this technology is interesting but impractical in this setting where no sequential tissue biopsies were available. Technologies focussing on sequencing of ctDNA are now entering the mainstream e.g. Avenio ctDNA Targeted Kit (Roche, Basel, Switzerland) which is optimised for lung and colorectal cancers.

4.6.3 Absence of variants in PTEN, RET and LYN

Despite sequencing multiple regions of interest in genes such as *PTEN, RET* and *LYN* there was a notable absence of variants detected. No *PTEN* variants were identified despite 70% of the gene being covered by the CHPv2 panel. At the time of panel selection very little was known about the frequency of *RET* and *LYN* variants in patients with ER+ endocrine resistant ABC although from general genomic studies it could be extrapolated that they were not genes frequently containing variants. However, from the pre-clinical work reviewed in chapter 1 it seemed reasonable to think that one or two samples may contain a variant.

During the course of this project large datasets reported on the specific frequency of variants in *RET* and it became clear that it was very unlikely that any would be picked up in the cohort in this project as the overall frequency of SNVs in breast cancer was 25/9693 (0.25%) and the majority of these were in patients with ER- tumours (Paratala et al. 2018). It seems clear that it is not genetic variants in *RET* that is the cause of RET overexpression nor are genetic variants occurring in response to treatment with vandetanib.

While the CUSTOM panel only covered a small number of hotspots in *LYN* the amplicons covering these hotspots spanned around 85% of the gene. Similarly, to *RET*, very little was known about the

prevalence of variants in *LYN* in patients with breast cancer at time of panel selection. Subsequently a few studies have specifically noted inclusion of *LYN* in targeted sequencing panels. It was included in the MSK-IMPAKT dataset accessed in CBioPortal, where the frequency of SNVs in *LYN* was 2/1756 (0.1%); both patients were ER+ and the tumour sequenced was a metastatic deposit (Razavi et al. 2018). The rate was slightly higher in the TCGA dataset although variants were still only present in 4/482 (0.8%) from a mix of breast cancer subtypes. This low frequency makes me question the probability of Schwarz et al detecting a variant in *LYN* in just four patients tested even if they had pre-selected patients who showed evidence of primary endocrine resistance (Schwarz et al. 2014). Amplification of *LYN* was more common in both datasets but still was infrequent at 5% in the TCGA samples (majority ER+/HER2-) and 0.5% in the MSK-IMPAKT dataset (majority ER+/HER2- subtype).

4.7 Chapter summary

In this chapter the results of next generation sequencing of 48 primary tumour FFPE samples and 41 cfDNA plasma samples representing the onset of metastatic endocrine resistant disease have been presented. Key findings from the analysis of the FFPE samples include the positive correlation between tumour percentage and extracted DNA concentration along with a negative correlation between final library concentration and number of variants detected. Conversely there was no relationship between the extracted DNA concentration and final library preparation which may suggest that technique and laboratory conditions are a greater contributing factor to final library concentration along with the fact that a proportion of the extracted DNA may be highly fragmented and thus not suitable for PCR in the library preparation stage of sequencing.

Variants were ascribed a tier relating to potential pathogenicity. Overall, 65% of FFPE samples contained a tier II variant, most commonly in *PIK3CA, TP53* or *AKT1*. When compared to larger datasets, constructed using larger sequencing panels, the variant frequencies by gene from the CHPv2 panel were slightly lower in *PIK3CA* and *TP53* but higher in *AKT1*.

cfDNA samples were less likely to contain a tier II variant, with only 29% of samples containing a tier II variant as called by the automated bioinformatic pathway, this rose to 50% if variants at ~1% VAF as detected by manual inspection were included. The concordance between FFPE primary tumour and cfDNA sample was 50% which compares well with the 49.9% reported in the MOSCATO trial (Jovelet et al. 2016).

Nine tier III variants were identified in genes of interest in this project. Interesting variants were identified in RET; a missense variant V899I and a frameshift deletion where in 2/3 patients it potentially resulted in decreased phosphorylated RET expression in the corresponding FFPE sample.

In chapter 5 ddPCR will be used to investigate ESR1 variants across the whole trial population and copy number variations in key breast cancer genes as a major limitation of the panels chosen here is their inability to look at copy number variation which has been shown to be prevalent and targetable in metastatic ER+ breast cancer. In chapter 6 this data, along with that generated from the immunohistochemistry and ddPCR work will be paired with clinical outcome data

5 Using ddPCR to detect single nucleotide variants and copy number alterations with a focus on ESR1, MYC and FGFR1

5.1 Chapter overview

Droplet digital PCR is a complementary technique to NGS and in this project has been used to expand areas of interest (*ESR1* SNV detection across a larger range of samples), confirm NGS findings (SNV detection of common variants in *PIK3CA, ERBB2* and *AKT1* – reported in Chapter 4) and explore CNAs in two genes commonly amplified in ER+ breast cancer (*MYC* and *FGFR1*). The methods used are described in detail in Chapter 2. While there are reasonably well-established protocols for SNV analysis using ddPCR, analysis of CNA remains experimental with no test having moved into routine clinical use. A large proportion of the CNA section of this chapter covers the challenges involved with sample analysis, both in FFPE and cfDNA samples when investigating *MYC* and *FGFR1* amplifications.

5.2 Chapter Aim

To establish the frequency of *ESR1* variants and *MYC* and *FGFR1* amplification in cfDNA samples representing patients with endocrine resistant metastatic breast cancer who had received treatment with fulvestrant +/- vandetanib in preparation for correlation with survival outcomes in Chapter 6.

5.3 Chapter Objectives

To establish the frequency of *ESR1* variants in cfDNA samples taken from patients treated with fulvestrant +/- vandetanib.

To determine the copy number ratio of *MYC* and *FGFR1* compared to the reference gene *AGO1* in cfDNA samples from patients treated with fulvestrant +/- vandetanib

To compare methods of deciding whether a sample is amplified or non-amplified

If amplification is present, to determine whether the corresponding primary tumour sample also shows amplification of *MYC* and/or *FGFR1*

5.4 Materials

For detection of *ESR1* variants and CNA in *MYC* and *FGFR1* cfDNA extracted from plasma samples taken at the end of trial treatment (likely to be at the point where the patient's disease was progressing and thus may contain higher concentrations of ctDNA) was selected. While *MYC* and *FGFR1* CNA have been demonstrated in both endocrine sensitive and resistant disease *ESR1* variants are rarely detected in endocrine sensitive primary tumours. It is noted that the frequency of CNA in *MYC* and *FGFR1* are higher in metastatic disease than primary tumours offering further reason as to the selection of cfDNA metastatic samples rather than FFPE primary tumour (see Chapter 1 figure 6) as the main sample of reference. DNA extraction methods from plasma and FFPE samples are detailed in Chapter 2.

81 participants in the FURVA clinical trial were identified where the lab had received an end of treatment trial (EOT) sample. 68 of these were available for use. Some samples had arrived at the lab outside the agreed processing window[11] (n=4) while others were unable to be located in the -80°C freezer (n=4). 3 samples were submitted without the relevant paperwork, one was collected in the wrong bottle and one no reason was given (see Figure 39).

EOT samples were selected over other time points due to the potential for higher DNA concentrations. If potential predictive biomarkers were identified prospective validation would be required to see if they were detectable in samples collected prior to treatment.

[11] Samples were to reach the central processing lab within 96 hours of collection

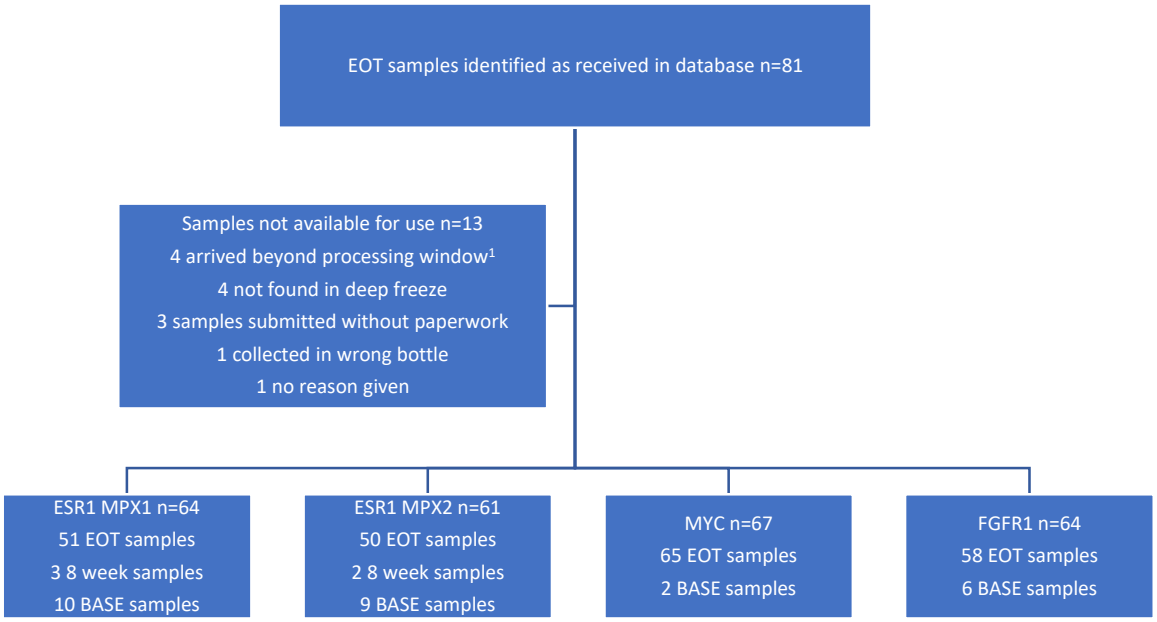


Figure 39 CONSORT diagram showing samples available for use in ddPCR experiments. Number of samples varies for each biomarker as availability depended on previous use e.g. for NGS. EOT = end of treatment, BASE = baseline sample prior to treatment with fulvestrant +/- vandetanib, MPX1 or 2 = multiplex 1 or 2[12]

In a small number of patients, the extracted DNA from the EOT sample had been used up during previously run experiments, in this situation if cfDNA was available from another time point representative of endocrine resistant metastatic disease then this was substituted (n=2 for *MYC*, n=6 for *FGFR1*, n= 13 for *ESR1* MPX1 and n= 11 for *ESR1* MPX2).

5.5 Methods

Methods for ddPCR are detailed in chapter 2 but briefly comprise the creation of individual reactions in wells of a 96 well plate including Supermix, FAM labelled probes for the gene of interest and HEX labelled probes for the control sequence along with up to 20ng of DNA. Using the QX200 droplet generator up to 20,000 droplets are created using oil. The DNA in these droplets then undergo PCR and finally the droplets are read by the droplet reader.

[12] MPX1 is a set of multiplexed ddPCR probes covering variants E380Q, Y537C, D538G and L536R while MPX2 covers S463P, Y537S and Y537N.

Droplet cluster assignment was performed using Quantasoft Analysis Pro version 1.0.596. A dataset was then exported in .csv format for further analysis.

5.5.1 Single nucleotide variant (SNV) detection

Once droplets had been assigned to the correct cluster a CSV file was downloaded containing details of the number of droplets containing DNA, total number of droplets and sample concentration. For QC purposes the sample had to meet the following criteria: total droplet count >10000, total WT containing droplets >300 and double positive droplets <10% of total droplet number. Samples meeting these criteria with ≥5 positive droplets were recorded as positive. As samples were tested using multiplexed probes where each assay included probes for a number of different variants it is not possible to accurately determine the variant allele frequency of any variant present as three or four clusters of paired wild type droplets are present thus all VAFs presented here represent the minimum VAF. The higher the DNA input into each assay the more accurate the minimum VAF estimate becomes. The minimum VAF is calculated within the Quantasoft analysis software and presented with a 95% confidence interval using the formula $VAF = a/(a + b)$ where a = concentration of probes containing variant and b= concentration of probes containing WT DNA.

5.5.2 Copy number alteration (CNA) detection

Droplets were allocated to the correct cluster both as per the control samples run and manually and the resulting CSV file downloaded and the results compared. For QC purposes the sample had to meet the following criteria: 10000 droplets generated, at least 400 droplets containing DNA from the reference gene and 400 containing DNA from the gene of interest. For samples passing the QC checks the ratio of gene of interest: reference gene was recorded. Figure 40 shows a typical sample; in this case the sample used a positive control for MYC. The sample is an FFPE sample from a patient with a MYC amplified haematological malignancy with the amplification confirmed by FISH. It is easy to see that the number of blue droplets (representing MYC DNA containing droplets) far outnumber the green droplets (representing the control; AGO1 DNA containing droplets). Unlike in cfDNA

samples there are droplets whose fluorescence amplitude falls between those containing no DNA (grey droplets) and those containing MYC amplified droplets, these droplets are colloquially referred to as 'rain' and it is not possible to confidently assign as droplets containing DNA from the gene of interest. This is a common challenge of ddPCR when using FFPE samples containing degraded DNA and its effect on the final amplified status of the sample is explored in later sections of this chapter. Attempts can be made to minimise rain by selecting the correct annealing temperature and increasing the number of PCR cycles but it often persists to a degree particularly in samples with a higher starting concentration of DNA.

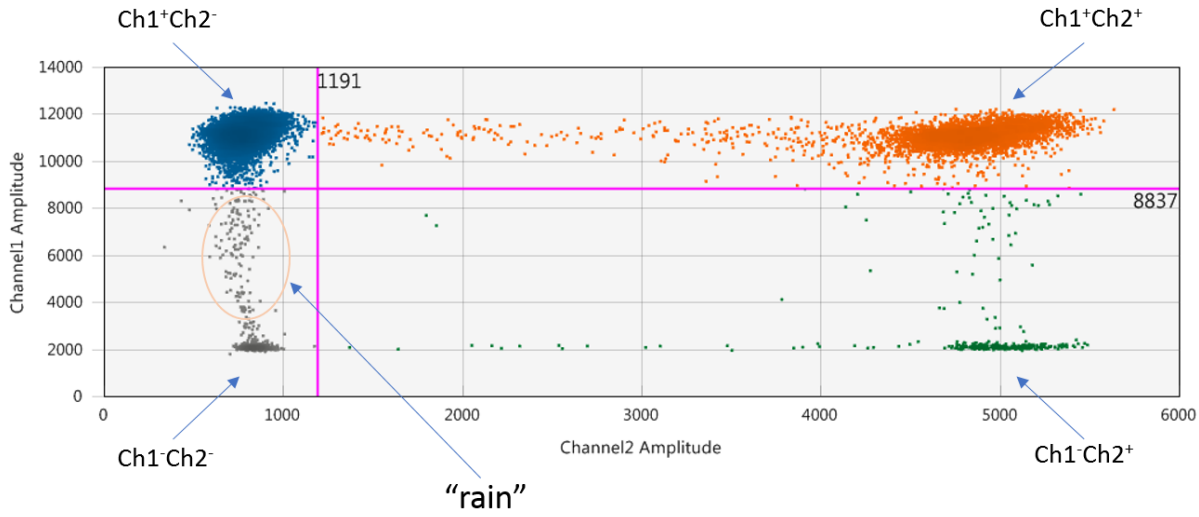


Figure 40 Quantasoft Analysis showing droplet generation for MYC positive control. This control sample (FFPE) contained 8 copies of MYC. Ch1 represents MYC and Ch2 represents the reference gene AGO1. This chart also shows the presence of "rain" where droplets fall between neat clusters. Despite the presence of the "rain" this sample is clearly amplified as the number of droplets containing MYC DNA (blue droplets) is much larger than the number containing AGO1 DNA (green droplets).

For FFPE samples any sample with a ratio of ≥2 was considered amplified as per Garcia-Murillas et al (Garcia-Murillas et al. 2013). A ratio of ≥2 should ensure that a sample that was 100% tumour was correctly identified as amplified. Outlining of the area with the highest tumour percentage when cells were scraped from slides as part of the DNA extraction process aimed to keep the tumour DNA as close to 100% as possible. However, a number of samples had tumour content below the ideal

100% even when an area of maximal tumour density was identified. To allow for this variation any FFPE sample with a ratio of >1.7 was considered borderline amplified unless it was known that the tumour percentage in the sample was ≥90%.

Determining whether cfDNA samples showed amplification of the gene of interest is challenging as it has not been possible to determine the tumour fraction of DNA present in each sample in this project. For a small proportion this can be estimated if they have had a variant identified on NGS. However, many of the variants detected may be sub-clonal thus their variant frequency does not necessary correspond to the percentage of tumour DNA in the sample. There are methods that have been used to determine tumour fraction but they were not possible to use in this setting. Applying a ratio of >2 to cfDNA samples means that many amplified samples will be missed as the DNA content based on an average of VAF detected by NGS lies at around 20%. Therefore, a ratio of 1.2 or such may indicate amplification. However, it is not known whether low level amplification is clinically meaningful, thus, it is reasonable when looking for clinically relevant biomarkers to apply higher cut offs. Meaning that samples with very low tumour percentage or very low amplification levels may be excluded from the analysis.

5.6 Detection of ESR1 variants using ddPCR

Samples with ≥5 droplets containing *ESR1* variant DNA were recorded as positive. VAF was noted but as it was not possible to identify the corresponding WT cluster the VAF should be interpreted with caution and is likely higher than that recorded. It is not possible to identify which variant is present due to the similar amplitude of each probe and the number of regions multiplexed in each test. Each multiplex contained one variant that occurs significantly more frequently in large datasets than others (highlighted in green in Table 35). Frequency of variants in Table 35 is derived from all the ESR1 variants present in patients with ER+/HER2- breast cancer included in the MSK-IMPAKT breast cancer dataset in CBioPortal. 94 patients with an *ESR1* variant were described.

Multiplex 1 (MPX1)	Frequency of variant	Multiplex 2 (MPX2)	Frequency of variant
E380Q	11/94 (11.7%)	S463P	2/94 (2.1%)
Y537C	10/94 (10.6%)	Y537S	21/94 (22.3%)
D538G	37/94 (39.3%)	Y537N	5/94 (5.3%)
L536R	1/94 (1.0%)		

*Table 35 Distribution of variants amongst patients whose tumour DNA tested positive for an ESR1 variant by NGS in the **MSK-IMPAKT** breast cancer dataset. 87/94 (93%) of the ESR1 variants detected in the **MSK-IMPAKT** cohort are covered by the two multiplex reactions. Green shading shows the most common variant in each multiplex.*

5.6.1 Results MPX1 (covering variants D538G, E380Q, Y537C and L536R)

64 samples were identified as having sufficient volume of extracted DNA available for testing. All patients had an EOT sample recorded as available for testing however for 13 patients this was no longer available thus 51 EOT, 3 8-week and 10 baseline samples were tested. Overall, a variant was detected in **15/64 (23%)** of samples. As per the distribution of variants in Table 35 it is likely that around 40% of these variants are the common D538G variant with one or two E380Q and Y537C variants.

Although VAF cannot be accurately measured using multiplexed probes the median minimum VAF using the MPX1 probes was 1.7% (range 0.1% to 15%).

