Pharmacogenetic study of Fc Gamma Receptor and HER2 genes in breast cancer

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Abstract

Breast cancer is a complex set of diseases with different biological and clinical characteristics. An important contribution to this diversity is provided by germ-line genetic variations. The HER2-positive breast cancers have been extensively studied with particular regard to their biology and targeted treatments. However, the influence of pharmacogenetic (PG) factors on these aspects remains largely unexplored. This research focused on the possible effects of common single nucleotide polymorphisms (SNPs) on specific aspects of HER2-positive disease.

Initially we analysed two coding SNPs in the HER2 gene (Ile655Val and Ala1170Pro) in breast cancer patients and evaluated their potential association with HER2 expression in tumour samples. The proline variant of the Ala1170Pro SNP was associated (odds ratio = 1.7, p = 0.01) with HER2 over-expression/amplification in over 360 breast cancer patients. In contrast, Ile655Val was not associated with HER2 over-expression/amplification. Bioinformatics tools predict that Ala1170Pro might affect the structure or function of the HER2 protein. The same variants were explored in the context of DNA extracted from the patients' primary tumours in 241 patients. We hypothesized that the proline allele of Ala1170Pro could undergo allele-specific amplification during the development of HER2-positive tumours. This hypothesis, however, was not confirmed. Although the association of the proline allele of Ala1170Pro with HER2 positivity is intriguing, the role of the two SNPs in HER2 over-expression/amplification remains to be elucidated.

Trastuzumab has radically changed the treatment of HER2-positive breast cancer. However, resistance to treatment and toxicity can limit its effectiveness. The second objective of this project was the analysis of PG, biomarker and pharmacokinetic (PK) parameters in trastuzumab-treated patients. Fc Gamma Receptors (FcγRs) are key proteins in the trastuzumab-induced Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) and two coding SNPs in these genes (FCGR2A His131Arg and FCGR3A Phe158Val) were analysed.

The measurement of trastuzumab in plasma was made possible by the development of a novel cell-based ELISA.

Only 28 patients with advanced disease treated with trastuzumab were recruited. However, we observed a possible association of the valine allele of the FCGR3A Phe158Val SNP with a longer time to progression ($p = 0.03$). Cardiac toxicity was assessed in a group of 139 patients treated with adjuvant trastuzumab. Although a role of germ-line genetic variants could not be demonstrated, the analysis highlighted the challenges and limitations encountered in the conduct of an observational pharmacogenetic study.

This project leaves a legacy archive composed of germ-line DNA samples, tumour DNA samples, plasma samples and tumour FFPE blocks from over 360 breast cancer patients. These samples and data are available for the exploration of further potential factors which might influence the biology of the disease and/or its response to treatment.

To Iliana, Stefania and Emma

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Abbreviations

ADCC	Antibody-Dependent Cell-Mediated Cytotoxicity
ACT	Adriamycin plus Cyclophosphamide, Paclitaxel
ACTH	Adriamycin plus Cyclophosphamide, Paclitaxel and Trastuzumab
AKT	Akt (Ak Thymoma) kinase, also known as Protein Kinase B (PKB)
Ala	Alanine
ALTTO	Adjuvant Lapatinib and/or Trastuzumab Treatment Optimisation
Arg	Arginine
ASCO	American Society of Clinical Oncology
ATP	Adenosine-5'-triphosphate
BCIRG	Breast Cancer International Research Group
BMI	Body Mass Index
BRCA1	gene enconding for the BReast CAncer type 1 susceptibility protein
BRCA2	gene enconding for the BReast CAncer type 2 susceptibility protein
CAP	College of American Pathologists
CD20	B-Lymphocyte Antigen (Cluster of Differentiation 20)
CEP17	Chromosome Enumeration Probe 17
CGH	Comparative genomic hybridization
CHF	Congestive Heart Failure
CISH	Chromogenic In Situ Hybridization
CRUK	Cancer Research UK
C_T	Threshold cycle
CubeX	Cubic Exact Solution
CV	Coefficient of Variation
CYP2D6	Cytochrome P450 2D6
DCIS	Ductal Carcinomas In Situ
DFS	Disease-Free Survival
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNP	Dinitrophenol
DPD	Dihydropyrimidine dehydrogenase
EBCTCG	Early Breast Cancer Trialists' Collaborative Group
ECD	Extra-Cellular Domain
EGFR	Epidermal Growth Factor Receptor

ELISA	Enzyme-Linked ImmunoSorbent Assay
EMA	European Medicines Agency
ErbB	Avian Erythroblastosis Oncogene B
EudraCT	European Union Drug Regulating Authorities Clinical Trials
FcγR	Fc (Fragment Crystallizable Region) Gamma Receptor
FCGR2A	gene enconding for the FcγR IIa protein
FCGR3A	gene enconding for the FcγR IIIa protein
FCS	Foetal Calf Serum
FEC	Fluorouracil, Epirubicin and Cyclophosphamide
FFPE	Formalin-Fixed Paraffin-Embedded
FISH	Fluorescent In Situ Hybridization
Glu	Glutamic acid
GWAS	Genome-Wide Association Study
H&E	Hematoxylin & Eosin
HER	Human Epidermal Growth Factor Receptor
HER2	Human Epidermal Growth Factor Receptor 2
HERA	HERceptin Adjuvant trial
His	Histidine
HR	Hormone Receptor
HRP	Horseradish Peroxidase
HTA	Human Tissue Act
H-W	Hardy-Weinberg equilibrium
ICH	International Conference on Harmonisation
IEF	Isoelectric Focusing
IGF-1R	Insulin-like Growth Factor Type 1 Receptor
IgG	Immunoglobulin G
IHC	Immuno-Histo-Chemistry
Ile	Isoleucine
ISH	In Situ Hybridization
LCM	Laser Capture Microdissection
LD	Linkage Disequilibrium
LLQ	Lower Limit of Quantification
LN	Lymph Node
LOH	Loss of Heterozygosity
LVEF	Left Ventricular Ejection Fraction
mAb	Monoclonal Antibody

MAF	Minor Allele Frequency
MAPK	Mitogen-Activated Protein Kinase
MIDAS	Multiallelic Interallelic Disequilibrium Analysis Software
mTOR	Mammalian Target Of Rapamycin
MUC4	Mucin-4 cell surface associated
MUGA	Multi Gated Acquisition
NCBI	National Centre for Biotechnology Information
NCCC	Northern Centre for Cancer Care
NHS	National Health Service
NICR	Northern Institute for Cancer Research
NOS	Not Otherwise Specified
NST	No Special Type
NTC	No Template Control
ORR	Overall Response Rate
OS	Overall Survival
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pCR	pathological Complete Response
PD	Pharmacodynamic
PEN	Polyethylene Naphthalate
PFS	Progression-Free Survival
PG	Pharmacogenetic
Phe	Phenylalanine
PI3K	Phosphatidylinositol 3-kinase
PK	Pharmacokinetic
Pro	Proline
PTEN	Phosphatase and Tensin homolog
QC	Quality Control
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RAS	Rat Sarcoma family of protein
REC	Research Ethics Committee
RECIST	Response Evaluation Criteria In Solid Tumors
RFLP	Restriction-Fragment Length Polymorphism
rhu4D5	Recombinant Humanized Monoclonal Antibody 4D5 (trastuzumab)
Rn	Normalized Reporter

RPMI	Roswell Park Memorial Institute Medium
RT	Room Temperature
SD	Standard Deviation
SDS	Sequence Detection System
SEER	Surveillance, Epidemiology and End Results
SERM	Selective Estrogen Receptor Modulator
SISH	Silver Enhanced In Situ Hybridization
SNP	Single Nucleotide Polymorphism
TBS	Tris Buffered Saline
TCH	Docetaxel plus Carboplatin, Trastuzumab
TMA	Tissue Microarray
TMB	3,3′,5,5′-Tetramethylbenzidine
TOP2A	DNA Topoisomerase 2-alpha
TOT	Time on Trastuzumab
TPMT	Thiopurine Methyltransferase
TTP	Time to Progression
UGT1	Uridine 5'-diphospho-glucuronosyltransferase
UV	Ultraviolet
Val	Valine

Chapter 1. Introduction

1.1 Breast cancer

1.1.1 Epidemiology and risk factors

Breast cancer is the second most common malignancy worldwide (the most common among women) after lung/bronchus neoplasms, with 1,383,500 estimated new cases in 2008 (Jemal *et al.*, 2011). It caused 458,400 estimated deaths worldwide in 2008, being the fourth cause of death from cancer overall (after lung/bronchus, stomach and liver) and the first cause among women.

While female gender is the strongest and most obvious risk factor, many other genetic and environmental elements interact and contribute to the development of this disease. On the one hand, besides the long-known mutations in genes such as BRCA1 and BRCA2 which are associated with the occurrence of the relatively rare hereditary breast cancers (Hall *et al.*, 1990), multiple more common low-penetrance genetic variations have been proposed as possible determinants of a small increase in risk in different populations (Zhang *et al.*, 2011). On the other hand, well-established environmental risk factors include age, a long menstrual history, nulliparity or late age at first birth, use of postmenopausal hormone replacement therapy or oral contraceptives and alcohol consumption (Hulka and Moorman, 2008). The wide variation in the incidence rates seen among different world areas (highest in Western Europe and North America, lowest in sub-Saharian Africa and Asia) has been at least in part explained by differences in reproductive/hormonal factors and the availability of early detection services, but it could also be influenced by the different genetic predisposition of the various ethnicities.

In the United Kingdom in 2008 48,034 new cases and 12,116 deaths were reported (CRUK, 2011). As in other Western countries the incidence rate of breast cancer has increased over the last 30-40 years due to the introduction of screening programmes. This implementation, together with the establishment of more effective treatment, also contributed to a significant reduction in the mortality rate (Figure 1.1).

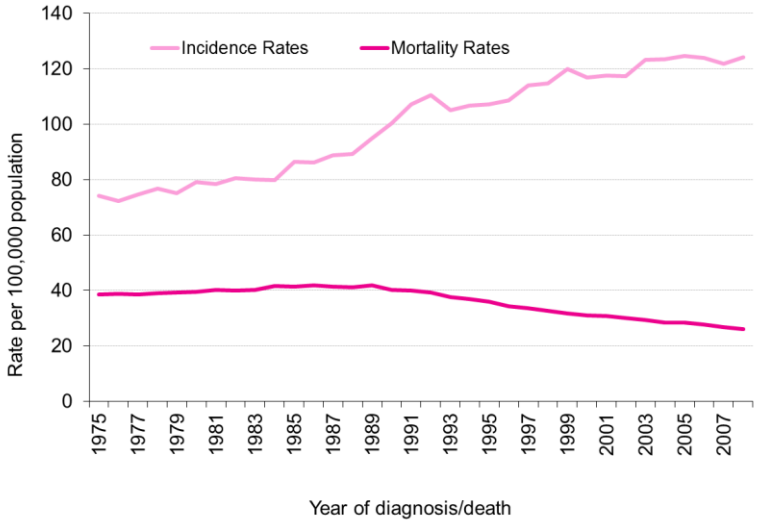

Figure 1.1: Breast cancer incidence and mortality rates in GB, females, 1975 - 2008.
From CRUK (2011)

The same effect is also well illustrated by the rise in the ten-year survival rate from approximately 40 % to over 70 % over the last 30 years of the twentieth century (Figure 1.2).

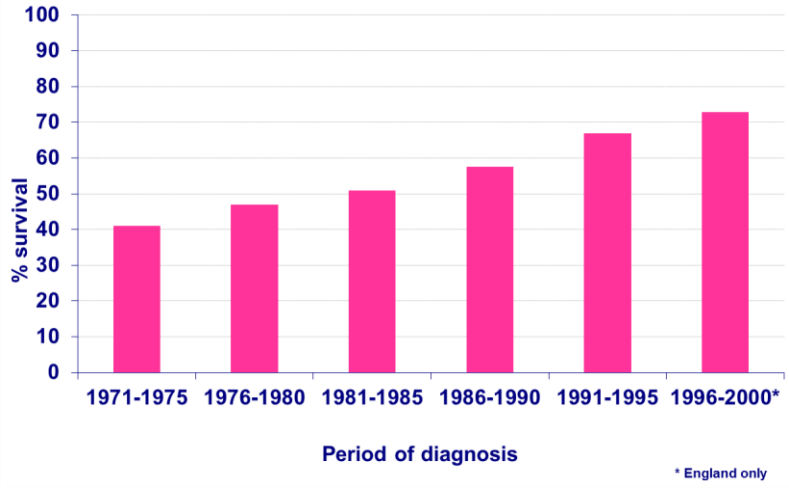

Figure 1.2: Ten-year relative survival rates, females breast cancer, England and Wales, 1971 - 2000.
From CRUK (2011)

1.1.2 Biology

The most common histologic type of cancer of the breast is the carcinoma, which arises from the tubulo-alveolar unit of the mammary gland. “Breast cancer” and

"breast carcinoma" are often treated as synonyms in the literature and they are considered as such in this thesis. Like many other epithelial neoplasms, the development of breast carcinomas is characterized by a sequence of progressive steps which include flat epithelial atypia, atypical hyperplasia, *in situ* carcinoma and invasive carcinoma. This evolution is supported by a large amount of immunohistochemical, genomic and transcriptomic data (Bombonati and Sgroi, 2011). Once the tumour cells acquire the capacity to invade the basal membrane, they can enter the lymphatic system and the cardiovascular system. Such cells can potentially give rise to metastases, initially to regional lymph nodes and subsequently to distant organs (more frequently bone, skin, liver and lungs).

Within this common framework, however, breast cancer does not appear to be a homogenous disease. Rather, from many perspectives, it emerges as a complex and numerous set of distinct entities. Firstly, many histopathologic subtypes have been described, of which the most frequent are the ductal and lobular types. In addition, there are several relatively rare variants which include tubular, cribriform, mucinous, neuroendocrine, papillary, apocrine, metaplastic and other even rarer forms. Furthermore, the grade of differentiation classifies the cancers as well, moderately and poorly differentiated on the basis of the microscopic similarity of the tumour cells to normal breast tissue, evaluated in terms of tubule formation, nuclear pleomorphism, and mitotic count (Elston and Ellis, 1991).

Besides the different microscopic appearance of the tumours, another important discriminating factor is the presence of particular markers which are also fundamental therapeutic targets. The expression of oestrogen and/or progesterone receptors, which occurs in up to 80% of cases, is historically the first biologic factor which has been extensively evaluated and taken into account as a possible driver of treatment choice (Cleator *et al.*, 2009). The expression of the Human Epidermal growth factor Receptor 2 (HER2) and its biological and pharmacological implications will be discussed in Sections 1.2, 1.3 and 1.4.

More recently high-throughput technologies have allowed the emergence of different molecular classifications based on differential expression of sets of genes (Perou *et al.*, 2000) and investigations on the possible role of gene-silencing by epigenetic modifications are currently under way (Orlando and Brown, 2009).

The biologic heterogeneity of breast cancer, highlighted by all the scientific approaches included in this incomplete list, may at least in part explain the wide variation seen in the clinical behaviour of the disease. Indeed, the marked differences observed in terms of presence/absence of metastases, time required to metastasise, sites of metastases, time pattern of disease progression, responsiveness to different anti-cancer drugs etc. have been interpreted as resulting from tumour diversity. However, the diversity in the host's biology and its interactions with the tumour are also of fundamental importance and constitute a major focus of this thesis.

1.1.3 Overview of multi-modal treatment

The possible therapeutic strategies for breast cancer are traditionally subdivided into the three broad categories of surgical, radiation and systemic approaches. The surgical treatment, essentially aimed at the removal of the primary tumour, has undergone an important process of refinement during recent decades, which has led to more effective and, when possible, less destructive operations (Luini *et al.*, 2007). Radiotherapy is mainly based on the use of ionizing radiation, which causes damage to the DNA of the tumour cells and subsequent cellular death. As with surgery, the technique of radiotherapy has significantly improved over time, obtaining a more accurate targeting of the tumour areas, with a lower dose of radiation delivered to the normal tissues (Van Limbergen and Weltens, 2006). Finally, systemic treatment includes a wide range of anti-cancer drugs. Chemotherapy was first employed in the 1950s and currently there are tens of agents approved for clinical use in the Western countries and thousands of new compounds under pre-clinical or clinical development worldwide.

The choice among these different therapeutic strategies and their combination or sequence depends on many interacting factors which are schematically illustrated in Figure 1.3.

The stage of the tumour, which discriminates between early disease (in which the spread of the tumour is confined to the breast and regional lymph nodes) and advanced disease (in which distant metastases are present) is a first fundamental determinant. In the case of early breast cancer the strategy has a curative intent and usually consists of a surgical operation which can be accompanied by radiotherapy

and/or systemic treatments which aim at reducing the risk of recurrence (adjuvant treatments). The efficacy and toxicity of adjuvant treatments have been demonstrated by countless studies over recent decades. A quinquennial analysis is performed by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG), based on updated data on individual patients randomised into the main trials of the treatment of early breast cancer (Clarke *et al.*, 2005; EBCTCG, 2005; CTSU, 2011). In the case of advanced breast cancer the intent of the treatment is generally palliative, although it is possible in some cases to obtain a control of the disease which lasts for several years. In this setting systemic treatments usually play the main role, but radiation and surgical options can still be adopted in order to achieve a better palliation or prolong the patients' survival in selected cases (Pockaj *et al.*, 2010).

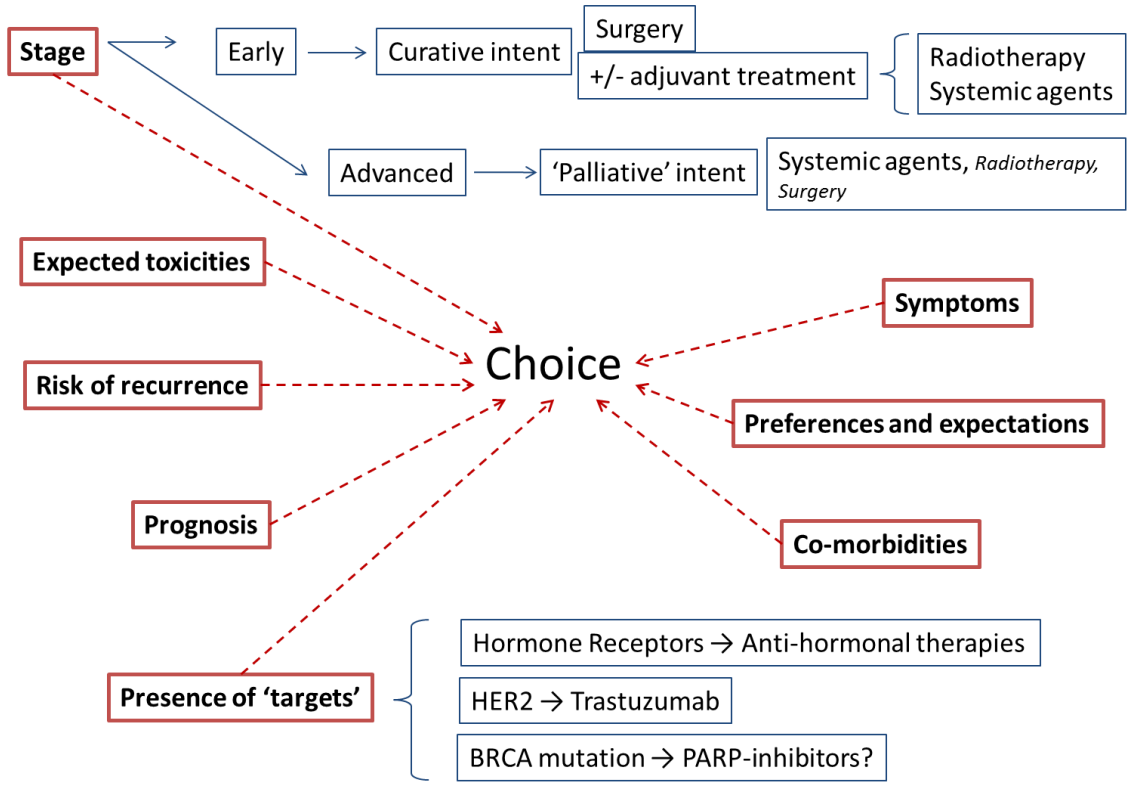

Figure 1.3: Factors influencing therapeutic choice in breast cancer

A second factor influencing therapeutic choice is the presence of biologic targets on the tumour. One of the most important targets is the expression of hormone receptors (HR), which allows the use of a wide range of anti-hormonal agents, in both the adjuvant and the palliative settings (Pritchard, 2003; Cigler and Goss, 2007). A second

well-validated target is the HER2 receptor, which is the main focus of this thesis and will be discussed in detail in the next sections. There are also several potential biomarkers which have been recently described and are currently being validated in clinical trials. One of these is the presence of mutations in the BRCA genes (Tutt *et al.*, 2010), which is being evaluated as a potential indicator of sensitivity to approaches that target DNA repair deficiency, such as Poly (ADP-ribose) polymerase (PARP) inhibitors. Other important determinants of the therapeutic choice are listed in Figure 1.3.

1.1.4 Individualization of treatment

It has been clear for many years that not all breast cancer patients receiving the same treatment would respond in the same way, showing a wide variability in terms of both efficacy and side effects. The accumulation of a variety of therapeutic options, especially among systemic treatments, with different efficacy and toxicity spectra, has prompted efforts toward a better individualization of breast cancer therapy. Many studies have therefore been conducted in order to identify factors which could predict the different outcomes of patients treated with particular drugs. As exemplified in Figure 1.4, a predictive factor can influence the outcome of a group of patients who receive a given treatment, but it has no effect on patients who do not receive the same treatment (Figure 1.4b).

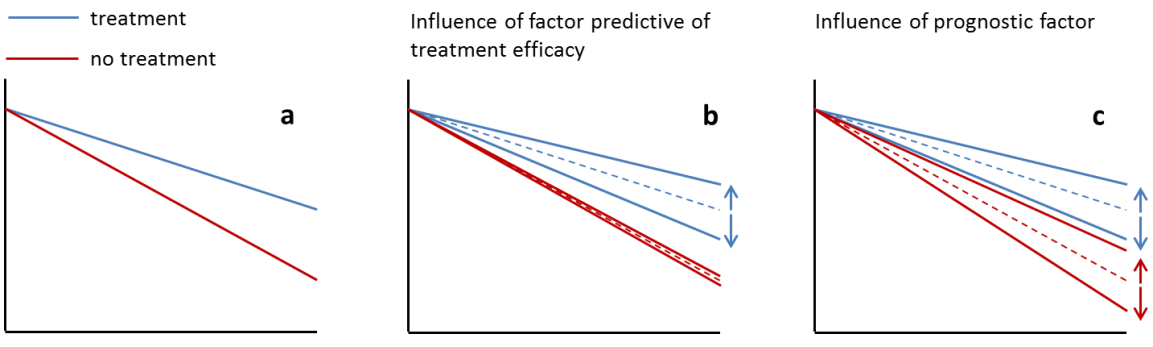

Figure 1.4: Influence of a predictive or prognostic factor on outcome of patients receiving a given treatment.
X-axis: time, Y-axis: probability of patients' survival. a) Patients who receive an effective treatment have a longer survival compared to patients who don't receive the treatment; b) If patients are stratified according to the presence of a predictive factor the group of treated patients is split in a subgroup of patients who benefit more and a subgroup of patients who benefit less from the treatment, whereas the untreated patients have the same outcome irrespectively of the presence of the factor; c) If patients are stratified according to the presence of a prognostic factor both treated and untreated groups are split in subgroups of patients with better or worse prognosis

An analogous effect can be observed with regard to the toxicity profile of a therapeutic approach. In contrast, a prognostic factor can affect the survival of patients irrespective of their treatment (Figure 1.4c).

The use of tamoxifen, a selective estrogen receptor modulator (SERM), can be regarded as an early example of treatment individualization. While this drug was initially prescribed to all postmenopausal breast cancer patients, it became gradually clear that only the patients whose tumour expressed hormone receptors would achieve a clinical benefit and tamoxifen use was restricted to HR-positive breast cancer patients, irrespective of their age (EBCTCG, 1998). The use of HER2-targeted therapies is also regarded as a successful strategy of treatment individualization and will be described in the next sections.

Among the efforts towards a better personalization of anti-cancer therapy, pharmacogenetics (PG) could play a major role. Pharmacogenetic studies investigate the influence of heritable genetic factors on drug response and toxicity and could offer key benefits in the field of oncology, where commonly used therapeutics are often characterized by a narrow therapeutic window and a wide inter-individual variability in terms of both anti-tumour activity and side effects. Several well-known examples include the role of thiopurine methyltransferase (TPMT) polymorphisms in treatment with thiopurines (Wang and Weinshilboum, 2006), the effect of genetic variations of Uridine 5'-diphospho-glucoronosyltransferase (UGT1A1) on irinotecan toxicity (Innocenti and Ratain, 2006) and the influence of dihydropyrimidine dehydrogenase (DYPD) variants on 5-fluorouracil toxicity (van Kuilenburg, 2006).

In breast cancer, the most extensively studied PG relationship is that between cytochrome P450 2D6 (CYP2D6) variations and tamoxifen activity and toxicity, whose clinical relevance and applicability is still under debate (Kelly and Pritchard, 2012; Rae *et al.*, 2012; Regan *et al.*, 2012). Other emerging potential determinants of response and/or toxicity are being evaluated with regard to several systemic treatments, including aromatase inhibitors, anthracyclines, taxanes, bevacizumab and, as detailed in the next chapters, trastuzumab (Marsh and Liu, 2009; Longo *et al.*, 2010; Jamieson and Boddy, 2011).

1.2 HER2

1.2.1 HER family and HER2

The HER2 (Human Epidermal growth factor Receptor 2) protein is part of a family of four transmembrane growth factor receptors (ErbB protein family, or Epidermal Growth Factor Receptor - EGFR - family, or HER family) which function to activate intracellular signalling pathways in response to extracellular signals. HER proteins are widely expressed and important for the development and function of many organs and systems, including brain, skin, lung, gastrointestinal tract and heart.
Their structure, illustrated in Figure 1.5, consists of an extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase domain.

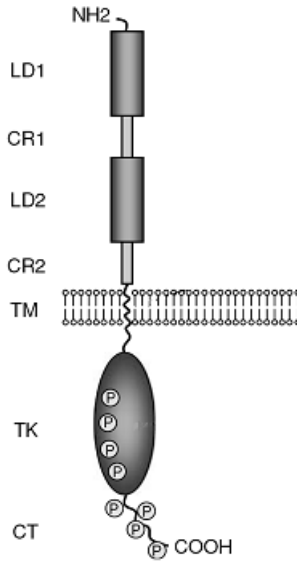

Figure 1.5: Structure of HER proteins.
Modified from Moasser (2007)

The binding of a ligand induces a conformational change in the extracellular domain of ErbB proteins that promotes their dimerization and consequent transphosphorylation.
Unlike the other members of the family, the extracellular domain of HER2 exists in a constitutively active conformation. HER2 in fact lacks ligand-binding activity and its signalling function is engaged by its ligand-bound heterodimeric partners HER1, HER3 and HER4 (Moasser, 2007).
HER2 plays a key role in many physiological processes such as cell growth, cell death, differentiation and tissue development, but it is also involved in carcinogenesis and metastasis.
The transforming potential of the rodent cellular homologue of HER2 - *neu* - was first demonstrated in the 80s, when a point mutation - a valine residue to a glutamic acid residue at position 664 - in the transmembrane domain (Bargmann *et al.*, 1986) was found to be essential for tumour formation. Subsequently, several other deletion mutations within the extracellular juxtamembrane region that promote dimerization and enhanced kinase activity (Siegel and Muller, 1996) were described.

Differently from mice, human tumours appear to show over-expression of the receptor as the main or the only tumorigenic mechanism. The diverse and interacting downstream pathways involved in HER2-induced tumorigenesis have been reviewed by Moasser (2007).

1.2.2 HER2 and breast cancer

HER2 is abnormal in approximately 20% of breast cancers (Owens *et al.*, 2004; Yaziji *et al.*, 2004; Choritz *et al.*, 2011) and this abnormality consists in the amplification of the region where the gene is located (chromosome 17, long arm - 17q12-q21). In the vast majority of cases, amplification leads to an over-expression of the protein on the tumour cell membrane.

A number of studies suggest that amplification of HER2 is a very early event in the development of invasive carcinomas and it is usually maintained throughout the natural history of the tumour (Liu *et al.*, 1992; Park *et al.*, 2006). Furthermore, gene-expression analyses have shown that HER2-amplified breast cancers are likely to represent one of the four or five distinct molecular subtypes of the disease (Perou *et al.*, 2000).

These tumours have been clearly demonstrated to have an overall poorer prognosis in comparison with the HER2-negative group (Slamon *et al.*, 1987; Andrulis *et al.*, 1998). They also have particular biological and clinical characteristics, including poor differentiation, high histologic grade, high proliferation rate (Prati *et al.*, 2005) and a typical pattern of metastases to the brain, as reviewed by Leyland-Jones (2009).