5.6.1.1 Samples tested in duplicate

To ensure accuracy of the result 16 samples were tested in duplicate (see Table 36); all positive samples with DNA remaining for testing (n=8), all unassigned (<5 droplets positive) samples with DNA remaining for testing (n=2) and 6 WT samples. Of the positive samples on first test 7/8 had a confirmed positive result on the second test; albeit with one sample only having 2 positive droplets, however this is likely due to the sample running out as the total number of positive droplets was <100. One sample which had 5 positive droplets in the first test only had a single positive droplet in the second; it is likely that this represents a very low frequency variant. The negative samples

included a sample with very high DNA concentration which can increase the risk of false positives. Both unassigned samples contained a single positive droplet on the first test and had >400 WT DNA containing droplets in both tests. The sample that had a single positive droplet in both tests is likely to contain a very low-level variant while the other is more likely to be artefact. However, for the purposes of this study neither samples were counted as positive. Firstly; due to the question over whether a single droplet is artefact and secondly even if the positive droplet is a true representation of a very low-level variant there is little evidence that this would be clinically meaningful.

	Sample ID	First test MPX1			Second test MPX1		
		***ESR1*^{var} droplets**	***ESR1*^{WT} droplets**	**min VAF**	***ESR1*^{var} droplets**	***ESR1*^{WT} droplets**	**min VAF**
Samples with ≥5 droplets positive on first test	Patient 87	310	2675	10.84	283	2033	12.57
	Patient 90	10	2504	0.51	7	1302	0.51
	Patient 107	455	2596	15.99	213	3704	5.80
	Patient 118	35	1028	3.19	2	84	2.32
	Patient 119	42	2206	1.99	59	1811	3.18
	Patient 21	43	2092	2.08	33	1947	1.86
	Patient 159	29	2377	1.30	38	2740	1.39
	Patient 3	5	1273	0.37	1	786	0.12
Samples with 1-4 positive droplets on first test	Patient 17	1	1264	0.08	1	486	0.20
	Patient 19	1	1884	0.05	0	1436	0.07
Samples WT on first test	Patient 24	0	160	0.00	0	211	0.00
	Patient 11	0	295	0.00	0	973	0.00
	Patient 15	0	1403	0.00	0	2167	0.00
	Patient 16	0	1110	0.00	0	712	0.00
	Patient 51	0	4932	0.00	0	9655	0.00
	Patient 85	0	244	0.00	0	471	0.00

Table 36 Samples tested in duplicate using MPX1 probes. Dark grey highlighting shows non-concordant results between tests. Sample Patient 118 (highlighted in light grey) only had a tiny volume left for the second test reflected in the low number of positive droplets for both the variant and WT probes. Number of positive droplets for both ESR1^{var} and ESR1^{WT} are shown along with the minimum VAF for the variant identified.

5.6.2 Results MPX2 (covering variants Y537N, Y537S and S463P)

62 samples had enough DNA remaining to be tested. The tested samples comprised of 50 EOT samples, 3 8-week samples and 8 baseline samples. Overall a variant was detected in **10/62 (16%)**

samples. It is likely that the majority of the 10 variants detected are Y537N. The median minimum VAF using the MPX2 probes was 0.8% (range 0.2%-22%).

12 samples were tested in duplicate (shown in Table 37); four samples that were positive on the first test, 2 that were unassigned and 6 that were WT. 2/12 samples showed non-concordance the first was positive on initial testing but then only contained two positive droplets on the second test. It is likely that this is a true low frequency variant. Patient 1 tested unassigned with 4 positive droplets on first test, when the DNA input was slightly higher on the second test this increased to 6 positive droplets. This again is likely to represent a sample with a variant at low frequency.

	Sample ID	First test MPX2			Second test MPX2		
		***ESR1*^{var}**	***ESR1*^{WT}**	**min VAF**	***ESR1*^{var}**	***ESR1*^{WT}**	**min VAF**
Samples with ≥5 droplets positive on first test	Patient 91	153	1288	11.49	222	1697	12.31
	Patient 107	6	3157	0.22	2	2414	0.08
	Patient 119	9	1100	1.07	14	2111	0.70
	Patient 159	287	2777	10.83	244	2593	9.69
Samples with 1-4 positive droplets on first test	Patient 1	4	1037	0.37	6	1337	0.43
	Patient 90	2	2120	0.13	1	1475	0.19
Samples WT on first test	Patient 85	0	284	0.00	0	246	0.00
	Patient 87	0	3076	0.00	0	2213	0.00
	Patient 108	0	3280	0.00	0	1924	0.00
	Patient 129	0	2202	0.00	0	1581	0.00
	Patient 148	0	1438	0.00	0	919	0.00
	Patient 24	0	133	0.00	0	72	0.00

Table 37 Duplicate testing of samples with probes for MPX2. Light grey shading indicates discordant result. Number of positive droplets for both ESR1^{var} and ESR1^{WT} are shown along with the minimum VAF for the variant identified.

5.6.3 Overall results for *ESR1* variants detected using ddPCR

Of the 62 patients who had data available for both MPX1 and MPX2 25 variants were detected in 20 patients. Giving an overall frequency of *ESR1* variants in the cohort of **20/62 (32%)**. 5/62 (8.1%) had dual *ESR1* variants (shown in Table 38); detected by both MPX1 and MPX2. Additional patients may have had dual variants but if both variants were covered by the same multiplex then this would not have been detected. In 3/5 samples with dual variants there was a dominant clone with a higher VAF; one with a MPX1 dominant clone and 2 with a MPX2 dominant clone. For samples Patient 21 and Patient 151 both clones were found at similar VAF. All samples had fairly well-matched overall concentrations which is helpful when comparing VAF.

	MPX1			MPX2		
Sample ID	***ESR1*^{var}**	***ESR1*^{WT}**	**min VAF**	***ESR1*^{var}**	***ESR1*^{WT}**	**min VAF**
Patient 107	6	3157	0.22	334	3150	10.90
Patient 119	14	2111	0.70	50	2008	2.59
Patient 21	78	2050	3.69	38	2019	1.97
Patient 151	8	2407	0.34	11	2429	0.45
Patient 159	265	2685	10.26	33	2558	1.34

Table 38 Samples with variants present in both MPX assays. Green shading denotes samples had sufficient sample for duplicate testing. Number of positive droplets for both ESR1^{var} and ESR1^{WT} are shown along with the minimum VAF for the variant identified.

5.6.4 Comparing *ESR1* variants detected using NGS and ddPCR

In chapter 4 samples from 41 patients who had received treatment with fulvestrant and vandetanib were tested for the presence of ESR1 variants (amongst other genes) using NGS. 4 samples contained a variant (10%). When samples from patients who had received treatment with fulvestrant and vandetanib were tested with ddPCR the frequency of ESR1 variants was 6/28 (21%) with the same four samples being identified as positive and an additional two samples containing an ESR1 variant with a low VAF (0.4% and 1.15% minimum VAF respectively). Neither were detected using NGS, even when the BAM files were reviewed manually. The NGS panel did not contain amplicons covering variants E380Q or S463P which were contained in MPX1 and MPX2 respectively, thus this discrepancy could be explained if the variants detected by the MPX probes were either E380Q or S463P.

5.7 Detection of copy number variation (CNA) of *MYC* using ddPCR

5.7.1 cfDNA

67 participants in the FURVA clinical trial were identified with sufficient volume of sample for *MYC* amplification testing. All samples were EOT samples apart from 2 baseline samples due to

insufficient EOT sample remaining. Stringent QC was applied (>10,000 total droplets with at least 400 droplets containing the region of interest for *MYC* and the control gene *AGO1*). Low DNA concentration meant that only 39 (58%) samples met the QC criteria. Of these 39 samples four had sufficient volume and concentration to allow repeat testing for further validation.

Of the 39 samples the median ratio of *MYC:AGO1* was 1.19 (range 0.89-2.49). The data was not normally distributed. 36/39 samples had a ratio ≤1.5. The ratio will be examined as a continuous variable along with the clinical outcome data in chapter 6 using the maxstat method to determine the optimum ratio that correlates with progression free survival.

Two samples had a ratio >2 meaning that there were double the number of copies of *MYC* in the samples compared to the reference gene; thus these samples are amplified. As previously discussed, given that the tumour percentage is likely to be less than 100% we should not only be calling samples with a ratio >2 amplified. Many of those with a ratio below 2 will also be amplified. However, this project was not able to determine the tumour fraction in the sample and thus the best analysis here will be a retrospective cut point analysis using the survival data to determine the cut point with the best predictor for significant difference in the relevant outcome between a *MYC* 'amplified' and a *MYC* 'non amplified' group.

The maxstat method (Hothorn and Zeileis 2008) was used to determine the optimum cut point to enable dichotomisation of the data into two groups *MYC* 'amplified' and *MYC* 'non amplified'. From the 39 samples meeting the QC thresholds a cut point was determined at 1.345. This resulted in 11/39 (28%) samples being deemed 'amplified'.

5.7.1.1 Reproducibility of cfDNA samples passing QC

Four samples were tested in duplicate as shown in Table 39.

Sample ID	Test 1	Amplified by maxstat	Test 2	Amplified by maxstat	Mean ratio
Patient 119	1.435	YES	1.397	YES	1.416
Patient 15	1.469 (EOT)	YES	1.345 (BASE)	YES	1.407
Patient 64	1.521	YES	1.408	YES	1.464
Patient 143	1.179	NO	1.073	NO	1.126

Table 39 Samples tested in duplicate for MYC CNA. All were from the same time point except Patient 15 where one sample was taken post treatment and one pre-treatment.

Although there was slight variation between samples, likely due to varying DNA concentrations and droplet assignment all samples tested in duplicate showed concordance in the interpretation of the result when a cut point was applied.

5.7.2 FFPE

58 samples were tested, 46 samples met QC criteria for assessment as detailed in section 5.7.1.

'Rain' (see section 5.5.2) was a significant challenge in FFPE samples, this was largely independent of sample concentration. Samples with minimal rain often did not contain enough amplified DNA to meet the QC checks. Thus, assignment of droplets to the appropriate cluster was difficult. Two approaches were investigated (shown in with a worked example from a single sample showing both methods in practice). Firstly, assigning a tight cut off for *MYC* positive droplets based on the control sample and applying this in a blanket fashion to other samples (see plot A). This strategy ensured that *MYC* positive droplets were stringently identified at the trade-off that the number of *AGO1* positive droplets was likely to be over called as the 'rain' from droplets containing DNA from both *MYC* and *AGO1* (shown in orange) were included in this count thus hypothetically decreasing the chances of a final amplified result. Secondly, droplets were manually assigned a cluster (see plot B);

this strategy allowed strict control of both *MYC* and *AGO1* positive droplets but at the trade-off of less droplets containing DNA from the genes of interest being included in the final analysis. This strategy hypothetically increases the chance of an amplified sample being called although in practice this is a low risk because more 'rain' is created from *MYC* containing droplets than *AGO1* containing droplets due to the concentration of the probes in the assay.

The manual assignment of droplets is likely to give a more accurate representation of droplets containing DNA from either the target or reference gene and so will be used for both the *MYC* and *FGFR1* FFPE analysis in this project. When comparing the two methods only one sample was deemed amplified by one method while non-amplified by the other (see Table 40)

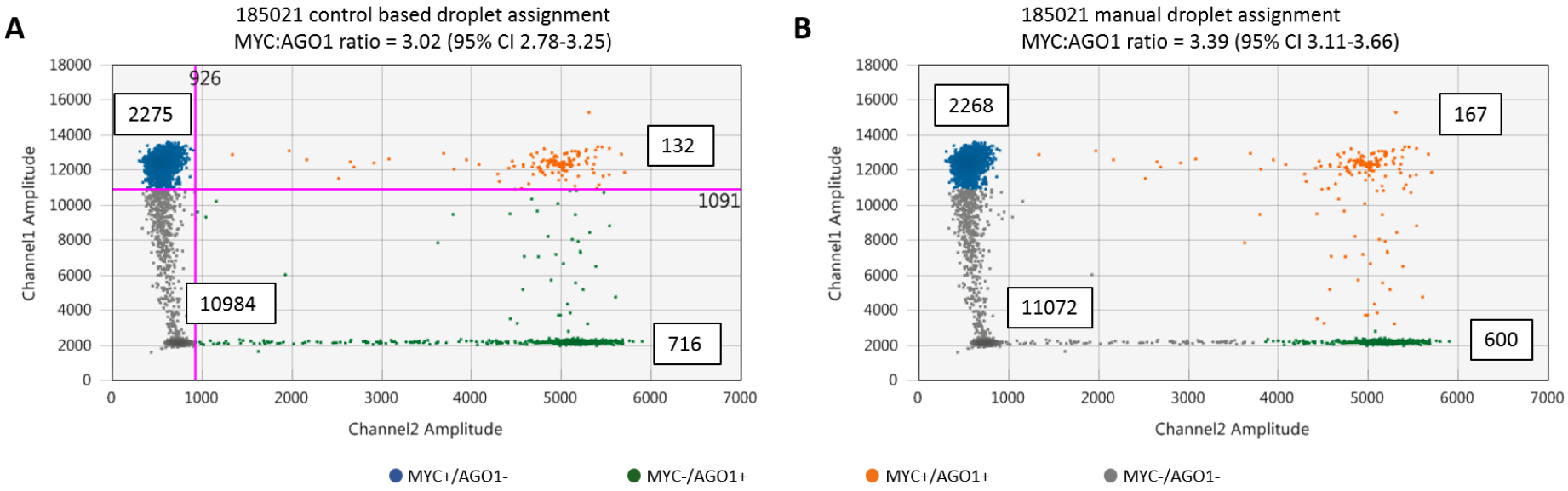


Figure 41 Same sample analysed using both the control based method (A) and the manual assignment method (B). The numbers of droplets assigned to each cluster is noted in the white superimposed boxes.

When samples were analysed using both assignment methods there was little difference between the two methods (see). Four more samples passed QC when droplets were assigned based on the control sample (50 vs 46 samples) but the number of amplified samples identified remained similar (14 amplified samples vs. 15 amplified samples). 13 samples were amplified by both methods. Where the result differed between the two methods, a single sample passed QC by control-based droplet assignment but failed by manual assignment, while two samples had a ratio >2 in the manually assignment method group but did not in the control-based assignment. The ratio difference was small for one sample (1.93 vs 2.08) but larger for the other (1.86 vs 2.38). No obvious explanation was identified as to why manual assignment resulted in a higher ratio in this sample although purely mathematically there must have been more rain droplets present from *AGO1* cluster than in other samples.

	Droplets assigned based on control	**Droplets assigned manually**
Samples passing QC	50	46
Samples amplified by MYC:AGO1 ratio >2	14	15

Table 40 Comparing methods of assigning droplets and the resulting interpretation of results

Moving forwards the results from the manual assignment of droplets will be used. As shown in Table 40 above this does not alter the number of amplified samples significantly, this is likely to be because the *MYC* probes produce more 'rain' than the *AGO1* probes. Thus, the frequency of *MYC* amplification in FFPE samples is **15/54 (28%)**.

5.7.2.1 Reproducibility (FFPE)

16 samples were available for testing in duplicate. The results are presented in Table 41. The tests were performed in separate batches but generally the number of DNA containing droplets from each test was similar. In two samples the amount of DNA was deliberately reduced in the second test to see if a similar ratio was seen with a reduced amount of DNA input (Patient 18 and Patient 27) in these cases despite halving the DNA input the ratios were similar; 3.26 vs 3.34 and 1.39 vs 1.43. Only one sample (shaded in grey in Table 41) had discordant results between the two tests; Patient 58 showed likely non-amplification in the first test but a borderline result in the second test with a ratio of 1.87. The estimated tumour content of this FFPE sample was 60% so it is possible that it had low level amplification present.

	Sample ID	Test 1			Test 2		
		MYC	***AGO1***	**Ratio**	***MYC***	***AGO1***	**Ratio**
Amplified ratio ≥2 on first test	Patient 18	4454	1216	3.26	2478	698	3.34
	Patient 22	1902	814	2.25	1698	754	2.22
	Patient 119	856	421	2.02	1754	883	1.95
Borderline amplified ratio ≥1.7 on first test	Patient 62	1380	740	1.84	1541	788	1.92
	Patient 156	1007	564	1.77	1542	785	1.93
	Patient 136	1979	1142	1.71	1545	801	1.90
Non-amplified on first test, ratio <1.7	Patient 139	1495	872	1.68	1325	787	1.66
	Patient 58	901	544	1.65	1169	616	1.87
	Patient 12	1793	1098	1.61	1923	1199	1.58
	Patient 24	1089	687	1.57	1253	778	1.60
	Patient 143	2083	1401	1.47	2135	1325	1.58
	Patient 27	3466	2178	1.39	1440	990	1.43
	Patient 153	1282	942	1.35	1404	966	1.46
	Patient 64	1401	1033	1.34	1255	935	1.33
	Patient 28	803	804	1.00	1009	968	1.04
	Patient 25	2087	2202	0.95	1939	1999	0.97

Table 41 Concordance of FFPE samples tested in duplicate for MYC amplification. Table shows number of positive droplets for each target gene and then shows the ratio. Samples are grouped by their ratio. Sample highlighted in grey is the only sample where the interpretation of the results changed from test 1 to test 2 where on test 2 the result was interpreted as borderline amplified.

5.8
5.8 Detection of copy number alterations (CNA) of FGFR1 using ddPCR

5.8.1 cfDNA

64 patients were identified as having a sample available for testing. 58 samples were taken at EOT, 5 from BASE and one from an unknown time-point but likely to have been on treatment by the dates of the associated samples. After QC (total droplets >10,000 and *FGFR1* and *AGO1* containing droplets >400) 31 high DNA concentration samples were analysed.

Of these 31 samples the median ratio of *FGFR1:AGO1* was 1.17 (range 0.712 to 4.17). The data was not normally distributed. Only three samples had a ratio ≥2.

Using the maxstat methodology (Hothorn and Zeileis 2008) a cut off of ratio of ≥1.27 was used to identify an amplified sample. Using this methodology **11/31 (35%)** samples were amplified. Two samples had a ratio of 1.27.

In chapter 6 this data will be explored in more detail; combining the data on ratios and amplification status with clinical outcome data from the FURVA trial.

5.8.1.1 Reproducibility of cfDNA samples passing QC

3 samples were tested in duplicate. One sample remained non-amplified at all thresholds, two remained amplified throughout as shown in Table 42.

Sample ID	Test 1	Amplified by maxstat	Test 2	Amplified by maxstat	Mean ratio
Patient 9	0.78	NO	0.77	NO	0.78
Patient 10	3.60	YES	3.68	YES	3.64
Patient 64	1.52	YES	1.44	YES	1.48

Table 42 cfDNA samples tested in duplicate for FGFR1 amplification using ddPCR

5.8.2 FFPE samples tested for *FGFR1* amplification

As per FFPE analysis for *MYC* amplification two methods of droplet assignment were compared (tabular data not shown). Assigning droplets based on the positive control resulted in 43 samples passing QC with 6 amplified samples identified. By manually assigning droplets 42 samples passed QC with the same 6 amplified samples identified. This gives an overall frequency of **6/42 (14%)** *FGFR1* amplification in FFPE samples.

5.8.2.1 Reproducibility of FFPE FGFR1 samples

6 samples were tested in duplicate and met QC standards (results shown in Table 43). Two samples were the selected internal controls which performed consistently though multiple tests. Although there was some variability in the ratio of *FGFR1:AGO1* this is likely due to the varying DNA concentration in each sample; the overall results as to whether the sample was amplified or not was the same in all four tests. In clinical samples that were tested twice ¾ showed concordance between tests. The samples from patient 62 showed discrepancy between the two tests; both tests had very similar starting DNA concentrations, the starting tumour content of the samples was ~80% and both tests had >17000 droplets generated, however the results did show a true discordance. Unfortunately, the sample ran out and no further DNA material was available for further testing.

Sample ID	Test 1	Test 2	Test 3	Test 4
Patient 2	0.94	0.74		
Patient 62	1.58	1.98		
Patient 24	0.73	0.79		
Patient 27	0.83	0.78		
Patient 9	0.79	0.91	0.83	1.01
Patient 10	3.03	3.49	3.81	3.59

	Amplified
	Borderline amplified
	Non-amplified

Table 43 Reproducibility of amplification status in FFPE samples tested for FGFR1 amplification. The samples from patient 9 and 10 were the control samples.

5.9 Concordance between paired cfDNA and FFPE samples

5.9.1 MYC

21 patients had both a cfDNA and FFPE sample that had been tested for MYC amplification and had passed QC checks.

Sample ID	cfDNA; amplified if ratio >1.35	FFPE; amplified if ratio >2
Patient 127	2.5	2.67
Patient 119	1.44	2.18
Patient 22	1.35	2.82
Patient 64	1.52	1.41
Patient 91	1.48	1.39
Patient 15	1.47	1.3
Patient 85	1.45	1.36
Patient 12	1.35	1.85
Patient 129	1.32	2.2
Patient 88	1.25	4.67
Patient 151	1.21	2.85
Patient 121	1.19	1.54
Patient 20	1.18	1.06
Patient 143	1.18	1.68
Patient 153	1.16	1.73
Patient 118	1.13	1.29
Patient 137	1.01	1.84
Patient 162	**0.98**	**1.86**
Patient 59	**0.97**	**1.45**
Patient 136	**0.96**	**1.84**
Patient 3	**1.34**	**1.33**

Amplified
Non amplified

Table 44 MYC amplification in cfDNA and FFPE samples. 21 participants had both a cfDNA and FFPE sample available for analysis. For cfDNA samples were amplified if their ratio was ≥1.35 as per the maxstat methodology. For FFPE status any sample with a MYC:AGO1 ratio >2 was counted as amplified

There is a mixture of concordance patterns, as seen in Table 44; patients whose tumours show amplification both in primary tumour and cfDNA (representing metastatic disease), n=3, those who do not show amplification in either setting, n= 10 and those where the amplification status changes over time n=8. 5 patients had an amplification detected in cfDNA but not in primary FFPE tumour suggesting that it may have occurred during the disease course. Three patients had a detectable amplification in tissue but this was not detected in cfDNA. This could either be due to clones with amplification not having become part of the metastatic tumour or, more likely, that if amplification was present in metastatic disease then it was either very low level or the patients circulating ctDNA concentration was very low.

5.9.2 FGFR1

18 patients had both an FFPE and a cfDNA sample that met strict QC criteria (see Table 45). 17/18 samples were concordant with the same amplification status in both FFPE and cfDNA samples. A single sample showed unassigned amplification in cfDNA but was non-amplified in FFPE.

Sample ID	cfDNA amplified if ratio >1.27	FFPE amplified if ratio >2
Patient 10	3.58	3.48
Patient 21	1.89	2.50
Patient 64	1.48	1.53
Patient 15	1.36	1.18
Patient 121	1.33	0.85
Patient 119	1.29	1.33
Patient 60	1.27	1.29
Patient 151	1.27	1.24
Patient 12	1.24	0.89
Patient 143	1.20	0.88
Patient 51	1.17	0.98
Patient 91	1.15	0.77
Patient 153	1.12	1.00
Patient 137	1.00	1.25
Patient 127	0.90	0.85
Patient 22	0.90	1.52
Patient 129	0.88	0.91
Patient 9	0.78	0.88

Amplified
Non amplified

Table 45 FGFR1 amplification in cfDNA and FFPE samples. 18 participants had both a cfDNA and FFPE sample available for analysis. For cfDNA samples were amplified if their ratio was ≥1.27 as per the maxstat methodology. For FFPE status any sample with a FGFR1:AGO1 ratio >2 was counted as amplified

Overall the rate of FGFR1 amplification as determined using the methodology detailed in chapter 2 in FFPE tumours representing primary tumours was 14%. Where samples were available (n=18) to investigate correlation between FFPE and cfDNA samples FGFR1 amplification was detected in both FFPE and cfDNA samples in 2 patients, in 6 patients in cfDNA samples but not in FFPE samples and in 10 samples no amplification was detected in either FFPE or cfDNA samples. This is summarised in Table 45. This data suggests that FGFR1 amplification can occur at any point during the disease

course but may be more common in the metastatic disease setting. However, given the small numbers of patients in which both samples are available this should be interpreted with caution, it should also be noted that two of the cfDNA samples sat just on the amplification cut point.

5.10 Chapter discussion

5.10.1 *ESR1* variants

The overall frequency of *ESR1* variants in cfDNA in this cohort of patients with endocrine resistant breast cancer was 32%. This compares well with other published literature as detailed in Table 46 where the studies that most closely resemble the participants in the FURVA study showed frequencies of between 24% and 39% detected using ddPCR. The study that offers the best comparison where the same probes were used and patients were recruited to the trial at the same point in their disease course was the PALOMA-3 subgroup of the Fribbens paper where the frequency of variants was 25.3% with 7% of all samples tested (total samples tested = 360) showing polyclonal *ESR1* variants (Fribbens et al. 2016). The fact that the frequency in the FURVA patients was slightly higher could be related to the fact the samples were tested after exposure to treatment with fulvestrant +/- vandetanib thus potentially meaning that the majority of tumours were progressing at the point of sample collection previously undetectable sub-clones were now reaching detectable levels. It could also be due to the smaller numbers of patients involved.

Despite other studies (detailed in Chapter 2 section 2.5.4.1) calling samples positive with less than 5 droplets containing DNA with the variant of interest a strict cut off ≥5 droplets was needed to call a positive sample in the samples used in this project. The reasons for this are two-fold; first several samples had very low DNA concentrations which results in wide 95% confidence intervals particularly in samples with low DNA input *and* low VAF. In these samples applying a ≥ 5 should have reduced the risk of a false positive call. Second; the aim of detecting these samples containing an *ESR1* variant is to explore whether they affect response to treatment with fulvestrant +/- vandetanib and little is known about what VAF an *ESR1* variant must be present at to influence treatment

response. Samples with less than 5 positive droplets are likely to have a low VAF (although this depends on the starting DNA input) and it is unknown at what VAF the presence of an *ESR1* variant begins to influence clinical outcomes.

The identification of 5 samples (see Table 38) with dual *ESR1* variants is interesting. The VAF in the samples is not sufficient to sequence the samples by pyrosequencing or similar to ascertain which specific variants are present nor is their sufficient sample remaining to establish which variants are present by ddPCR. Could it be possible that in a similar manner to dual *PIK3CA* variants predicting for enhanced response to PI3K inhibitors (Vasan et al. 2019) dual *ESR1* variants would lead to an enhanced response to fulvestrant? Of the 5 patients whose samples contained more than one *ESR1* variant PFS varied from 53 to 580 days (median 62 days) suggesting that if anything rather the predicting for enhanced response to treatment the presence of more than one variant was a poor prognostic sign.

Study	Stage of disease	Variants tested for	Number of patients tested	Overall frequency of ESR1 variants	Frequency of double mutants	Comments	Reference
BOLERO-2[13] clinical trial	1st line metastatic disease (mixture of progression on endocrine therapy and those who had previously received an AI but had not shown resistance)	D538G and Y537S	541	28.8%	5.5%	Both variants and double mutated disease predicted for shorter OS compared to WT status. HR 1.59 (1.26-2.00) p = <0.001. (multivariate analysis) Y537S was a stronger predictor than D538G and those patients with double mutant disease had the worst overall prognosis	(Chandarlapaty et al. 2016)
SoFEA[14] and PALOMA-3[15] clinical trials	Patients with prior exposure to AI who had developed endocrine resistant disease	Same MPX probes used as used in	161 from SoFEA and 360 from	39% in SoFEA and 25.3% in PALOMA-3	17% of all samples tested in SoFEA and	In the SoFEA study patients who had an ESR1 variant and received fulvestrant had a better PFS than those who received exemestane HR 0.52 (95% CI 0.30-0.92, p=0.02). In the PALOMA-3	(Fribbens et al. 2016)

[13] BOLERO-2 was a double-blind placebo controlled randomised phase 3 study comparing exemestane and placebo to exemestane and everolimus (an mTOR inhibitor) in patients with ER+/HER2- ABC that had progressed on a non-steroidal AI

[14] SoFEA (Study of fulvestrant versus exemestane with or without anastrazole) was a randomised phase III study in the setting of disease progressing after prior endocrine therapy.

[15] PALOMA-3 (Palbociclib combined with fulvestrant in hormone receptor-positive HER2-negative metastatic breast cancer after endocrine failure) was a randomised phase III study comparing fulvestrant and placebo to fulvestrant and palbociclib (a CDK4/6 inhibitor).

		this project	PALOMA-3		7% in PALOMA-3	study there was no difference in response to fulvestrant and CDK4/6 inhibition in patients with $ESR1^{mut}$ or $ESR1^{WT}$ disease.	
Laboratory study	Advanced breast cancer	L536R, Y537S, Y537N, Y537C, D538G	171	10.5% (14% in ER+ disease)	Not stated	ESR1 variants predicted shorter PFS in patients taking aromatase inhibitors in the setting of advanced disease HR 3.1 (1.9-23.1; p = 0.0004 by log rank test) n=45	(Schiavon et al. 2015)
Laboratory study	Patients with ER+ metastatic disease	K303R, S463P, Y537C, Y537N, Y537S, D538G	29 cfDNA samples tested	24%	Not stated	0.2%-13.7% VAF in cfDNA. D538G was the most common variant. 6/7 patients in whom a variant was detected had been treated with an AI, one had only received a SERD.	(Wang et al. 2016)

Table 46 Studies in which ddPCR was used on cfDNA samples to assess ESR1 variant frequency

5.10.2 Using ddPCR to determine copy number variation in cfDNA and FFPE samples

The methods used in this chapter were based on for assessment of *ERBB2* copy number alteration in breast cancer cells (Garcia-Murillas and Turner 2018). In FFPE samples a comparative gold standard test of samples using FISH or similar method would have been ideal. Comparison between ddPCR and the gold standard would be necessary for implementation of ddPCR as a valid clinical test to asses copy number changes in FFPE samples. However, I believe the results here are valid as stringent cut offs have been applied and great pains have been taken to ensure that droplets have been correctly assigned and interpreted. The probes and the selection of a reference gene were designed by Biorad and purchased without modification. The validation of the probes by Biorad extended to wet lab demonstration of the presence of two copies of the target gene using *AGO1* as a reference gene in two cell line samples, this does leave a possibility that *AGO1* as a choice of reference could have been improved. Additionally, if fresh frozen tissue samples could have been used as starting material the problem of 'rain' occurring may have been minimised as the DNA extracted would have been less fragmented (Hughesman et al. 2016).

In cfDNA samples it is accepted that ctDNA will make up a small proportion of total circulating DNA. To determine this percentage is challenging but can be done with next generation sequencing (Adalsteinsson et al. 2017). Looking to the future when CNA detection by ddPCR has potential as a routine clinical test it may not be practical to assess ctDNA as a percentage of cfDNA by NGS for each patient therefore it is not unreasonable to ask whether we can determine the presence of copy number variation without knowing the proportion of ctDNA in cfDNA. Assuming that the reference gene is never amplified and that the probes use a sequence of DNA unique to each gene of interest one can be confident that the ratio is true and the methodology correct. The subtlety comes in interpreting what ratio constitutes amplification; this will vary between samples based on the total input DNA but also the percentage of cfDNA derived from ctDNA which is very challenging to establish even with advanced technologies and high concentrations of input DNA.

The risk is that samples where the ctDNA component of cfDNA is high will potentially result in false positive calls e.g. if the ctDNA component of cfDNA was 90% then only samples with a ratio of >1.9 should be called positive. This an extreme example as it is generally accepted that ctDNA represents a smaller proportion of cfDNA. Certainly, in these patients if the range of *ESR1* variant frequencies offers a conservative estimate of ctDNA then all samples would likely be below 30% offering scope for setting the threshold for calling a positive sample around 1.3. Some estimates can be made at the likely tumour fraction (i.e. the % of cfDNA made up of ctDNA) in patients with metastatic breast cancer according to Adalsteinsson et al's methodology which developed a technique for assessing tumour fraction using ultra low pass WGS in cfDNA samples. Here of the 391 patients included in the study (of which around 60% were ER+) only 32% of samples had a tumour fraction of ≥10% (Adalsteinsson et al. 2017) when a single sample was tested. Bearing in mind that at least 30% of these samples were ER- and therefore likely to have higher tumour fraction than patients with ER+ breast cancer and that DNA was extracted from 4mls of plasma it is likely that the tumour fraction in the samples used in this project is <10% for most patients.

5.10.3 *MYC* amplification

MYC amplifications were detectable in both cfDNA and FFPE samples. There were significant challenges with both sample types. CfDNA samples were often very low in concentration, this meant that samples often did not pass QC and that they were exhausted quickly. If *MYC* amplification status was the sole aim after extraction of cfDNA then one could increase the sample concentration using vacuum based evaporation e.g. Speedvac. However, as the samples used here were needed for multiple tests this was not performed. Alternatively, if prospective testing was planned more than 2mls of plasma could be used to extract from.

FFPE samples were also difficult to work with as significant 'rain' was present meaning that droplets did not split into easily identifiable clusters. Work was undertaken to try and explore how this affected the final status of the sample and two methods resulted in little change in the number of

amplified samples leading to an acceptance that while droplet assignment was not perfect the overall amplification status of the sample was unlikely to change.

The frequency of *MYC* amplifications in FFPE samples was higher than the best available comparator dataset; 28% in this dataset vs. 9.3% (Razavi et al in CBioPortal limited to patients with ER+/HER2- metastatic disease). However, the time points at which the samples were taken may have differed significantly and the MSK-IMPAKT dataset used NGS to determine CNA rather than ddPCR. Stringent QC was applied to the FURVA dataset so I do not believe that the difference in frequency is wholly due to technical differences between methods although this cannot be completely excluded. A meta-analysis by Qu et al identified 29 studies that had looked at *MYC* 'overexpression' in patients with breast cancer. Overexpression was a mixture of CNA as determined by FISH right through to protein expression as determined by IHC. When the 29 studies are limited to those that looked specifically at amplification (i.e. those that used FISH, CISH (chromogenic in situ hybridisation), qPCR or ddPCR) in patients with breast cancer (excluding studies in patients with DCIS) the range of 'over-expression' was 5.25% (Al-Kuraya et al. 2004) to 38.46% (Sadeghi et al. 2017). However, on further investigation Sadeghi et al determined 'overexpression' from RNA extraction from FFPE samples which was then converted into cDNA and tested for *MYC* amplification using qPCR. This methodological difference may account for the high rate of 'over expression'. In papers where DNA direct from FFPE tissue has been examined rates generally compare with those in the Razavi dataset e.g. Rodriguez-Pinilla et al who used CISH on FFPE samples to establish a frequency of *MYC* amplification of 8.75% in patients with luminal breast cancer (Rodriguez-Pinilla et al. 2007). As to whether a specific level of amplification influences clinical outcome there are many conflicting reports with no clear conclusion drawn (Blancato et al. 2004).

One may hypothesise that ddPCR would be a more sensitive method of detecting amplification particularly in low DNA samples and this could account for higher frequencies of amplification than other methods. This section of work would benefit significantly if samples had also been tested for

amplification using FISH, however no further sample was available for this testing to be carried out. This would form an essential part of any prospective validation if, when correlated with clinical outcomes, *MYC* showed potential as a prognostic or predictive biomarker.

In cfDNA the frequency of detection of *MYC* amplification was 28%. Comparing the ranges of *MYC:AGO1* ratios to those quoted in *ERBB2* CNA work by Gevensleben et al the ratios for the *MYC* CNA are lower. This would suggest that few samples are strongly amplified, many samples have low tumour fraction or a combination of both. Focussing initially on those that demonstrate strong amplification i.e. those that remain amplified even when stringent thresholds are applied in the analysis is a reasonable place to start when investigating their potential as biomarkers in this context and will be the approach taken in chapter 6.

A major limitation of the *MYC* amplification work, particularly in cfDNA was the lack of sample availability for duplicate testing. Of the 39 samples meeting strict QC only 4 had sufficient volume and concentration to be tested in duplicate. There was 100% concordance suggesting that the test is reliable. However, testing only 10% of samples in duplicate is sub-optimal but does tentatively lend support to the idea that if the sample concentration has allowed good amplification of the regions of interest (i.e. >400 droplets containing DNA sequence for *MYC* and *AGO1*) then a low threshold for calling amplification is acceptable.

5.10.4 *FGFR1* amplification

Rates of *FGFR1* amplification were lower in FFPE samples (14%) but higher in cfDNA samples (35%) than *MYC* amplifications. The frequency of amplification detection in FFPE samples is in keeping with the MSK-IMPAKT breast cancer dataset (Razavi et al. 2018). FGFR1 amplifications in ER+ breast cancer are often noted as part of larger genomic analyses but are rarely the main focus of a study. Review articles suggest a frequency range of 7.5-27% in breast cancers (André and Cortés 2015).In a paper that looked specifically at *FGFR1* in 880 breast cancers using CISH the overall rate of amplification was 8.7%, amplification was more common in ER+ tumours but did not reach statistical

significance. Amplifications were defined as samples where >50% of neoplastic cells contained > 5 copies of the gene. In patients with ER+ disease its presence predicted for poorer OS on univariate analysis (Elbauomy Elsheikh et al. 2007). In a more recent study *FGFR1* amplification in FFPE samples from patients with ER+ ABC predicted for shorter PFS when patients were taking endocrine therapy alone (Drago et al. 2019). In this study FISH was used on FFPE samples and samples were called amplified if the *FGFR1* to centromere 8 ratio was ≥2.

Where samples from both primary tissue and metastatic cfDNA were available amplifications were detected at both time points suggesting that *FGFR1* amplification could be an early event and that clones harbouring amplifications do not respond to subsequent therapies in particular endocrine therapies.