The potential influence of HER2 status on the response to various anticancer agents - anthracyclines, taxanes, tamoxifen, aromatase inhibitors - has been extensively studied, but with mixed results (Pegram *et al.*, 1997; Schiff *et al.*, 2005; Pritchard *et al.*, 2006; Hayes *et al.*, 2007; Rasmussen *et al.*, 2008). In particular, the potential role of HER2 in sensitivity to anthracyclines has been attributed by several authors to the occurrence of co-amplification of the TOP2A gene (Di Leo *et al.*, 2002; Press *et al.*, 2011). This gene, encoding for the topoisomerase IIα enzyme (target of anthracycline therapy), is also located on 17q12-q21 and genetic aberrations of this region were described in a significant proportion of HER2-amplified tumours (Jarvinen *et al.*, 1999). However, data do not seem to be conclusive (Romero *et al.*, 2012) and a recent meta-

analysis of individual data conducted on more than 3,000 patients from five randomised adjuvant trials does not support the use of HER2 and TOP2A as molecular markers to predict anthracycline activity (Di Leo *et al.*, 2011).

HER2 is currently tested on all newly diagnosed breast cancers using Immuno-Histo-Chemistry (IHC) and/or In Situ Hybridization (ISH), according to the recommendations issued by the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) (Wolff *et al.*, 2007) (Figures 1.6 and 1.7). Although the ASCO/CAP guidelines certainly provided an important framework for the standardization of HER2 testing, a number of issues are currently being debated. These include, among others, the assessment and the potential role of the chromosome enumeration probe 17 (CEP17) and chromosome 17 polysomy (Moelans *et al.*, 2011; Tse *et al.*, 2011) and the evaluation of varying degrees of HER2 amplification/overexpression within the same tumour (HER2 intra-tumoral heterogeneity) (Ohlschlegel *et al.*, 2011).

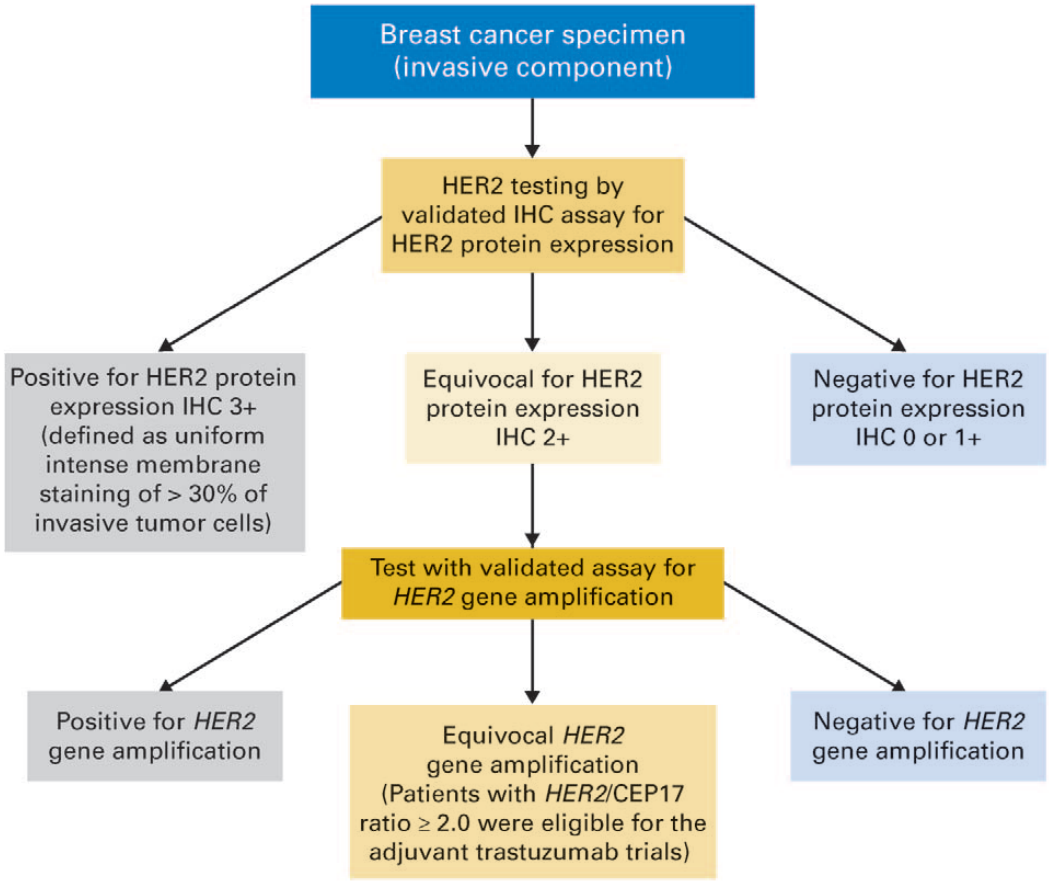

Figure 1.6: Optimal algorithm for HER2 testing (IHC).
From Wolff et al. (2007)

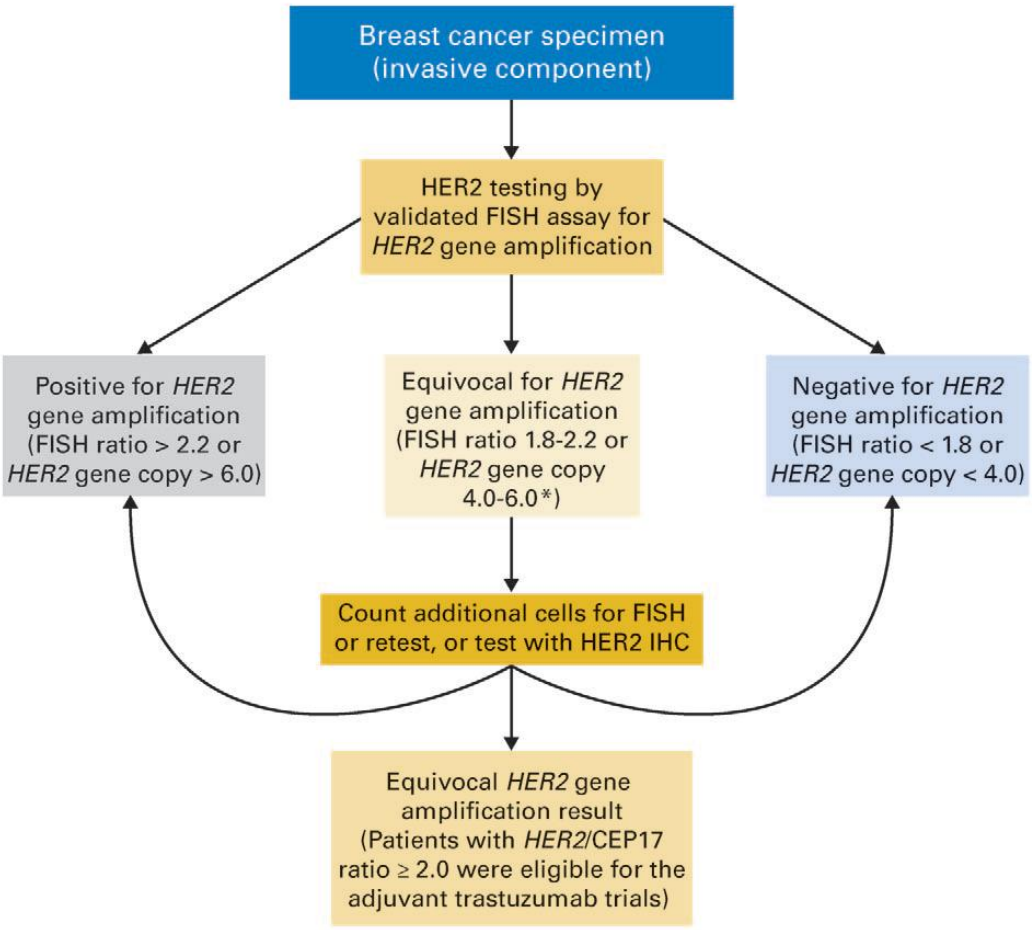

Figure 1.7: Optimal algorithm for HER2 testing (ISH).
From Wolff et al. (2007)

Most importantly, HER2 is the target of the monoclonal antibody trastuzumab (commercial name Herceptin™), which will be reviewed in Section 1.3.

1.2.3 HER2 Extra-Cellular Domain

The Extra-Cellular Domain (ECD) of HER2 can be cleaved from the surface of breast cancer cells by matrix metalloproteases and released into the circulation (Zabrecky *et al.*, 1991; Leitzel *et al.*, 1992), where it can be detected and measured by immunological techniques (Payne *et al.*, 2000; Meenakshi *et al.*, 2002).
In a review of the literature published by Leary *et al.* (2009) HER2 ECD has been reported to be present in serum of 23% to 62% (median 36.5%) unselected (i.e. HER2-

positive and HER2-negative) metastatic breast cancer patients and in 3.1% to 34% (median 11.4%) unselected early stage disease patients.

HER2 ECD has been extensively evaluated over the last 20 years with regard to its potential use in many different clinical situations. These include determination of HER2 status in unselected patients, estimation of prognosis, prediction of response to chemotherapy or hormonal therapy, trastuzumab and lapatinib, and follow-up of individual patients. Importantly, the absence of standardization in terms of assay techniques and cut-off (which is applied to distinguish between 'normal' and 'raised' concentrations) as well as the marked heterogeneity of the studies make the comparison of these results problematic and jeopardize the validity of any systematic review (Leyland-Jones and Smith, 2011).

With this limitation in mind, the comprehensive literature review published by Leyland-Jones and Smith (2011) has recently confirmed the conclusions reported by Leary *et al.* (2009) and Lennon *et al.* (2009). In summary:

- evidence so far does not support the use of HER2 ECD to determine the HER2 status of a primary tumour or metastases;
- high levels of HER2 ECD have been demonstrated to be an independent marker of poor prognosis in metastatic patients, but not in the early stage setting;
- in terms of therapeutic outcome, results are inconsistent with regard to its potential role as a predictive marker of response to cytotoxic or endocrine treatments, and pre-treatment level does not seem to predict for outcome in trastuzumab treated patients;
- a rise in HER2 ECD concentration during HER2-targeted treatments appears to be a marker of bad outcome, but its negative predictive value is generally low.

In conclusion, current evidence is insufficient to support routine clinical use of HER2 ECD outside of a clinical trial (Harris *et al.*, 2007; Leary *et al.*, 2009; Lennon *et al.*, 2009; Leyland-Jones and Smith, 2011).

1.2.4 Single Nucleotide Polymorphisms in HER2

Although rare HER2 mutations have been reported in lung cancers, no mutations within the transmembrane domain or other regions of the receptor have ever been

described in naturally occurring breast cancers. However, sequence analysis of human cDNA clones has identified several Single Nucleotide Polymorphisms (SNPs) within the gene and some have been extensively studied in relation to breast cancer.

The first one to be discovered is related to a residue in the transmembrane region at codon 655 (Papewalis *et al.*, 1991) and encodes either isoleucine (Ile; ATC) or valine (Val; GTC). Interestingly, this SNP occurs near the position where mutations are observed in the rat *neu* homologue (Val664Glu), within a consensus sequence that is known to induce transmembrane helix dimerization (N-terminal dimerization motif). Using computational methods Fleishman *et al.* (2002) have proposed a model in which the HER2 homodimer can switch from one 'active' conformation to an 'inactive' conformation without crossing exceedingly unfavourable states. This model may explain why the Ile655Val SNP may have a role in carcinogenesis: whereas activating Val664Glu mutation (in rat *neu*) would shift the equilibrium toward the active dimeric form, the substitution of Val for a bulkier Ile residue in this position would destabilize the formation of active dimers that are mediated by the N-terminal dimerization motif. Consequently, receptor activation will be reduced, even under conditions of HER2 overexpression.

Ile655Val (Reference SNP Cluster Report rs1136201 in the National Centre for Biotechnology Information - NCBI - SNP database) is a relatively common polymorphism, with a substantial variability of the Minor Allele Frequency (MAF) across different populations (from 2% to 50%). In particular the valine allele appears to be more frequent in Caucasians, less frequent in Asian populations and extremely rare in African groups (NCBI_a, 2009). These data have been confirmed by Kallel *et al.* (2010), who discussed their findings in a Tunisian population in the context of several previous reports from different ethnicities (Figure 1.8).

Since a pivotal paper (Xie *et al.*, 2000) suggested an increased risk of breast cancer among carriers of the Val allele in a Chinese case-control study, many epidemiological studies have evaluated this potential association in different populations, resulting in inconsistent or even contradictory data.

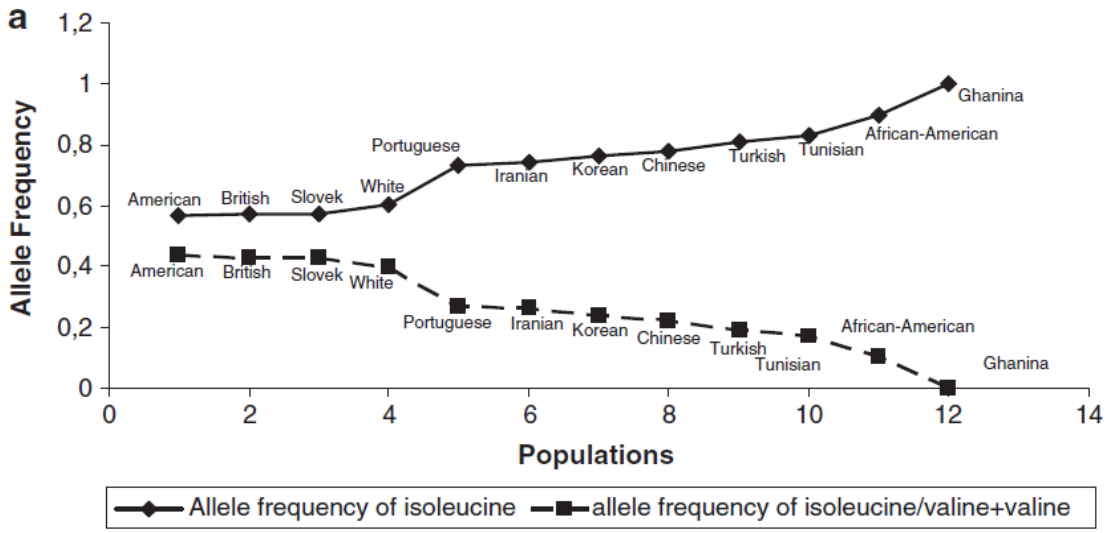

Figure 1.8: Allele frequencies of the Ile655Val polymorphism in controls.
From Kallel et al. (2010)

Several recent meta-analyses (Tao *et al.*, 2009; Lu *et al.*, 2010; Dahabreh and Murray, 2011; Ma *et al.*, 2011) have collected data from the numerous published reports (ranging from 20 to 33 reports per analysis), with total numbers ranging from 9,209 cases and 10,132 controls in Ma *et al.* (2011) to 20,461 cases and 23,832 controls in Dahabreh and Murray (2011). Taken together, these analyses were not able to consistently demonstrate an association between the Ile655Val Val allele and an increased risk of breast cancer.

A second common coding SNP (Reference SNP Cluster Report rs1058808) encodes either alanine (Ala; GCC) or proline (Pro; CCC) at residue 1170, within a C-terminal, intracellular regulatory domain (Xu *et al.*, 2000). The frequency of the proline allele varies from more than 50% in European series to approximately 10-20% in African populations (NCBI_b, 2009). This polymorphism has not been evaluated for its potential biologic function so far, but two studies have assessed its influence on breast cancer risk, both focusing on Caucasian cases of familial tumours. In particular, in the first paper (Frank *et al.*, 2005) the authors did not find any association of the Ala1170Pro SNP with occurrence of familial breast cancer. Likewise, the second group (Tommasi *et al.*, 2007) observed no difference in allele frequencies between cases and controls, but the proline variant was associated with the presence of oestrogen receptors and with two missense polymorphisms in the BRCA1 gene. These two SNPs

have been examined in several studies which reported inconclusive results on their role in breast or ovarian cancer risk (Dombernowsky *et al.*, 2009).

After the discovery of the 'Haplotype Blocks' structure of the human genome (Gabriel *et al.*, 2002), a few groups have applied a 'tagging SNP' approach in order to study linkage disequilibrium and haplotype patterns of different HER2 genetic variants in relation to breast cancer risk (Han *et al.*, 2005; Benusiglio *et al.*, 2006; Einarsdottir *et al.*, 2006). In particular, Han and co-workers (2005) showed that in a Korean population the two Ile655Val and Ala1170Pro SNPs could be used as haplotype-tagging variants and the three haplotypes defined by their combinations would account for 94% of the total predicted haplotype variation (it should be noticed, however, that this finding is not generalizable to all ethnicities). None of these studies showed any significant association between HER2 haplotype patterns and risk of breast cancer.

HER2 SNPs and HER2 expression

Both SNPs have been evaluated by several authors for their potential role in HER2 over-expression or amplification and the results of these analyses are summarized in Table 1.1. As described in the table, one major confounding factor can be that the methods used for assessing the HER2 status of tumours vary significantly across the studies, and the definitions of "HER2-positivity" are often much broader than the accepted standard definition illustrated in Figures 1.6 and 1.7. Moreover, the numbers of patients in these studies are sometimes small and do not allow an adequately powered statistical analysis.

With these caveats, inconsistent results seem to emerge from the synopsis, for both the Ile655Val and the Ala1170Pro SNP. In two of the papers listed in Table 1.1 (Benz *et al.*, 2006; Puputti *et al.*, 2006) the authors also considered the differences in genotype between germ-line DNA and tumour DNA. Both groups found that, in most cases of heterozygous germ-line genotype, only one of the two alternative alleles of the two SNPs was detectable in the context of the HER2-amplified breast tumours. These observations inspired the piece of research described in Chapter 5 and will be discussed in detail in Section 5.7.

Ref	Method for HER2 expression	Method for HER2 amplification	SNP	Method for SNP	Pts no.	Findings
Zheng *et al.* (2001)		FISH (amplification ratio ≥ 2.0 for HER2 gene over chromosome 17 centromere)	Ile655Val	RFLP based	134	Border-line association between HER2 amplification and Val allele. (P=0.095)
Millikan *et al.* (2003)	IHC ("unambiguous membrane staining in at least 10% inv cells showing a weak, moderate or strong staining intensity")		Ile655Val	TaqMan based	611	Non-significant trend for association between HER2 positivity and Val allele. (P=0.17)
		Differential PCR	Ile655Val		506	No association between HER2 amplification and Val allele. (P=0.96)
An *et al.* (2005)	IHC (3 classes: +3 / +2 / 0-1)		Ile655Val	RFLP based	169	Non-significant trend for association between HER2 negativity and Ile allele. (P=0.11)
Puputti *et al.* (2006)	IHC (+3 = positive)	CISH	Ile655Val	sequencing	27	No test performed No obvious influence of SNP on HER2 amplification or expression
Kallel *et al.* (2010)	IHC ("at least 5% of tumour cells stained")		Ile655Val	RFLP based	89	Border-line association between HER2 positivity and Ile allele. (P=0.091)
Benz *et al.* (2006)		CGH and qRT-PCR	A1170Pro	Single base primer extension method	673	No test performed HER2-pos and HER2-neg from two different populations No obvious difference in distribution of genotypes between HER2-pos and HER2-neg
Han *et al.* (2005)	IHC (2 classes: +0-1 / +2-3)		Haplotype analysis	Single base primer extension method	1094	Found 7 haplotypes, using 6 SNPs in HER2 3 of these 7 haplotypes had frequency > 5% and comprise 93.8% of the total variation These 3 haplotypes were distinguishable with only two SNPs: Ile655Val and A1170Pro Haplo I: Ile655Val A A1170Pro C Haplo II: Ile655Val A A1170Pro G Haplo III: Ile655Val G A1170Pro G In Haplo I patients (both homo and heterozygous) HER2 protein overexpression (2+ and 3+) in tumours was 1.5 times higher than others (P=0.009); excluding HER2 2+ cases OR increased to 1.8 (P=0.004)

Table 1.1: HER2 SNPs and expression of HER2 in primary tumours.
Legend: RFLP (Restriction-Fragment Length Polymorphism), CISH (Chromogenic in situ hybridization), CGH (Comparative Genomic Hybridization), qRT-PCR (Quantitative Real Time Polymerase Chain Reaction)

1.3 Trastuzumab

1.3.1 Introduction and mechanisms of action

Trastuzumab (Herceptin™ - Roche) is a humanised Monoclonal Antibody (mAb) of the immunoglobulin G1 type directed against the extracellular juxtamembrane portion of HER2. It can be administered alone or in combination with different chemotherapeutic agents, showing a remarkable synergistic effect. Trastuzumab is the first mAb which has been shown to prolong life in patients with an epithelial tumour and is currently approved by the American and European regulatory authorities for both advanced and early stage HER2-positive breast cancers. A comprehensive review of its pharmacology and its use in breast cancer was prepared and published during the conduct of this project and is presented in Appendix 1 (Cresti and Jamieson, 2010).

Various mechanisms of action have been described, including:

- inhibition of signal transduction pathways (e.g. the RAS-MAPK pathway and the PI3K-AKT-mTOR pathway);
- blocking of cleavage of the extracellular domain;
- endocytosis and degradation of the receptor;
- inhibition of angiogenesis;
- inhibition of DNA damage repair;
- immune-mediated response.

The large volume of laboratory data on trastuzumab mechanism of action has been recently reviewed (Hudis, 2007; Valabrega *et al.*, 2007; Spector and Blackwell, 2009). Although the discussion on the relative contribution to the efficacy of trastuzumab is still open, these different mechanisms are currently being further evaluated and exploited by many groups, with the aim of improving trastuzumab efficacy. A partial list of these strategies includes conjugation of the antibody with cytotoxic agents, co-administration with 'biologic' agents (such as anti-angiogenic agents, immunologic agents, inhibitors of different epitopes of HER2 or other ErbB receptors, inhibitors of other signal transduction pathways) and construction of trastuzumab mutants with greater immunological activity (Pegram *et al.*, 2006; Karagiannis *et al.*, 2009; Blackwell *et al.*, 2010; Burris *et al.*, 2011; Baselga *et al.*, 2012).

1.3.2 Clinical Development

The early clinical development of trastuzumab, unlike most anti-cancer agents, was characterized by an exclusive focus on a particular subset of patients, i.e. the HER2-positive breast cancer patients. This pioneering effort towards treatment personalization may at least in part explain the outstanding successes achieved by the drug during its clinical development. After three Phase I (Shak, 1999) and four subsequent Phase II trials (Baselga *et al.*, 1996; Pegram *et al.*, 1998; Cobleigh *et al.*, 1999; Vogel *et al.*, 2002) which showed that trastuzumab was safe and had interesting activity in pre-treated and untreated patients, a randomized clinical trial was conducted in patients with previously untreated, HER2-positive, metastatic breast cancer (Slamon *et al.*, 2001). Patients received chemotherapy (either anthracyclines or paclitaxel) alone or in combination with the antibody. The addition of trastuzumab conferred to patients a clinically-significant increase in time to disease progression (4.6 vs 7.4 months), objective response rate (50% vs 32%), duration of response (9.1 vs 6.1 months) and median survival (25.1 vs 20.3 months). A second randomized trial of docetaxel alone or with trastuzumab achieved similar results (Marty *et al.*, 2005) and various uncontrolled and controlled trials have recently shown the efficacy and relative safety of trastuzumab either alone or in combination with many other chemotherapeutic and biologic agents.

When used as adjuvant treatment of early stage HER2-positive breast cancers, trastuzumab reduces the risk of recurrence by approximately 40 to 50% (Piccart-Gebhart *et al.*, 2005; Romond *et al.*, 2005; Slamon *et al.*, 2006; Smith *et al.*, 2007; Joensuu *et al.*, 2009). Results were consistent across four major trials and several smaller studies, in spite of the differences among the studies; these differences included duration of treatment (one year, two years, nine weeks), type of associated adjuvant chemotherapy (anthracycline-containing, anthracycline-free, taxane-containing, not protocol-defined), and timing of administration (start concomitant to chemotherapy or after chemotherapy). A recent update of one of these large studies (HERA trial) (Goldhirsch *et al.*, 2012) has demonstrated that two years of treatment do not provide any additional benefit compared to the current standard duration of one year for adjuvant trastuzumab. With a relatively short follow-up in all these studies, a

significant advantage in terms of overall survival is already clearly measurable (Figure 1.9).

In the pre-operative setting, trastuzumab has also been shown to be extremely active in combination with chemotherapy and targeted agents (Buzdar *et al.*, 2005; Gianni *et al.*, 2010; Baselga *et al.*, 2012). Interestingly, this setting has proved extremely useful for the evaluation of early biological and clinical end-points as part of the development of novel HER2-targeted combinations.

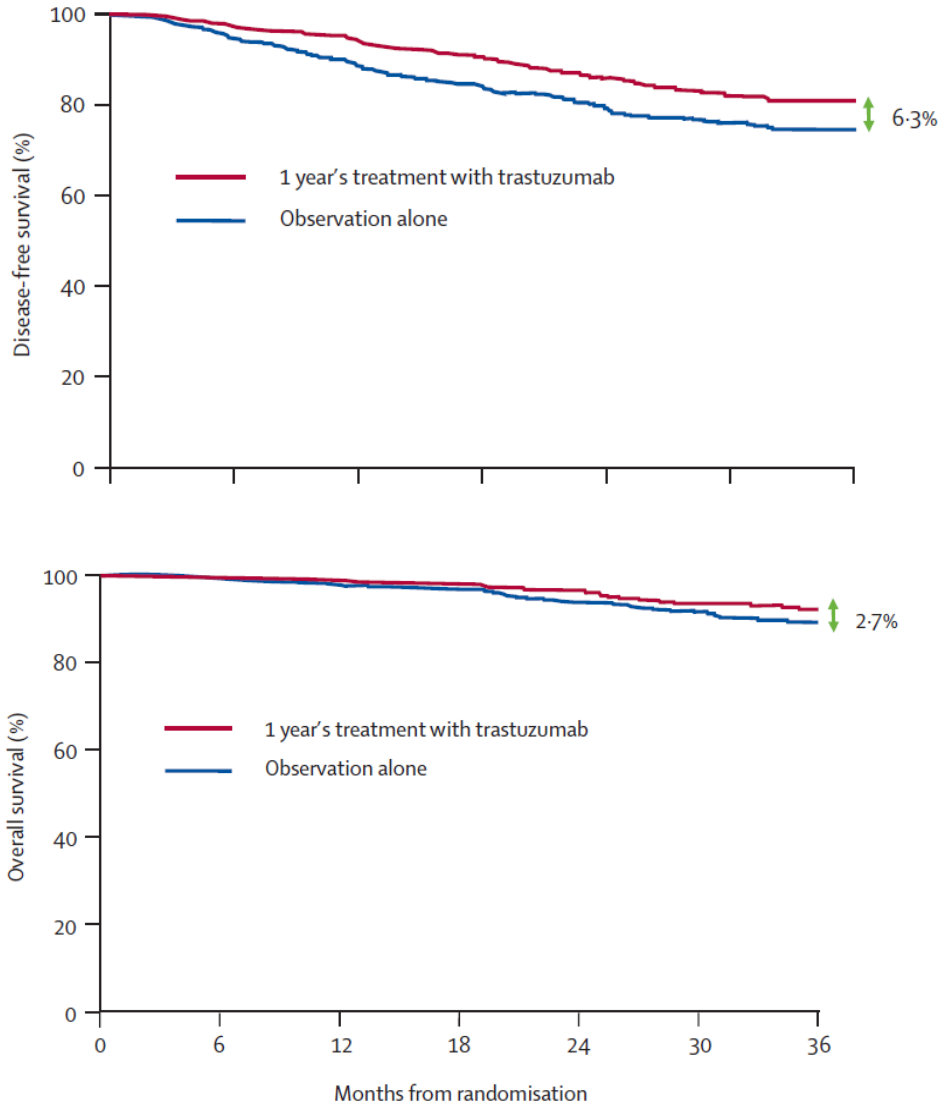

Figure 1.9: Kaplan-Meier estimates of disease-free survival and overall survival with a median follow-up of 2 years in the HERA study.
From Smith et al. (2007)

Although some questions are not entirely resolved (e.g. mono-therapy versus combination, use beyond progression of disease, association with hormone treatments, optimal use in the neo-adjuvant setting, role in patients with brain metastases) trastuzumab has become a mainstay of treatment in many countries.

1.3.3 Safety

Trastuzumab is generally well tolerated and the most frequent acute adverse event is a hypersensitivity-like infusion reaction (EMA, 2012). However, the occurrence of cardiac dysfunction can be a major concern in a minority of patients and early treatment discontinuation might favour disease recurrence or progression. Clinically, trastuzumab-related cardiotoxicity can span a range of clinical situations, from asymptomatic variations in heart contractility (measured as left ventricular Ejection Fraction - LVEF) to severe and sometimes fatal cardiac failure. Unusual features of trastuzumab cardiotoxicity include the absence of ultrastructural changes in the heart muscle and a general tendency to reversibility. Furthermore, trastuzumab-induced heart dysfunction is not always easy to diagnose in metastatic cancer patients, because concurrent factors can contribute to generate dyspnoea, oedemas and fatigue, which constitute the main clinical symptoms.

In the pivotal registration trial (Slamon *et al.*, 2001), congestive heart failure (CHF) was reported in an unexpectedly high proportion of patient: its incidence in the paclitaxel plus trastuzumab and the anthracycline plus trastuzumab arms was respectively 13% and 27%. In the four large adjuvant trials (Piccart-Gebhart *et al.*, 2005; Romond *et al.*, 2005; Slamon *et al.*, 2006), where cardiac eligibility criteria were stringent and trastuzumab was interrupted or discontinued in response to the development of cardiac dysfunction, the incidence of CHF ranged from 1 to 4%.