FGFR1 inhibitors are entering clinical practice. The phase 2 FINESSE study used lucitanib as a single agent in three cohorts of patients with ER+/HER- breast cancer. The minimum number of responses as determined by RECIST 1.1 was only reached in the *FGFR1* amplified group where amplification was determined by FISH and experimentally using ddPCR (no details given about what cut-off value was applied) (Hui et al. 2019) suggesting that *FGFR1* amplification may be an important biomarker to explore in further trials using FGFR directed therapies.

5.11 Chapter summary

The data presented in this chapter shows that it is possible to detect both SNVs and CNAs from FFPE and cfDNA samples using ddPCR. For the more well validated assays such as *ESR1* the frequency of *ESR1* variants in the FURVA study participants was 32%. By using ddPCR the detection rate is improved compared to NGS although it is not known if detecting variants at low VAF is clinically meaningful. In the copy number assays which are less methodologically developed the frequency of *MYC* amplification in cfDNA and FFPE was 28% although not always with concordance between cfDNA and FPE samples. For *FGFR1* the frequency of amplification in cfDNA samples was 35% and 14% in FFPE samples. Although numbers were small it appeared that *MYC* amplifications could occur

in primary tumour and as a potential mechanism of resistance in cfDNA samples representing metastatic disease, for the *FGFR1* amplifications identified where both FFPE and cfDNA samples were available both occurred in the primary tumour and persisted in the metastatic setting suggesting that *FGFR1* amplification is an early event in the disease course.

Overall the data in this chapter has increased the number of samples tested in this project for *ESR1* variants which should allow for a better clinical analysis of the effects of *ESR1* variants on response to treatment with fulvestrant +/- vandetanib. Furthermore, new data has been generated regarding the potential and challenges of detecting gene amplifications in *MYC* and *FGFR1* using ddPCR technology in plasma samples.

The data produced in this chapter along with data regarding RET protein expression from chapter 3 and NGS SNV data from chapter 4 will now be paired with clinical outcome data from the FURVA clinical trial in chapter 6.

6 Clinical correlation

6.1 Chapter Overview

In this chapter data created and explored in the prior three results chapters will be paired with clinical outcome data from the FURVA clinical trial to assess whether individual or combined results have potential for further investigation as biomarkers in the setting of endocrine resistant metastatic breast cancer treated with fulvestrant +/- vandetanib. Each category of potential biomarker will be considered in turn; immunohistochemical analysis of RET protein expression, detection of SNVs by NGS and ddPCR and detection of CNVs by ddPCR.

Following each individual analysis an overall recommendation will be made as to whether any of the biomarkers explored should be taken forward for validation studies in this setting.

6.1.1 Chapter Aim

To establish whether any of the biomarkers investigated have potential to act as predictive biomarkers of response to either treatment with fulvestrant +/- vandetanib with regards to progression free survival.

6.1.2 Chapter objectives

To explore the response to treatment with fulvestrant +/- vandetanib based on the hypotheses and pre-planned exploratory analyses stated in chapter 1. From these new hypotheses will be generated for future work.

- Hypothesis 1: High expression of t-RET and/or p-RET will predict shorter progression free survival (PFS)
- Hypothesis 2: Response to vandetanib will depend on high t-RET or p-RET expression

- Exploratory analysis 1: Variants in key proteins downstream from RET and their individual and collective relationships with progression free survival in patients treated with fulvestrant +/- vandetanib
- Exploratory analysis 2: The relationship between clinical outcome data and SNVs detected using NGS and ddPCR as predictors of patient's clinical outcomes when treated with fulvestrant +/- vandetanib
- Exploratory analysis 3: Copy number alterations in *MYC* and *FGFR1* in FFPE samples representing primary tumour and cfDNA samples representing metastatic disease and their relationship with progression free survival in patients treated with fulvestrant +/- vandetanib

As this project is designed to be hypothesis generating statistical tests with a p value of <0.1 will be noted as of interest and marked in *italics*. If results meet statistical significance; p value ≤0.05 these will be noted in ***bold italics***.

6.1.3 Materials

6.1.3.1 Clinical data

The clinical data used in this chapter was provided by referring centres recruiting participants into the FURVA trial. Information was captured using clinical report forms (CRFs), the data from which was then manually coded to form a dataset of clinical variables by participant by the trial team in Centre for Trials Research (CTR), Cardiff University. The data was shared via an SPSS workbook. Some data was further summarised for the purpose of statistical analysis by ZH. For example, instead of reporting number of sites of metastatic disease further sub-categories were introduced e.g. bone only disease. Progression free survival data (PFS) was calculated from date of randomisation to date of reported clinical or radiological (by RECIST 1.1) progression or death. If patients withdrew from the study, either through choice or toxicity, they were censored at their last date of RESCIST or clinical assessment. Where participants were lost to follow up the date they were

last known to be alive was used. The analysis performed in this chapter is based on the data available as of 12/03/2020 using the methods detailed in Chapter 2 section 2.6.

Recruitment to the trial closed on 30th October 2017 and at the time of data analysis (March 2020) there had been 130 progression events. The trial was designed to detect a hazard ratio of 0.65 for progression free survival with 90% power and one-sided significance of 20% assuming an overall loss to follow up of 10%. It was deemed by the trial statistician that 98 events would need to occur before this could be assessed.

Participants in the FURVA trial were all post-menopausal at time of trial entry and had clinical evidence of endocrine resistance. The trial randomised patients in a 1:1 ratio between treatment with fulvestrant and placebo and fulvestrant and vandetanib. The randomisation was double blinded and used the minimisation method to ensure patient characteristics were balanced between the two arms for primary or secondary resistance to an AI and measurable vs. non measurable disease.

Other potential poor prognostic clinical markers were also relatively evenly distributed between the two arms (Table 47)

		Treatment	
		Fulvestrant and placebo	**Fulvestrant and vandetanib**
		Count	Count
Cancer Type	IDC	66	56
	ILC	10	16
	Other	8	8
	Data not available	1	0
Stage of Breast cancer	III inoperable	2	3
	IV	83	77
Prior adjuvant chemotherapy?	Yes	46	46
	No	39	34
First adjuvant endocrine therapy	Tamoxifen	51	42
	AI	25	22
	Other	1	4
	Data not available	8	12
Disease relapse while on adjuvant endocrine therapy?	Yes	29	29
	No	48	39
	Data not available	8	12
Lines of endocrine therapy	0	4	8
	1	49	39
	2	18	17
	3	14	16
Lines of chemotherapy in the metastatic setting	0	51	51
	1	33	29
	2	1	0
Bone only disease	Yes	19	17
	No	66	63

Pattern of metastatic disease	Bone only	19	17
	Lymph node only	4	6
	Visceral disease	55	48
	Bone and lymph node only	6	7
	Other	1	2

Table 47 Clinical characteristics of trial participants. IDC = invasive ductal carcinoma. ILC = invasive lobular carcinoma. Visceral disease includes liver and/or lung metastases. Other sites of metastatic disease included skin and pleural effusion.

It is important to note a number of clinical characteristics of the patients who supplied samples for this study. Firstly, while all trial participants were post-menopausal at the time of trial enrolment the use of tamoxifen as the first line of endocrine therapy in 93/145 (64%) participants with data available could suggest that the majority of the women were pre-menopausal at the time of diagnosis as tamoxifen is generally only used for pre-menopausal women in the clinical setting. However, it could also reflect the era within which the patients received their primary treatment. Secondly, 103/165 (62%) participants had visceral disease at trial entry suggesting relatively advanced disease although no significant impairment on end organ function which would have prohibited entry into the trial. In addition to this 58/145 (40%) participants, where data was available, relapsed while on adjuvant endocrine therapy which suggests that these patients already had intrinsic endocrine resistance potentially through different mechanisms to those that acquired resistance over time once they had developed metastatic disease. However, no dates were available to analyse at what point in their adjuvant therapy they relapsed; the biology of disease when someone relapses in the first year is likely to be very different to those patients who relapse in the latter years of extended endocrine therapy.

6.1.3.2 Sample data

6.1.3.2.1 FFPE samples for immunohistochemistry

The materials and methods used for the generation of the t-RET and p-RET expression by immunohistochemistry have been detailed previously (chapter 2 and 3). In summary, all samples

where the tissue block received contained primary tumour were used for the clinical correlation analysis (n=115).

6.1.3.2.2 FFPE and cfDNA samples for NGS

The data regarding presence or absence of SNVs and CNVs in patient samples was obtained from the experiments detailed in chapter 4 and 5. A small number of additional baseline cfDNA samples were sequenced early in the project, these patients did not have corresponding FFPE samples and therefore were not included in the analysis in chapter 4. They have been included here where they account for knowledge of patients *PIK3CA/AKT1/TP53* variant status that has not been determined by testing of another time point (n=6).

Some trial participants had more than one sample available. Where a variant was present in more than one sample per patient it was only counted once. As many patients did not have samples available for testing there are potential sources of bias as we do not know why a sample was not provided. It may be that patients with rapidly worsening disease were not inclined to provide a blood sample. Another potential bias is that some centres recruiting patients to the trial collected more samples than others and thus samples may be clustered from certain areas of the UK. For both FFPE and cfDNA samples there were a range of time points at which samples were taken. Effort was made to standardise this and the majority of FFPE samples were from primary tumour rather than metastases and most cfDNA samples were taken at the point of stopping treatment either due to disease progression or toxicity.

6.1.4 Methods

Data was received from the trial statistician via an SPSS workbook. Data analysis was performed in SPSS Version 25.0.0.1 (IBM, Armonk, NY, USA) Chi-squared and Cox proportional hazard tests were performed in SPSS. Figures were produced using the packages ‘Survival’ (Therneau 2015), ‘Survminer’ (Kassambara, 2019) and ‘ggplot2’ (Wickham 2009) in R Studio version 1.1.456 using R programming language. Analysis was performed using the pre-planned statistical methods detailed

in chapter 2 where the biomarker being studied was present in ≥ 5 patients. In addition, there are sections of descriptive analysis where it was felt that performing statistical analysis on very small numbers of samples could lead to a misleading result. Where survival analyses are performed differences between the comparative groups are shown using Kaplan Meier plots with a hazard ratio and two sided p value calculated using Cox Proportional Hazards.

6.2 Clinical correlation of t-RET and p-RET expression

HYPOTHESIS 1: HIGH EXPRESSION OF T-RET AND/OR P-RET WILL PREDICT SHORTER PROGRESSION FREE SURVIVAL (PFS) AND HYPOTHESIS 2: RESPONSE TO VANDETANIB DEPENDS ON HIGH T-RET OR P-RET EXPRESSION

Firstly, a dataset was created using a binary variable representing whether a sample was deemed 'RET-high' or 'RET-low' by the three methods of defining 'RET-high' samples explored in chapter 3. This data was combined with progression free survival data from the clinical trial and Cox proportional hazards model was used in SPSS to explore whether any of the methods could demonstrate a difference in survival between patients with 'RET-high' or 'RET-low' tumours. In addition to this, to try and maximise the data, the maxstat method (Hothorn and Zeileis 2008) was applied to H scores as a continuous variable and the data regarding PFS. The maxstat method identified a score of 166 as the optimum cut point to distinguish a difference in PFS between T-RET-high and T-RET-low samples and 85 in P-RET-high and P-RET-low samples.

	T-RET					P-RET				
		95% CI for HR					95% CI for HR			
		HR	Lower	Upper	Sig		HR	Lower	Upper	Sig
Method A	High/Low = 60/55	0.833	0.540	1.284	0.407	High/Low = 61/54	0.837	0.548	1.279	0.412
Method B	High/Low =30/85	0.785	0.478	1.291	0.341	High/Low = 24/91	0.836	0.485	1.439	0.517
Method C	High/Low = 75/40	0.646	0.416	1.004	*0.052*	High/Low = 64/51	0.881	0.578	1.343	0.557
Maxstat	High/Low = 47/68	0.558	0.359	0.866	***0.009***	High/Low = 79/36	0.567	0.358	0.900	***0.016***

Table 48 RET expression (t-RET and p-RET) and progression free survival (PFS) explored using three different cut-points and evaluated using Cox-Regression modelling. Shaded results met pre-specified significance.

Using a cut point method based on cut points for percentage of cells showing 2+ or 3+ staining (Methods A, B and C[16]) no cut point could predict for a statistically significant different in PFS between the two groups created for either T-RET or P-RET. Method C for identifying T-RET high tumours came close to statistical significance with a p value of 0.052. However, for both assays the maxstat method, which used overall H-score as a continuous variable was better able to identify a cut point that resulted in a statistically meaningful difference in PFS between patients with high and low T-RET and P-RET expressing tumours.

When the maxstat method was applied to the data and the data visualised using a Kaplan Meier plot it was shown that, contrary to hypothesis 1, patients with t-RET-high or p-RET-high tumours, on average, had a longer PFS.

[16] Method A (>10% of cells showing 3+ staining), Method B (>30% of cells showing 3+ staining), Method C (>10% of cells showing 3+ staining OR >30% showing 2+ staining).

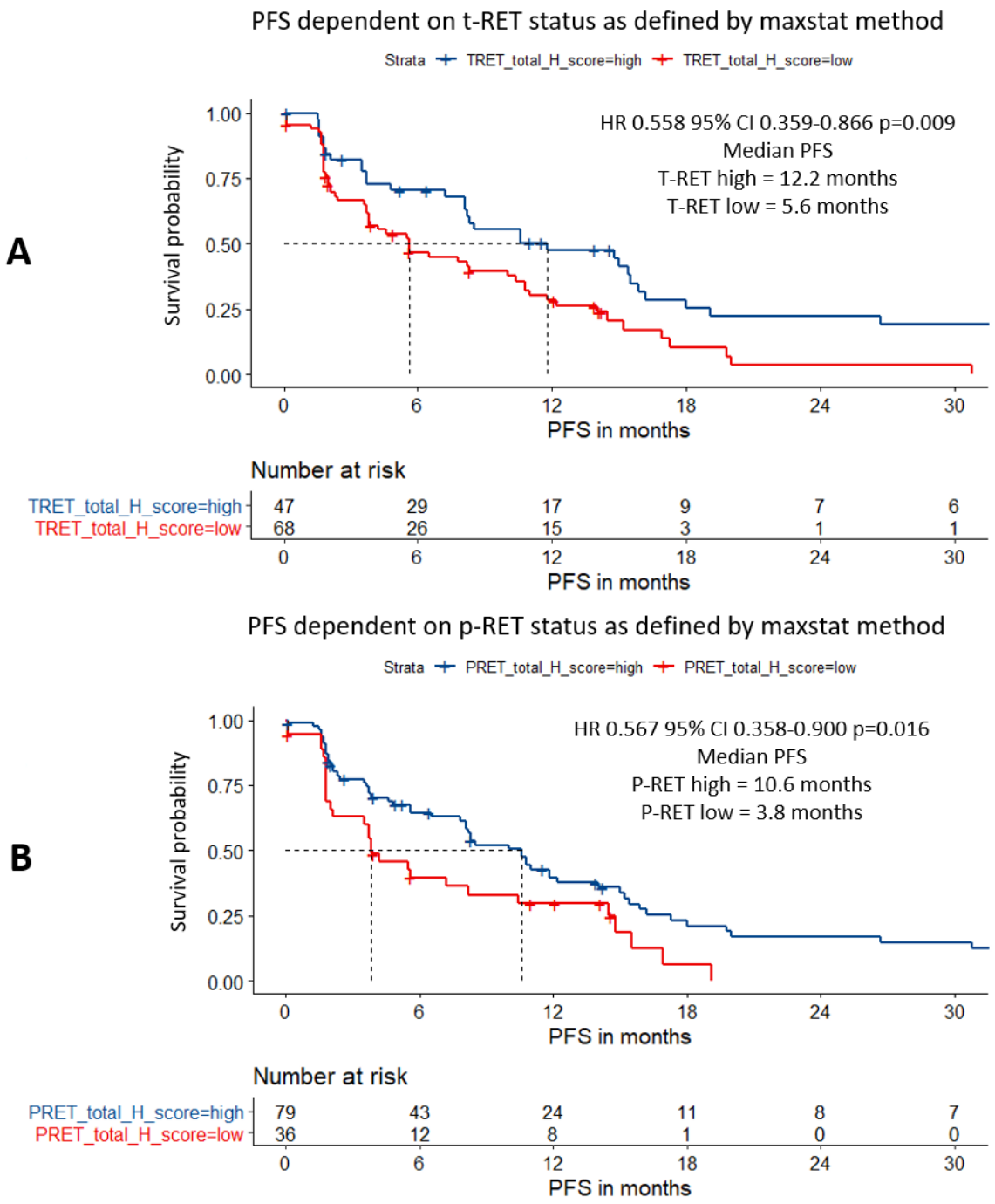


Figure 42 Kaplan Meier plots showing the difference in PFS when patients are grouped by (A) t-RET status and (B) p-RET status. Here the RET status has been determined using the maxstat method with total H-scores > the maxstat defined cut off being deemed 'high' and those ≤ 'low'. N=115 primary tumour FFPE samples with RET expression having been determined using the methodology discussed in chapter 3.

One possible explanation for this would be that patients with t-RET-high and p-RET-high tumours had greater benefit from RET inhibition with vandetanib (hypothesis 2). To explore this cox

regression analysis was performed for both potential biomarkers in both treatment groups (Table 49). This data is also shown in the Kaplan Meier plots in Figure 43.

	T-RET				**P-RET**			
		95% CI				95% CI		
	HR	Lower	Upper	p-value	HR	Lower	Upper	p-value
Arm A: Fulvestrant and Placebo	0.529	0.302	0.928	***0.026***	0.768	0.418	1.413	0.397
Arm B: Fulvestrant and vandetanib	0.591	0.291	1.200	0.145	0.371	0.176	0.781	***0.009***

Table 49 Cox regression analysis of both RET based biomarkers ability to predict for PFS (progression free survival)

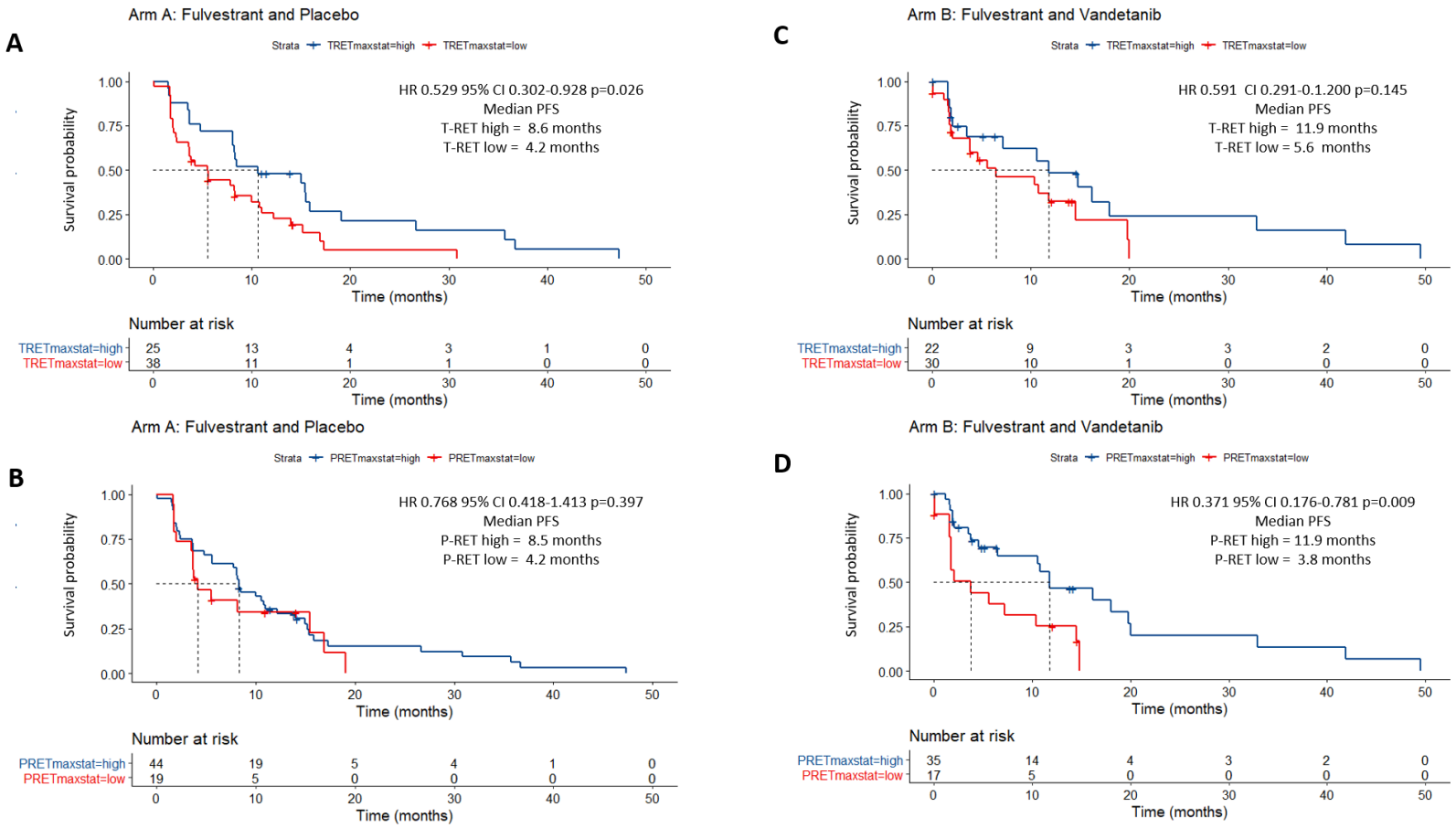


Figure 43 Kaplan Meier plots showing RET based biomarkers ability to predict PFS split by treatment received and biomarker. In plots A and B treatment received was fulvestrant and placebo while in plots C and D treatment was with fulvestrant and vandetanib. Hazard ratios and confidence intervals calculated using cox regression analysis in SPSS while median PFS was calculated using Kaplan Meier methodology. Time (months) refers to PFS (progression free survival). 63/85 (76%) patients in Arm A had a primary tumour FFPE samples analysed. 52/80 (65%) patients in Arm B had a primary tumour FFPE sample analysed.

The data in both Table 49 and Figure 43 tentatively suggest that there may be a relationship between a high P-RET score and improved PFS when receiving treatment with fulvestrant and vandetanib. There was no statistically significant difference in PFS between patients with high or low P-RET scores when treated with fulvestrant and placebo. However, for T-RET this was reversed, a statistically significant difference was noted in the group treated with fulvestrant and placebo while there was no difference when treated with fulvestrant and vandetanib, although the HR between the two arms was less marked. It should be noted that small numbers of patients may have generated a false positive signal as patients with high P-RET and T-RET scores did better in both arms of the trial but reached statistical significance only in one of the arms.

However, when the relationship between RET status, treatment received and PFS was explored using a multivariate cox regression analysis it was demonstrated that only RET status (t-RET or p-RET) was significant. This is illustrated in the Kaplan Meier plots in Figure 44.

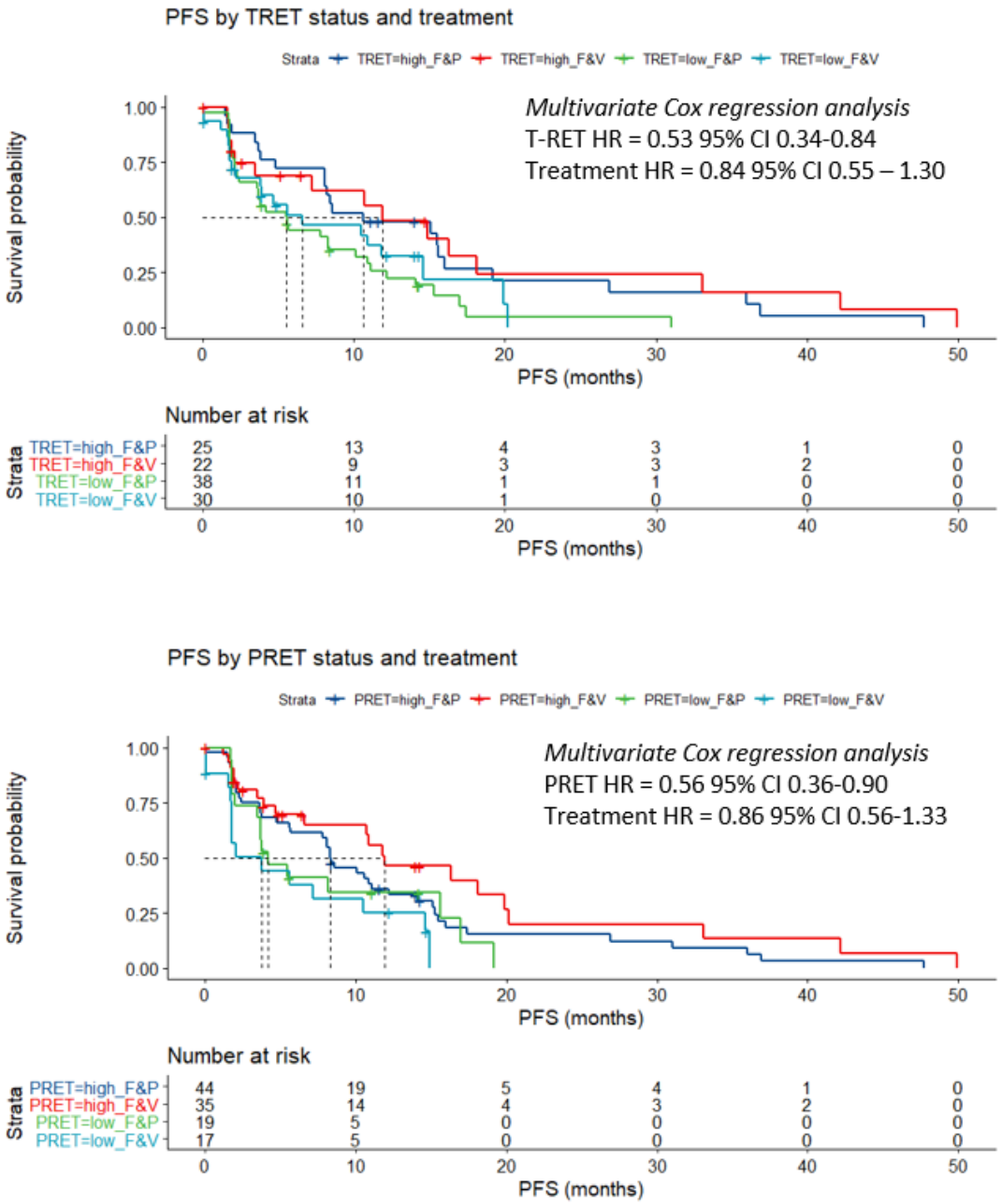


Figure 44 Kaplan Meier plots and multivariate cox regression analysis examining the relationship between PFS and RET status by treatment received. HR = hazard ratio. CI = confidence interval. F&P = fulvestrant and placebo, F&V = fulvestrant and vandetanib.

6.2.1 Exploring the relationship between T-RET high status and longer PFS

The relationship between T-RET high status and PFS did not reach statistical significance when patients were treated with fulvestrant and vandetanib but did demonstrate a relationship when patients were treated with fulvestrant alone. However, when examined using a multivariate analysis

it was deemed that this perceived difference was incidental and that the true difference was in PFS dependant on T-RET status rather than T-RET status as a predictor of outcome. This leads to exploration of whether T-RET is a surrogate marker for a tumour that is sensitive to the treatment strategy employed in the FURVA trial. This is explored in Table 50.

		T-RET high	T-RET low	Chi-square	Significance
Cancer Type	IDC	39	46	2.901	0.234
	ILC	12	6		
	Other	7	5		
Liver or lung metastases	Yes	40	31	2.587	0.108
	No	18	26		
Bone only disease	Yes	9	12	0.590	0.442
	No	49	45		
Chemotherapy for metastatic disease?	Yes	16	17	0.070	0.791
	No	42	40		
Primary or Secondary endocrine resistance	Primary	11	6	1.625	0.202
	Secondary	47	51		
Measurable or non-measurable disease	Measurable	43	40	0.225	0.635
	Non-measurable	15	17		

Table 50 Potential prognostic variables and their relationship with T-RET status. Tested for independence using Pearson Chi-Square test in SPSS. No adjustment made for multiple testing. Data was available for all patients who had a primary tumour sample tested for T-RET expression. IDC = invasive ductal carcinoma, ILC = invasive lobular carcinoma.

T-RET high status is not more frequent in any of the potential prognostic clinical indicators explored in Table 50. Using Cox regression analysis across the whole data set three of the six variables were predictive of a change in PFS across all trial participants, see Table 51.

Likely prognostic biomarker	HR	95% CI for HR		Sig
		Lower	Upper	
Cancer Type ***(IDC, ILC, other)***	0.902	0.677	1.202	0.480
Liver and/or lung metastases ***(Yes, No)***	1.544	1.062	2.247	***0.023***
Bone only disease ***(Yes, No)***	0.536	0.323	0.889	***0.016***
Chemotherapy for metastatic disease? ***(Yes, No)***	0.770	0.519	1.14	0.192
Primary or Secondary endocrine resistance	0.719	0.440	1.177	0.190
Measurable or non-measurable disease	0.605	0.397	0.923	***0.020***

Table 51 Cox regression analysis of potential prognostic biomarkers in the FURVA trial participants across both trial arms.

The presence of visceral metastases, bone only disease and measurable disease are prognostic when PFS is examined using cox-regression modelling. All are largely measuring the same thing; the presence or absence of disease burden in key organs. The numbers of patients with ILC (n=26), primary endocrine resistance (n=25) and those who had received chemotherapy for metastatic disease (n=42) were relatively small which may explain why there was no significant difference in PFS between those patients where the biomarker was present vs. those where it was absent.

Given that the finding of improved PFS in patients whose tumours had high T-RET expression was unexpected and somewhat contrasting to previously published literature one has to question whether the way T-RET status was determined could explain the result. The methodology for this and the results are presented in chapter 2 and 3 and are believed to be thorough and accurate. The antibody selected had been shown to be selective for RET although not in breast tissue prior to use in this project. Another possibility is that contrary to the published literature (Gattelli et al. 2013) T-RET status is a surrogate for another marker of good prognosis disease. Information regarding original tumour size which has been shown to correlate with RET-high status (Gattelli et al. 2013) was not available.

Another possibility is that the statistical methodology used to determine a cut point has created a false positive by forcing a retrospectively determined, and hence potentially biased cut point on the data. Interestingly the relationship between PFS and T-RET expression by treatment still exists when the pre-planned method C is used but the result does not reach statistical significance (see Table 52)

	T-RET			
	95% CI			
	HR	Lower	Upper	p-value
Overall	0.633	0.421	1.046	0.077
Arm A: Fulvestrant and Placebo	0.626	0.359	1.091	0.098
Arm B: Fulvestrant and vandetanib	0.690	0.331	1.438	0.322

Table 52 Cox regression analysis of T-RET status by method C (>10% of neoplastic cells showing strong staining or >30% of neoplastic cells showing moderate staining) and then interrogated by treatment received.

Assuming that the antibody chosen was specific for RET, the methodology of the assays and interpretation of the scores was sound and that high-RET expression is not a surrogate for another driver of prognosis then this finding merits further consideration. A larger series examined prospectively would be a natural next way in which to explore this. In particular, if one assumes that vandetanib has been a largely inactive drug in this trial, as deemed by the overall negative trial result, then one could hypothesise that RET expression may have a relationship to response to fulvestrant.

6.2.2 Exploring the relationship between P-RET high status and longer PFS

This relationship is biologically and pharmacologically more plausible as one would expect the greatest impact of vandetanib to be on those tumours where RET was active, as demonstrated here by the expression of phosphorylated RET. As demonstrated in Figure 43 panel D there was a

statistically significant relationship between high P-RET expression and improved PFS when receiving treatment with fulvestrant and vandetanib that was not demonstrated in the patients receiving fulvestrant and placebo. Again though, when multivariate analysis methods were applied it did not appear that the difference was dependant on treatment received.

However, again we must exercise caution in interpreting the relationship as the cut-point was retrospectively applied and none of the pre-determined cut points resulted in a statistically meaningful relationship between p-RET status and PFS. Potentially those patients with highly active p-RET could be experiencing benefit from vandetanib but the numbers of patients in the trial with very high p-RET expression was too small for a signal to be detected. If further clinical trials were designed prospective analysis of p-RET status and potentially enriching the study population with patients with high p-RET expressing tumours in a pre-planned subgroup analysis could be an interesting translational strategy.

6.3 Clinical correlation of downstream signalling from RET

Exploratory analysis 1: variants in key proteins downstream from RET and their individual and collective relationships with progression free survival in patients treated with fulvestrant +/- vandetanib

Pathogenic variants in three key downstream genes were examined in detail; *PIK3CA, AKT1* and *ESR1*. Cox regression modelling was used to investigate whether the presence of variants in any of the genes showed a statistically significant relationship with PFS. Table 53 shows that only the detection of *PIK3CA* variants in cfDNA correlated with a statistically significant difference in PFS between those that had the variant detected and those that did not. However, it should be noted that the number of samples with a variant detected was only 6. Overall 64 patients had data

available from either tissue or cfDNA for *AKT1* and *PIK3CA* and 54 for *ESR1*[17]. RET has also been shown to activate the MAPK pathway but no variants were detected in either FFPE or cfDNA samples[18].

Gene	DNA source	HR	95% CI		p-value
			Lower	Upper	
PIK3CA	Tissue	1.104	0.557	2.188	0.777
	cfDNA	3.552	1.285	9.819	***0.015***
	Either	1.037	0.539	1.994	0.914
AKT1	Tissue	0.677	0.237	1.932	0.466
	cfDNA	1.754	0.527	5.832	0.359
	either	1.116	0.452	3.006	0.751
ESR1[19]	cfDNA	1.318	0.717	2.423	0.373

Table 53 Variants in key downstream genes from RET and their relationship to PFS

Where data was available both about the presence or absence of downstream variants and the RET status of the patient's primary tumour sample chi squared analysis was performed to investigate whether there were genetic variants that correlated with RET status. As shown in Table 54 there was no statistically significant difference in the distribution of downstream variants in patients whose samples showed T-RET high or low expression. Although it should be noted that for a number of the genes the numbers of samples where a variant was detected was too small to accurately apply the

[17] These numbers differ as for AKT1 and PIK3CA the presence or absence of a variant was investigated using NGS. All NGS work was carried out on samples from patients receiving fulvestrant and vandetanib. ESR variants were largely detected by ddPCR and samples came from patients in both arms of the trial. See Chapters 4 and 5 for more information.

[18] The cancer hotspot panel covered common variants in KRAS, NRAS, HRAS and BRAF

[19] ESR1 variants were only tested in cfDNA samples as they are known to be very rare in primary tumours Jeselsohn, R. et al. 2015. ESR1 mutations—a mechanism for acquired endocrine resistance in breast cancer. *Nat Rev Clin Oncol* 12(10), pp. 573-583. doi: 10.1038/nrclinonc.2015.117

chi squared test. It is interesting to note that although not statistically significant there were fewer *ESR1* variants detected in patients with T-RET low tumours compared to T-RET high (3 versus 11).

6.3.1 The relationship between T-RET status and variants in downstream genes

In Table 54 overall numbers of variants detected between T-RET high and low groups are too small for accurate statistical analysis. However, descriptively it is noted that the presence of *ESR1* variants is more common in the T-RET low group which had an overall shorter PFS. The numbers of samples tested for ESR1 status which also had data available for T-RET status is relatively small (n=39) so this result should be interpreted cautiously and it should be noted that it did not meet statistical significance.

Gene	DNA source		T-RET low	T-RET high	Chi squared	Significance
PIK3CA	Tissue	Present	7	6	0.001	0.978
		Absent	19	16		
	cfDNA	Present	3	1	0.417	0.519*
		Absent	21	15		
	Either	Present	7	6	0.065	0.799
		Absent	22	16		
AKT1	Tissue	Present	3	4	0.684	0.408*
		Absent	23	18		
	cfDNA	Present	1	0	0.422	0.516*
		Absent	23	16		
	Either	Present	3	3	0.131	0.718*
		Absent	26	19		
ESR1	cfDNA	Present	11	3	1.987	0.159
		Absent	14	11		

*Table 54 Exploring correlation between T-RET status and variants in downstream pathways. Pearson Chi Squared test applied to test independence of variables. *insufficient numbers for accurate assessment of independence using Chi squared testing. Only samples where data was available for both RET status and the presence or absence of key variants have been used in this analysis.*

The analysis in Table 54 was repeated with the P-RET data. The presence of variants was evenly distributed between those patients with P-RET high and P-RET low tumours (data not shown).

The association between T-RET-low status and the presence of an *ESR1* variant could offer some explanation as to why there is a difference in PFS between patients with RET-high and RET-low

tumours. I.e. if a proportion of those with T-RET low tumours also had an *ESR1* variant then these patients may be expected to do poorly as *ESR1* variant detection has been strongly associated with endocrine resistance and impairment of response to standard first line endocrine therapies suggesting that resistance mechanisms to endocrine directed therapies such as fulvestrant may already be established in the tumour (Angus et al. 2017).

However, based on the translational data from the SoFEA[20] trial one might expect patients with *ESR1* variants to gain more benefit from fulvestrant than those with *ESR1*^{WT} status. Analysis of plasma samples from the SoFEA clinical trial showed that patients taking fulvestrant had a longer PFS (HR 0.52 95% CI 0.30-0.92 p=0.02) after taking fulvestrant (n=45) than exemestane (n=18) if they had an *ESR1* variant present. Plasma samples were analysed using ddPCR using the same probes as were used here (Fribbens et al. 2016). It should be noted in the SoFEA trial the findings were based on small numbers of samples and, to my knowledge, have not been replicated.