Exhaustive analyses of large clinical trials both in the advanced and early settings have shown that older age, pre-existing cardiac diseases or risk factors and use of anthracyclines can all contribute to an increased likelihood of developing trastuzumab-related cardiotoxicity (Perik *et al.*, 2007).

The potential mechanistic explanations of this significant side effect have been recently studied by different research groups. There is abundant laboratory evidence that HER2 has an important role in cardiomyocyte development and function (Fuller *et*

al., 2008). HER2 in fact functions as a co-receptor for two other ErbB receptor tyrosine kinase family members, HER3 and HER4, and their peptide ligands, the neuregulins, all of which are expressed in cardiac tissue (Fuller *et al.*, 2008). According to one of the most widely-accepted models, the inhibition of HER2 signalling would lead to mitochondrial dysfunction and ATP depletion and, in turn, to reduced contractility of the cardiomyocyte (Force *et al.*, 2007; Fuller *et al.*, 2008).

1.3.4 Resistance

Only 20 to 30% of patients with HER2-positive metastatic breast cancer respond to trastuzumab administered as a single agent, whereas the response rate when used in combination with taxanes is approximately 60 to 70%. This suggests that a proportion of patients are primarily resistant to trastuzumab. Moreover, virtually all treated patients with advanced disease will eventually face a progression of their disease, developing an acquired resistance. In the adjuvant setting, there is also a significant proportion of patients who experience a recurrence of their breast cancer despite having received trastuzumab.

These clinical observations have prompted several researchers to examine in depth the biological mechanisms of trastuzumab resistance (Nahta *et al.*, 2006).

Increased signalling from other HER family receptors and from Insulin-like Growth Factor Type 1 Receptor (IGF-1R) has been observed by Motoyama *et al.* (2002) and Nahta *et al.* (2005) in the context of trastuzumab-resistant cells. Activation of the PI3K pathway has been shown to be important in trastuzumab downstream effects and can be regulated by Phosphatase and Tensin homolog (PTEN). *In vitro* and clinical studies suggest that activation of this pathway and loss of PTEN function may contribute to trastuzumab unresponsiveness (Nagata *et al.*, 2004). The membrane-associated glycoprotein MUC4, which may hinder trastuzumab binding to its epitope on HER2, has also been proposed as an additional marker of resistance (Nagy *et al.*, 2005). Some of these pathways and mechanisms are currently being evaluated as potential therapeutic targets in pre-clinical and clinical studies (Jones and Buzdar, 2009; Tsang and Finn, 2012). While lapatinib in combination with capecitabine (a HER2/EGFR dual tyrosine kinase inhibitor) is the only approved drug in trastuzumab-resistant patients

(Geyer *et al.*, 2006; Cameron *et al.*, 2010), many other therapeutic strategies are currently under investigation for HER2-positive breast cancer. They include:

- targeting of HER receptors with different monoclonal antibodies, antibodies conjugated with cytotoxic drugs or small molecules (Burstein *et al.*, 2010; Burris *et al.*, 2011; Baselga *et al.*, 2012);
- targeting of angiogenesis (Pegram *et al.*, 2006; Hurvitz *et al.*, 2009b);
- targeting of the heat-shock protein molecular chaperones (Modi *et al.*, 2011);
- modulation of the PI3K/AKT/mTOR pathway (Andre *et al.*, 2010; Jerusalem *et al.*, 2011);
- targeting of IGF-1R (Tsang and Finn, 2012).

1.3.5 Schedule and pharmacokinetics

Trastuzumab is generally administered either weekly (4 mg/kg loading dose followed by 2 mg/kg) or every three weeks (8 mg/kg loading dose followed by 6 mg/kg). The pharmacokinetics of weekly trastuzumab were investigated in phase I, II and III studies (Baselga *et al.*, 1996; Pegram *et al.*, 1998; Cobleigh *et al.*, 1999; Slamon *et al.*, 2001) as part of the clinical development program, and then re-analysed using a population-based approach by Bruno *et al.* (2005). In the final model, the long-term accumulation of trastuzumab is described by a linear two-compartment pharmacokinetic model with clearance of 0.225 l/d, volume of distribution of 2.95 l and a terminal half-life of 28.5 days. The steady-state predicted peak and trough plasma concentrations were respectively 110 and 66 μg/ml, well above the suggested therapeutic threshold concentration of 20 μg/ml. Both the pharmacokinetics and the observed variability are typical of the IgG1 immunoglobulins (Bruno *et al.*, 2005).

Cobleigh *et al.* (1999) observed that tumour response was associated with a 1.6-fold higher mean trough trastuzumab concentration (at weeks 7 and 8 of a weekly regimen) than that seen in non-responders.

Two studies have directly evaluated the pharmacokinetics of three-weekly trastuzumab, either in combination with paclitaxel (Leyland-Jones *et al.*, 2003) or as a single agent (Baselga *et al.*, 2005). They show that the pharmacokinetics of three-weekly trastuzumab is similar to that of the weekly schedule: in particular terminal half-life was found to range between 16 and 27 days, mean C_{max} and trough

concentration at the steady state were respectively up to 70% higher and 20% lower than the same parameters in the weekly regimen. With both the weekly and the three-weekly schedule trastuzumab has been shown to reach steady-state concentrations after approximately 20 weeks (EMA, 2012). A recent study by Leyland-Jones *et al.* (2010) successfully evaluated the use of an intensive loading regimen (6 mg/kg intravenously on days 1, 8 and 15 followed by 6 mg/kg every 3 weeks from day 22), with the intent of reaching higher serum concentrations during cycle 1.

Many studies have investigated whether trastuzumab could potentially affect the kinetics of other anticancer drugs (paclitaxel, cyclophosphamide, cisplatin, gemcitabine and lapatinib) administered concurrently, and vice versa. No drug-drug interactions have been reported, in line with the characteristics of other mAbs currently used in clinical practice (Shak, 1999; Leyland-Jones *et al.*, 2003; Lunardi *et al.*, 2003; Zinner *et al.*, 2004; Bruno *et al.*, 2005; Storniolo *et al.*, 2008). Analysis of combination treatment with a different mAb such as bevacizumab (Pegram *et al.*, 2004) showed no alteration in the pharmacokinetic profile of either drug.

Interestingly, several studies have reported that trastuzumab low trough concentrations, faster clearance or shorter half-life may be associated with high circulating HER2 ECD in plasma (Baselga *et al.*, 1996; Pegram *et al.*, 1998; Baselga *et al.*, 2005; Bruno *et al.*, 2005). Several hypotheses were formulated by the authors to explain these findings:

- an interference between the trastuzumab and HER2 ECD assays (both Enzyme-Linked ImmunoSorbent Assay - ELISAs) could be responsible for artifactually low trastuzumab levels;
- the higher concentration of HER2 ECD could favour the formation of more antigen-antibody complexes in serum, resulting in a more rapid clearance from the circulation;
- the higher concentration of HER2 ECD might simply reflect a higher disease burden, which would result in an increased trastuzumab binding by tumour cells and, in turn, in a shortened trastuzumab half-life.

1.4 Antibody Dependent Cell-mediated Cytotoxicity

1.4.1 Overiew

Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) is an immune response mechanism in which antigen-specific antibodies direct immune effector cells of the innate immunity to the killing of the antigen-expressing target cells. It requires three components: 1) target cells expressing antigens on the surface; 2) antibodies of the IgG isotype to the target antigen, and 3) effector cells bearing Fc gamma receptor (FcγR). The antibodies bind to the antigen on the target cell, the effector cells then bind to the antibody-coated target cells through the FcγR and this triggers the toxic process (Iannello and Ahmad, 2005) (Figure 1.10).

Besides having a role in many *in vivo* responses against infections, ADCC has been recognized as one of the main immune-mediated responses for anticancer monoclonal antibodies (Wang and Weiner, 2008; Weiner *et al.*, 2009).

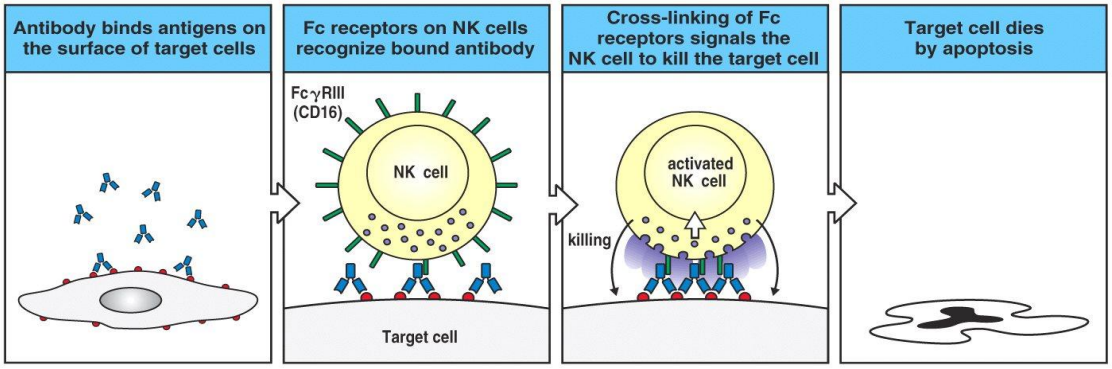

Figure 1.10: Mechanism of ADCC.
From Janeway et al. (2005)

In particular, trastuzumab and rituximab (a chimeric IgG1 monoclonal antibody targeting the B-cell differentiation antigen CD20 used in therapy of B-cell lymphomas) have been shown to work, at least in part, through ADCC.

1.4.2 ADCC and Trastuzumab

Many experimental and translational publications have evaluated and clarified the role of ADCC in trastuzumab therapy. First, Tokuda *et al.* (1996) showed that cytotoxicity of human peripheral blood mononucleated cells against a variety of HER2-positive human

tumour cell lines was significantly augmented in the presence of rhu4D5 (trastuzumab). Then Cooley *et al.* (1999) confirmed that trastuzumab is able to mediate potent ADCC *in vitro* and that this process is FcγR-dependent. Clynes *et al.* (2000) demonstrated the same dependence in syngeneic and xenograft models. Subsequent investigations documented this process in primary operable breast cancers (Gennari *et al.*, 2004; Arnould *et al.*, 2006; Varchetta *et al.*, 2007) and correlated ADCC intensity and trastuzumab efficacy (Figure 1.11).

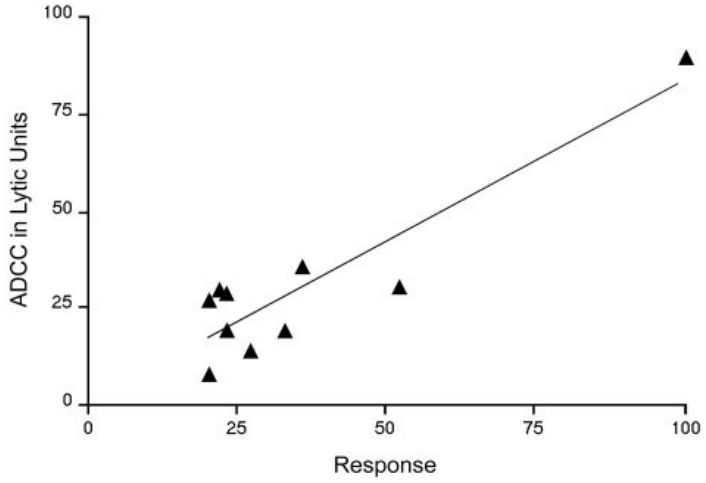

Figure 1.11: ADCC activity and trastuzumab efficacy in primary operable breast cancers.
From Gennari et al. (2004)

A recent study has evaluated the role of natural killer mediated cytotoxicity in metastatic patients (Beano *et al.*, 2008). Taken together, all these data support the position of ADCC as one of the contributory mechanisms of action of trastuzumab.

1.4.3 Fc Gamma Receptors

Fc Gamma Receptors (FcγR) are a family of receptors for the Fc region of IgG. They belong to the Ig-super family and are present on most cells of the immune system, including lymphocytes, dendritic cells, macrophages, monocytes, natural killer (NK) cells, neutrophils and mast cells (Cohen-Solal *et al.*, 2004).

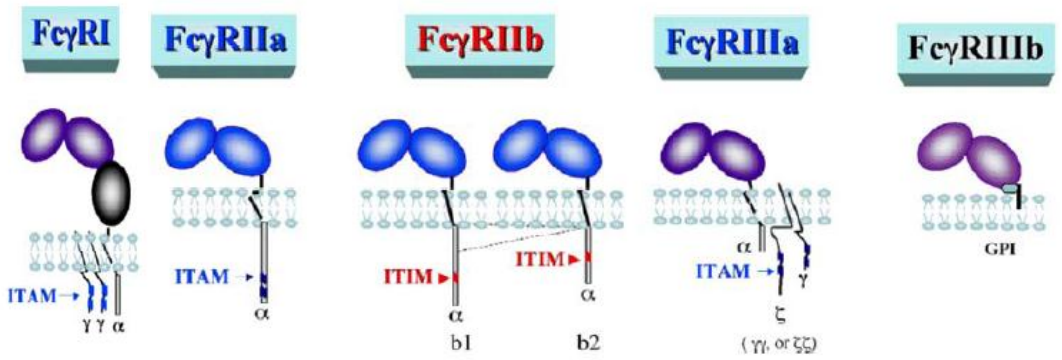

Figure 1.12: Family of FcγR.
From Cohen-Solal et al. (2004). ITAM: immunoreceptor tyrosine-based activation motif. ITIM: immunoreceptor tyrosine-based inhibition motif.

Three classes of receptors have been described and different members of the family can have opposite effects (activating or inhibitory) in the immune response, depending on differences in their cytoplasmic region (Figure 1.12).

FcγRs also differ in terms of their affinity for monomeric IgG (FcγRI Kd is 10^{-8}M, whereas FcγRII and FcγRIII Kds range from 10^{-5} to 10^{-7} M) (Cohen-Solal *et al.*, 2004). Most cell types express both activating and inhibitory receptors, so that the cellular response depends on the relative expression, which is in turn finely modulated by the cytokine environment.

FcγRs are thought to control the balance between autoimmunity and peripheral tolerance (Cohen-Solal *et al.*, 2004). In addition, because of their absolute requirement for antibody-dependent effector cell responses, they play a major role in the therapeutic effect of monoclonal antibodies.

1.4.4 SNPs in Fc Gamma Receptor genes

Two common functional SNPs have been described in two of the Fc Gamma Receptor genes. Their related proteins (FcγRIIa, encoded by FCGR2A and FcγRIIIa, encoded by FCGR3A) are low-affinity, activating receptors and differ in their pattern of expression on immune cells (Cohen-Solal *et al.*, 2004). In particular, FcγRIIIa is expressed predominantly on the surface of macrophages and NK cells, whereas FcγRIIa is the most widespread Fc Gamma Receptor.

A functional polymorphism in FCGR2A was initially described by Anderson *et al.* (1987), who classified 27 healthy individuals as 'responders' or 'non-responders' on the basis of the capacity of their mononuclear cells to respond to murine IgG1. They showed that the 40 kD receptor responsible for this binding was equally expressed in 'responders' and 'non-responders' and had the same electrophoretic mobility, but the isoelectric focusing (IEF) patterns of the two classes were different, suggesting the existence of two allelic forms of the receptor. Subsequently Warmerdam et al. (1990; 1991) demonstrated that these two variants are due to a genetic polymorphism which encodes either arginine (R; CGT) or histidine (H; CAT) at residue 131, within the extracellular region of FcγRIIa, specifically in the second Ig-like domain (Reference SNP Cluster Report rs1801274) (NCBI_c, 2009). This change alters the ability of the receptor to bind different human IgG subclasses (Parren *et al.*, 1992), as the histidine allele is associated with a higher affinity for IgG2 than the arginine allele. A very recent study evaluating the three-dimensional structure of the receptor has supported these early observations (Ramsland *et al.*, 2011). Interestingly, Shashidharamurthy *et al.* (2009) suggested that the His variant might also have higher binding affinity for human IgG1 and IgG3, although this finding is in apparent contrast with previous reports (Bredius *et al.*, 1994; Bruhns *et al.*, 2009).

A functional polymorphism in FCGR3A was observed by Ravetch and Perussia (1989) and then attributed to a SNP in the membrane-proximal, IgG-binding domain of the receptor: phenylalanine (F; TTT) or valine (V; GTT) at amino acid position 158 (Reference SNP Cluster Report rs396991) (NCBI_d, 2009) (Koene *et al.*, 1997). In IgG binding experiments the valine allele was associated with a higher affinity than the phenylalanine allele for human IgG1 and IgG3, and a gene dosage effect was present. For the sake of clarity, it should be specified that FCGR2A His131Arg and FCGR3A Phe158Val are in some studies referred to as, respectively, FCGR2A His166Arg and FCGR3A Phe176Val, in accordance with the most recent versions of the Genome Reference Consortium human genome assembly (GRC, 2012).

The clinical implications of these two SNPs have been analyzed with special regard to inflammatory response to infections and autoimmune diseases, such as Systemic Lupus Erythematosus, Rheumatoid Arthritis and Guillain-Barré syndrome (Bournazos *et al.*, 2009).

Furthermore, many authors have considered the role of these genetic variants in the response to monoclonal antibody therapies. In a pivotal study Cartron *et al.* (2002) found a significant association between the valine allele of the FCGR3A Phe158Val SNP and better clinical and molecular responses to rituximab in 49 non-Hodgkins lymphoma patients (Figure 1.13). Many subsequent papers have examined the potential effect of both SNPs on rituximab treatment in different clinical settings, sometimes obtaining apparently contradictory results (Weng and Levy, 2003; Farag *et al.*, 2004; Carlotti *et al.*, 2007; Galimberti *et al.*, 2007; Mitrovic *et al.*, 2007; Ahlgrimm *et al.*, 2011; Fabisiewicz *et al.*, 2011).

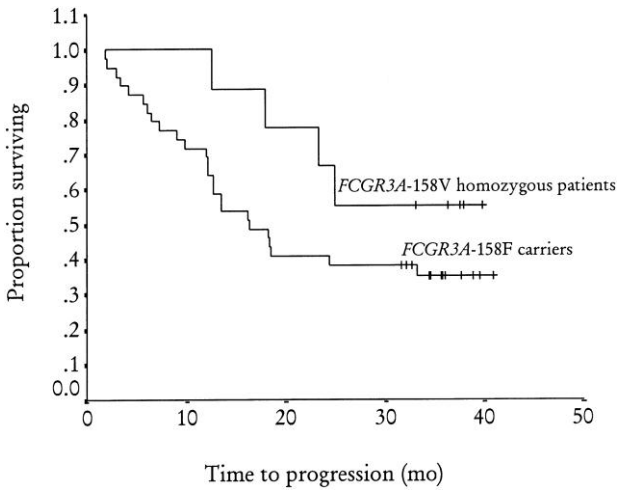

Figure 1.13: Progression-free survival after rituximab treatment by FCGR3A Phe158Val genotype. From Cartron et al. (2002)

Other recent works have also looked at the potential role of these variants in several cancers including colorectal carcinoma treated with cetuximab, a chimeric IgG1 monoclonal antibody directed against EGFR (Zhang *et al.*, 2007; Bibeau *et al.*, 2009; Etienne-Grimaldi *et al.*, 2012) and neuroblastoma treated with hu14.18-IL2 IC, a humanized anti-GD2 monoclonal antibody linked to human interleukin-2 (Delgado *et al.*, 2010).

1.5 Aims of the research

Taken together, the literature data reviewed in this chapter show how breast cancer is increasingly being studied as a complex set of diseases with different biological and clinical characteristics. An important contribution to this diversity is provided by germ-line genetic variations, which may play a role, not only in the pathogenesis and natural history of the tumours, but also in their varying response to different treatments.
The HER2-positive breast cancers certainly constitute a unique subset of tumours and have been extensively and successfully studied with particular regard to their biology and possible targeted treatments. However, the influence of pharmacogenetic (PG) factors on these aspects remains by and large unexplored.
Therefore, the general aim of this research is to focus on the possible effects of several common single nucleotide polymorphisms (SNPs) on specific aspects of HER2-positive disease.
A prospective observational pharmacogenetic study was designed in order to address two main issues:

1. In the overall population of breast cancer patients the objective of the study was to determine the frequency of SNPs in HER2 gene (reviewed in Section 1.2.4) and evaluate their potential association with HER2 expression in tumour samples in both early and advanced disease patients.

2. In a subset of HER2-positive breast cancer patients receiving trastuzumab, the objective was to determine the influence of four common coding SNPs in Fc Gamma Receptor genes (reviewed in Section 1.4.4) and in the HER2 gene on:

- the outcome of patients in the advanced setting;
- the reduction of risk of recurrence in the adjuvant setting;
- the occurrence of side effects in both settings;
- the potential pharmacokinetic and pharmacodynamic parameters related to trastuzumab treatment (reviewed in Sections 1.2.3 and 1.3.5).

In order to meet these objectives we identified a list of key parameters which would be determined and analysed as part of the study. They included:

a) frequency of genotypes for HER2 and Fc Gamma Receptor SNPs in patient blood samples;
b) frequency of genotypes for HER2 and Fc Gamma Receptor SNPs in breast cancer tissue samples;
c) HER2 expression in breast tumours;
d) response to trastuzumab therapy, in terms of objective radiological variation, symptoms relief, time to progression, overall survival, toxicity in patients with advanced disease; time to recurrence and toxicity in early-stage patients;
e) trough trastuzumab and HER2 ECD concentrations in plasma of patients receiving trastuzumab treatment.

The following chapters describe in detail the methods used for the determination and the evaluation of these parameters (Chapters 2 and 3). The results of the research are presented as well as their interpretation in the context of the current literature (Chapters 4, 5 and 6). Finally a concluding chapter (Chapter 7) will summarize the key findings of the whole study and suggest potential future directions for research in this promising field.

Chapter 2. Methods

2.1 Approval and sponsorship

The project, entitled 'Pharmacogenetic study of Fc Gamma Receptor and HER2 genes in relation to treatment of breast cancer' was approved in 2007 by both the Newcastle & North Tyneside 2 Research Ethics Committee (REC number 06/Q0906/155) and the Medicines & Healthcare products Regulatory Agency (EudraCT number 2006-002532-24). The study was approved and sponsored by the Newcastle upon Tyne Hospitals NHS Foundation Trust through its Research & Development Department (Trust reference number 3737).

2.2 Patients and samples

The target population of this trial was composed of breast cancer patients attending the Breast Out-Patient Clinic and the Chemotherapy Day Unit of the Northern Centre for Cancer Care in Newcastle (Newcastle upon Tyne Hospitals NHS Foundation Trust). Key inclusion criteria for the study were:

- Age ≥ 18 years;
- Histological confirmation of primary breast cancer with HER2 testing;
- Written voluntary informed consent.

Only patients who had been tested for HER2 over-expression/amplification on their tumour were offered study participation (HER2 has been routinely tested on every newly diagnosed breast tumour since 2005).

HER2 testing

The testing of HER2 on tumour samples was performed by the Department of Cellular Pathology of the Newcastle upon Tyne Hospitals NHS Foundation Trust according to the recommendations issued by the American Society of Clinical Oncology and the College of American Pathologists (Wolff *et al.*, 2007), as mentioned in Section 1.2.2. The expression of HER2 was initially scored using IHC. Score 0 and 1 tumours were considered 'negative' and score 3 tumours were considered 'positive' on the basis of

protein expression only. Score 2 samples were further investigated using ISH. The classical Fluorescent In Situ Hybridization (FISH) was the method of choice in the laboratory until 2009. FISH is a robust and standardized technique which uses fluorescent DNA probes to detect the HER2 gene and chromosome 17 centromere. However, it requires specialised fluorescence microscopy equipment and is characterized by fading of fluorochromes, which limits long-term stability. In addition, this method has a long learning curve and is subject to significant inter-observer variability, mainly due to the difficult signal-to-noise interpretation. In order to overcome these disadvantages in 2009 the Silver-enhanced In Situ Hybridisation (SISH) method was introduced (Dietel *et al.*, 2007). SISH is an automated method which uses dinitrophenol (DNP) labelled probes, a rabbit anti-DNP primary antibody and a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP) as the chromogenic enzyme. The chemistry of the chromogenic reaction is driven by the sequential addition of silver acetate, hydroquinone and H_2O_2. The reduction of silver ions (Ag+) to metallic silver atoms (Ag) by hydroquinone is fuelled by the substrate for HRP, H_2O_2. The silver precipitation is deposited in the nuclei and a single copy of the HER2 gene is visualised as a black dot. The specimen is then counterstained with hematoxilin for interpretation by light microscopy. This method showed high concordance with FISH and low inter-observer variability even among pathologists who were interpreting SISH results for the first time (Dietel *et al.*, 2007).

The vast majority of HER2-positive patients went on to receive trastuzumab as either adjuvant or palliative treatment. They were tested for genetic polymorphisms in germ-line DNA from peripheral nucleated blood cells and were investigated for pharmacokinetic and pharmacodynamic parameters on plasma samples. HER2-negative patients and HER2-positive patients who were not to receive trastuzumab were tested only for HER2 polymorphisms in blood cells (Figure 2.1).
For patients who were not to receive trastuzumab, one 10 ml blood sample was taken following consent to the study, when most convenient for the patient. For patients who were receiving trastuzumab, the initial design of the study planned a single on-treatment 'trough' sample to be preferably drawn immediately before the 4^{th} cycle in a 3-weekly schedule (or 10^{th} cycle in a weekly schedule); if this timing was unfeasible (dose already given or patient unlikely to continue treatment up to this time), the

sample was drawn immediately before the next administration was due. After an amendment approved in June 2010 a pre-treatment sample to be drawn immediately before commencing trastuzumab was added to the sampling schedule.

For all patients (HER2-positive and HER2-negative) who gave their specific consent to genetic analyses being performed on their archived tumour samples, a Formalin-Fixed Paraffin-Embedded (FFPE) block was requested from the Pathology Department (Figure 2.1).

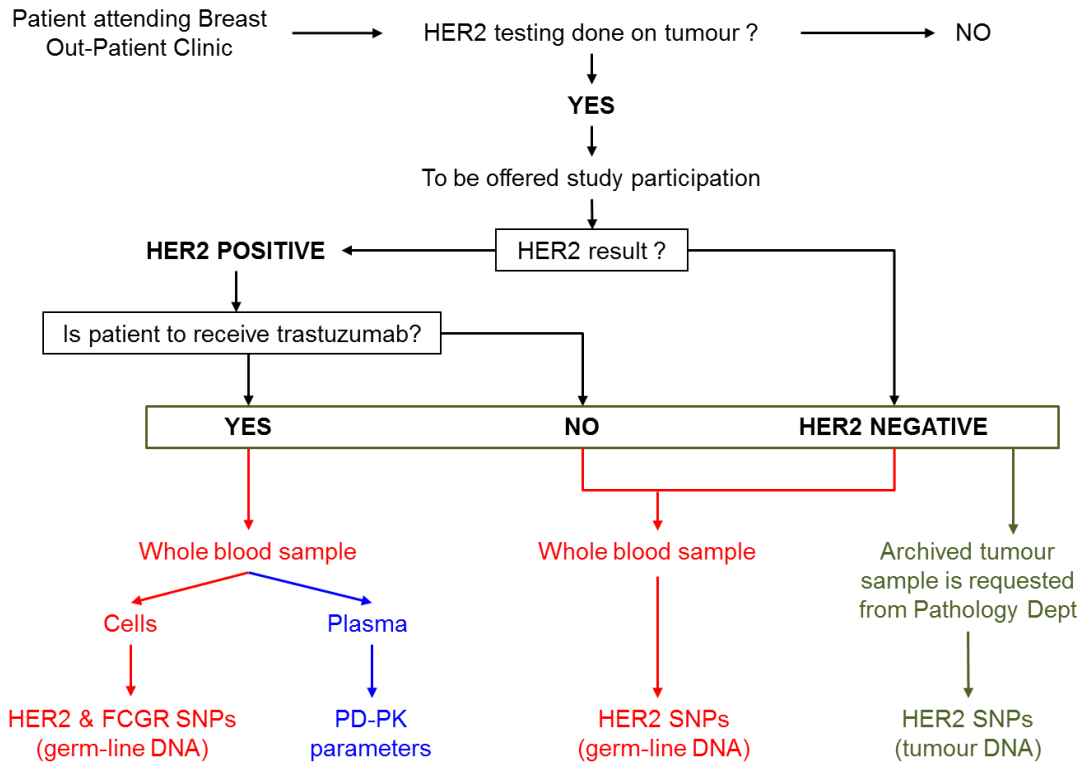

Figure 2.1: Flow-chart summarizing recruitment of patients and collection/analysis of samples

All samples were collected, transferred, stored and analysed according to the regulations of the Human Tissue Act (HTA, 2004) and entered into the Northern Institute for Cancer Research Central Tissue Resource. All laboratory work was performed in keeping with the principles of the International Conference on Harmonisation's Good Clinical Practice (ICH, 2011).

This prospective observational cohort study did not alter the treatment of the patients recruited, nor were the results of the study used to determine any subsequent treatment of patients in the study.

Patients with metastatic disease were to be followed up indefinitely and those who received adjuvant therapy were to be followed up for a minimum of five years in the Breast Out-Patient Clinic. Demographic data and pathological characteristics of the tumours were collected from clinical notes on all patients. Data on all surgical, radiation and systemic therapies received, objective responses, toxicities, dates of disease recurrence, disease progression and death were collected from clinical notes on patients treated with trastuzumab only.

2.3 Materials

2.3.1 Equipment

- Gilson pipettes models P10, P20, P100, P200, P1000, P5000, Microman M10, M100 and M1000 and disposable pipette tips (supplied by Anachem, Luton, UK). The Microman pipettes and tips were used only for PCR preparation in order to limit potential DNA cross-contamination;
- NucleoClean decontamination wipes (Chemicon International, Temecula, USA) were used to clean surfaces and instruments during PCR preparation;
- 7 ml Sterilin bijous (Fisher Scientific UK, Loughborough, UK);
- 0.5 ml Flat Cap Microcentrifuge tubes (Starlab UK, Milton Keynes, UK);
- 1.5 ml Flat Cap Microcentrifuge tubes (Sarstedt, Leicester, UK);
- 50ml CellStar conical centrifuge tubes (Greiner Bio-One, Stonehouse, UK);
- Clifton Cyclone Vortex Mixer (Scientific Laboratory Supplies, Hessle, East Riding of Yorkshire, UK);
- Grant Water bath OLS200 (Fisher Scientific UK, Loughborough, UK);
- Eppendorf microcentrifuge Model 5415 D (Eppendorf AG, Hamburg, Germany)
- Centrifuge Falcon 6/300 (Fisons, Crawley, UK);
- ELGA PURELAB Ultra machine (Elgastat, High Wycombe, UK) was used to produce Ultra-High Purity (UHP) water;
- Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, USA);
- Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA);

- Sigma 4K15C Laboratory Centrifuge (DJB Labcare, Newport Pagnell, UK);
- Glass slides covered with polyethylene naphthalate (PEN) membrane for LCM: MembraneSlide 1.0 PEN (D) (Carl Zeiss Ltd., Welwyn Garden City, UK);
- PALM Microbeam System, Version – 1206; Microscope stage PALM ROBOstage II; PALM CapMover II (P.A.L.M. Microlaser Technologies GmbH, Bernried, Germany);
- Hitachi CCD Color Camera, Model: HV-D30P (Hitachi Kokusai Electric Europe GmbH, Erkrath, Germany);
- Zeiss Axio Observer inverted microscope (Carl Zeiss Ltd., Welwyn Garden City, UK);
- 0.5 ml PCR tubes, cap filled with opaque adhesive material for buffer-free sample capture: AdhesiveCap 500 opaque (D) (Carl Zeiss Ltd., Welwyn Garden City, UK);
- M4000-D light microscope (Swift. Optical Instruments, San Antonio, TX, USA);
- UV sterilisation PCR cabinet (Wolf Laboratories Limited, York, UK);
- Fisherbrand Twin Frosted Microscope Slides and Slide coverslips (Fisherbrand Fisher Scientific UK, Loughborough, UK);
- Thermo Scientific Richard-Allan MICROM HM 315 Rotary Microtome (Fisher Scientific UK, Loughborough, UK);
- Section Dryer: Thermo Scientific Raymond A Lamb Drying Hotplate (Fisher Scientific UK, Loughborough, UK)

2.3.2 Reagents

- Phosphate buffered saline (PBS) solution was prepared by dissolution of PBS tablets (Sigma-Aldrich, Poole, Dorset, UK);
- Ethanol, absolute (Fisher Scientific UK, Loughborough, UK);
- Reagents and other consumables used in the DNA extraction protocols were part of the QIAamp DNA Micro Kit (QIAamp_Micro, 2010) and the QIAamp DNA Blood Maxi Kit (QIAamp_Midi/Maxi, 2010);
- TaqMan Genotyping PCR Master Mix, TaqMan Genotyping Assay Mix, MicroAmp Fast Optical 0.1ml PCR plates and MicroAmp Optical adhesive films (Applied Biosystems, Foster City, CA, USA).
- Xylene (Fisher Scientific UK, Loughborough, UK);
- Eosin Y Solution, Aqueous (Sigma-Aldrich, Poole, Dorset, UK);
- Hematoxylin Solution, Mayer's (Sigma-Aldrich, Poole, Dorset, UK)

2.4 Blood cells and plasma separation

In case of pre-treatment samples and on-treatment 'trough' samples obtained from patients treated with trastuzumab, blood cells and plasma were separated from whole blood samples by centrifugation at 2000 g for 10 minutes and then stored at -20°C.

2.5 DNA extraction from blood cells

Extraction of DNA from peripheral nucleated blood cells was performed using the QIAGEN® QIAamp DNA Blood Maxi Kit, which provide silica-membrane-based DNA purification. The detailed procedure is described in the QIAmp DNA blood midi/maxi kit handbook (QIAamp_Midi/Maxi, 2010). Briefly, whole blood samples or blood cell samples after separation from plasma (4 - 10 ml) were brought to room temperature and made up to either 5 ml or 10 ml, as appropriate, using PBS. Five hundred microliters of protease K was firstly mixed with the blood sample, before the addition of 6 ml (or 12 ml in case of initial volume > 5 ml) lysis buffer. The resulting solution was incubated in a 70°C water bath for at least 10 minutes before 5 ml (or 10 ml in case of initial volume > 5 ml) 96-100% ethanol was thoroughly mixed in. This solution was spun through a QIAamp Maxi Spin Column at 1850 g for 5 minutes, followed by two 4500 g wash spins with two different 5 ml buffers (AW1 and AW2), the first for 3 minutes and the second for 15 minutes. DNA was then eluted in 600 µl (or 1 ml) Buffer AE at 4500 g for 5 minutes. The concentration of the eluted DNA (ng/µl) was determined using the Nanodrop Spectrophotometer running Nanodrop 3.0.1 software. DNA aliquots were stored at -20°C.

2.6 DNA extraction from cell lines

The DNA from four established human cancer cell lines (CCRF-CEM: T-cell acute lymphoblastic leukaemia; K562: chronic myeloid leukaemia; SKBR3 and MCF7: both breast cancer) was used as control for the genotyping experiments. Extraction of DNA was performed using the QIAGEN® QIAamp DNA Mini Kit, whose principle is analogous to the one of the Blood Maxi Kit. The detailed procedure is described in the QIAamp

DNA Mini and Blood Mini Handbook (QIAamp_Mini, 2010). Briefly, cell pellets in a 1.5 ml microcentrifuge tube (approximately 10^6 cells) were brought to room temperature and re-suspended in 200 μl PBS. Twenty microliters of protease K was firstly mixed with the blood sample, before the addition of 200 μl lysis buffer. After mixing by pulse-vortexing the resulting solution was incubated in a 56°C water bath for 10 minutes, before 200 μl 96-100% ethanol was thoroughly mixed in. This solution was spun through a QIAamp Mini Spin Column at 6000 g for 1 minute, followed by one 6000 g wash spin with 500 μl AW1 buffer for 1 minute and one 20000 g wash spin with 500 μl AW2 buffer for 3 minutes. DNA was then eluted in 200 μl AE buffer at 6000 g for 1 minute. The concentration of the eluted DNA (ng/μl) was determined using the Nanodrop Spectrophotometer and DNA aliquots were stored at -20°C.

2.7 Laser Capture Microdissection

A comprehensive review of this methodology accompanied by a detailed reference protocol was published by Espina *et al.* (2006). All the LCM-related procedures were performed by Dr Despina Televantou, pathologist at the Northern Institute for Cancer Research.

2.7.1 Histological evaluation

An initial histological evaluation on Hematoxylin & Eosin (H&E) sections received from the available FFPE blocks was performed. Sections (one from each block) were received using the microtome at 4 μm depth, mounted on twin frosted slides and stained for H&E. Mayer's Hematoxylin and Eosin Aqueous Solutions were applied for 2 min and 20 sec, respectively. Hematoxylin staining was preceded by section deparaffinization in xylene, followed by tissue hydration, using ethanol of gradually decreasing grade (100%, 95% and 70%). Staining was followed by tissue dehydration, using ethanol of progressively increasing grade (70%, 95% and 100%) and final xylene application, before covering the section. H&E sections were evaluated using light microscope.

2.7.2 Preparation of sections for LCM

PEN-membrane slides were UV-treated in the PCR cabinet for 30 min, prior to sectioning, as proposed by the manufacturer. Sections of 6 µm in thickness were cut from FFPE blocks using the microtome and mounted on PEN-membrane slides.
For most blocks, only one section was cut; for a limited number of blocks 2 or 3 sections were cut, according to the number of tumour cells on the section as previously assessed (>1000 cells: 1 section, 500-1000 cells: 2 sections, 300-500 cells: 3 sections). Slides were incubated at 56°C for 3-6 hours in the section dryer before staining in order to obtain deparaffinization and better adhesion of the section to the slide. Slides were then stained according to the above H&E protocol. After section dehydration (final step), the sections were not covered but air-dried instead and submitted for LCM as quickly as possible (better results were obtained when sections were stained on the same day of LCM). Every effort was made during this process to avoid contamination.

2.7.3 LCM procedure

Invasive tumour tissue, non-invasive components and normal tissue (in selected cases) were collected using the PALM Microbeam System in conjunction with the Zeiss Axio Observer inverted microscope. The microscope was also fitted with a PALM RoboStage II and PALM CapMover for precise movement of the specimen and collection vessel respectively. All components were controlled using the PALM RoboSoftware (version 4.0.0.10). The tissue of interest was identified under the microscope and then circumscribed using the PALM RoboSoftware. The circumscribed area was then automatically cut and catapulted by the laser into an AdhesiveCap 500 opaque. The tube was mounted on the CapMover such that the inside of the cap could be positioned directly above the tissue to be catapulted. When the catapulted tissue came into contact with the inside of the cap, it adhered to it. Multiple pieces of microdissected tissue could be captured in one cap.
The area of tissue captured for each sample was between 1 mm^2 and 3 mm^2. The dimensions of the individual elements to be dissected were based on the size of tumour aggregates and on the number of inflammatory or other normal cell

admixtures. Their area varied from 300 to 200,000 μm^2. Magnification used for LCM was X50 for elements > 40,000 μm^2 and X200 for smaller elements.

2.8 DNA extraction from Laser Microdissected tissue

Extraction of DNA from microdissected tissue was performed using the QIAGEN® QIAamp DNA Micro Kit. The procedure described in the QIAmp DNA Micro handbook (QIAamp_Micro, 2010) at pages 28 - 30 was followed with minor modifications. Briefly, after equilibration of samples and reagents to room temperature, a mixture of 15 μl Buffer ATL and 10 μl protease K was prepared for each sample. Fifteen microliters of this solution was dispensed into the AdhesiveCap 500 opaque tube cap. The tissue was carefully dislodged from the adherent cap surface with the tip of the pipette and the tube was briefly centrifuged to collect tissue and liquid in the bottom. The step was then repeated with the remaining 10 μl of the solution. After addition of 25 μl Buffer ATL a mixture of 50 μl Buffer AL and 1μl RNA (prepared in advance and mixed gently to avoid bubbling) was added to each sample and the tube was vortexed for 15 sec, ensuring that a homogeneous solution was obtained. After addition of 50 μl 96-100% ethanol the tube was vortexed for 15 sec, incubated for 5 min at room temperature and then briefly centrifuged to remove drops from the lid. The entire lysate was spun through the QIAamp MinElute column at 6000 g for 1 minute, followed by two 6000 g wash spins with two different 500 μl buffers (AW1 and AW2), both for 1 minute. The column was then centrifuged at 20,000 g for 3 min to dry the membrane completely. The column was placed in a clean 1.5 ml microcentrifuge tube, 25 μl pure water was applied to the centre of the membrane and, after incubation at room temperature for 1 min, DNA was eluted at 20,000 g for 1 min. After measurement of DNA concentration with Nanodrop the aliquot was stored at -20°C.

2.9 Genotyping

A Fluorogenic 5’ nuclease assay (TaqMan assay) was used in order to detect SNPs in DNA extracted from both peripheral nucleated blood cells and breast cancer samples.

Principle of the assay

This assay exploits the 5' to 3' exonuclease activity of Taq DNA polymerase. Each reaction contains a gene-specific primer and uses two allele-specific oligonucleotides (fluorogenic probes) to which a 5’ reporter (R) dye and a 3’ quencher (Q) dye are attached; two different reporter dyes (VIC and FAM) are attached to the two probes (Figure 2.2).

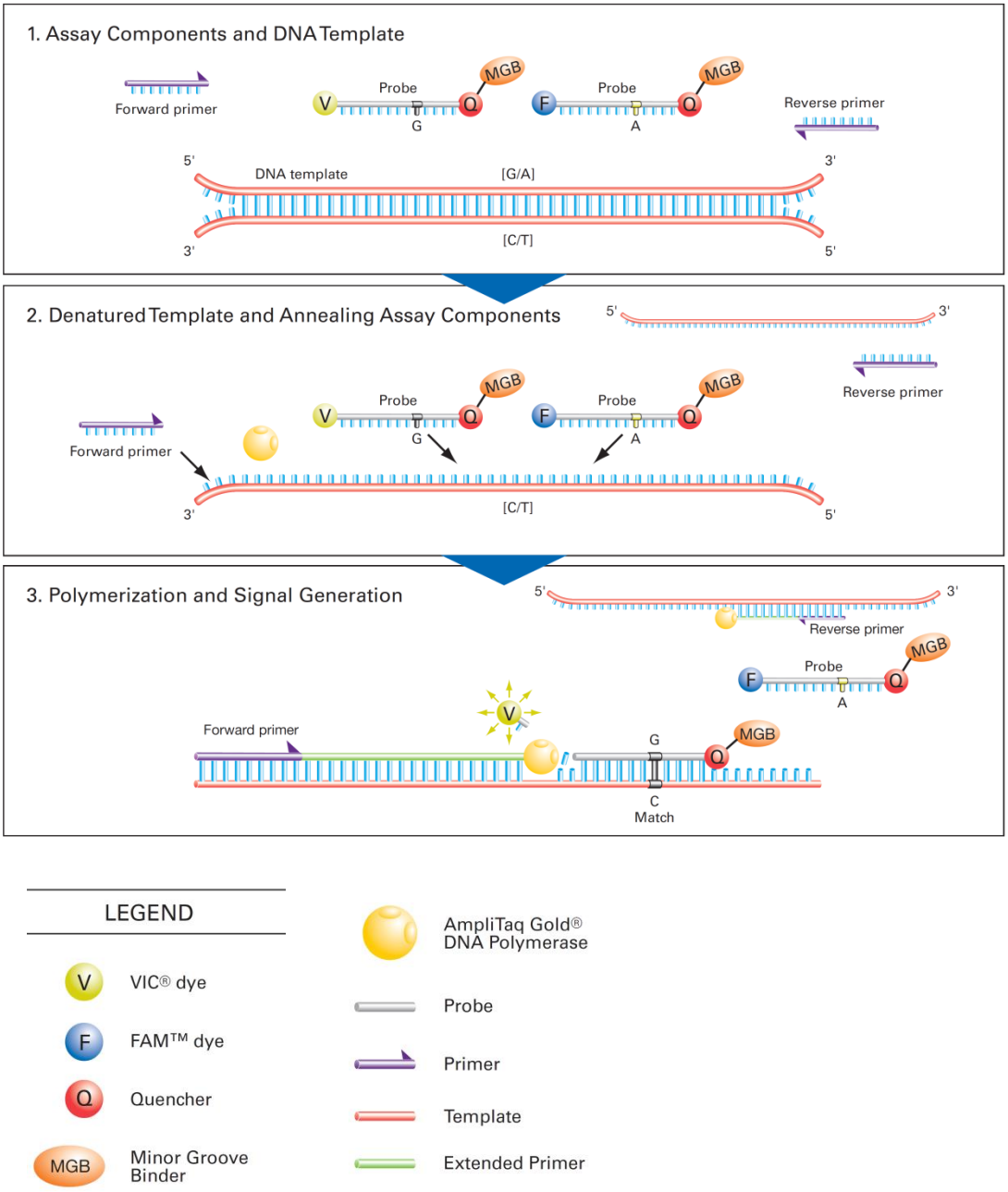

Figure 2.2: Chemistry of TaqMan assay.
From TaqMan® SNP Genotyping Assays Protocol (TaqMan_SNP_Assays_protocol, 2010)

The 3'-end is also blocked to prevent extension during PCR. The probe with complete homology anneals to the target of interest, whereas the other probe doesn't. When both dyes are attached to the probe, the reporter dye emission is quenched. If hybridization occurs, the probe is displaced and then cleaved by the 5' nuclease activity of the Taq polymerase. This separates the reporter dye from the quencher dye, generating a fluorescent signal. During each extension cycle, the Taq DNA polymerase cleaves the reporter dye from the probe if there is complete homology between the probe and the template, resulting in an increase in fluorescence (Figure 2.3).

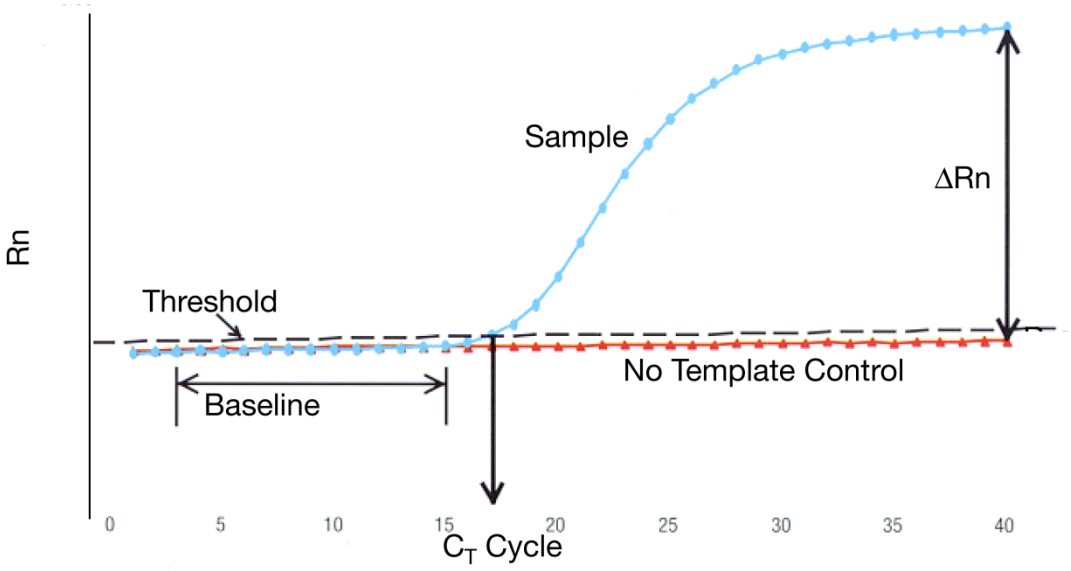

Figure 2.3: Model of a single-sample amplification plot from the Real-Time PCR Systems Chemistry Guide.
From PCR_Chemistry (2005). Baseline = initial cycles of PCR, in which there is little change in fluorescence signal; Rn (Normalized reporter) = ratio of the fluorescence emission intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye; ΔRn = magnitude of the signal generated by the given set of PCR conditions (ΔRn = Rn - baseline); Threshold = arbitrary level of fluorescence chosen on the basis of the baseline variability. A signal that is detected above the threshold is considered a real signal that can be used to define the threshold cycle (C_T) for a sample; C_T (Threshold cycle) = cycle number at which the fluorescence passes the threshold.

If a DNA sample is homozygous for one of the two alleles only the fluorescence signal generated by that allele-specific reporter dye will be detected, whereas in a heterozygous sample both VIC and FAM signals will be detected (Figure 2.4).
At the end of the amplification process an allelic discrimination plot is obtained, in which X allele-homozygous, Y allele-homozygous and heterozygous samples can be clearly distinguished (Figure 2.5) (PCR_Chemistry, 2005).

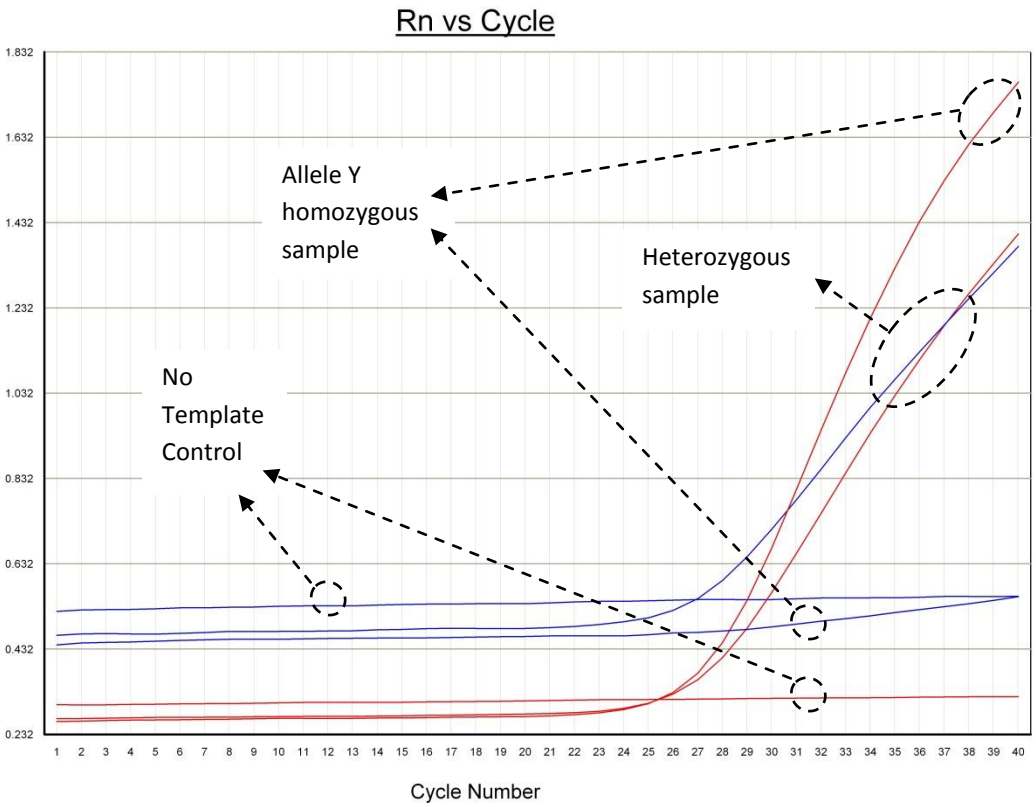

Figure 2.4: Example of amplification plot from the Sequence Detection System (SDS) Software version 1.4.
Three samples are shown: one NTC, one heterozygous, 1 Y homozygous. Red line = Allele Y; Blue line = Allele X.

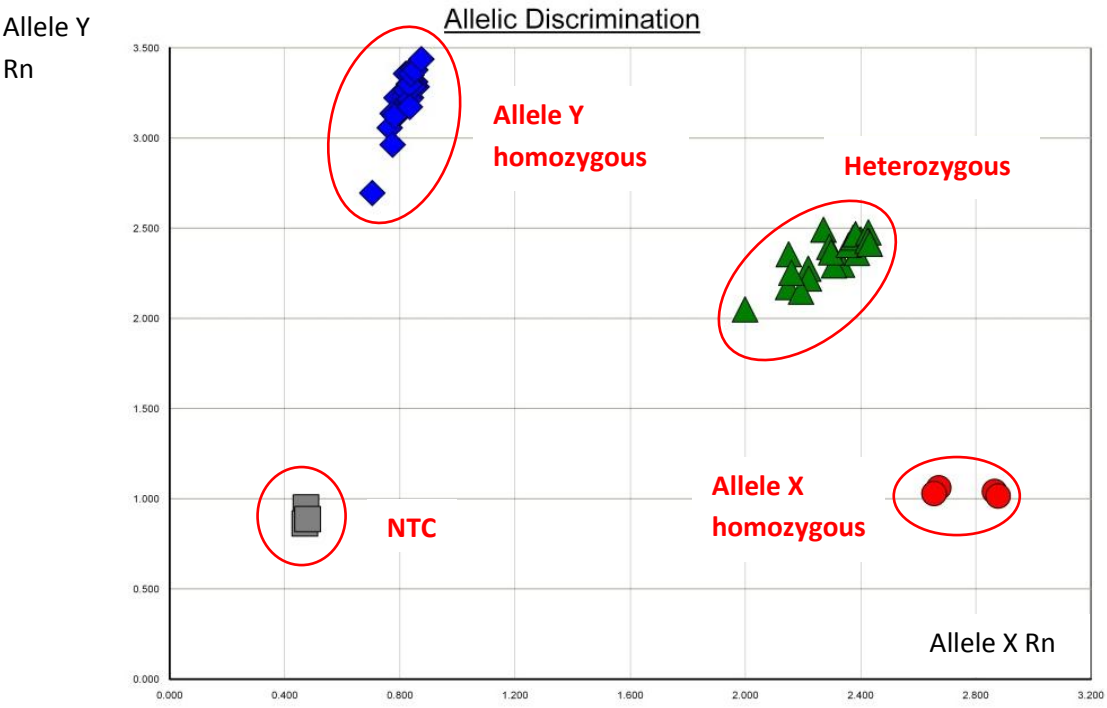

Figure 2.5: Example of Allelic Discrimination Plot from the Sequence Detection System (SDS) Software version 1.4

The procedure was performed using the Applied Biosystems® 7500 Fast Real-Time PCR System operated through the Sequence Detection System (SDS) Software version 1.4 according to the manufacturer instructions (TaqMan_SNP_Assays_AD_Guide, 2010; TaqMan_SNP_Assays_protocol, 2010).

Each polymerase chain reaction contained 5 - 20 ng of purified DNA, 1X TaqMan Genotyping PCR Master Mix and 1X SNP Genotyping Assay Mix made up to 25 µl volume with sterile UHP water. Reactions were run on 96-well MicroAmp Fast Optical 0.1 ml PCR plates covered in MicroAmp Optical adhesive film which were centrifuged at 1500 g for 1 minute prior to placing on the real-time PCR machine. Each plate contained 2 No Template Controls (NTCs) plus known genotype controls where available.

The default conditions on the 'Absolute Quantitation' plate documents were used for all TaqMan real-time PCRs runs (initial hold at 95°C for 10minutes, then a total of 40 cycles between 92°C for 15 seconds and 60°C for 1 minute).

Specific primers and probes for the TaqMan genotyping method were available from Applied Biosystems for all the four SNPs (Table 2.1).

Gene	SNP	Assay ID	Context sequence
HER2	Ile655Val rs1136201	C___7452451_1_	Forward: CGCCCCCAGCCCTCTGACGTCCATC[A/G]TCTCTGCGGT GGTTGGCATTCTGCT
	Ala1170Pro rs1058808	C___1551672_20	Reverse: ACCTGCTGGTGCCACTCTGGAAAGG[C/G]CCAAGACTC TCTCCCCAGGGAAGAA
FCGR2A	His131Arg rs1801274	C___9077561_20	Forward: AATGGAAAATCCCAGAAATTCTCCC[A/G]TTTGGATCC CACCTTCTCCATCCCA
FCGR3A	Val158Phe rs396991	C__25815666_10	Forward: TCTGAAGACACATTTTTACTCCCAA[C/A]AAGCCCCCTG CAGAAGTAGGAGCCG

Table 2.1: TaqMan® genotyping assays

2.10 Measurement of trastuzumab and circulating HER2 in plasma

The quantification of trastuzumab and circulating HER2 in plasma samples was performed by means of two different Enzyme-Linked ImmunoSorbent Assays (ELISAs). A detailed description of these two assays, including the equipment and reagents employed and the results of the validation experiments, is presented in Chapter 3.

2.11 Statistics

Statistical analyses were performed using SPSS software, version 17.0. Details regarding the types of statistical testing performed are included where relevant in the results chapters.

The initial power calculations for the study were divided into two parts.

The first part was relative to the first aim of the research, focusing on HER2 status of breast tumours and HER2 SNPs (Section 1.5). The whole sample size was calculated based on these assumptions:

- 20% of patients were expected to be HER2-positive (Section 1.2.2);
- the frequency of the Ile/Val plus Val/Val allele for the HER2 Ile655Val SNP was expected to be between 25% and 50% (Section 1.2.4).

In order to achieve a 90% power at the 5% significance level, a cohort of between of 500 and 700 patients was required to demonstrate a two-fold increase in the presence of the Val allele of Ile655Val for patients with HER2-positive tumour relative to those with HER2-negative tumours.

The second part was relative to the subgroup of HER2-positive advanced patients treated with trastuzumab. The relevant assumptions were:

- study duration = 2 years, with constant accrual;
- Progression-Free Survival (PFS) rate at 2 years = 35%, based on survival curves of a metastatic breast cancer study which was open in the NCCC at that time (Verrill *et al.*, 2007);
- 5% of patients lost to follow-up;
- Frequency of Val/Val plus Phe/Val allele for the FCGR3A Phe158Val SNP = 46% (Cartron *et al.*, 2002);

The hazard function was empirically generated so that the survival function matched that of similar groups of breast cancer patients. In order to achieve a 90% power at the 5% significance level, a cohort of 140 - 150 patients was required to demonstrate a two-fold decreased risk (hazard ratio) for carriers of the Val allele relative to the Phe homozygous patients.

Chapter 3. Results I: Measurement of trastuzumab and HER2 in plasma

3.1 Introduction

One of the objectives of this project was to obtain pharmacokinetic (PK) and pharmacodynamic (PD) information from the subset of patients treated with trastuzumab. The measurement of trastuzumab in patient plasma, previously included in a limited number of published clinical trials (reviewed in Section 1.3.5), was chosen as a reasonable PK parameter. Using the same samples it was planned to evaluate the concentration of circulating HER2, which had been suggested by many research groups as a potential biomarker and could be linked to PK and HER2 pharmacogenetics (Section 1.2.3).