A translational analysis of the PALOMA-3[21] trial showed that *ESR1* Y537S variants were more common in EOT (12%) samples than those taken at baseline (4%) (p=0.0037, McNemar test *q*=0.047, Bonferoni correction). This difference persisted when analysed by treatment arm where 10% of those in the fulvestrant and palbociclib arm and 6% of those in the fulvestrant and placebo arm acquired an *ESR1* Y537S variant during treatment. Suggesting that *ESR1* Y537S could confer resistance to fulvestrant and possibly palbociclib (O'Leary et al. 2018).

When this theory is applied to the data in this thesis it is noted that of the 19 that contained a variant, up to 10 contained a MPX1[22] variant (of which Y537S is by far the most common) while up to 14 contained a MPX2[23] variant (of which D538G is the most frequent). So, if we assume that Y537S

[20] The SoFEA trial compared fulvestrant and anastrazole with exemestane in post-menopausal women with ER+ breast cancer who had progressed on an AI.
[21] The PALOMA-3 trial compared fulvestrant and palbociclib with fulvestrant and placebo in women with ER+ breast cancer who had progressed on endocrine therapy
[22] MPX1 is a ddPCR multiplex assay for ESR1 variants D538G, E380Q, Y537C and L536R
[23] MPX2 is a ddPCR multiplex assay for ESR1 variants Y537N, Y537S and S463P

may confer resistance to fulvestrant up to half of the patients with an *ESR1* variant could have been resistant to fulvestrant.

To further explore the relationship between the presence of an *ESR1* variant, low t-RET score and poorer PFS the six patients with *ESR1* variants detected where data was also available for additional variant analysis by NGS and t-RET data was available (5/6 cases) were examined in more detail (see Table 55). In 4/6 cases an *ESR1* variant was the only variant detected in cfDNA. A single case had a good PFS approaching 1 year (case 1006) however, here we note a relatively low VAF *ESR1* variant that potentially could have been one of the less common and potentially less pathogenic variants in the multiplex e.g. S463P. This sample had been sequenced using NGS, in addition to ddPCR testing, covering the Y537 region in *ESR1* and on manual inspection of the region in IGV there was no evidence of a variant present; this adds some weight to the fact that the variant detected by MPX may have been a less common and potentially less pathogenic variant as these were not covered in the NGS panel.

Patient	t-RET H-score (max 300)	cfDNA *ESR1* variant	cfDNA additional variant 1	Variants detected in tissue	TISS variant 1	TISS variant 2	PFS (days)
Patient 22	55	MPX1 15%	None	Yes	*TP53* C238Y 3%	None	47
Patient 51	100	MPX2 22%	*TP53* R158L 72%	Yes	*AKT1* E17K 50%	*TP53* R158L 37%	54
Patient 21	115	Both MPX positive at 2% and 3.5%	None	No	None	None	62
Patient 3	95	MPX1 0.25%	None	Yes	*AKT1* E17K 4%	None	89
Patient 19	140	MPX2 1%	None	Yes	*PIK3CA* C420R 14%	None	116
Patient 1	NA	MPX2 0.5%	*AKT1* E17K 7%	NA	NA	NA	333

Table 55 Patients with an ESR1 variant detected who had additional sequencing by NGS and primary tumour tested for t-RET status

A further complexity should be noted here; most samples tested for *ESR1* variants were from EOT thus we do not know for sure if the *ESR1* variant developed in primary tumour, during first line endocrine treatment in the metastatic setting or during treatment with fulvestrant. Here review of the literature can aid somewhat. It is well established that *ESR1* variants are very rare in primary tumours (Jeselsohn et al. 2015). By the time patients have progressed on endocrine therapy they are common as demonstrated in retrospective analyses of the SoFEA trial (Fribbens et al. 2016) and the

BOLERO-2 trial (Chandarlapaty et al. 2016). In the FURVA samples, of the 20 samples where an *ESR1* variant was detected two samples were tested at both baseline and EOT and in primary FFPE tumour tissue and the variant was present in both cfDNA samples but absent in primary tumour showing that, at least for these two cases, the variant had occurred after exposure to first line endocrine therapy but before treatment with fulvestrant.

ESR1 variants were the only SNV tested for in both arms of the trial due to their suitability for ddPCR multiplex testing. They were also the only SNV that had been shown, albeit not unanimously, in studies to impact response to fulvestrant, hence their selection over *PIK3CA* or *AKT1* for extended testing.

		Treatment			
		Fulvestrant and placebo		**Fulvestrant and vandetanib**	
		MPX1	MPX2	MPX1	MPX2
Source of samples tested (all cfDNA)	EOT	32	32	13	15
	8-week	0	0	4	4
	BASE	0	0	5	3
	TOTAL	**32**	**32**	**22**	**22**

Table 56 Source of cfDNA samples tested for ESR1 variants using two multiplexed probe sets by ddPCR.

All patients receiving treatment with fulvestrant and placebo had an EOT sample tested while those receiving treatment with fulvestrant and vandetanib had a mixture of time points tested although the majority of samples for both MPX1 and MPX2 came from EOT samples (56% and 63% respectively), see Table 56. This disparity is due to the samples from patients receiving treatment with fulvestrant and vandetanib having already been tested for other markers and used for NGS in this study thus not all had an EOT sample available.

The distribution of the 20 patients in whom one or more *ESR1* variants was detected from their samples is uneven between the two treatment arms; in patients receiving treatment with fulvestrant

and placebo 13/32 (41%) patients had one or more ESR1 variants while for patients receiving treatment with fulvestrant and vandetanib only 7/22 (32%) patients had one or more ESR1 variant detected. In the fulvestrant and vandetanib arm variants were detected in samples from all time points making it less likely that the lower frequency is purely down to the mixture of time points tested. However, there is always the possibility that using an non EOT sample, particularly if it is an on treatment sample, may result in a lower cfDNA concentration therefore making it more challenging to detect a variant if one is present.

6.3.2 Summary of RET status and relationship with downstream genes

The number of patients with data available for both RET status and the presence or absence of variants in downstream genes was small. However, it was noted that *ESR1* variants were more common in patients with T-RET low status compared to T-RET high status (11 vs 3). When this association was tested using chi-squared methodology the result did not meet statistical significance but opens interesting questions around the interplay between *ESR1* variants and RET expression. This may be linked to RET being an oestrogen regulated gene (Gattelli et al. 2013).

6.4 Comparing clinical outcomes and potential genomic biomarkers by prognostic grouping

Exploratory analysis 2: The relationship between clinical outcome data and SNVs detected using NGS and ddPCR as predictors of patient's clinical outcomes when treated with fulvestrant +/- vandetanib

Previous sections looked at whether biologically pertinent biomarkers could be used to predict progression free survival across both arms of the FURVA trial. In this section biomarkers are explored by looking specifically at patient's clinical response to treatment with fulvestrant and vandetanib.

In patients with endocrine resistant metastatic breast cancer treated with fulvestrant, the expected PFS is around 5 months (Cristofanilli et al. 2016; Krop et al. 2016; Baselga et al. 2017). To explore whether there are different patterns seen in the biomarkers explored in this project between different prognosis patients who had received treatment with fulvestrant and vandetanib two key groups of interest were examined. The two groups selected were patients with a PFS ≤ 8 weeks (62 days) and PFS > 10 months (312 days). The first group represents patients who had progressed at or before the time of their first CT scan representing a clinical group that had not derived benefit from treatment. The second group represents a doubling of the predicted PFS for patients entering the trial.

Patients data was analysed if they had data available from NGS, if they had additional data available regarding T-RET, P-RET and *ESR1* status available this was also considered. If this part of the study was limited to patients with a complete dataset only 13 patients would have been suitable for analysis.

In addition to the *PIK3CA, AKT1* and *ESR1* variants discussed in previous sections due to their direct relationship to RET here additional variants in alternative growth pathways and cell cycle machinery such as those in *TP53* and *ERBB2* will be included. 64 patients had data available for *PIK3CA, AKT1* and *TP53* status. Of these 51 had data available for T-RET and P-RET status and 19 had data available on *ESR1* status.

By using the groups detailed above 22 (7 censored) patients had a PFS event recorded on or prior to 62 days. 22 patients had a PFS event recorded after 312 days on treatment. Of these 5 were censored (two were still receiving trial treatment). The presence or absence of variants in either cfDNA or tissue samples is detailed in Table 57.

Gene and sample source		PFS ≤62 days	PFS >312 days
***PIK3CA* TISSUE**	Detected	5	6
	Not detected	15	13
***PIK3CA* cfDNA**	Detected	4	0
	Not detected	11	17
***AKT1* TISSUE**	Detected	3	2
	Not detected	18	16
***AKT1* cfDNA**	Detected	1	1
	Not Detected	14	16
***TP53* TISSUE**	Detected	3	3
	Not detected	17	16
***TP53* cfDNA**	Detected	2	0
	Not detected	13	17
***ESR1* cfDNA**	Detected	2	2
	Not detected	5	1
Total tissue	Detected	9	10
	Not detected	11	9
Total cfDNA	Detected	8	2
	Not detected	7	15

Table 57 Descriptive comparison of pathogenic SNVs found in each prognostic group. Light blue shading indicates the two parameters where there was a difference of ≥2 events between prognostic groups.

While numbers of samples tested for each marker are small there is a demonstrable difference in the frequency of cfDNA detected variants in the two groups. 8 cfDNA variants were detected in the poor prognosis group while 2 were detected in the good prognosis group. This is not seen in variants detected in FFPE samples. *PIK3CA* and *TP53* variants were only detected in the poor prognosis group (≤62 days), whereas *ESR1* and *AKT1* variants were detected in both prognostic groups. The numbers

here are very small but tentatively suggest that the detection of a *PIK3CA* or *TP53* variant in cfDNA is a poor prognostic sign. Alternatively, one could interpret this to say that the detection of ctDNA (independent of variant detected) is a poor prognostic sign.

CfDNA detection as a predictor of poor response to fulvestrant and vandetanib was then explored using all available data to perform a survival analysis. The results of this are presented in Figure 45.

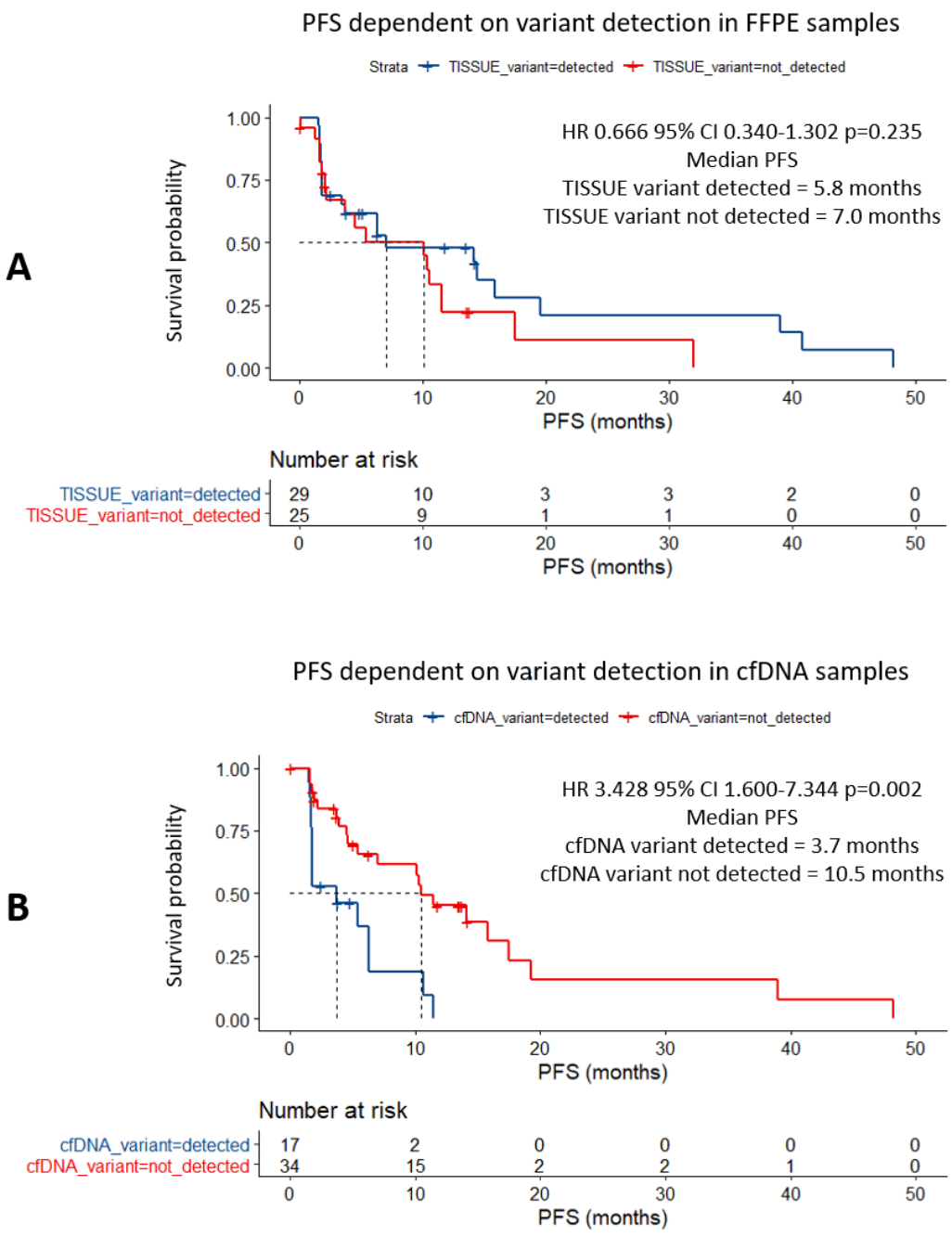


Figure 45 KM curves demonstrating the relationship between PFS and the detection of variants in either tissue or cfDNA samples.

In Figure 45 there is a statistically significant and clinically meaningful difference in PFS when a variant is detected in cfDNA compared to when no variant is detected. Of the 17 variants detected 7 were in *ESR1*, 6 in *PIK3CA*, 4 in *TP53* and 3 in *AKT1*. Of these 8 were in the poor prognosis group (PFS ≤62 days) group and thus are potentially the key drivers in the overall poor PFS of patients where

variants were detected in cfDNA. Samples tested were not all from the same time point, however the majority were taken at the point of disease progression on fulvestrant and vandetanib (*TP53*, *PIK3CA*, *AKT1*) and fulvestrant +/- vandetanib (*ESR1*).

6.4.1 Patients with a detectable *PIK3CA* variant in cfDNA

Patients who had a detectable *PIK3CA* variant were examined in more detail as they represented the majority of the cfDNA variants detected in the poor prognosis group.

All 6 variants detected in cfDNA were also present in primary tumour. One patient had an additional cfDNA variant present. There was no obvious relationship between VAF and PFS although it is noted that the sample with the highest *PIK3CA* VAF also had one of the shortest PFS. In this patient (Patient 64) the *PIK3CA* variant was present at high VAF in both tissue and cfDNA suggesting that this variant was present in the majority of tumour cells and that the primary tumour was shedding a large amount of DNA into the blood stream. No tissue samples had an additional variant present except the sample from patient 60 where two *PIK3CA* variants were detected. This tentatively suggests that the *PIK3CA* variant was the driver variant in these tumours. There did not appear to be any relationship between the presence of a *PIK3CA* variant and a high or low T-RET or P-RET score.

Sample ID	cfDNA variant (VAF%)	cfDNA additional variant	TISS variant present	TISS variant (VAF%)	t-RET H-score	p-RET H-score	PFS (days)
Patient 64	H1047R (75%)	No	Yes	H1047R (69%)	180	85	54
Patient 15	E545K (5%)	No	Yes	E545K (27%)	140	49	53
Patient 12	E545A (4%)	No	Yes	E545A (4%)	NA	NA	56
Patient 2	E542K (6%)	No	Yes	E542K (27%)	120	125	56
Patient 77	H1047R (20%)	No	Yes	H1047R (15%)	117	120	148
Patient 60	E545K (4%)	*ESR1* D538G 4%	Yes	E545K (31%) and E435Q (31%)	NA	NA	166

Table 58 Patients with a PIK3CA variant detected in cfDNA

Although only the relationship between presence of a *PIK3CA* variant in cfDNA and shorter PFS reached statistical significance it seems likely that other variants also contribute to a short PFS phenotype but their numbers were too low to apply a meaningful statistical analysis (Table 53).

6.4.2 Presence of variants and prognostic group

This data can also be illustrated in a more detailed way looking not only at whether a variant is present or absent in cfDNA but at what VAF it is present. In Figure 46 each bubble represents a variant in a trial participant. The colour and size of the bubble relate to which gene and at what VAF

the variant is present in. Trial participants are grouped according to PFS based on the clinically meaningful groups detailed previously. Patients whose samples contained variants but were censored during the survival analysis were not included.

There are more variants detected in patients for whom PFS was short, both in cfDNA and tissue samples. The number of variants detected in the longer PFS group was low, only two cfDNA variants and four tissue variants. The VAF was also higher in cfDNA samples in the shorter PFS group compared to the longer PFS groups. This could be indicative of a driver variant being present. Interestingly the two out of three *PIK3CA* variants detected in tissue samples (E545K and H1047R) in the group with a PFS ≥312 days were also detected in patients with short PFS suggesting that it may not only be the variant itself but the frequency at which it occurs and the presence or absence of other prognostic factors.

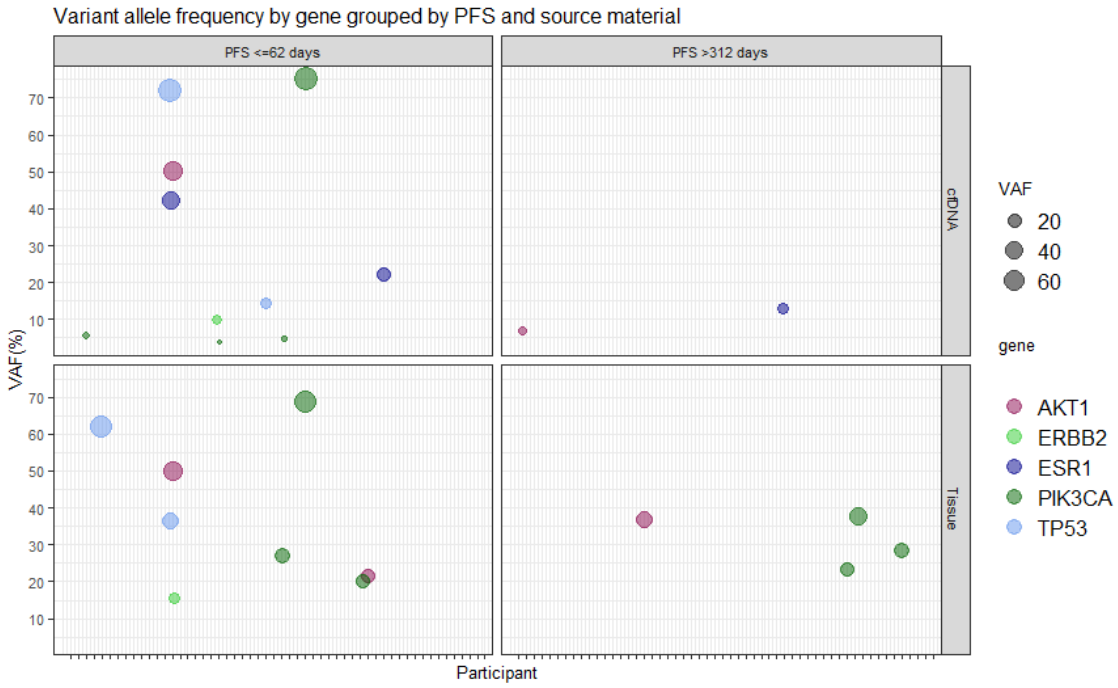


Figure 46 Bubble plot showing variants detected by PFS grouping and DNA source

These findings align with reports in the literature of the presence of ctDNA being a prognostic biomarker (Hrebien et al. 2019). It is interesting here that the effect is marked even over a relatively short disease course. While previous studies have looked at time to relapse or overall survival one

hypothesis that could be generated from these results is that the presence of ctDNA is of use in identifying patients who are likely to have a shorter PFS on fulvestrant and thus perhaps should be offered alternative treatments at the point of endocrine resistant disease.

Overall 17/51 (33%) patients had a variant detected in cfDNA. If we take this data as our pilot study with patients with a variant detected in cfDNA having a mean PFS of 102 days and those without a variant detected in cfDNA as having a mean of 244 days then we can perform a power calculation using Cohen's T-test; with an alpha level set at 0.05. Using a 2 tailed test we can calculate that we would need 28 samples to achieve 80% power to detect a difference in PFS between the two groups. If this test is retrospectively applied to the data here we find that with the samples available there was 96% power to detect a difference between the two groups.

6.4.3 Summary of clinical outcomes and variant detection

The key finding in this section is that the presence of a detectable tier II variant in cfDNA correlates with shorter PFS in patients treated with fulvestrant and vandetanib. From the data available it has not been possible to conclude which genes or variants are the likely key drivers of this. One could tentatively suggest that it is not the variant per sae as some of the variants were present in both the short and longer PFS groups but at very different VAF. Hence, it may the quantity of detectable ctDNA rather than the variant itself that is the potential biomarker.

6.5 Clinical correlation of CNA

Exploratory analysis 3: copy number alterations in MYC and FGFR1 in FFPE samples representing primary tumour and cfDNA samples representing metastatic disease and their relationship with progression free survival in patients treated with fulvestrant +/- vandetanib

6.5.1 Overview of samples tested

DNA extracted from FFPE primary tumour and cfDNA representing metastatic disease was tested using ddPCR probes to examine copy numbers of the gene of interest and a reference gene; in both cases *AGO1*. After QC as detailed in chapter 5 number of samples were eligible for further analysis are shown in Table 59.

	MYC	***FGFR1***
FFPE	46	42
cfDNA	39	31
Both FFPE and cfDNA	22	18

Table 59 Number of samples available for analysis after QC

6.5.2 Copy number alterations in MYC

Copy number ratios from cfDNA samples which passed QC as detailed in chapter 5 (n=39) were used along with corresponding PFS data to determine the optimal clinically relevant cut point. Using the maxstat calculation in the R package 'survival' ratios >1.345 were called as amplified. When this cut point was applied and survival analysis for PFS performed using cox regression analysis it resulted in HR 1.723 (95% CI 0.823-3.606, p = 0.149). This is shown in the Kaplan Meier plot below (Figure 47) with median PFS between the two groups determined using the log rank test.

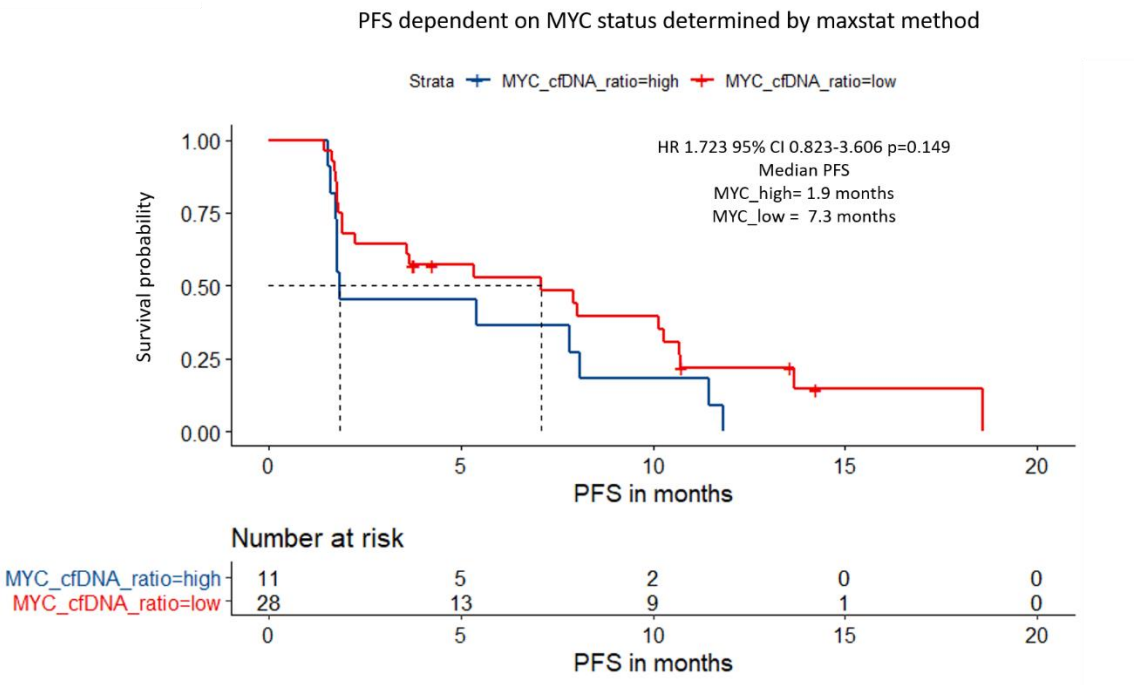


Figure 47 Kaplan Meier plot showing PFS when cfDNA samples grouped by MYC copy number ratio where the cut point has been determined by the maxstat method.

FFPE samples were considered amplified if the ratio of *MYC* to the reference gene AGO1 were ≥2. At this cut-point there was no correlation between *MYC* status and PFS by Cox Regression analysis; HR =0.762, 95% CI 0.383-1.517 p=0.440, 8 samples were identified as amplified (range of *MYC:AGO1* ratios 2.812-4.675). Even if the maxstat method is applied to determine a cut off (>2.718) a statistically significant difference cannot be detected; HR 0.418, 95% CI 0.162-1.083, p=0.073.

Numbers here are small but using descriptive analysis it appears there may be a trend towards reduced PFS in patients with *MYC* amplification detectable in cfDNA when stringent cut points such as those determined by the maxstat methodology are used to determine 'amplified' samples.

It was noted in chapter 5 that *MYC* amplification potentially changed (or at least the ability to detect it did) during the course of disease. Table 60 explores the changes in *MYC* amplification between samples and pairs this with the corresponding PFS data.

Patient ID	FFPE	cfDNA	PFS in days
Patient 127	2.67	2.5	168
Patient 119	2.18	1.44	176
Patient 88	4.67	1.25	247
Patient 129	2.2	1.32	320
Patient 91	1.39	1.48	252
Patient 15	1.30	1.47	53
Patient 12	1.85	1.35	56
Patient 85	1.36	1.45	244
Patient 62	1.86	0.98	316
Patient 136	1.84	0.96	166
Patient 137	1.84	1.01	250
Patient 121	1.54	1.19	334
Patient 59	1.45	0.97	415
Patient 3	1.33	1.34	89
Patient 118	1.29	1.13	69

Table 60 MYC amplification in FFPE and cfDNA paired with PFS data. Light blue shading indicates non-amplified sample and dark blue represents amplified sample. FFPE samples amplified if ratio ≥2, while cfDNA amplified if ratio >1.345 (as determined by maxstat).

Of those patients where *MYC* amplification was detected in primary tumour (n=4) 2 of these patients also had detectable *MYC* amplification in cfDNA. The FFPE sample with the highest level of *MYC* amplification did not have *MYC* amplification detected in cfDNA. 4 patients had *MYC* amplification detectable in cfDNA but had no amplification present in their corresponding FFPE sample. Of these 50% had a short PFS (53 and 56 days respectively) while the other two had PFS in the region of 250 days. Both patient 12 and patient 15 also had a *PIK3CA* variant detected in cfDNA which, as reviewed in section 6.4 may contribute to a shorter PFS.

Thus in summary, this small dataset has not shown any convincing evidence that *MYC* amplification is a prognostic biomarker in this setting either when detected in FFPE or cfDNA samples.

6.5.3 Copy number alterations in FGFR1

31 cfDNA samples resulted in data that passed QC for further analysis of *FGFR1* amplification status. When the maxstat methodology was applied to determine a cut point samples with a *FGFR1:AGO1*

ratio >1.273 were called amplified. When this data was combined with information regarding PFS and cox regression modelling used to determine whether there was a relationship between detection of an *FGFR1* amplification in cfDNA the HR was 0.436, 95% CI 0.167-1.141, p=0.091. Furthermore, when amplification status in FFPE samples (defined as a FGFR1:AGO1 ratio ≥2) was used to divide the patients into those with *FGFR1* amplified and *FGFR1* non-amplified tumours there was no difference seen in PFS between the two groups; HR 0.454, 95% CI 0.171-1.203 p=0.112) by Cox regression analysis. This remained unchanged when the maxstat methodology was applied to determine a cut-point as it identified the cut-point as 2.1.

Patient ID	FFPE	cfDNA	PFS in days
Patient 10	3.48	3.58	359
Patient 21	2.5	1.89	358
Patient 64	1.53	1.48	55
Patient 15	1.18	1.36	54
Patient 121	0.85	1.33	335
Patient 119	1.33	1.29	370
Patient 60	1.29	1.27	196
Patient 151	1.24	1.27	581
Patient 12	0.89	1.24	57
Patient 143	0.88	1.2	112
Patient 51	0.98	1.17	55
Patient 91	0.77	1.15	253
Patient 153	1	1.12	60
Patient 137	1.25	1	251
Patient 22	1.52	0.9	48
Patient 127	0.85	0.9	169
Patient 129	0.91	0.88	321
Patient 9	0.88	0.78	51

Table 61 FGFR1 amplification in FFPE and cfDNA samples and corresponding PFS. Shading indicates amplification present by defined cut-offs.

Table 61 explores the relationship between FGFR1 amplification status in FFPE and cfDNA samples. The majority of patients (6/8) who had an *FGFR1* amplification detected in either FFPE or cfDNA samples had a prognosis >180 days, the two exceptions to this were patients Patient 64 and Patient 15. Patient 15 and Patient 64 had a *PIK3CA* variants detected in cfDNA at a VAF >20% suggesting that their disease may be driven by a dominant *PIK3CA* variant clone. Thus from this small dataset there

is no obvious relationship between PFS and *FGFR1* amplification detected in either FFPE or cfDNA. It is noted that FGFR1 amplification as defined by the maxstat methodology is more frequent in cfDNA samples. However, two samples only just met the criteria for amplification with an FGFR1: AGO1 ratio of 1.27.

6.6 Chapter Discussion

6.6.1 High T-RET or P-RET expression as a predictor for improved PFS

The first key finding in this chapter is that high t-RET and p-RET expression predict for a statistically significant improved PFS likely independent of treatment received.

The literature around the ability of RET to predict for clinical outcome is limited. The ability for t-RET expression to predict for survival has been noted previously (Gattelli et al. 2013). The Gattelli series included 89 patients and showed that high RET correlated with shorter metastasis free and overall survival in patients with primary breast cancer who received treatment between 1988 and 1994. Of the 89 patients 42 had ER+/HER2- breast cancer. In the Gattelli et al dataset the strongest correlation was between patients with TNBC and high RET expression (22/30 (73%)), high RET expression was also found in luminal A breast cancers (24/36 (67%).

However, in a later paper RET expression was evaluated using IHC in 990 patients with breast cancer and t-RET expression did not correlated with overall survival in either all comers or patients with luminal A or luminal B breast cancer (Mechera et al. 2019). However, it did correlate strongly with the presence of ER. The RET ligand GDNF has been shown to be increased in luminal B breast cancers and has then correlated with shorter relapse free and overall survival (Morandi et al. 2013).

The initial hypothesis in this thesis was that high RET expression would result in clinical response to treatment with vandetanib and hence an overall improvement in PFS. To an extent this was true but only reached statistical significance in the p-RET high subgroup (p=0.009). There was a trend towards longer PFS in the t-RET high subgroup when treated with fulvestrant and vandetanib (p=0.145) but the relationship between t-RET high status and longer PFS was stronger in patients treated with fulvestrant and placebo (p=0.026). However, on multivariate cox regression analysis these trends disappeared suggesting that there may not be a relationship between RET expression and response

to vandetanib. As both clinical trial arms contained fulvestrant, which is known to be active in patients with ER+ breast cancer, it is possible that RET expression could influence response to fulvestrant.

In this chapter the overall findings from the FURVA trial have been reviewed demonstrating that adding vandetanib to fulvestrant did not result in increased progression free survival. There is evidence that often drugs that have been effective in one tumour type do not work in a tumour agnostic manner (Carlisle et al. 2016). In this case vandetanib has been used to successfully treat patients with thyroid cancer; however, most patients had *RET* activating genetic variants, in particular the M918T variant. There was reasonable pre-clinical evidence that RET was over-expressed in endocrine resistant breast cancer and that this over-expression could be targeted with vandetanib; in cell line models and in mice (Esseghir et al. 2007; Gee et al. 2014; Spanheimer et al. 2014).

A further finding of note was the predominance of *ESR1* variants in the t-RET low group; 11/14 patients with at least one *ESR1* variant identified were in patients with t-RET low tumours. *PIK3CA* and *AKT1* variants were evenly distributed between t-RET high and t-RET low tumours (see Table 54). Samples used to determine t-RET status were from primary tumours whereas *ESR1* variant status was determined from plasma samples taken once endocrine resistance had developed. Biologically this could suggest that upregulation of RET and the presence of *ESR1* variants are largely mutually exclusive events. This may be in part due to RET being an ER regulated gene (Griseri et al. 2016) hence high expression only occurring in the presence of a functional ER.

ESR1 variants can co-occur with variants in *PIK3CA* and/or *AKT1* as seen in work here and backed up by a much larger dataset using the MSK-IMPAKT NGS panel for tumours from patients with metastatic breast cancer (Razavi et al. 2018). Razavi et al were able to demonstrate that variants in the MAPK pathway were mutually exclusive to variants in *ESR1*, whereas *ESR1* variants could co-exist with those in *PIK3CA* and *AKT1*. It would be interesting to obtain samples of tumours with *ESR1*

variants to prospectively examine their RET status to establish whether *ESR1* variants uniformly result in low RET expression.

6.6.2 SNV detection in FFPE and cfDNA samples

When examining cfDNA based potential biomarkers only the presence of *PIK3CA* variants detected in cfDNA showed a statistically significant ability to predict for shorter PFS (Table 53). This fits with recently published literature such as a study by Jacot et al where detection of PIK3CA variants in cfDNA using ddPCR after one month of treatment with hormone deprivation therapy was a negative prognostic factor for 1 year PFS (Jacot et al. 2019). However, when descriptive analysis examined the presence of all variants detected in cfDNA (Table 57) it was shown that the presence of a ctDNA as defined by the presence of a *PIK3CA* or *TP53* variant, seemed to point towards an increased likelihood of short PFS. This leads to a discussion point around whether it is the variant itself that is the biomarker or whether it is its detectable presence. This has been noted previously and is now being used to try a detect early relapsed disease (Garcia-Murillas et al. 2019). Garcia-Murillas et al demonstrated that detection of ctDNA (as determined by the presence of a tier II pathological variant) in patients with ER+ disease could predict disease free survival. In a cohort of 24 patients where cfDNA samples were collected using serial follow up samples (taken at 2-4 weeks post-surgery and then every 6 months), the 6 patients that had detectable ctDNA during the study period relapsed within the first 2 years post treatment of their primary tumour. It is notable that all patients in this study had received neo-adjuvant chemotherapy identifying them as patients who already had poorer prognosis disease. All variants used to predict recurrence were category II variants i.e. those that were deemed to be pathological.

6.6.2.1 Using ctDNA as a predictive biomarker

CfDNA enters the blood stream through a combination of necroptosis, apoptosis and secretion (Schwarzenbach et al. 2011). When each of these mechanisms in probed in more detail the picture surrounding the production, release and clearance of cfDNA is much more complicated (Aucamp et

al. 2018). The mechanism involved may vary depending on the cell of origin; for example when ctDNA released from breast cancer cell lines was investigated it was noted that ctDNA levels did not correlate with the number of cells that were apoptotic or necrotic but instead was an active process associated with viable cells, thus likely to be occurring via active secretion (Wang et al. 2017).

CtDNA can be released from established tumour sites but can also be released from micro metastatic disease and circulating tumour cells (Schwarzenbach et al. 2009). The body naturally clears circulating nucleic acids through the liver and kidneys and as such it may be that there are significant peaks and troughs in the levels of ctDNA during treatment and potentially over short time windows (Fleischhacker and Schmidt 2007).

To put this into context the detection of ctDNA from patients in the FURVA trial relies on a number of variables; patients giving a sample, the sample being taken and processed according to protocol, the extraction of the DNA, the quality of the lab preparation, the quantity of ctDNA in cfDNA which is closely linked to the manner and quantity in which it is released into the bloodstream.

It was notable that only SNVs detected in cfDNA appeared to confer a shorter PFS. One possible explanation could be that only those clones containing variants which create the more aggressive phenotypes or more genetically unstable cells are then detected in ctDNA. The trend demonstrated with this data is that having a variant detectable in cfDNA is a marker of poorer prognosis, particularly if the variant is detected at a high VAF. However, this dataset is unable to answer whether it is the variant itself or simply the fact that tumour is shedding ctDNA that is the true biomarker.

When we consider the sites of action of the drugs involved in the FURVA trial one could hypothesise that driver variants in *PIK3CA, AKT1* or *ESR1* may render RET inhibition ineffective unless RET overexpression itself was a bigger driver of disease than its downstream components. Certainly, patients with detectable variants in downstream genes have a poorer PFS than those without when treated with fulvestrant and vandetanib (Figure 45). One hypothesis that would be interesting to

explore going forward is that patients with detectable variants in cfDNA prior to starting treatment in the setting of endocrine resistant disease should be offered treatments that target the cell cycle rather than the ER axis.