As both analytes were macromolecules, the most appropriate method for their quantification was a ligand-binding assay (or immuno-assay), which is based on the capacity of an antibody to bind selectively to one specific molecule (antigen). For circulating HER2 one among the several commercially available Enzyme-Linked ImmunoSorbent Assays (ELISA) was chosen. For trastuzumab a novel method was developed, validated and published (Appendix 2) (Jamieson *et al.*, 2009).

For both PK and PD assays the method validation is an essential step in order to confirm reliability for the intended application (ICH, 2005). Therefore standard guidelines and recommendations were followed for both the HER2 and trastuzumab assays (DeSilva *et al.*, 2003; Kelley and DeSilva, 2007), taking into account the potential differences in the validation variables to be considered between bioanalytic assays and biomarker assays (Chau *et al.*, 2008). In our case, given the analogies between these methods, the two validation processes were carried out in parallel, as described in the following sections.

3.2 Cell-based ELISA for the measurement of trastuzumab in plasma

3.2.1 Background

The majority of the early published studies on the pharmacokinetics of trastuzumab (Baselga *et al.*, 1996; Cobleigh *et al.*, 1999; Tokuda *et al.*, 1999) refer to a single ELISA method carried out by Genentech®, but do not provide sufficient information to allow

independent researchers to reproduce the assay. In one clinical trial (Pegram *et al.*, 1998) the procedure of this test was described, but the capture antigen employed was a purified HER2 protein provided by the manufacturer that is not commercially available. Even in more recent works (Leyland-Jones *et al.*, 2003; Baselga *et al.*, 2005) on the three-weekly trastuzumab schedule the authors adopted the same Genentech® ELISA. Maple *et al.* (2004) published a detailed description of a different trastuzumab immunoassay using a cell lysate HER2 ELISA standard purchased from Oncogene Research Products® as the capture antigen. Unfortunately this reagent can be supplied only as part of a commercially-available HER2 ELISA kit and the quantities required for the assay allow the analysis of only a limited number of samples for each kit purchased, contributing to an unreasonable total cost.

The lack of commercially available and affordable methods led to the development of a novel immuno-assay in which the capture antigen would be provided by the widely used HER2 overexpressing SKBR3 cell line. The development and validation of this cell-based ELISA method are described in the next sections.

3.2.1 Reagents and equipment

Trastuzumab (Herceptin™) was purchased from Roche (Welwyn Garden City, UK). TMB (3,3',5,5'-tetramethylbenzidine) substrate kit was purchased from Pierce (Cramlington, UK). Foetal calf serum (FCS), HRP conjugated goat anti-human antibody and Alexa fluor488 conjugated goat anti human antibody were purchased from Invitrogen (Paisley, UK). Goat serum was purchased from Millipore (Herts, UK). Human plasma from nine individuals was purchased from the Blood Transfusion Service (Newcastle upon Tyne, UK). Phosphate buffered saline, Tris base, sodium chloride, concentrated hydrochloric acid, Tween 20, sodium azide, poly-D-lysine, Hoescht 33342, RPMI 1640, Formalin and Costar 96 well tissue culture plates were all purchased from Sigma (Poole, Dorset, UK). Sterile μ-clear black-walled optical 96 well plates were purchased from Greiner (Gloucestershire, UK). SKBR3 breast adenocarcinoma cells and MDA MB231 breast adenocarcinoma cells were gifts from Dr. Felicity May at the NICR, UK. The pieces of equipment employed included: Pathway HT Inverted fluorescent microscope (BD, Oxford, UK), Coulter Z1 cell counter (BD, Oxford, UK), Centaur 2 bench top centrifuge (MSE, London, UK), Gyro rocker STR9 (Bibby Scientific, Stone, UK), DSG

Titertek titer plate shaker (Flow Laboratories, Hertfordshire, UK), Spectramax 250 plate reader (Molecular Devices, Sunnyvale, CA, USA).

3.2.2 Development of the assay

The scheme in Figure 3.1 shows the steps of the assay, which is similar to the vast majority of the published cell-based ELISAs.

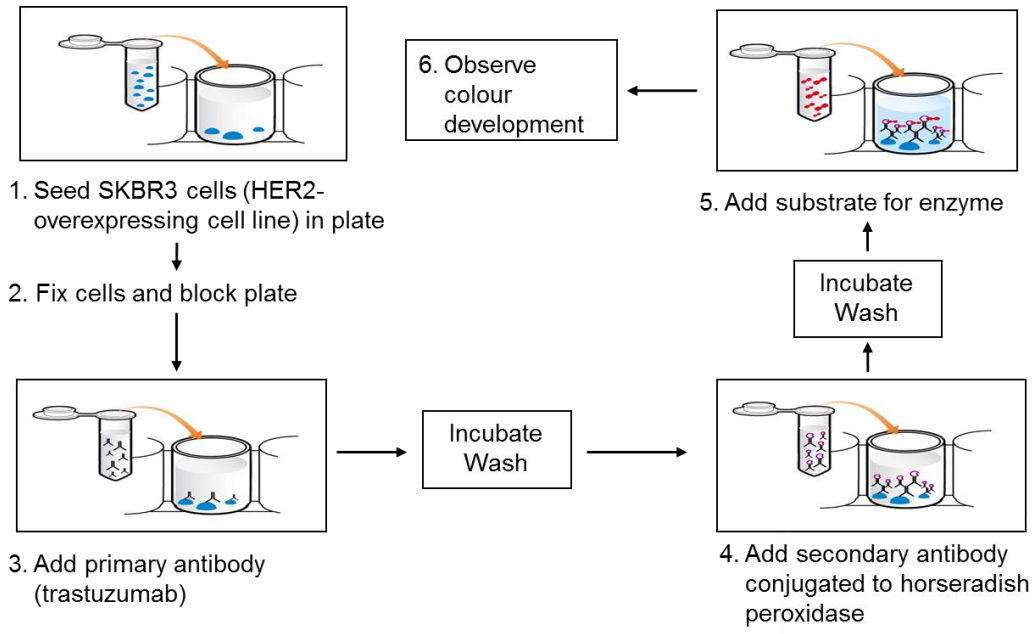

Figure 3.1: Steps of the cell-based trastuzumab ELISA.

The preliminary stage of the development was aimed to visualize the specificity of the interaction between HER2 overexpressed on the surface of the SKBR3 cells and trastuzumab at pharmacological concentrations in a plasma matrix. In order to achieve this objective an immunocytochemistry experiment was set up using the SKBR3 cell line and the HER2-negative MDA MB 231 cell line as antigens. The two cell lines were plated at 5000 cells per well in two separate optical 96 well plates and allowed to adhere for 24 h at 37 °C and 5% CO_2. After washing with PBS, cells were fixed with 10% formalin for 20 min. The plates were blocked for 1 h with 20% goat serum in Tris buffered saline (TBS) with 0.1% Tween 20 for 1 h at room temperature and subsequently incubated with human plasma spiked with trastuzumab and diluted 1/4000 in wash buffer (TBS with 0.1% Tween 20 and 1% goat serum).

Each well was washed 3 times with wash buffer, then incubated for 1 h at room temperature with an Alexafluor 488 tagged goat antihuman antibody diluted 1/500 in wash buffer with 10 µg/ml Hoescht 33342. Wells were washed 3 times with PBS and imaged with a ×20 objective on the fluorescent microscope.

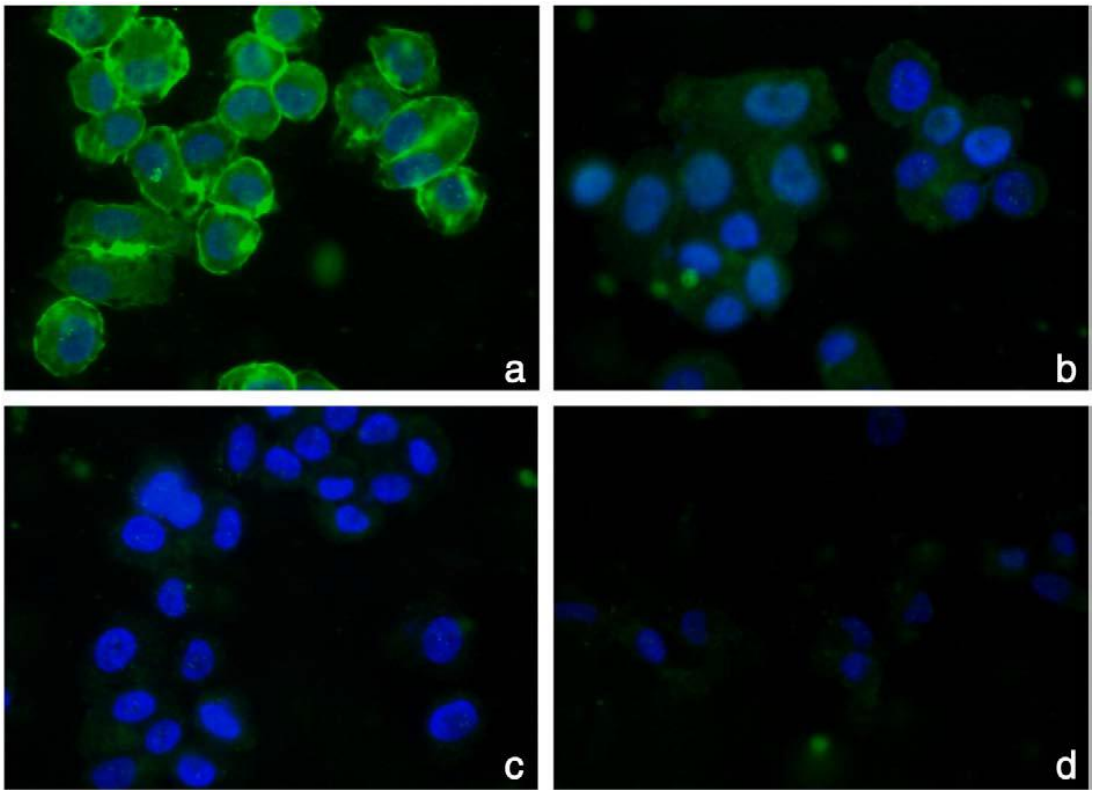

Figure 3.2: Immunocytochemistry detection of trastuzumab in human plasma.
Trastuzumab in human plasma was used as the primary antibody at 120 µg/ml (a and d), 10 µg/ml (b) or 0 (unspiked human plasma, c) with formalin fixed HER2+ SKBR3 cells (a, b, c) or HER2- MDA MB 231 cells (d) as the capture antigen.

When SKBR3 breast carcinoma cells, which over-express HER2, was used as the antigen a strong fluorescent signal was observed when the cells were incubated with 120 µg/ml trastuzumab in human plasma diluted 1/4000 in wash buffer (Figure 3.2a), and a signal above background detected when 10 µg/ml trastuzumab in human plasma diluted 1/4000 in wash buffer was used as the primary antibody (Figure 3.2b). No signal was detected when SKBR3 cells were incubated with unspiked human plasma diluted 1/4000 (Figure 3.2c). When MDA MB 231 cells, which do not over-express HER2, were incubated with 120 µg/ml trastuzumab no fluorescence above background was observed (Figure 3.2d).

During the development process a series of factors which could potentially affect the reliability of the ELISA were identified and several solutions were suggested.

SKBR3 cells as a reagent

Firstly, the use of cultured breast cancer cells as capture antigen was seen as a potential obstacle to the reproducibility of the assay. To overcome this problem it was decided to prepare single-use aliquots of SKBR3 cells. SKBR3 cells were propagated in RPMI 1640 with 10% FCS at 37 °C and 5% CO_2, maintained and trypsinised at approximately 90% confluence, and split 1 in 2 successively until thirty two 175 cm^2 tissue culture flasks were obtained with cells at approximately 90% confluence. The cells were trypsinised, pooled and counted on the cell counter. The resuspended cells were centrifuged at 1000 rpm, the medium was aspirated and the pellet resuspended in RPMI 1640 with 10% FCS and 10% DMSO at a concentration of 2.6×10^7 cells ml^{-1}. This suspension was stored at -80 °C in 0.5 ml aliquots until needed for the assay.
The final concentration of cells in wells had also to be fixed in order to get uniform results. It was decided to employ the cell density which would result in a confluent mono-layer 24 hours after the seeding from -80 °C storage (1×10^5 cells per well).

Background absorbance and signal-to-noise ratio

One of the major problems in the development of a ligand-binding assay is the occurrence of non-specific secondary antibody interactions, which can be a source of high absorbance background and poor signal-to-noise ratio.
In order to assess this issue the effect of different blocking agents was firstly investigated. Twenty four hours after seeding at 1×10^5 cells per well, SKBR3 cells were fixed and then incubated overnight at 4 °C under four different conditions (Figure 3.3). Plates were flicked dry and trastuzumab 100 μg/ml in human plasma (or unspiked human plasma) diluted 1/2000 in wash buffer was added. The plate was incubated for 1 h at room temperature on the Gyro rocker at 20 rpm. After washing three times with wash buffer, 100 μl per well HRP goat anti-human antibody diluted 1/1000 in wash buffer was added and the plate was incubated for 1 h at room temperature on the Gyro rocker at 20 rpm. Each well was then washed 3 times with 300 μl wash buffer. HRP activity remaining was detected with TMB Substrate Kit (described in the protocol

sub-section). The absorbance at 450 nm of each well was read on the plate reader. The results showed that 20% goat serum had the best blocking effect, with a signal-to-noise ratio of 5.

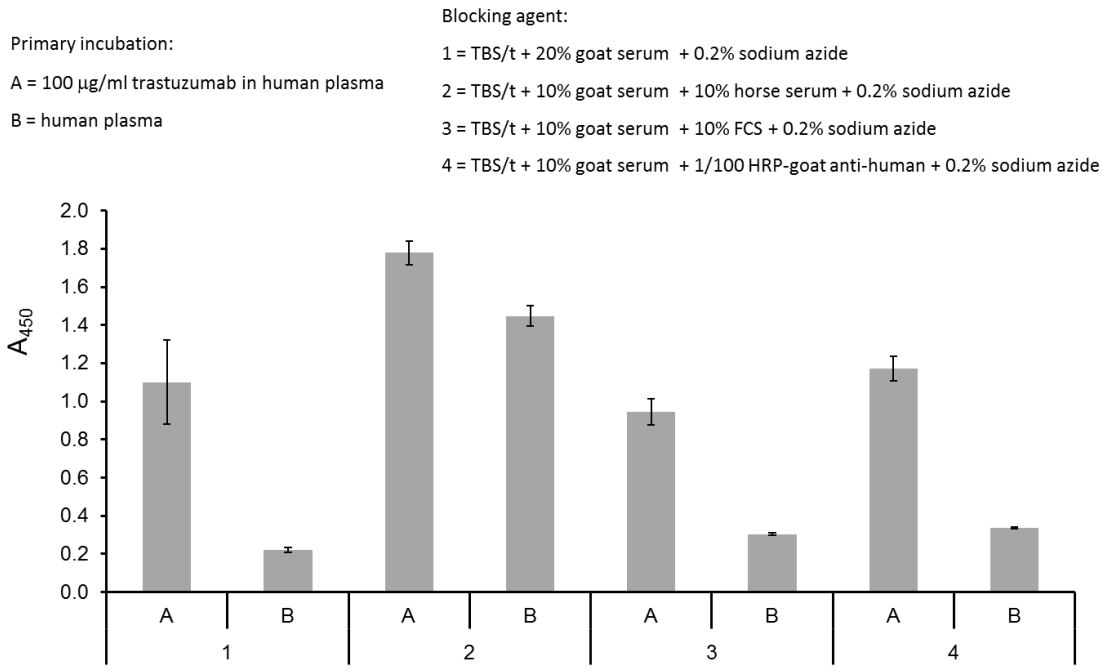

Figure 3.3: Effect of four different blocking agents on signal-to-noise ratio in the trastuzumab cell-based ELISA

A second experiment was set up in order to assess the effect of different number of washes and different washing procedures.

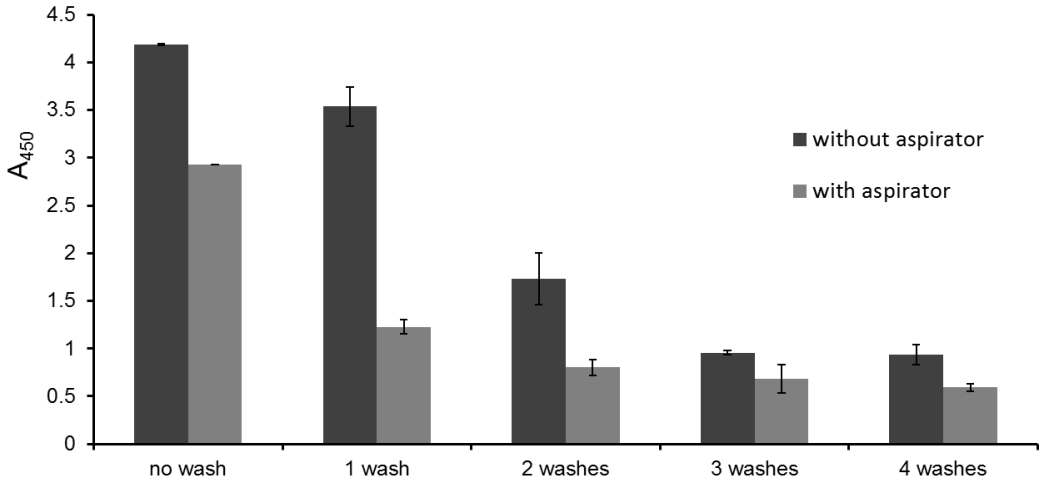

Figure 3.4: Effect of different number of washes and washing procedure on background absorbance

The results in Figure 3.4 show that at least three washes after the primary incubation and the use of the aspirator after the secondary incubation were able to reduce background absorbance. The results of further experiments suggested that the use of the aspirator had to be extended to both wash cycles and the best number of washes after the secondary incubation was five.

Early assessment of the concentration-absorbance response

The effect of different dilutions of the secondary antibody was evaluated within the same experiment described in the previous section. Figure 3.5 shows that 1/1000 dilution of the HRP goat anti-human antibody resulted in the best concentration-signal response.

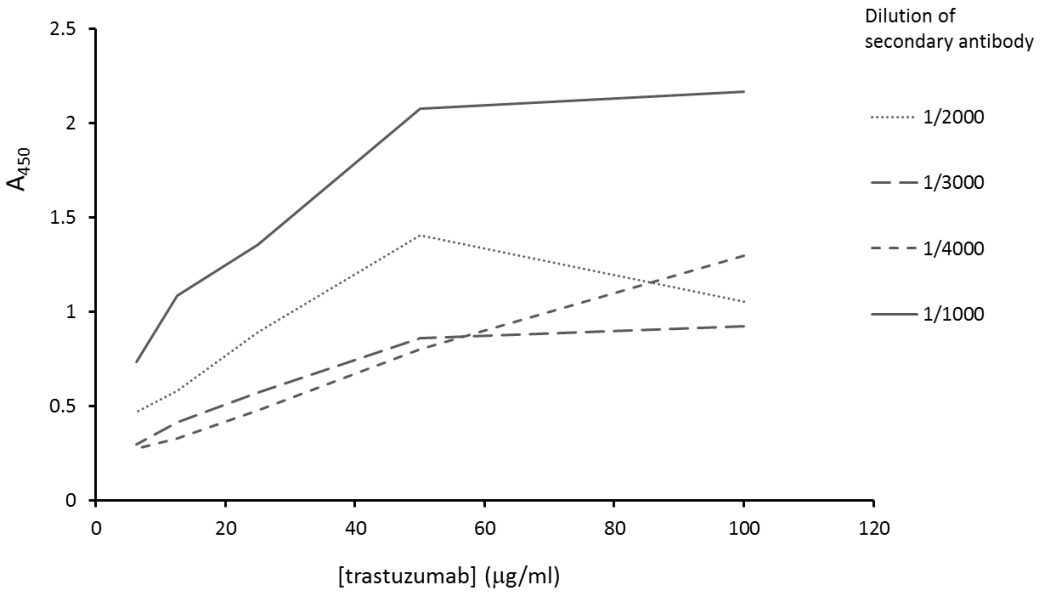

Figure 3.5: Effect of different dilutions of secondary antibody

A good correlation was observed except for the absorbance values at 100 µg/ml trastuzumab. This was attributed to the detachment of cells caused by the aspiration which led to a lower number of HER2 receptors available on the plate and, consequently, to a saturation of the receptors by trastuzumab. An effective solution to the problem was the use of poly-D-lysine as a pre-coating agent in order to improve the adhesion of SKBR3 cells to the plate. This led not only to a better linearity of the assay, but also reduced the intra-assay variability resulting from lack of uniform cell density across the wells.

The effects of different dilutions of the analyte in wash buffer and different enzymatic reaction times on the assay concentration-absorbance response were also investigated (Figure 3.6). One in four thousand dilution of the analyte and 20 minutes of incubation with the TMB substrate achieved the best outcome.

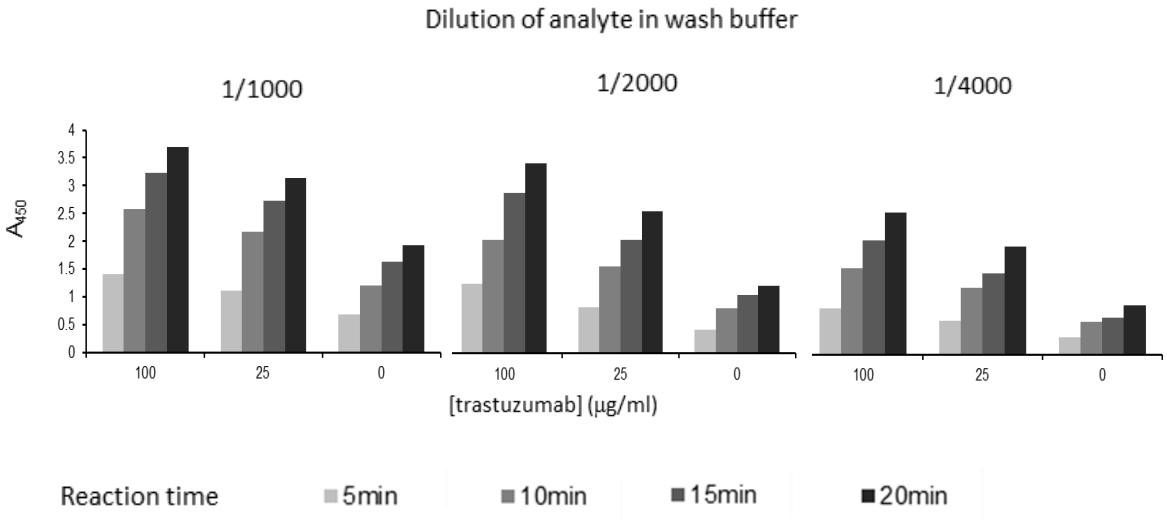

Figure 3.6: Effect of different dilutions of analyte and different enzymatic reaction times

Description of the protocol

The results of the previous optimization experiments allowed the generation of a protocol which was used for the validation experiments and for the measurement of clinical samples. The assay was carried out over three days. On day one, the inner 60 wells of two 96 well tissue culture plates were incubated with 100 µl per well of 100 µg/ml 70,000–150,000 kDa poly-D-lysine for 5 min at room temperature under sterile conditions. The poly-D-lysine was aspirated and each well was washed with sterile deionised water and the plates were allowed to dry under sterile conditions. Once dry, a single-use aliquot of SKRB3 cells was thawed and re-suspended to a total volume of 13 ml in RPMI 1640 with 20% FCS and a concentration of 1×10^6 ml^{-1}. This cell suspension was distributed into the poly-D-lysine coated wells of the previously-coated plates at a density of 1×10^5 cells per well in 100 µl volumes. The plates were incubated for exactly 24 h at 37 °C and 5% CO_2 to allow the cells to recover and adhere to the poly-D-lysine coated substrate. On day two of the assay the medium was gently aspirated by vacuum and the cells were washed with cold PBS and fixed with 100 µl per well 10% formalin for 20 min. The formalin was aspirated and the cells were

washed 3 times with 100 µl PBS per well. Following washing, the fixed cells were incubated overnight at 4 °C in 100 µl blocking buffer (Tris buffered saline with 0.1% Tween 20, 20% goat serum and 0.2% sodium azide). On day 3 of the assay all samples, standards and QCs were diluted 1/4000 in wash buffer (TBS with 0.1% Tween 20 and 1% goat serum). The block buffer was aspirated from the plate, replaced with 100 µl per well of the diluted samples, standards and QCs in replicates and incubated for 1 h at room temperature on the Gyro rocker at 20 rpm. Following the primary incubation the samples were removed by aspiration and each well was washed 3 times with 100 µl wash buffer. The final wash was replaced with 100 µl per well HRP goat anti-human antibody diluted 1/1000 in wash buffer and the plate was incubated for 1 h at room temperature on the Gyro rocker at 20 rpm. Each well was then washed 5 times with 300 µl wash buffer. HRP activity remaining was detected with TMB Substrate Kit as per manufacturer's instruction. Briefly 100 µl of the combined TMB substrate and H_2O_2 solution was added to each well and was incubated in darkness at room temperature for 20 min on the Titertek plate shaker on setting 6. After 20 min the reaction was stopped with the addition of 100 µl per well 180 mM sulphuric acid and agitation for 10 s on the plate shaker on setting 6. The absorbance at 450 nm of each well was read on the plate reader. Sample and QC values were interpolated from the standard curve using a Power equation in Excel (Microsoft Office Excel version 2010) ($y=cx^b$).

Dynamic range

A 150 mg vial of trastuzumab was dissolved in 2 ml sterile deionised water and aliquots stored at −80 °C. The 75 mg/ml solution was diluted to 1 mg/ml solutions in human plasma from one individual. For the initial determination of a dynamic range, trastuzumab at 1 mg/ml was diluted in human plasma by serial 1 in 2 dilutions to give a range of concentrations from 1 mg/ml to 60 ng/ml. These solutions were further diluted 1/4000 in wash buffer. A set of three experiments identified a dynamic range for the assay of between 8 and 125 µg/ml when samples had been diluted 1/4000 in wash buffer (Figure 3.7).

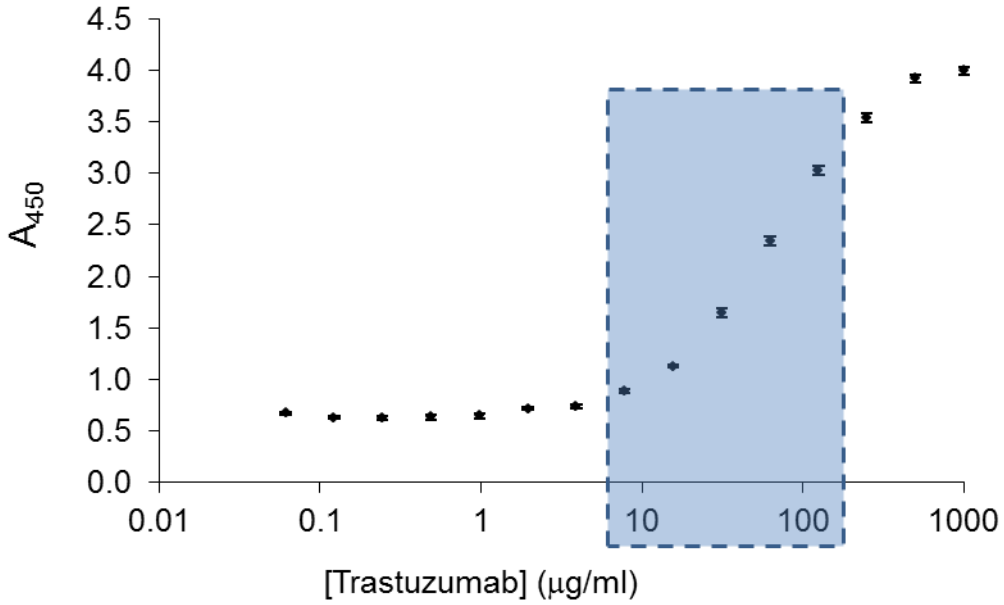

Figure 3.7: Representative experiment showing the dynamic range of the cell-based trastuzumab ELISA.

3.2.3 Validation of the assay

Seven trastuzumab concentrations within the dynamic range (120, 100, 80, 60, 40, 20 and 10 µg/ml) were used to generate standards and these concentrations were used in all subsequent validation experiments. The plot of signal versus concentration was best fitted by a power curve ($y=cx^b$) with an R^2 value of 0.996 (Figure 3.8).

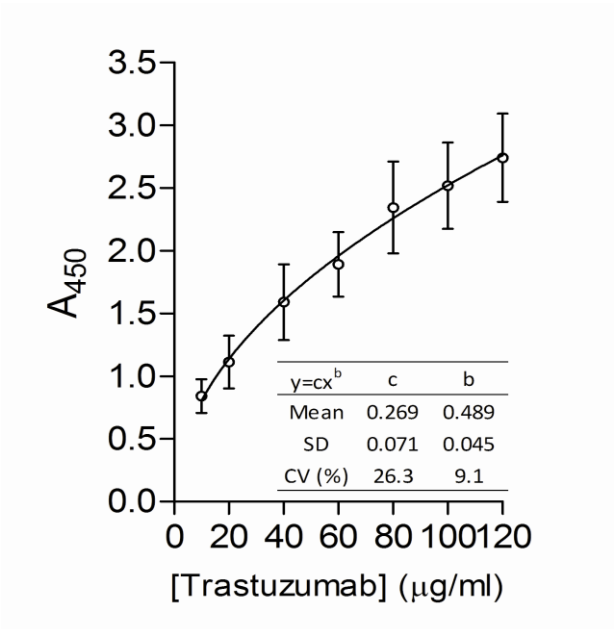

Figure 3.8: Standard curve of human plasma spiked with trastuzumab as determined by cell-based ELISA. The insert table shows the mean, standard deviation and CV of the constants from a power equation ($y=cx^b$). Figure represents mean and SD of 13 independent experiments.