6.6.3 CNV detection in FFPE and cfDNA samples

The data presented in this thesis show that neither *MYC* nor *FGFR1* amplification in cfDNA or FFPE correlated with PFS when patients were treated with fulvestrant +/- vandetanib. This has not been previously studied but other studies have looked at the potential of *FGFR1* amplification to be predictive and prognostic in the setting of ER+ ABC. For example, it has been shown to predict for shorter time to progression on first line endocrine therapy (HR 3.21 p=0.006) and endocrine therapy in combination with CDK4/6 inhibitors (Drago et al. 2019). In the study from Drago et al amplification was determined by FISH testing on FFPE tumour material in 110 patients (65 from metastatic specimens and 45 from primary tumour). A ratio of *FGFR1* to centromere 8 of ≥2 was considered amplified.

A further study looked at the frequency of *FGFR1* variants detected in both FFPE and cfDNA samples from 100 patients with metastatic breast cancer, no data was given on HER2 status but 88/99 evaluable patients had ER+ disease (Bourrier et al. 2020). In the study by Bourrier 20% of patient FFPE samples showed *FGFR1* amplification as determined by FISH. A ratio of *FGFR1* to centromere 8 of ≥2 was considered amplified. The presence of *FGFR1* amplification in metastatic tumour biopsy was correlated with overall survival by both univariate and multivariate analysis. Plasma was available for 10/20 of the patients where an *FGFR1* amplification had been identified in FFPE tissue. Detection of CNV from cfDNA samples was much more difficult despite the authors using advanced sequencing techniques to estimate tumour fraction. Where the allele frequency was >3% there was 100% concordance between FFPE and cfDNA samples (number of samples not given).

The clinical utility of *FGFR1* amplification as a biomarker has not currently lived up to expectations as the non-randomised phase 2 FINESSE study showed similar response rates to lucitanib (an FGFR

inhibitor) in both patients with *FGFR1* amplification and those without evidence of amplification as determined by FISH, with a gene: centromere ratio of ≥2. In an exploratory analysis there was a suggestion that patients with highly amplified tumours (gene: centromere ratio of ≥4) had a higher ORR than those with less or non-amplified tumours, similar was seen with amplification detected from cfDNA (Hui et al. 2019).

The results reported in this thesis cannot support the findings that FGFR1 amplification is a potential prognostic biomarker. The data from the Bourrier study highlights the challenge in working with ctDNA to determine copy number alterations as only samples with a tumour fraction of >3% could reliably confirm findings from tissue samples.

MYC amplification in ER+ breast cancer has not been interrogated in the same way as *FGFR1* amplification in the current literature; potentially in part, due to the lack of MYC targeted therapies and the complexity of its sphere of influence on cellular functions. The results presented in this thesis show a very minor trend towards high *MYC* amplification correlating with PFS (p=0.149), but at the current time, do not support further investigation in the translational setting at this time.

6.7 Chapter Summary

In this chapter despite the overall analysis of the FURVA study demonstrating no additional PFS benefit when vandetanib is added to fulvestrant. Interesting data has emerged around the relationship between both t-RET and p-RET expression status, the presence of variants in cfDNA; in particular, *PIK3CA* and *ESR1* variants and prognosis. Furthermore, it has been shown that the presence of ctDNA in the form of detectable variants in key cancer genes in ER+ ABC correlates with shorter PFS in patients treated with fulvestrant + vandetanib. To summarise relevant negative findings; both *MYC* and *FGFR1* amplification detected in cfDNA did not correlate in a statistically significant way with PFS in patients treated with fulvestrant +/- vandetanib nor did the detection of SNVs in DNA extracted from FFPE samples.

These findings, along with those from earlier chapters, will now be discussed in Chapter 7 focussing on the wider context alongside commentary and opinion of what this research has added to existing literature and how it could be used to inform future studies.

7 General discussion, conclusions and future directions

7.1 Rationale for the research and potential for impact

The development of endocrine resistance in ER+ breast cancer is the ultimate mechanism by which the cancer escapes control and results in significant morbidity and mortality in people living with ABC. Although ER+ breast cancer has a good prognosis compared to other breast cancer subtypes such as triple negative breast cancer its frequency in the population means that it impacts and shortens tens of thousands of lives annually.

By adding targeted therapies to try and overcome endocrine resistance, in this case by adding a RET inhibitor (vandetanib) to a selective oestrogen receptor down regulator (fulvestrant) it was hoped to prolong progression free survival and thus the need for chemotherapy. It has often been demonstrated that when using targeted treatments such as tyrosine kinase inhibitors not all patients benefit therefore the use of biomarkers is essential to try and understand patterns of response to treatment in a genomically heterogeneous population.

This thesis has detailed the search for predictive and prognostic biomarkers in the context of patients with ER+ ABC treated with fulvestrant +/- vandetanib. It has contributed to understanding around clinical response to RET inhibition and has provided a snapshot of genomic contributors to clinical outcomes in the setting of patients with ER+ ABC treated with fulvestrant and vandetanib.

7.2 Summary of research findings

7.2.1 RET expression shows potential as a prognostic biomarker

Overall the FURVA study is the second study to show no progression free survival benefit with the addition of vandetanib to conventional treatment in patients with ER+ ABC. This may suggest that vandetanib is not a sufficiently active drug in an unselected patient population with ER+ ABC to merit further studies. However, it was interesting that RET expression; both high t-RET and high p-

RET correlated with longer PFS. This may be independent of treatment; perhaps as a biomarker of inherently more indolent tumour biology or may be related to response to treatment with fulvestrant which is known to be an active drug in ER+ ABC and was the hormone directed backbone of both arms of the FURVA clinical trial.

7.2.2 High RET expression is not the result of SNVs in RET

In this project increased RET expression is not linked to the presence of common *RET* variants in patients with ER+ breast cancer. While the methods used did not sequence the entirety of the *RET* gene they covered hotspots associated with common RET activating variants and no RET variants were detected. This fits with the low number of *RET* variants reported in larger scale sequencing studies of breast cancer patients for example only 0.2% (Rich et al. 2019) in one study and up to 1.2% in another (Paratala et al. 2018). Both these studies coupled with the information presented in this thesis add weight the hypothesis that high RET expression is not a result of *RET* SNVs. Plaza-Menacho et al were able to demonstrate that there is good correlation between RET mRNA and RET expression. As this does not seem to be linked to a high frequency of SNVs in *RET* this would suggest that the increased mRNA expression is due either to changes in *RET* copy number or changes at an epigenetic level with the latter being more likely as *RET* amplification has also been shown to be infrequent in breast cancer samples; for example in one series it occurred in 81/9693 (0.8%) of cases when determined using targeted genomic profiling using a panel provided by Foundation Medicine (Cambridge, MA) (Paratala et al. 2018).

7.2.3 Absence of ctDNA is a potential biomarker of favourable disease when patients are treated with fulvestrant and vandetanib

PFS in patients treated with fulvestrant and vandetanib was closely linked to whether patients had a detectable tier II cancer variant in cfDNA. Trying to identify patients who have inherently favourable or un-favourable disease is key to escalating therapy where needed and de-escalating therapy for patients with favourable disease.

Evidence has begun to build during this project suggesting that the presence of ctDNA at any point of disease is a poor prognostic sign. Garcia-Murillas et al prospectively recruited 170 women with early stage breast cancer to a study where primary tumour samples were sequenced and a personalised ddPCR assay created to monitor for variants every 3 months for a year then every 6 months. In all types of breast cancer the presence of ctDNA at diagnosis or after initial treatment predicted strongly for shorter relapse free survival, this remained true when analysed by receptor status although numbers were small. In the 24 patients with ER+ breast cancer no patients in the ctDNA-negative group experienced relapse while all 6 with ctDNA detected did (Garcia-Murillas et al. 2019). In a further small prospective study the presence of ctDNA containing a PIK3CA variant one month after starting endocrine treatment was predictive for progression free survival at 1 year in patients treated with endocrine therapy as first line treatment for metastatic ER+ breast cancer ($p=0.0053$ by log rank testing). This study was limited by its small number of participants n=39 but adds weight to the argument that detectable ctDNA is a poor prognostic marker, particularly if it persists despite therapy (Jacot et al. 2019). Furthermore, the detection of ctDNA through personalised assays can predict disease relapse after surgery and adjuvant therapy up to two years before clinical relapse offering a potential window of opportunity for additional treatment (Coombes et al. 2019).

These studies support the retrospective analysis performed in this project where the presence of ctDNA (in many cases after exposure to treatment) was a predictor of shorter PFS potentially indicating both unfavourable disease biology and a lack of response to treatment with fulvestrant and vandetanib. Due to small numbers of samples with variants it was difficult to assess whether this was driven by one variant or simply the presence of ctDNA independent of the variant or gene it represented. The presence of a variant detected in cfDNA in *PIK3CA* predicted for shorter PFS. There was a trend towards the same for *ESR1* but it did not reach statistical significance. This is potentially due to the more sensitive detection method used for *ESR1* variants as potentially the detection of a

variant in cfDNA only predicts for shorter PFS above a certain VAF or there is a certain VAF that begins to represent clinical significance.

The frequency of *TP53* and *AKT1* variants in cfDNA was ~5% in this cohort of patients with ER+ breast cancer therefore larger samples sizes would have been required to be adequately powered to detect a clinically meaningful difference in PFS between patients who did or did not have the variant detected in cfDNA. However, by grouping patients into those who did or did not have ANY detectable variant in cfDNA this binary grouping was able to predict for poorer PFS with greater statistical significance than if only the presence or absence of *PIK3CA* variants was used. This prediction was made based on the results from key genes (*PIK3CA, AKT1, TP53* and *ESR1*). This offers the possibility that large gene panels or highly sensitive panels may not be required to give a clinically relevant biomarker. This also means that for samples with very low DNA concentrations focus and sequencing depth can be used on the most relevant genes. However, this risks oversimplifying patient's data and there is likely to be richness in a larger panel that will be useful for future research and potentially offer additional treatment options to patients as and when additional targeted drugs become available. Technology is also improving allowing larger gene panels to be used with low DNA concentration samples; particularly if the aim of the panel is to monitor for variants detected from high DNA concentration samples such as tissue samples rather than to identify new low frequency variants (McDonald et al. 2019).

7.2.4 CNA when detected from FFPE or cfDNA samples using ddPCR did not correlate with progression free survival

Detecting copy number alterations with ddPCR is an established technique and has been favourably compared to the gold standard of FISH in FFPE samples. Detecting CNAs from cfDNA samples is more experimental and in this study, while technically feasible no cut points were able to identify a threshold above which CNAs in *FGFR1* or *MYC* demonstrated potential as a prognostic biomarker. Amplifications were identified in both the FFPE and cfDNA samples. In the FFPE samples numbers of

samples with a gene of interest: reference gene ratio of ≥2 were identified but they were small in number and only one sample tested for *MYC* showed a highly amplified ratio e.g. a ratio of ≥4. In cfDNA samples the challenge was trying to interpret results without knowledge of the tumour fraction. Here, even with retrospective cut point analysis no statistically significant cut point could be identified. Potentially, in this study, this was related to the small number of samples and thus very few samples showing high levels of amplification. In future studies, an attempt at tumour fraction, even if only to a crude percentage e.g. >1%, >10% or >50% may make interpretation more robust. Other research groups have managed to develop bioinformatics solutions to estimate tumour fraction and if I were to look further into copy number alteration by ddPCR I would aim to include bioinformatic determination of tumour fraction in my project.

7.2.5 Translational results from the FURVA clinical trial in the context of the current treatment landscape

When considering the results presented in this thesis we must be mindful that the treatment landscape for patients with ER+ ABC has altered since the idea for the FURVA study was conceived with the success of CDK4/6 inhibitors in the setting of ER+ ABC (de Groot et al. 2017). Furthermore, there are now newer RET targeting agents such as LOXO-292 which have had much positive press around their ability to treat patients with tumours harbouring *RET* fusions or *RET* activating variants such as M918T and V804M, predominately in thyroid cancer and for patients with RET fusions in non-small cell lung cancer (Subbiah et al. 2018). In LOXO-292s drug development phase it was demonstrated that unlike vandetanib it is highly selective for RET (Seoane and Capdevila 2018). LOXO-292 is now part of an expanded access program in the USA for patients with “Evidence of a RET activating alteration”. As SNVs, gene fusions and amplifications of RET are very rare in breast cancer it is not known if a more selective RET inhibitor would be more successful in treating breast cancers with high levels of RET expression.

Personalised medicine in ER+ breast cancer via advanced genomic analysis has gained ground during the course of this project with success of PI3K inhibitors for patients with a tumour containing a *PIK3CA* variant (André et al. 2019) and promising phase 2 results for the AKT inhibitor capivasertib, although without a companion biomarker (Jones et al. 2020).

7.3 Future directions

7.3.1 RET targeted therapy in ER+ breast cancer

RET is an attractive target in ER+ breast cancer as there is strong pre-clinical evidence that it is upregulated in endocrine resistance. It is also a druggable target both with multi-tyrosine kinase inhibitors and those that are more RET specific. However, as it sits upstream and acts through two of the pathways most commonly found to contain genomic variants in breast cancer it may not be a strong enough driver of growth for inhibition alone to alter clinical outcomes. Through searching clinicaltrials.gov (accessed 29/6/2020) it did not appear that LOXO-292 was being investigated in breast cancer unless a variant had been identified that made the patient eligible for a tumour agnostic indication. *RET* is an active gene in ER+ breast cancer and a potentially targetable one but the evidence presented here and in the literature would suggest that we do not yet understand enough about how it interacts with other key pathways to determine how RET inhibitors may fit into the treatment pathway for ER+ ABC.

7.3.2 ctDNA as a predictive and prognostic biomarker

The work presented in this thesis has demonstrated that detectable ctDNA during or after treatment with fulvestrant and vandetanib correlates with a shorter PFS compared with patients with undetectable ctDNA. Combining these findings with others that have been published during the course of this project strongly support further investigation into how this knowledge can be applied clinically.

One significant clinical issue is how to identify patients who will survive for years with ER+ ABC and those that are likely to develop endocrine resistance early and require new ways of treating their

disease to prolong life. Using a liquid biopsy at the point of the development of metastatic breast cancer may be the most opportune moment as it could guide whether patients will respond to endocrine therapy alone or whether they may benefit from additional therapies such as CDK4/6 inhibitors or even upfront chemotherapy. This is earlier in the disease course than the samples used in this project but has significant potential to spare some patients the increased toxicity; both physical and potentially financial depending on the healthcare setting in which they are treated if absence of ctDNA can be shown to identify a group of patients who are at low risk of imminent disease progression when treated with endocrine therapy alone. This may be particularly prudent in patients who have significant comorbidities or personal preferences that mean they wish to avoid more toxic therapies.

Targeted therapies may be less appropriate in patients with detectable ctDNA as their tumours may be behaving in a more aggressive manner; perhaps for these patients' upfront chemotherapy should be considered or even immunotherapy if the tumour mutational burden is significant. If multiple variants are present, particularly if variants are present in key cell cycle regulatory genes such as TP53 then targeted therapies may not be sufficient to induce clinically meaningful control of disease and these patients should be considered either for upfront systemic non-targeted treatments or be considered for early reassessment of their disease if they opt for targeted treatment.

7.4 Project limitations

7.4.1 Sample limitations

All aspects of sample collection had been pre-determined within the clinical trial protocol prior to the commencement of this project. Pragmatically fresh biopsies were not a requirement for trial entry and submission of tissue blocks and plasma samples for cfDNA extraction were not mandated. This created many challenges in the practical aspects of the project for example the timing and running of assays and in the interpretation of the data created.

All FFPE samples were from primary untreated tumour while all cfDNA samples were taken in the metastatic endocrine resistant setting. Therefore, it was very difficult to tell whether the absence of variants in cfDNA samples was a true absence or simply that they were not detectable with the samples and technology used. The age of the primary biopsies was also likely to have contributed to challenges in extracting high quality DNA for sequencing. The gold standard for this project would have been to have paired FFPE (or ideally fresh frozen) primary and metastatic biopsies alongside 3 plasma samples from baseline, 8 week and EOT during trial participation. In retrospect even if this had only been possible for a small number of patients it may still have generated richer data than the patchwork of samples that were available for the work presented in this thesis. However, fresh frozen samples are logistically difficult to manage and asking patients to undergo a biopsy that will not directly change their treatment has ethical and practical implications.

In future studies, setting up a translational sub-study where additional research biopsies were mandated and particular commitment was shown to collecting all plasma samples would enable in depth analysis of the genomic landscape and its interaction with RET expression.

7.5 Potential future projects

The focus of this thesis was on trying to identify potential biomarkers in the context of patients with ER+ ABC treated with fulvestrant +/- vandetanib. RET expression has again shown potential as a biomarker in ER+ breast cancer although with some findings potentially contradictory to the current published literature. Future projects could include further validation work looking at prospective validation of the cut points determined in this project. This could be in the context of a further clinical trial or with validation dataset where FFPE tissue samples and survival data were available. From the perspective of further clinical trials; using a more potent RET inhibitor could be of interest particularly if the trial participants were limited to those with demonstrated high RET activity.

Taking these findings back to the bench it would be interesting to explore the role of RET in response to fulvestrant +/- vandetanib in more detail. For example, examining other markers of RET activity

such as the expression of its ligands such as GFRA alpha. It would also be interesting to look at alternative methods of identifying RET expression; potentially even non-invasively through extraction of mRNA from exosomes as understanding the expression of RET overtime and in particular at the time point when therapy is being used is important. It would be particularly interesting to identify the mechanisms by which RET becomes overexpressed and this may offer insight into it's potential as a drug target in future.

From the perspective of using ctDNA detection as a biomarker there is great clinical need to identify predictive biomarkers for patients with ER+ ABC as within this group there are a wide variety of disease timelines witnessed in the clinic. The data reported in this thesis supports the published literature that this is a potentially useful biomarker. Collaborative working and the use of multiple genetic and clinical outcome datasets could provide the data necessary to support basing treatment on genomic data. Ultimately I would like to see studies designed where treatments were escalated or de-escalated based on biomarkers such as the detection of ctDNA.

7.6 Final conclusion

Improving therapies for people living with advanced ER+ breast cancer will change the lives of millions of women worldwide. In the era of increasingly personalised medicine exploring targets that are druggable and found in subsets of patients with ABC is a valid approach. In this thesis RET has been the focus and its expression in FFPE samples is a potential biomarker for further investigation. The knowledge gained here will be useful in further trials using a RET directed investigational agent.

In addition, the finding that the presence of detectable ctDNA correlates with shorter PFS adds additional evidence to other recent studies that the liquid biopsy at strategic points during treatment for ABC has a prognostic and potentially predictive role to identify a group of patients who may need more aggressive upfront treatments rather than targeted therapies.

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Appendices

Appendix 1: FURVA REC approval form

Appendix 2: FURVA patient information sheet

Appendix 3: Summary table of sample availability and variants detected in chapter 4