Concentrations of 100 and 25 µg/ml were used for quality control samples. Standard curves were made up fresh for each experiment. Quality control samples were made as single use aliquots and were stored at −80 °C. These samples were used to validate the assay as described below. The disposition of standards, quality controls, samples and blanks in replicate wells is described in Figure 3.9.

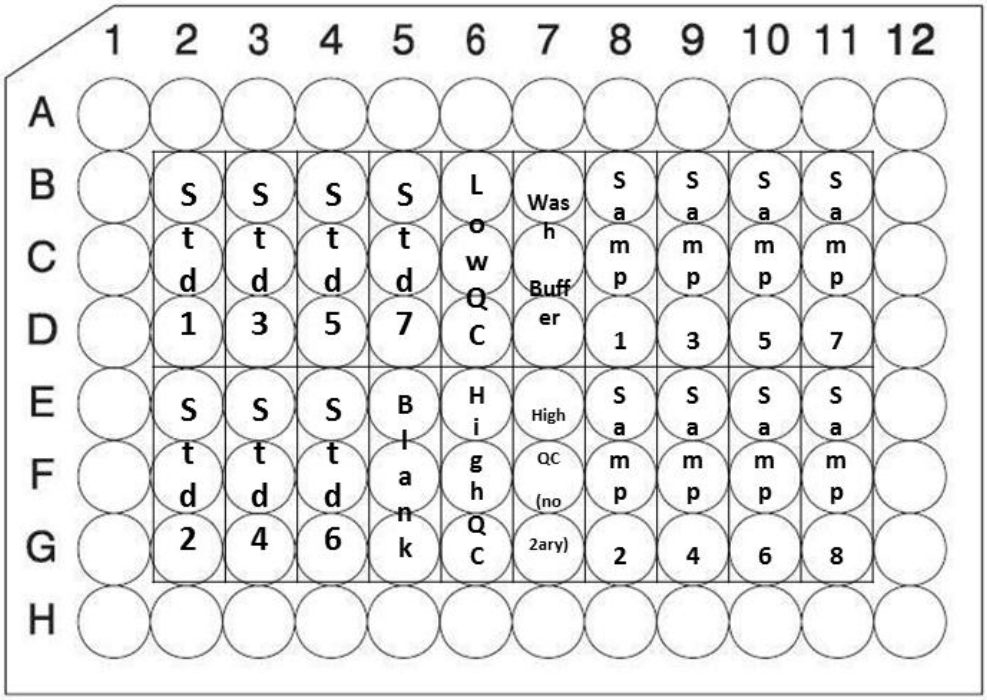

Figure 3.9: Costar 96-well plate showing the disposition of standards, quality controls, blanks and samples for the cell-based trastuzumab ELISA

Intra and Inter assay variability

Intra assay variability was determined by running 15 replicates of the low and high QC. This was repeated twice, for a total of 3 independent experiments, and the mean values were used to contribute towards the acquisition of data to determine inter assay variability. The mean intra assay variability was 9% and on one occasion out of six was above 15% (16% for the low QC) (Table 3.1). Inter assay variability was assessed over 11 independent experiments performed as part of the validation. Mean inter-assay variability was 12% for the low QC and 11% for the high QC. The variability of the back-calculated concentrations of trastuzumab from the standard curve were between 5 and 12% and all calculated values of the standards were within ± 8% of the intended

value (Table 3.1). The mean percentage recovery for the low and high QC samples was 98% and 95% respectively.

Intra-assay variability

	Low QC 1	Low QC 2	Low QC 3	Mean Low QC	High QC 1	High QC 2	High QC 3	Mean High QC
Mean trastuzumab (μg/ml)	23	28	29	27	84	96	106	95
N	15	15	15		14	15	15	
SD (μg/ml)	4	2	2		8	6	10	
CV (%)	16	8	6		9	6	10	

Inter-assay variability

	Low	High	std10	std20	std40	std60	std80	std 100	std 120
Mean trastuzumab (μg/ml)	25	95	11	19	39	56	87	101	121
N	11	11	13	13	13	13	13	13	13
SD (μg/ml)	3	11	1	2	5	4	7	5	7
CV (%)	12	11	11	12	12	8	8	5	6

Table 3.1: Cell-based trastuzumab ELISA intra- and inter-assay variability
(n = intra-assay replicates in individual experiments for the intra-assay variability determination and number of independent experiments for the inter-assay experiments)

Stability of trastuzumab QC samples in different storage conditions

The stability of trastuzumab under numerous storage conditions was determined. Both the high and low QCs were stable over 72 h at -20 °C (CV = 5 and 4% respectively) and 4 °C (CV = 4 and 5% respectively) (Figure 3.10).

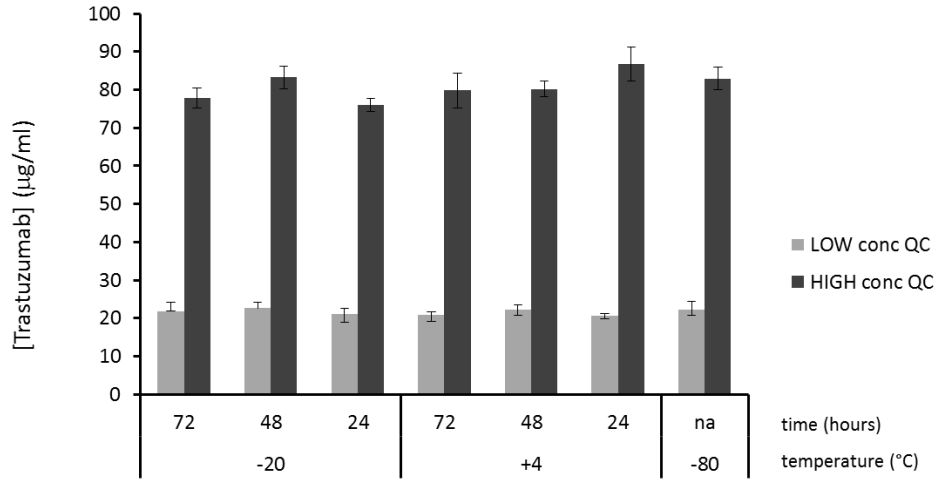

Figure 3.10: Stability of trastuzumab QCs at -20 °C and at -4 °C for 24, 48 and 72 h.
Error bars represent SD (intra-assay variability).

QCs were also stable for up to 10 weeks at −20 °C (CV = 4 and 9% respectively) (Figure 3.11) with no downward trend and for at least 6 months at −80 °C.

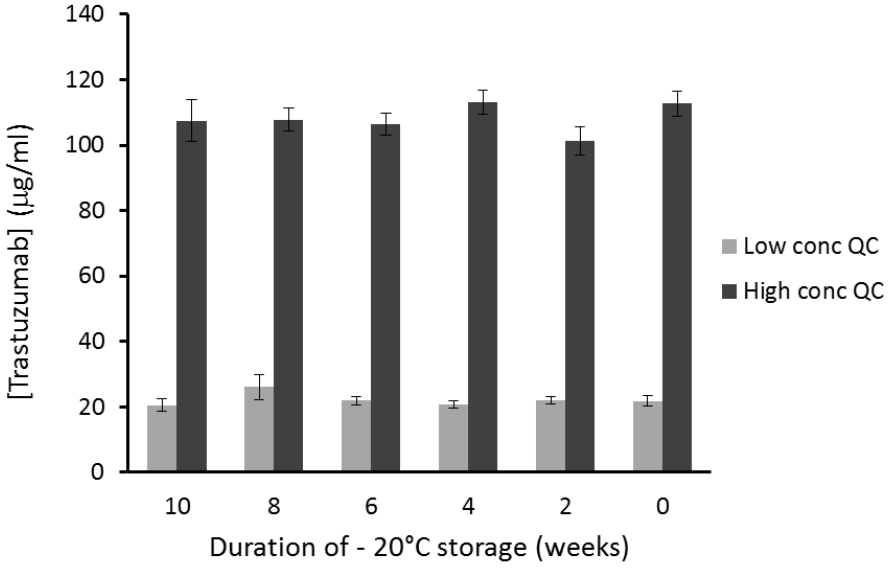

Figure 3.11: Stability of trastuzumab QCs at -20 °C for 2, 4, 6, 8 and 10 weeks.
Error bars represent SD (intra-assay variability)

There was no loss of signal over 4 freeze/thaw cycles from -80 °C to RT (Figure 3.12).

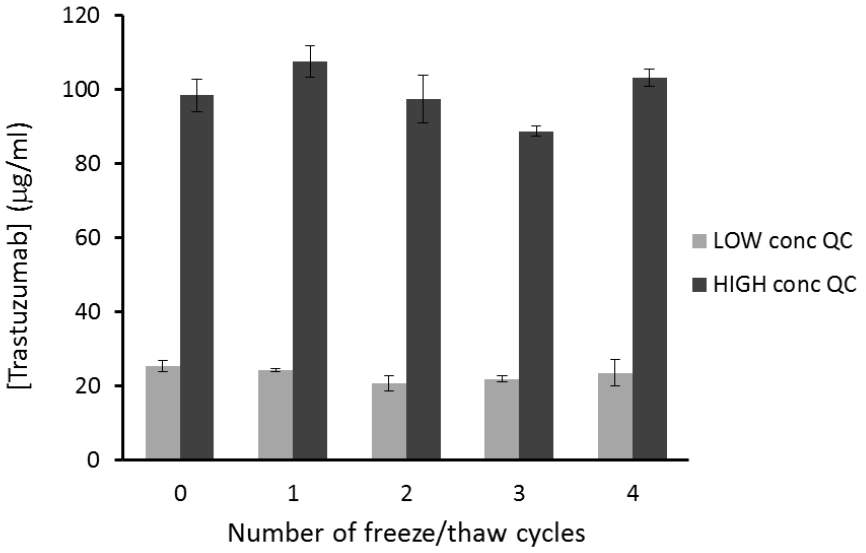

Figure 3.12: Stability of trastuzumab QCs over up to 4 freeze/thaw cycles.
Error bars represent SD (intra-assay variability).

There was a slight downward trend in the absorbance values of the QC samples over the period of the validation, but this had no effect on the calculated QC concentrations and was attributed to a decrease in the HRP activity of the secondary antibody. Absorbance values returned to those seen at the beginning of the method development when new secondary antibody was purchased.

Limit of quantification

The lower limit of quantification was determined by analysing the plasma of eight individuals who had not received trastuzumab. The apparent plasma concentration was 5.6 ± 1.1 µg/ml (Mean and SD). Therefore a concentration of 9 µg/ml trastuzumab (Mean + 3 SD) was established as the lower limit of detection and at this value it is predicted that less than 1 in 200 positive values will be false.

Dilutional linearity and parallelism

Dilutional linearity was demonstrated by spiking matrix with 1 mg/ml trastuzumab and serially diluting the spiked sample 1 in 2 followed by a 1/4000 dilution in wash buffer. No high dose 'hook effect' was observed up to 1 mg/ml (Figure 3.13).

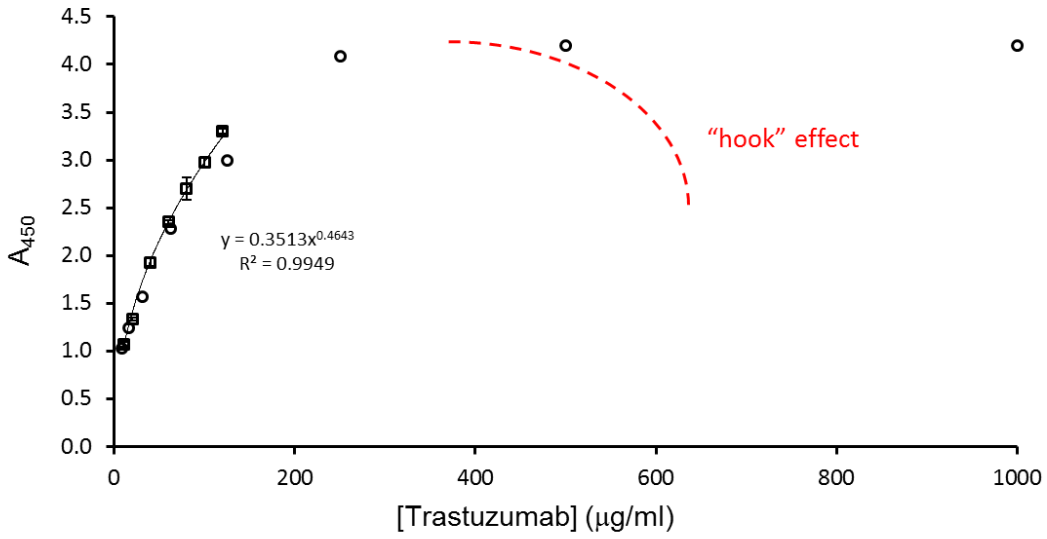

Figure 3.13: Absorbance of 1 in 2 Serial dilutions of 1 mg/ml trastuzumab (circles). Squares represents standards. Red dotted line shows a possible trend in the absorbance values if a "hook" effect were present. The high dose 'hook effect' refers to measured levels of analyte displaying a significantly lower absorbance than the actual level present in a sample. It occurs when the assay is saturated by a very high concentration of analyte binding to all available sites on both the capture antigen as well as the detection antibody and thereby preventing the sandwich-formation. The analyte-saturated detection antibodies in solution are washed off giving a falsely low signal. A "hook" is observed in the curve when data is plotted as a signal versus analyte concentration (Rodbard *et al.*, 1978).

Dilutions between 1/16 and 1/64 had predicted and measured results within the dynamic range of the assay and the trastuzumab concentrations corrected for dilution of these samples were within 20% of the predicted concentration (Figure 3.14).

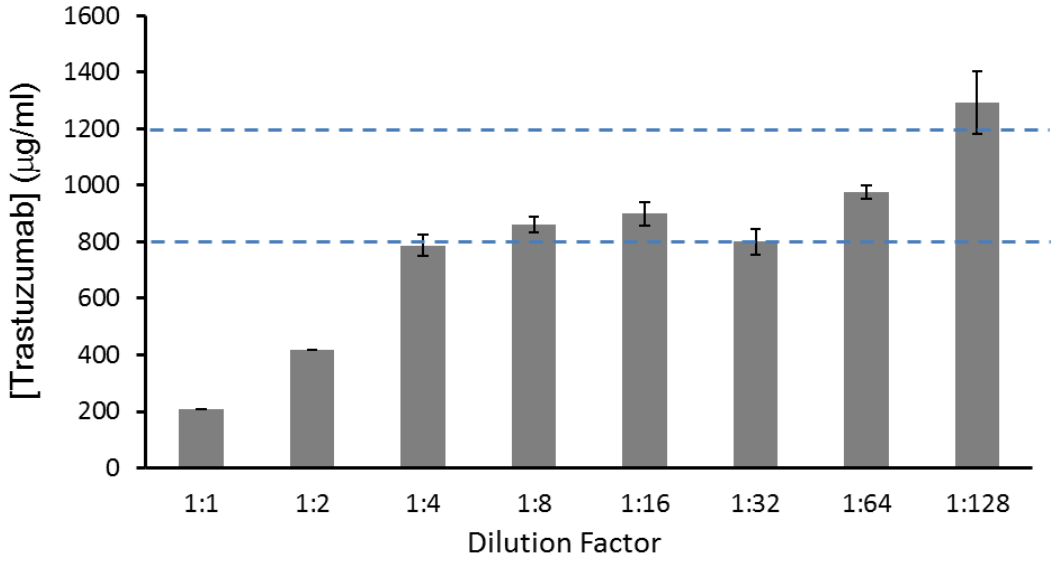

Figure 3.14: Back-calculated concentration of serial dilutions of 1 mg/ml trastuzumab.
The graph shows that there is parallelism in the concentrations that are diluted to be within the standard curve values (between 1/16 and 1/64).

Three patient samples with measured concentrations above the dynamic range of the assay were used to determine parallelism. The samples were serially diluted in matrix 1 in 2, followed by a 1/4000 dilution in wash buffer. For each sample the lowest dilution that resulted in a measured concentration within the dynamic range of the assay and a calculated concentration less than 20% different from the preceding concentration was reported as the determined concentration. In two of the patients the minimum required dilution was 1/4 and in one patient a 1/16 dilution was required.

3.2.4 Discussion

The data presented in the previous sections show that the cell-based trastuzumab ELISA was specific for the interaction between trastuzumab and HER2, as illustrated by the immunofluorescence experiments. No fluorescence signal was observed when a 1 in 4000 dilution of human plasma without trastuzumab was used as the primary antibody on the HER2-positive SKBR3 cells, nor when a 1 in 4000 dilution of human

plasma spiked with 120 µg/ml trastuzumab was used on the HER2-negative cell line MDA MB 231. The sensitivity of the assay was sufficient to discriminate plasma concentrations lower than the pharmacological target of a trough trastuzumab concentration of 20 µg/ml (Leyland-Jones *et al.*, 2003). The assay was comparable in terms of performance with the sandwich ELISA described by Maple *et al.* (2004), showing similar precision, dynamic range and accuracy, but was simpler to execute and is not limited by access to reagents of restricted availability.

The dynamic range of the assay of 10 to 120 µg/ml encompassed the expected mean trough trastuzumab concentration, as determined by previously published trastuzumab pharmacokinetic studies (Bruno *et al.*, 2005). The stability experiments show that trastuzumab in human plasma was stable over a range of conditions and manipulations. Finally, the results of the dilutional linearity and parallelism experiments implied that a patient sample that was above the upper limit of quantification could be diluted into the dynamic range of the assay and the calculated value was reliable. All these characteristics make the assay suitable for the purpose of measuring trough plasma concentrations in trastuzumab treated patients.

3.3 HER2 ELISA

3.3.1 Introduction

The measurement of plasma HER2 concentration was performed using a commercial kit which is based on the principles of a normal sandwich ELISA. Briefly the microwells of a 96-well plate had been previously coated with an anti-human HER2 antibody. The human HER2 present in the sample or standard bound to the antibodies adsorbed to the microwells. A HRP-conjugated anti-human HER2 antibody was added and bound to human HER-2 captured by the first antibody. Following incubation the unbound HRP-conjugated anti-human HER2 was removed during a wash step, and substrate solution reactive with HRP was added to the wells. A coloured product was formed in proportion to the amount of human HER2 present in the sample or standard. The reaction was terminated by addition of acid and absorbance was measured at 450 nm. A standard curve was prepared from seven human HER2 standard dilutions and human HER2 concentration was determined. The detailed procedures and the reagents and

equipment needed for the assay are described in the 'product information and manual' (Bender_Medsystems®, 2011). Although this assay had been validated by the manufacturer it appeared appropriate to conduct a series of further validation experiments before moving to the measurement of HER2 in patient samples.

3.3.2 Validation

The dynamic range of the assay was confirmed by preparing two-fold serial dilutions of the HER2 standard (10 - 5 - 2.5 - 1.25 - 0.63 - 0.31 - 0.16 - 0.16 ng/ml) as described in the product information and manual. 10 ng/ml and 0.16 ng/ml are the upper and lower limits of quantitation as described in the product information and manual. The average cumulative standard curve, obtained from 14 independent experiments (5 of them performed using a first batch of the assay, 9 using a second batch), was best fitted by a power curve ($y=cx^b$) (Figure 3.15).

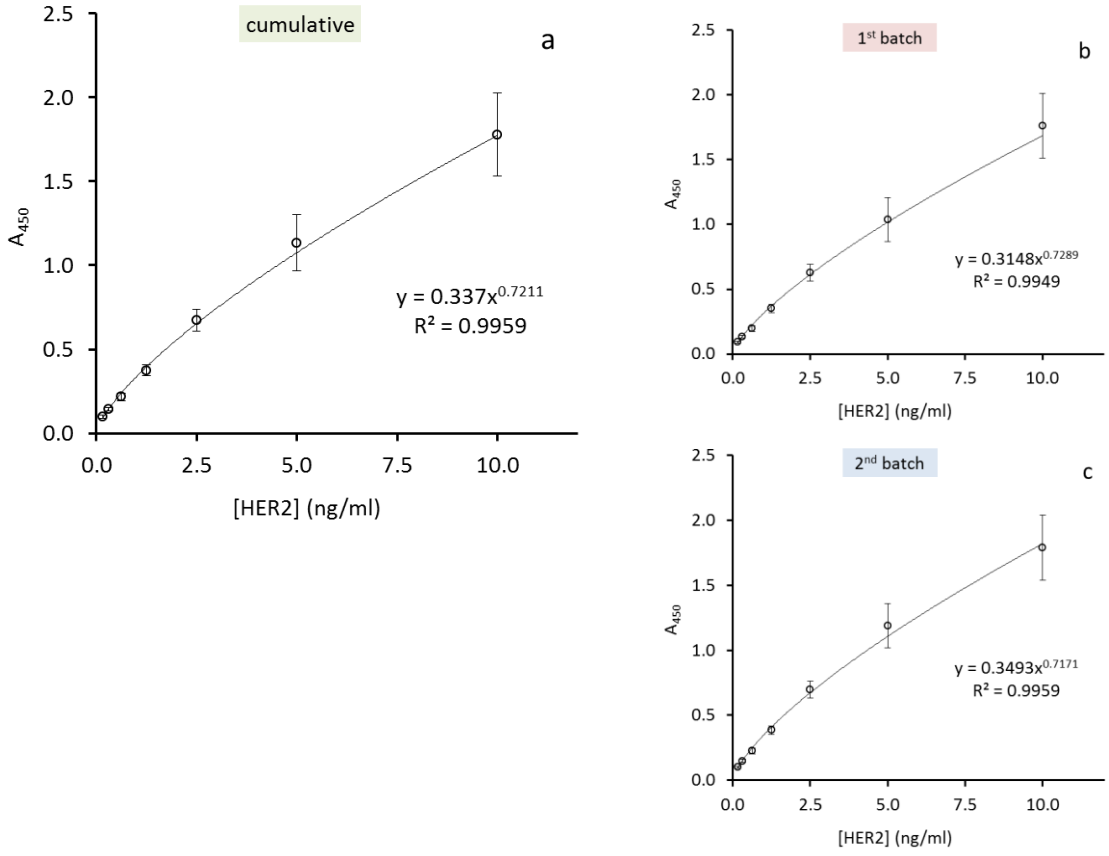

Figure 3.15: Standard curve of HER2 ELISA.
Graph a represents mean and SD of 14 independent experiments. Graphs b and c represents mean and SD of, respectively, 5 and 9 independent experiments using two different batches of the assay.

Preparation of Quality Controls

The high concentration and low concentration quality controls (high QCs and low QCs) were supplied by the manufacturer in the form of vials of lyophilised HER2 protein (one vial per plate), to be reconstituted in 100 µl distilled water in order to achieve a concentration range of 50 - 300 ng/ml and 5 - 30 ng/ml, respectively. Using the QCs as suggested by the product information and manual, however, would have resulted in the availability of a low number of QC samples at not exactly the same concentration and this would not have allowed the execution of the planned validation experiments and the necessary reproducibility. To overcome this issue, twenty vials of lyophilised low QC and twenty vials of high QC (from two 10-pack batches of the kit) were reconstituted in 100 µl of human plasma (per vial). These two sets of solutions were then mixed together in two separate tubes (one for low QC, one for high QC) and divided into multiple single-use aliquots, which were stored at -20°C.

Intra-assay, Inter-assay, Inter-batch variability

Intra assay variability was determined by running 16 replicates of the low and high QC, and this was performed three times for the first batch of the kit and three times for the second batch. The mean values were also used to contribute towards the acquisition of data to determine inter assay variability (Table 3.2).

		Intra-assay variability			**Inter-assay variability**		
	QC	Mean [HER2] (ng/ml)	n	Mean CV (%)	Mean [HER2] (ng/ml)	n	Mean CV (%)
Both batches (cumulative)	High	113.6	16x6	13.5	108.4	14	13.8
	Low	9.8	16x6	8.2	9.6	14	15.0
1st batch	High	122.1	16x3	13.3	124.7	5	7.0
	Low	10.4	16x3	7.9	11.0	5	9.3
2nd batch	High	105.1	16x3	13.8	99.3	9	8.3
	Low	9.1	16x3	8.5	8.7	9	9.6

Table 3.2: HER2 ELISA intra- and inter-assay variability
(n = intra-assay replicates per individual experiment for the intra-assay variability determination and number of independent experiments for the inter-assay experiments)

The mean intra assay variability was 13.5% for the high QC and 8.2 for the low QC. Inter assay variability was assessed over 14 independent experiments. Mean inter-assay variability was 13.8% for the high QC and 15% for the low QC. However, within the same batch the inter-assay variability was significantly lower (ranging from 7 to 9.6%).

Stability of HER2 QC samples in different storage conditions

Both high and low QCs were stable for up to 6 months at -20 °C with no downward trend and over 72 h at 4 °C (CV = 9 and 6% respectively) (Figure 3.16). The QCs were also stable over up to 5 freeze/thaw cycles from -20 °C to RT (Figure 3.17).

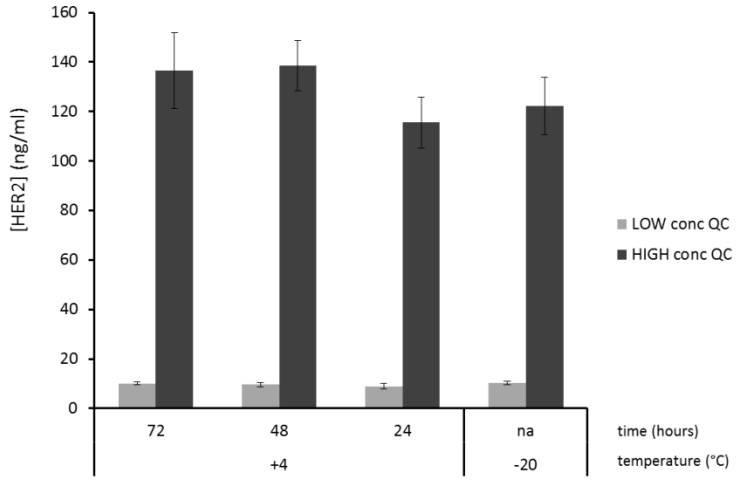

Figure 3.16: Stability of HER2 QCs at -4 °C for 24, 48 and 72 h.
Error bars represent SD (intra-assay variability)

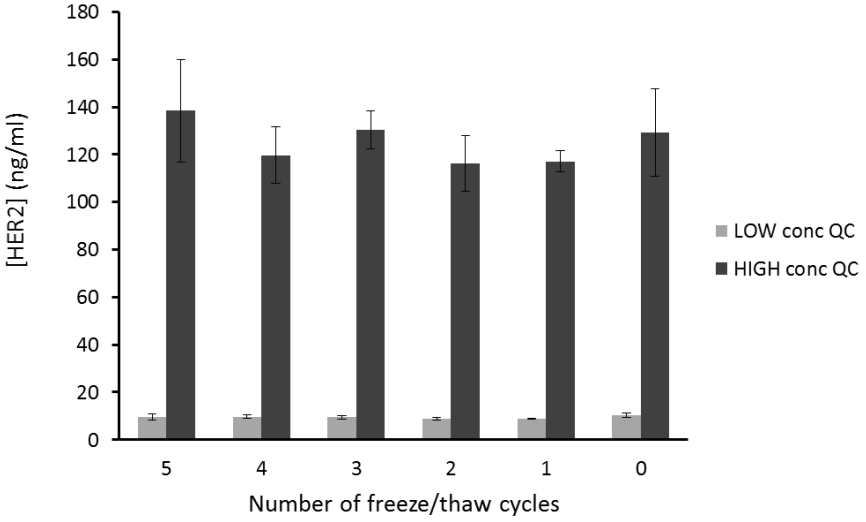

Figure 3.17: Stability of HER2 QCs over up to 5 freeze/thaw cycles.
Error bars represent SD (intra-assay variability).

Limit of detection

The limit of detection of the assay (0.13 ng/ml) was calculated as the mean plus 3 standard deviations of calculated values from blank wells of 14 different experiments.

Dilutional linearity

QCs and patient samples had to be diluted 1:20 in assay buffer as per the product information and manual. In order to assess dilutional linearity of the assay high and low QCs were serially diluted in assay buffer in order to obtain concentrations which would give absorbance signals within the dynamic range of the assay. Linearity was demonstrated in the case of the high QCs, where the HER2 concentrations corrected for the dilution were within 20% of the predicted concentration. However, this was not observed in the case of the low QCs, where dilution factors that were different from the recommended 1:20 gave unreliable results (Figure 3.18).

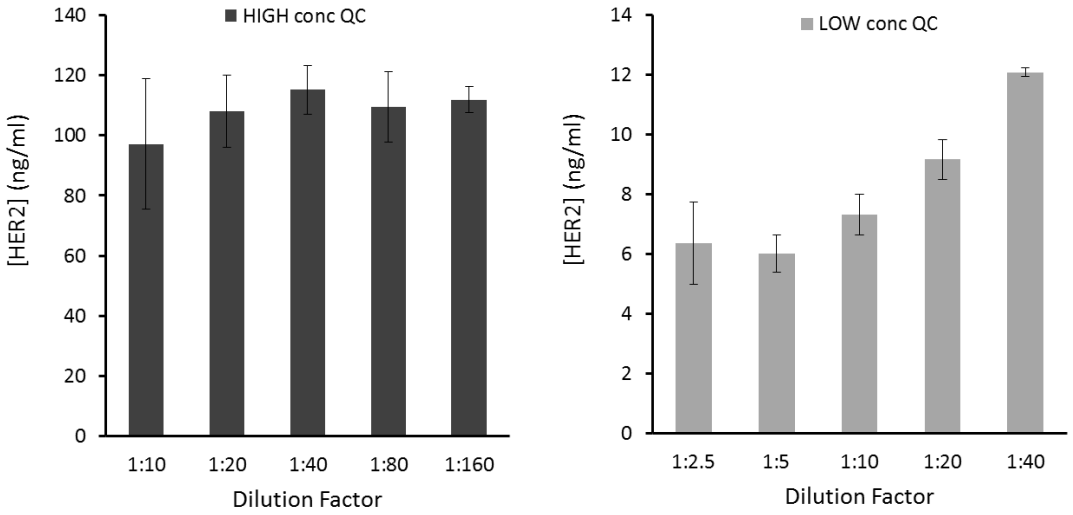

Figure 3.18: Back-calculated concentration of serial dilutions of HER2 high QCs and low QCs in assay buffer.
The graphs show that there is linearity with the high QC but not with low QC. Error bars represent SD (intra-assay variability).

Effect of haemolysis

In order to assess the potential effect of haemolysis on the quantification of HER2 two 5 ml blood samples in EDTA tubes were collected from a healthy volunteer. Haemolysis in one of the two samples was achieved by vigorous shaking and plasma from both

samples was prepared. An aliquot of high QC was diluted 3:4, 1:2 and 1:4 with either haemolysed or non-haemolysed plasma and HER2 concentrations were determined (Figure 3.19).

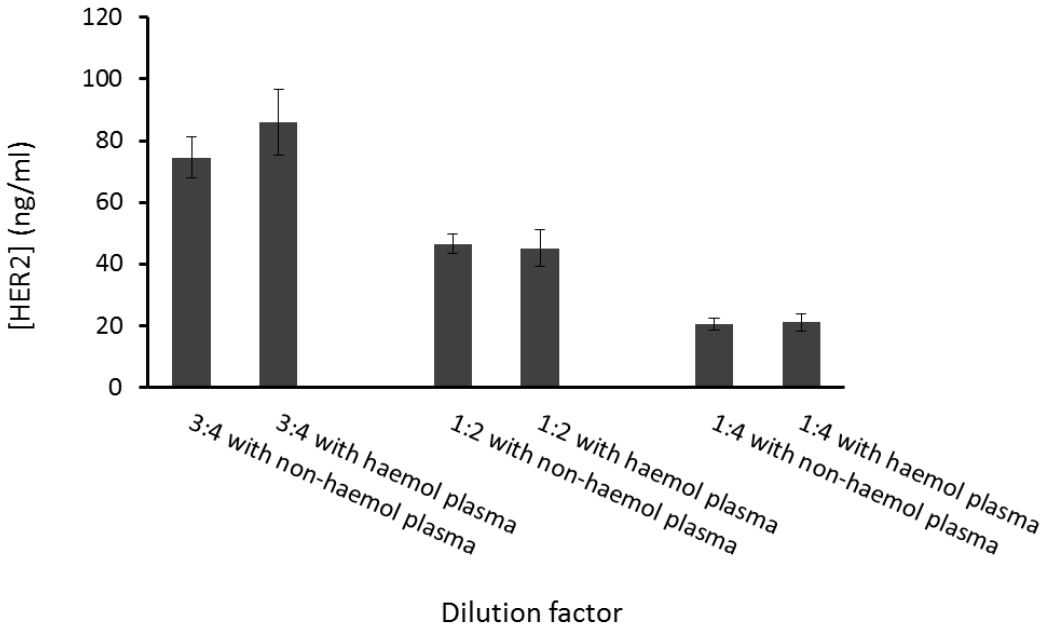

Figure 3.19: Concentration of HER2 high QCs diluted with either haemolysed or non-haemolysed plasma.
Error bars represent SD (intra-assay variability).

Effect of trastuzumab

Given that the molarity of trastuzumab in plasma of treated patients can range from 1 to 4 orders of magnitude higher than the HER2 plasma molarities reported in the literature, an experiment was set up in order to assess the potential interference of trastuzumab on the HER2 ELISA. Eight patient samples were spiked with either 10% water or 10% trastuzumab (concentrations 1000, 500, 250 and 125 μg/ml in water) to give final trastuzumab concentrations of 100, 50, 25 and 12.5 μg/ml. The concentration of HER2 were then determined (Figures 3.20 and 3.21).

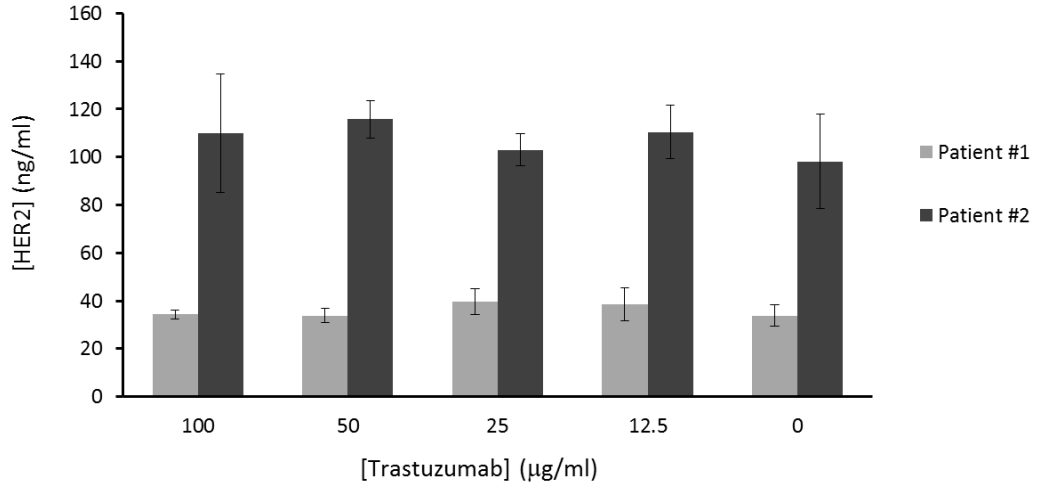

Figure 3.20: Concentration of HER2 in two patient samples spiked with trastuzumab at different concentrations.
X axis shows final concentration of trastuzumab in the sample. Error bars represent SD (intra-assay variability).

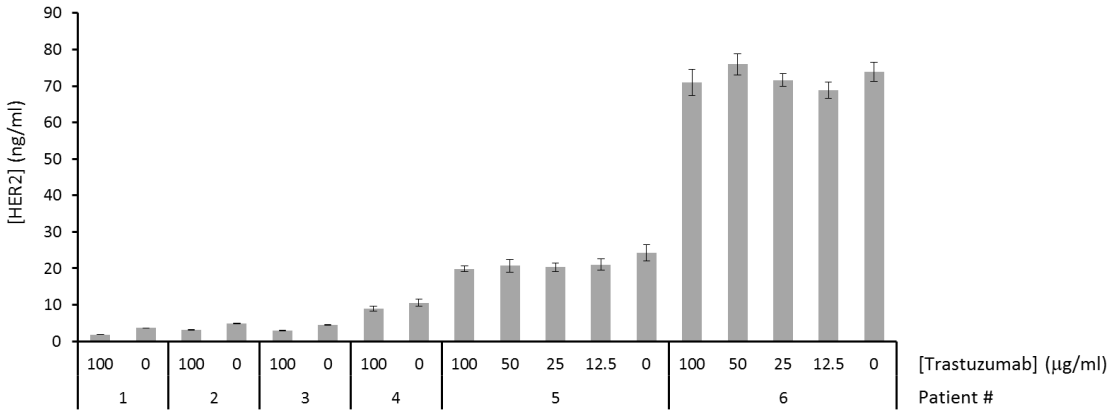

Figure 3.21: Concentration of HER2 in six patient samples spiked with trastuzumab at different concentrations.
X axis shows final concentration of trastuzumab in the sample. Error bars represent SD (intra-assay variability).

3.3.4 Discussion

The data presented in the previous sections confirm that the Bender-Medsystems® HER2 ELISA can detect HER2 plasma concentrations within the range reported in previously-published studies (Fornier *et al.*, 2005; Ludovini *et al.*, 2008). The intra assay, inter assay and inter batch variability experiments confirm an acceptable precision of the assay, with the only concern being the difference in the calculated

concentrations observed between the two batches. The stability experiments show that HER2 in human plasma is stable over a range of conditions and manipulations. Importantly, sample haemolysis does not seem to interfere with the assay. Pharmacological concentrations of trastuzumab in the sample had only a moderate effect on the measurement of values close to the lower limit of quantification of the assay, whereas higher HER2 values do not seem to be influenced. The results of the dilutional linearity experiments show that loss of parallelism can alter the measurement of low values as well. These two latter observations indicate that low patient values should be treated with caution.

3.4 Conclusion

The development and validation of a cell-based ELISA for the measurement of trastuzumab in plasma have been described. The performance of a similar assay for the measurement of HER2 in plasma samples has been evaluated by an analoguous validation process. Both the ELISAs have been shown to be suitable for the purpose of collecting pharmacokinetic and biomarker data from the HER2-positive breast cancer patients treated with trastuzumab in this study.

Chapter 4. Results II: HER2 SNPs in the whole population

4.1 Overview

This chapter initially discusses the process of patient recruitment, the characteristics of the whole study population and the clinical samples obtained. In the following sections the characteristics of the tumours are analysed, with a particular attention on the assessment of HER2 expression. The final sections of the chapter present the results of the HER2 SNPs genotyping in genomic DNA, their association with the tumour characteristics and the potential implications of these observations in the context of the relevant scientific literature.

4.2 Study population

4.2.1 Recruitment

From August 2007 to July 2011 a total of 367 patients from the medical oncology clinics of the Northern Centre for Cancer Care (NCCC) were recruited. Figure 4.1 shows the enrolment of patients over time. Since the final ethical approval was granted (August 2007) the recruitment process underwent two major delays, which coincided with the bureaucratic procedures necessary to obtain a first authorization to patient recruitment and a second, more comprehensive, honorary contract from the NHS Trust. The overall recruitment rate was 7.6 patients/month but, if the two periods of delay are excluded, the actual recruitment rate was 10.9 patients/month.

It is also evident from Figure 1 that the recruitment rate was faster in the period covered by the first NHS authorization than it was during the months following the grant of the honorary contract. This is because in this latter period a greater proportion of time was spent in the laboratory and time was also devoted to clinical duties within the breast cancer clinics at the NCCC. This led to a relative decrease in the time available for informing and consenting patients.

The allocation of patients to the categories of HER2-positive, HER2-negative and HER2 equivocal/discordant will be explained in detail in Section 4.3.1.

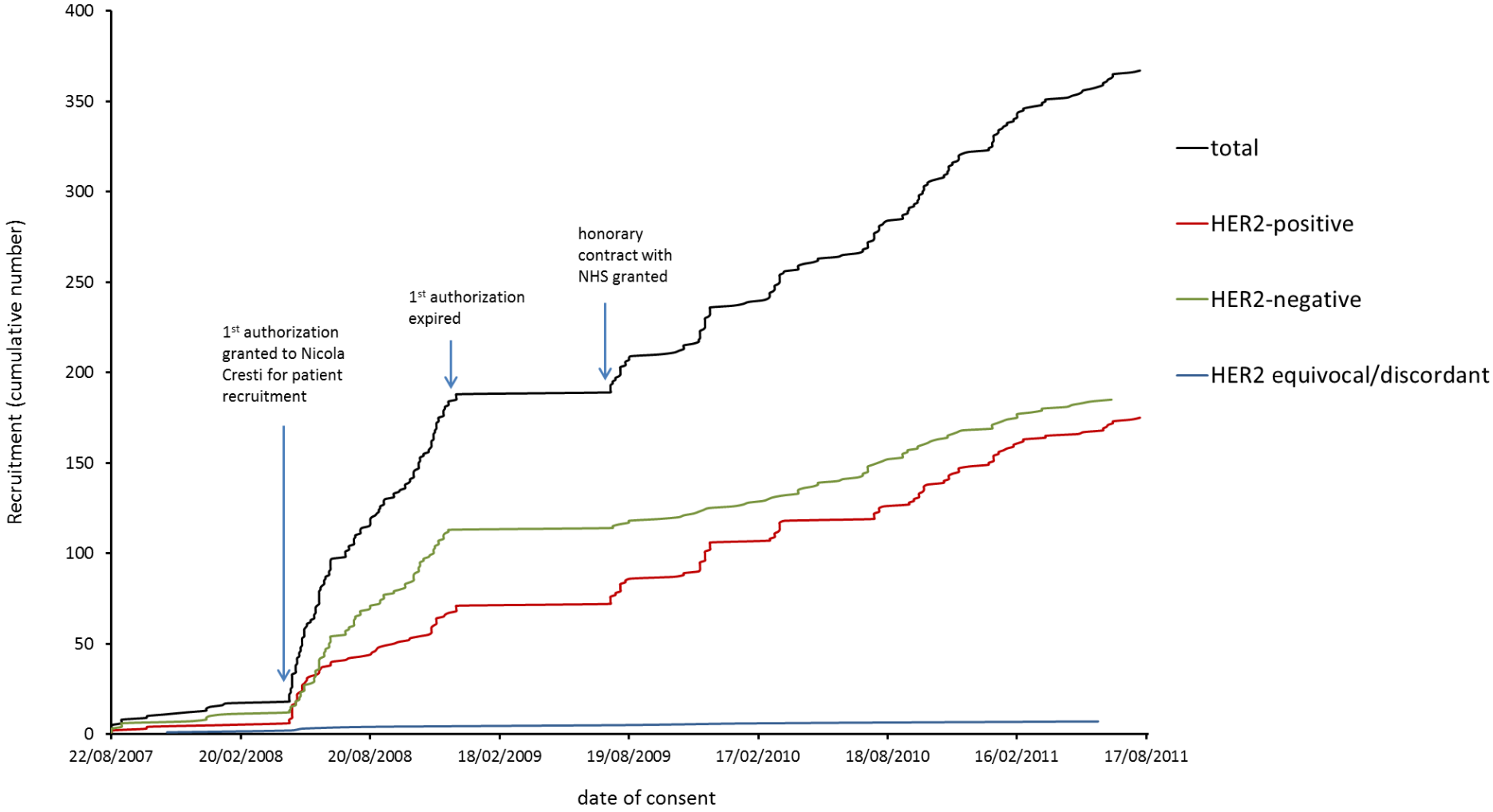

Figure 4.1: Patient recruitment over time

4.2.2 Characteristics of patients and samples collected

The demographic characteristics of the study population and the characteristics of samples collected are described in Tables 4.1 and 4.2, respectively. It is important to note that the vast majority of the patients were Caucasian and only one patient was male. With regard to the sample collection, blood drawing was not feasible in only six cases due to poor venous access. The analyses of tumour samples and plasma samples from trastuzumab treated patients are presented in Chapters Five and Six, respectively.

Total consented (number)	**367**	
Ethnicity	Caucasian (%)	97.5
	Non Caucasian (%)	2.5
Gender	Female (%)	99.7
	Male (%)	0.3 *
Age at date of consent	Median (years)	57
	Range (years)	29 - 82

Table 4.1: Demographic characteristics of study population
* 1 male patient

Total consented	**367**			
Blood sample collected	361			
Tumour sample collected	241			
Treated with trastuzumab	Yes	168	Plasma sample collected	138
	No	199		

Table 4.2: Characteristics of samples collected
(figures represent number of patients)

4.3 Characteristics of tumours

The histo-pathologic data of all tumours collected from pathology reports available in the patients' clinical notes and in the Trust laboratory and Patient Administration Service databases are presented in Table 4.3.

			number	%
Total			**367**	100
Histologic subtype	Ductal NST		299	81.5
	Lobular		24	6.5
	Carcinoma NOS		9	2.5
	Apocrine		4	1.1
	Mucinous		3	0.8
	Micropapillary		1	0.3
	Mixed or multiple histology		27	7.4
Histologic grade	1		12	3.3
	2		135	36.8
	3		193	52.6
	Discordant determinations		13	3.5
	Not assessed		14	3.8
Primary tumour	One	Unifocal	284	77.4
		Multifocal	63	17.2
	Bilateral (synchronous)		4	1.1
	Multiple diachronous		16	4.4
Diameter of major tumour	Occult		6	1.6
	≤ 2 cm		136	37.1
	2 - 5 cm		164	44.7
	≥ 5 cm		31	8.4
	Neo-adjuvant chemo*		18	4.9
	Unknown		12	3.3
Staging at date of first diagnosis	Early	Node-negative	139	37.9
		Node-positive	193	52.6
		Not assessed	20	5.4
	Distant metastases		13	3.5
	Suspicious distant metastases		2	0.5
Staging at date of consent	Early (no recurrence)		301	82
	Regional recurrence		13	3.5
	Distant metastases		51	13.9
	Suspicious distant metastases		2	0.5
Expression of hormone receptors	Positive (Quick score 4 - 8)		240	65.4
	Negative (Quick score 0)		97	26.4
	Low (Quick score 2-3)		18	4.9
	Discordant determinations		12	3.3

Table 4.3: Characteristics of tumours.
No. represents number of patients. * patient undergoing neo-adjuvant chemotherapy had tumours ≥ 5 cm by clinical measurement (calliper and/or ultrasound and/or mammogram)

As expected, the ductal histologic subtype represented the vast majority of the cancers (81.5%), with the lobular subtype being the second most frequent. As explained in Section 2.2, the patients recruited were not necessarily newly-diagnosed. In fact a significant proportion of patients had been diagnosed with more than one primary tumour and about 17% of patients had suffered from a regional or distant recurrence since their first diagnosis.

As described in the table, our sample is characterized by a relative enrichment in the expression of negative prognostic factors compared with large breast cancer databases (SEER, 2011). This may be due to the fact that most of the patients recruited were referred to the medical oncology breast clinic from the surgical department after surgery. The patients were to be considered for adjuvant chemotherapy, which is usually offered to patients with medium to high risk of recurrence (Goldhirsch *et al.*, 2009). Therefore most low-risk patients were not referred and could not be included in the study.

This selection effect can explain the fact that most tumours had a histologic grade of 2 or 3 (89.4%) and the proportion of grade 1 tumours (3.3%) was very low compared with large published series (Rakha *et al.*, 2008). In the same way, the relatively low proportion of small size (37.1% ≤ 2 cm) and node-negative (37.9%) tumours at first diagnosis is in apparent contrast with earlier detection of breast tumours facilitated by the screening policies implemented in the United Kingdom in recent years.

With regard to the expression of hormone receptors, this was determined in the vast majority of the cases using the 'Quick' scoring system, which ranges from 0 to 8 (excluding 1) (Leake *et al.*, 2000). The 'low' score reported for 4.9% of patients refers to a Quick score of 2 or 3. In 3.3% of patients hormone receptors assessed on multiple occasions (from multiple primary tumours and/or in primary and recurrent tumours) gave conflicting results and this subgroup was therefore isolated from the rest of the sample.

4.3.1 Determination of HER2 status

As pointed out in Section 2.2, the HER2 status in tumour samples was assessed using IHC and/or ISH.

IHC

The number of HER2 determinations using IHC and their results are shown in Table 4.4.

	no.		**no.**	**%**
IHC determinations	397	score 0	69	17.4
		score 1	66	16.6
		score 2	96	24.2
		score 3	142	35.8
		score not available	25	6.3

Table 4.4: HER2 determinations with IHC
(no. represents number of determinations)

As seen in the table, the total number of HER2 determinations exceeds the number of patients because several patients had more than one determination (in multiple primary tumours or in case of matched assessment in primary and recurrence). In 6.3% of cases the HER2 status was reported simply as 'positive' or 'negative' and it was not possible to retrieve the IHC score. Data on uniformity and intensity of membrane staining, as well as percentage of tumour cells stained were not available in pathology reports.

ISH

HER2 amplification using ISH was assessed in 110 cases (Table 4.5), the vast majority of which were subsequent to IHC score 2 determinations (96 cases, as shown in Table 4.4).

	no.		**no.**	**%**
ISH determinations	110	FISH	77	70
		SISH	33	30
ISH status		Amplified	50	45.5
		Not amplified	60	55.5
Chromosome 17 copy number/cell available	39			
HER2 copy number/cell available	98			
HER2/chromosome 17 ratio available	41			

Table 4.5: HER2 determinations with ISH
(no. represents number of determinations)

The 14 extra cases are accounted for as follows:

- multiple ISH determinations on the same tumour sample (IHC score 2);
- ISH determinations to confirm IHC negative or positive (0, 1 or 3) cases in single sample or in multiple samples in the same patient.

Two different methods (FISH and SISH in 70% and 30% of cases, respectively) were adopted for the HER2 amplification, reflecting a shift in the Pathology Laboratory procedures implemented over recent years (Section 2.2).

Detailed data on chromosome 17 and HER2 copy number were not always present in the pathology reports, as shown in Table 4.5. Figure 4.2 shows the distribution of the ISH results in the available cases. In only 39 cases were both chromosome 17 and HER2 copy number/cell available (Figure 4.3).

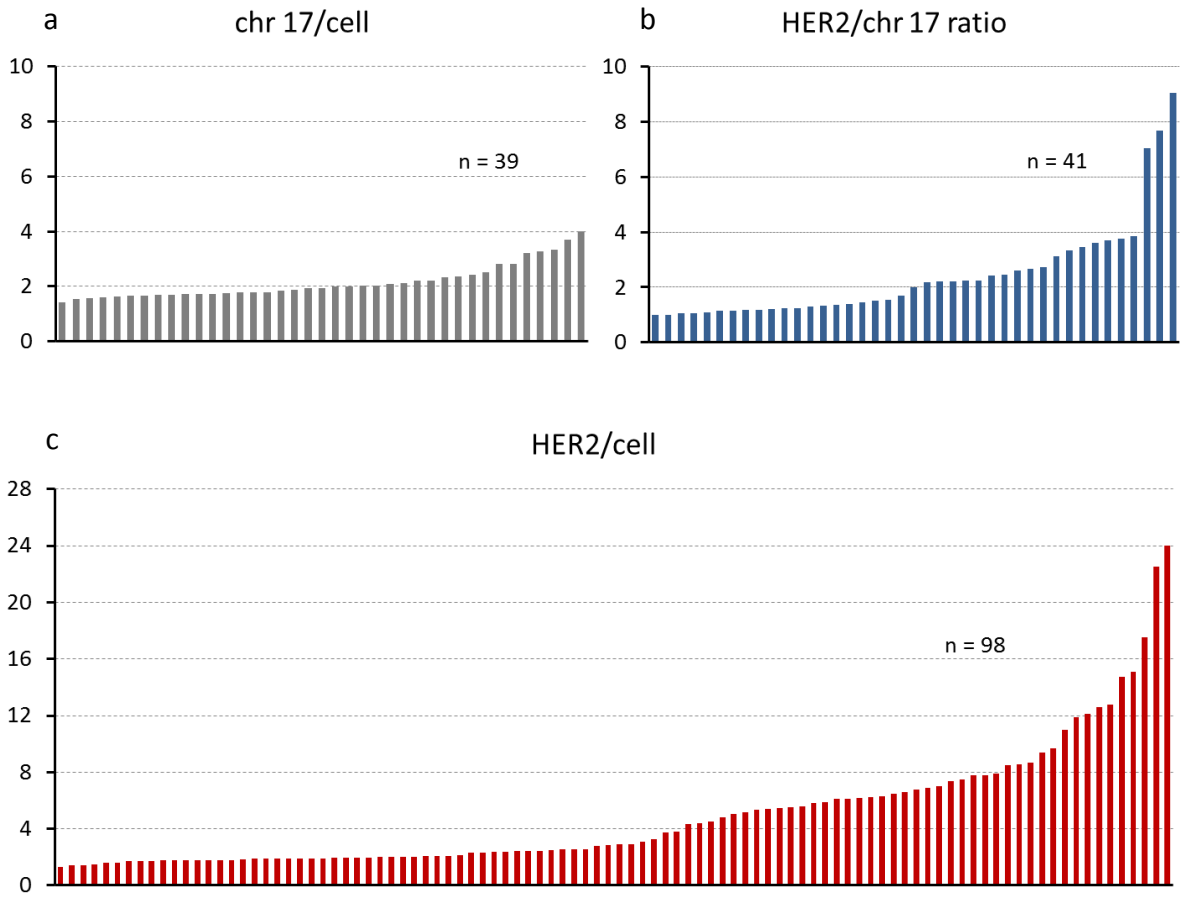

Figure 4.2: a) Chromosome 17 copy number/cell in 39 determinations; b) HER2/chromosome 17 ratio in 41 determinations; c) HER2 copy number/cell in 98 determinations

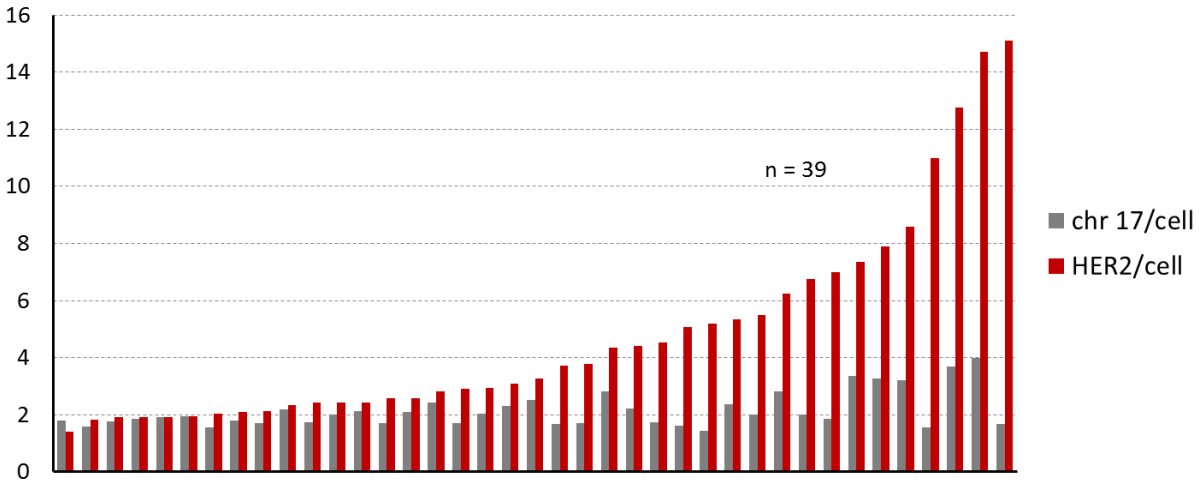

Figure 4.3: Matched chromosome 17 copy number/cell and HER2 copy number/cell in 39 determinations.

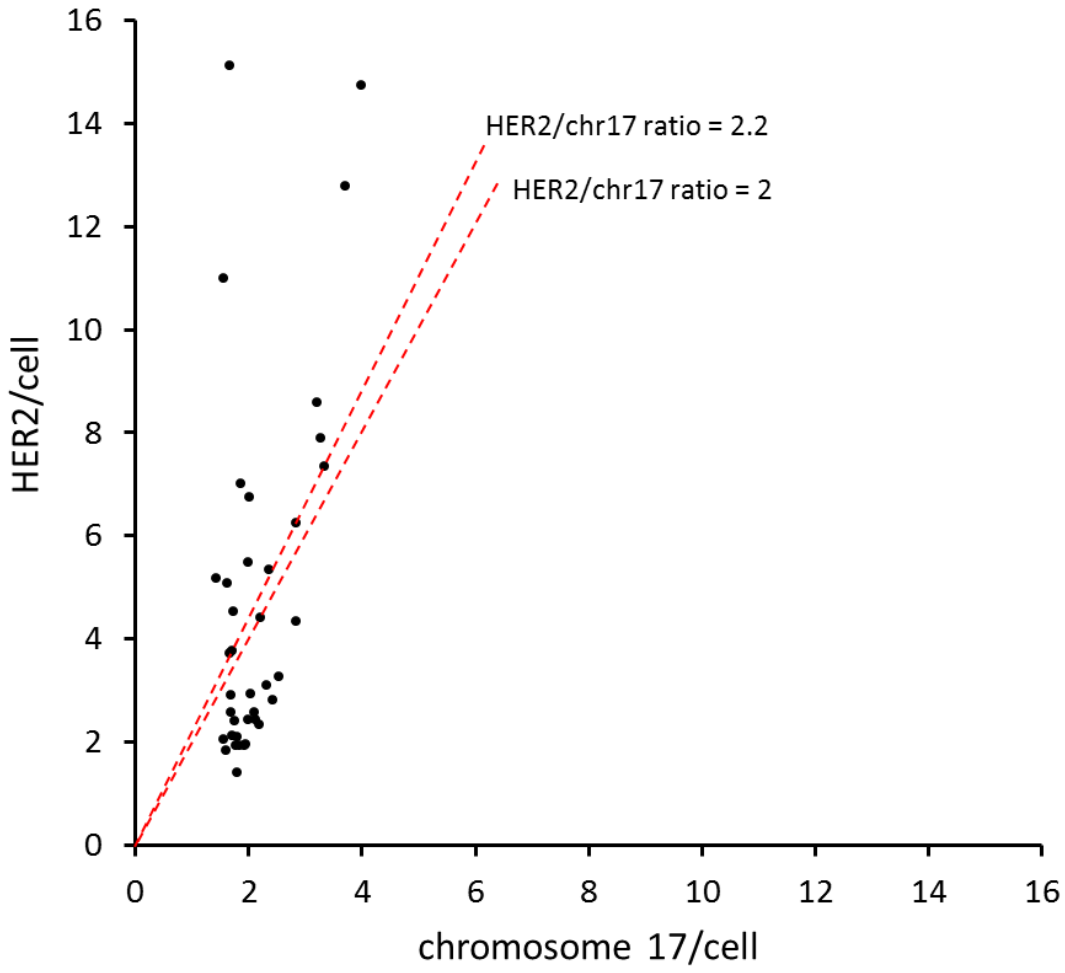

Figure 4.4: HER2 copy number/cell plotted against chromosome 17 copy number/cell in 39 determinations.
The two red dotted lines refer to HER2/chromosome 17 ratio cut-offs of 2 - adopted in this study - and 2.2 – as per Wolff *et al.* (2007)

As mentioned in Section 1.2.2, ISH standard cut-offs for HER2 amplification have been modified by recent guidelines and the currently accepted values are 2.2 for the HER2/chromosome 17 ratio and 6 for HER2 gene copy number/cell count (Wolff *et al.*, 2007). In this population, however, ISH ratio between 2 and 2.2 and HER2/cell count between 4 and 6 (which would have been regarded as 'equivocal' by current guidelines) were considered sufficient to define HER2 amplification. This was the case for six determinations when HER2/cell was chosen as the assay end-point (values ranging from 4.8 to 5.9) and for three determinations when the HER2/chromosome 17 ratio was considered (Figure 4.4).

HER2 determinations and patients

The number and sites (primary tumours or recurrences) of all HER2 determinations in the whole study population are reported in Table 4.6. Cases of patients with multiple determinations in the same sample or in different samples are included along with their matched results (concordant HER2-positive, concordant HER2-negative or discordant). Two cases in which the determination was performed more than once on a same tumour sample and gave discordant results are also reported (highlighted in red). This situation might have several possible explanations, including an error in HER2 determination, the effect of tumour heterogeneity and a change in HER2 status of the tumour due to anticancer treatments.
Categories 1, 2 and 3 describe different subsets of patients which take into account the presence of discordant determinations, the lack of HER2 determination in some samples and the sites of HER2 determination. Category 2, which include patients who had only one HER2 determination or multiple concordant determinations, was used to draw the graph in Figure 4.1 and was considered the most appropriate for the assessment of the potential effect of a SNP in the HER2 gene (see next sections).

Taken together, the data on HER2 determinations shown in Figure 4.1, Tables 4.4 and 4.5 show that there is a very high proportion of HER2-positive tumours in our population (slightly below 50%) compared with literature data (Slamon *et al.*, 1987). This enrichment in HER2-positive cases can be explained by the fact that part of the recruitment was conducted in a trastuzumab-dedicated clinic within the NCCC.

Number and site of HER2 determinations	No of patients	Notes	Category 1 All pts with unequivocal assessments	Category 2 Pts with single or concordant determinations	Category 3 Pts with single or concordant determinations (excluding pts with missing HER2 in one or more Ps)
1 on P	310		310	310	310
1 on R	18		18	18	18
2 on same P	4	4 pts concordant (3 HER2 neg, 1 HER2 pos); for 2 pts 1st determination pre-treatment, 2nd determination post neo-adjuvant treatment not including trastuzumab)	4	4	4
1 on axillary LN (occult P)	1		1	1	1
1 on R (or new P - uncertain)	1		1	1	1
2 on 2 Ps (synchronous)	11	7 pt concordant HER2 neg, 2 pt concordant HER2 pos, 2 pt discordant determinations	11	9	9
0 on 1st P, 1 on 2nd P (diachronous)	8		8	8	
1 on 1st P, 1 on 2nd P (diachronous)	3	1 pt concordant HER2 neg, 1 pt concordant HER2 pos, 1 pt discordant determinations	3	2	2
3 on 3 Ps (2nd and 3rd synchronous)	1	1st HER2 pos, 2nd HER2 neg, 3rd HER2 pos	1		
2 on 1st and 2nd P, 0 on 3rd (synchronous)	1	1st and 2nd concordant HER2 pos, 3rd HER2 not done	1	1	

Table 4.6 (continued on next page)

1 on 1st P, 1 on 2nd P (synchronous); 1 on 2nd P (repeated later)	1	both Ps concordant HER2 pos, later determination on 2nd P repeated after multiple treatments including trastuzumab: discordant with 1st determination (HER2 neg)			
0 on 1st P, 1 on 2nd P (synchronous) (performed later)	1	HER2 determined on one of the two Ps after failure of primary endocrine treatment	1	1	
0 on 1st P, 0 on 2nd P (diachronous), 1 on R	1		1	1	
0 on 1st P, 0 on 2nd P (or 1st R - uncertain determination), 0 on 2nd R, 2 on 3rd R	1	concordant HER2 pos (1st det pre-treatment, 2nd det post "neo-adjuvant" treatment including trastuzumab)	1	1	
0 on 1st P, 1 on 1st R, 1 on 2nd R	1	2 diachronous Rs, concordant determinations: HER2 neg	1	1	
1 on 1st P, 1 on R, 1 on 2nd P (diachronous)	1	two Ps and R, determinations all concordant: HER2 pos	1	1	1
1 on 1st P, 0 on 1st R, 1 on 2nd R	1	P and R, concordant determinations: HER2 pos	1	1	1
1 on P, 1 on R	1	discordant determinations: HER2 neg on P, HER2 pos on R	1		
1 on P, 3 on 1st R, 1 on 2nd R	1	P HER2 neg; 1st R: 1 determination HER2 pos, 2 determinations HER2 neg (tumour heterogeneity?); 2nd R HER2 neg			
Total	**367**		**365**	**360**	**347**

Table 4.6: HER2 determinations on patients.
The number and site of HER2 determinations, the presence of discordant determinations and the lack of HER2 determination in some samples were considered.
Legend: P = primary tumour; R = recurrence (loco-regional or distant); pt = patient

4.4 DNA extraction and quantification

The extraction of DNA from peripheral nucleated blood cells, performed as described in Section 2.5, was successful in all of the 361 patients with available blood samples. For each blood sample two different elution stocks of purified DNA were obtained (Table 4.7). The median concentrations were 197 ng/μl and 59 ng/μl, respectively.

		Eluate 1	**Eluate 2**
Total DNA samples (no.)		361	361
Concentration	**Median (ng/μl)**	197	59
	Mean (ng/μl)	199	69
	Range (ng/μl)	24 - 826	13 – 568
	CV (%)	44	62
260/280 ratio	**Median**	1.90	1.89
	Mean	1.90	1.89
	Range	1.77 - 2.04	1.62 - 2.06
	CV (%)	1.49	2.78
260/230 ratio	**Median**	2.32	2.14
	Mean	2.30	2.09
	Range	1.37 - 3.36	0.88 - 2.79
	CV (%)	8.78	14.16

Table 4.7: Characteristics of genomic DNA samples

With regard to DNA purity, the median ratio of absorbances at 260 nm vs 280 nm, which can be used to assess protein contamination (although with low sensitivity), was above the recommended cut-off of ~ 1.8. The 260/230 ratio, which may indicate contamination by polysaccharides, phenolate ions or thiocyanates, was also in the vast majority of cases above the 1.8 cut off. The 1st eluate stock showed the best purity ratios and was therefore used for the genotyping.

DNA was also successfully extracted according to the procedure described in Section 2.6 from the four cell lines (CCRF-CEM, K562, SKBR3 and MCF7) which were used as controls for the genotyping experiments.

4.5 Genotyping of HER2 SNPs in genomic DNA

The genotyping of the two SNPs in the HER2 gene, namely Ile655Val (rs1136201) and Ala1170Pro (rs1058808), was performed according to the method described in Section 2.9. It was always possible to allocate each individual patient sample to its distinct genotype by visual assessment. Two examples of allele discrimination plots from two representative experiments are reported in Figure 4.5.

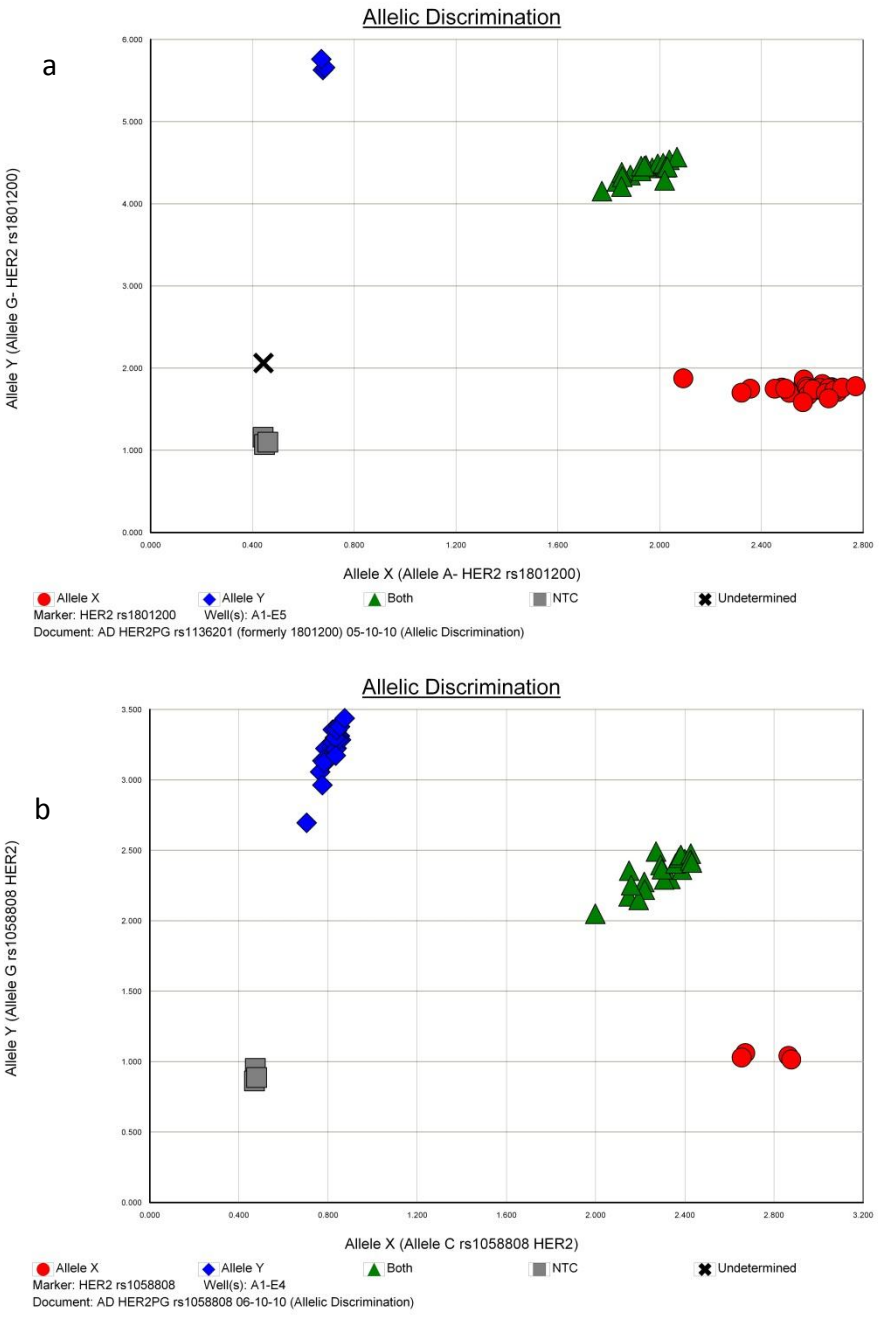

Figure 4.5: Allelic discrimination plots from two representative genotyping experiments; a) Ile655Val: A=Ile, G=Val; b) Ala1170Pro: C=Pro, G=Ala

Figure 4.6 shows the results of the genotyping of CCRF-CEM, K562, SKBR3 and MCF7 cell lines along with the 'no template control' values over ten consecutive experiments.

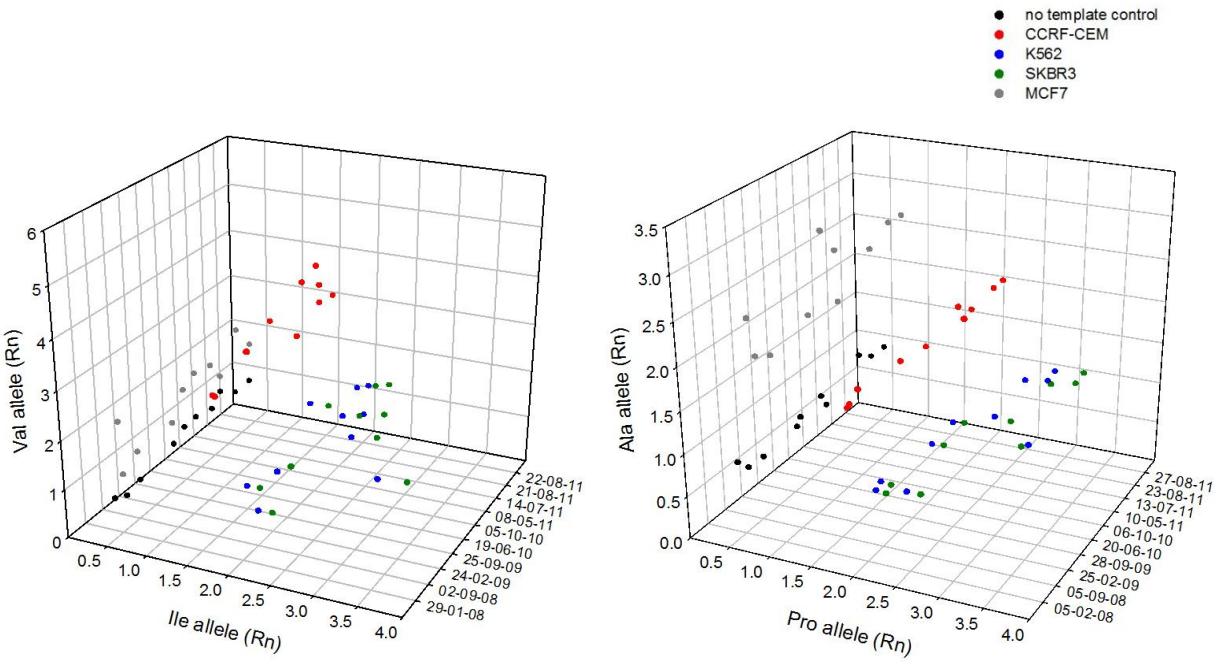

Figure 4.6: Genotyping of four cell lines for Ile655Val and Ala1170Pro across ten consecutive experiments

	Ile655Val						
	Mean (Rn)		**SD (Rn)**		**CV (%)**		**Genotype**
	Ile	Val	Ile	Val	Ile	Val	
ccrf-cem	1.29	2.70	0.32	0.65	25	24	**Ile/Val**
k562	1.81	0.49	0.38	0.33	21	68	**Ile/Ile**
skbr3	2.05	0.56	0.42	0.37	21	66	**Ile/Ile**
mcf7	0.01	0.84	0.04	0.42	633	50	**Undetermined (Val/Val?)**

	Ala1170Pro						
	Mean (Rn)		**SD (Rn)**		**CV (%)**		**Genotype**
	Pro	Ala	Pro	Ala	Pro	Ala	
ccrf-cem	1.49	1.08	0.26	0.22	17	20	**Ala/Pro**
k562	2.01	0.09	0.40	0.06	20	61	**Pro/Pro**
skbr3	2.19	0.10	0.42	0.05	19	55	**Pro/Pro**
mcf7	0.21	1.57	0.05	0.30	25	19	**Ala/Ala**

Table 4.8: Ile655Val and Ala1170Pro genotype.
Mean, standard deviation (SD) and coefficient of variation (CV) of Rn values after normalization to no template control of four cell lines across ten consecutive experiments

There was a degree of variation in the absolute Rn values across different experiments. These data were normalized to the ntc and the average values are shown in Table 4.8 along with standard deviation and coefficient of variation.

For some of the cell lines used as controls published genotypes for the two SNPs were available. The genotypes of SKBR3 for the Ile655Val SNP and of CCRF-CEM for both the HER2 SNPs were in accordance with published data (Tommasi *et al.*, 2004; Milani *et al.*, 2007). The genotyping of Ile655Val SNP in MCF7 cells showed no signal for the Ile allele and very low signal for the Val allele (Figure 4.6, Table 4.8), which caused an "undetermined" genotype call on the discrimination plot (Figure 4.5a). This phenomenon had been described and explained by Tommasi *et al.* (2004), who sequenced the region corresponding to the transmembrane portion of the receptor and observed that this cell line is Val homozygous for the Ile655Val SNP, but it also carries the extremely rare Val/Val variant of the adjacent Ile654Val SNP (rs1801201in the NCBI - SNP database) (NCBI_e, 2009), which may affect the alignment of the probe to the DNA sample and impair the PCR amplification.

The results of the genotyping experiments for the 361 patient genomic DNA samples were pooled after normalization to the ntc values and are shown in Figure 4.7. The genotyping of the four cell lines after normalization are also plotted in order to show that the dispersion of the results within the three genotype groups was comparable with the inter-assay variation. The distribution of genotypes for the two SNPs is shown in Table 4.9.

		No.	**%**	**MAF (%)**	**p for H-W**
	Ile/Ile	223	61.8		
Ile655Val	Ile/Val	124	34.3	21	0.21
	Val/Val	11	3.9		
	Ala/Ala	184	51		
Ala1170Pro	Ala/Pro	147	40.7	29	0.93
	Pro/Pro	30	8.3		
Tot		361	100		

Table 4.9: Distribution of genotypes for Ile655Val and Ala1170Pro SNPs in 361 patients.
H-W = Hardy-Weinberg equilibrium (p value for χ^2 test); MAF = minor allele frequency

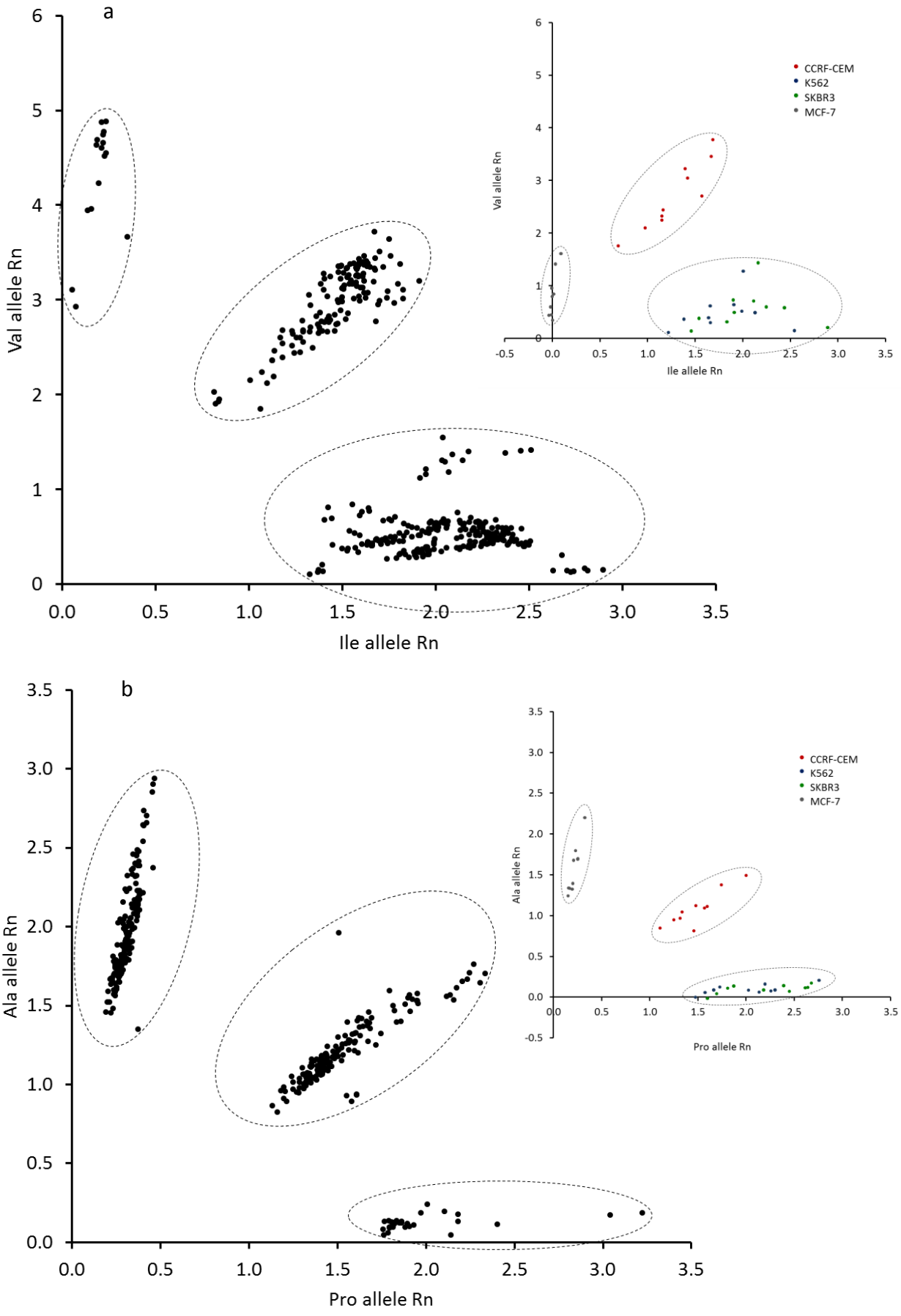

Figure 4.7: Pooled results of Ile655Val and Ala1170Pro genotyping experiments after normalization to ntc for 361 patients' genomic DNA samples; a) Ile655Val; b) Ala1170Pro

There was no significant deviation from Hardy-Weinberg equilibrium for both SNPs. The minor allele frequencies for Ile655Val and Ala1170Pro (respectively 21% and 29%) were comparable with published data in Caucasian populations (NCBI_a, 2009; NCBI_b, 2009).

The estimated haplotype frequencies and the linkage disequilibrium measures of the two SNPs were calculated using the exact solution to the cubic equation (CubeX) described by Gaunt *et al.* (2007) and the Multiallelic Interallelic Disequilibrium Analysis Software (MIDAS) (Gaunt *et al.*, 2006) and are shown in Table 4.10. The two parameters |D'| (linkage disequilibrium coefficient) and r^2 (correlation coefficient) are *both commonly used measures of Linkage Disequilibrium adjusted for the allele* frequencies. Whiles |D'| can range from 0 to 1 the upper limit of r^2 depends on the allele frequencies and is maximum (1) when alleles frequencies are equal. The lower these parameters the lower the likelihood of a non-random association of the alleles (Lewontin, 1964; Hill and Robertson, 1968).

The very low |D'| and r^2 values, together with the results of a classical χ^2 statistics comparing the observed and expected (according to the genotype frequencies in the whole population) diplotype frequencies (Table 4.11) suggest that the probability of a non-random associations of the alleles at the two loci is very low.

LD statistics according to MIDAS and CubeX

Haplotypes estimated frequency (%)		**|D'|**	**r^2**	**χ^2**	**p**
655Ile/1170Ala	56	0.072	0.0006	0.2	ns
655Ile/1170Pro	23				
655Val/1170Ala	15				
655Val/1170Pro	6				

Table 4.10: Linkage disequilibrium statistics for Ile655Val and Ala1170Pro SNPs in 361 patients |D'| = Linkage disequilibrium coefficient; r^2 = correlation coefficient. χ^2 and p values represent the significance of linkage disequilibrium; ns = not statistically significant. All values were calculated using MIDAS (Gaunt *et al.*, 2006) and Cubex (Gaunt *et al.*, 2007).

			Ala1170Pro			Total
			Ala/Ala	Pro/Ala	Pro/Pro	
Ile655Val	Ile/Ile	Count	112	91	20	223
		Expected Count	113.7	90.8	18.5	
	Ile/Val	Count	66	48	10	124
		Expected Count	63.2	50.5	10.3	
	Val/Val	Count	6	8	0	14
		Expected Count	7.1	5.7	1.2	
Total			184	147	30	361

p = 0.615

Table 4.11: χ^2 statistics comparing HER2 SNPs observed diplotype frequencies with expected frequencies calculated according to the observed genotypes in the whole population

4.6 HER2 SNPs and tumour characteristics

4.6.1 HER2 SNPs and HER2 expression

The set of patients with only one HER2 determination or with multiple (on different primaries or on primary and recurrence) and concordant (all HER2-positive or all HER2-negative) determinations (category 2 in Table 4.6) were evaluated with regard to the potential association between the HER2 SNPs and HER2 expression in tumours. The distribution of Ile655Val and Ala1170Pro genotypes are shown in Figure 4.8.
While the distributions of Ile655Val genotypes among HER2-positive patients and HER2-negative patients were very similar (Figure 4.8a), the distributions of Ala1170Pro genotypes stratified by HER2 status appeared to be different (Figure 4.8b).

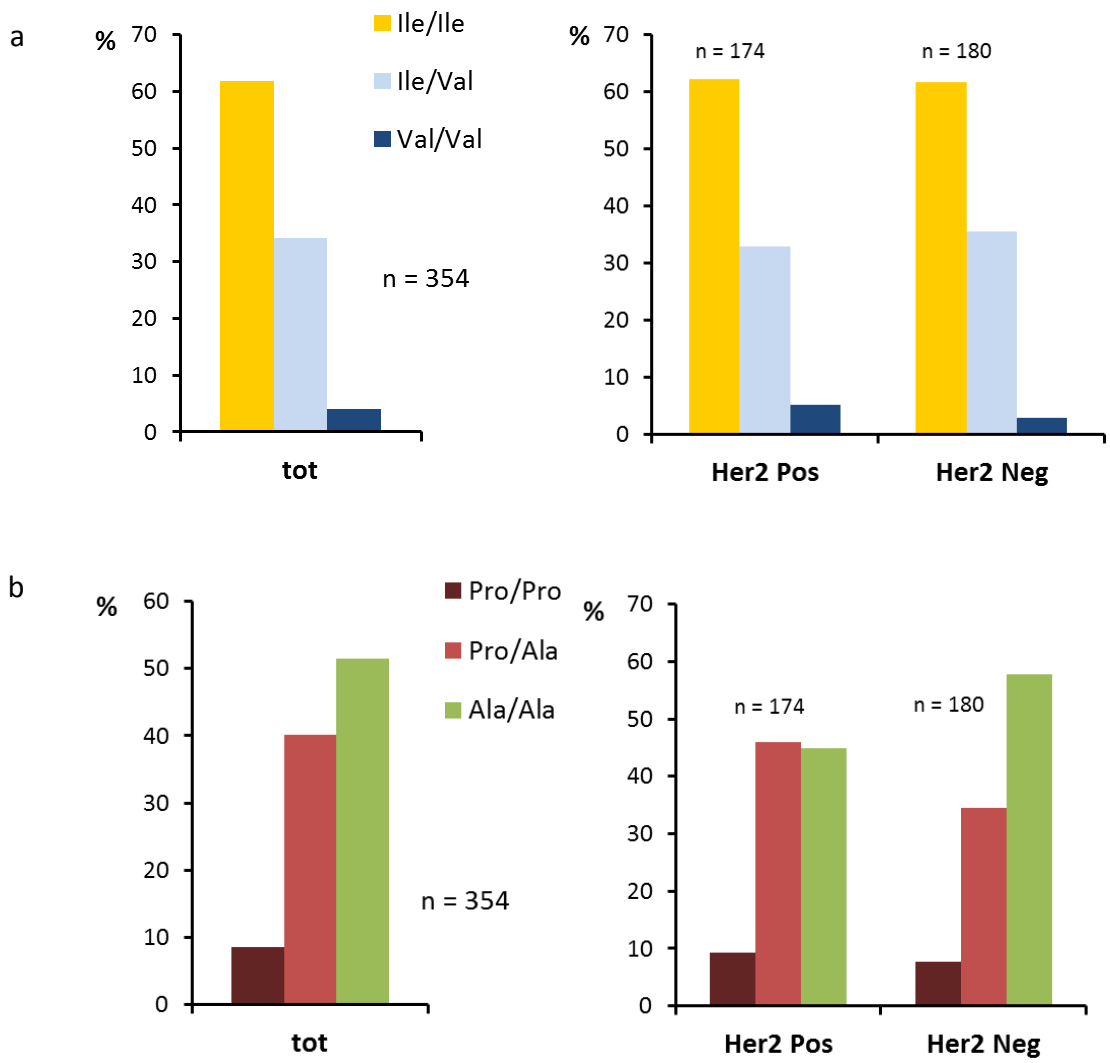

Figure 4.8: Distribution of Ile655Val genotypes (a) and Ala1170Pro genotypes (b) in the entire population and among HER2-positive and HER2-negative patients

When the carriers of minor allele and heterozygous patients were grouped together the proportions of HER2-positive tumours between Ile655Val Ile homozygous patients and carriers of the Val allele were exactly the same (49%, χ^2 statistics p value = 0.938) (Figure 4.9). In contrast, for the Ala1170Pro SNP the proportion of HER2-positive tumours was significantly higher among carriers of the Pro allele than it was among Ala homozygous patients (56% vs 43%, p = 0.015) (Figure 4.10).

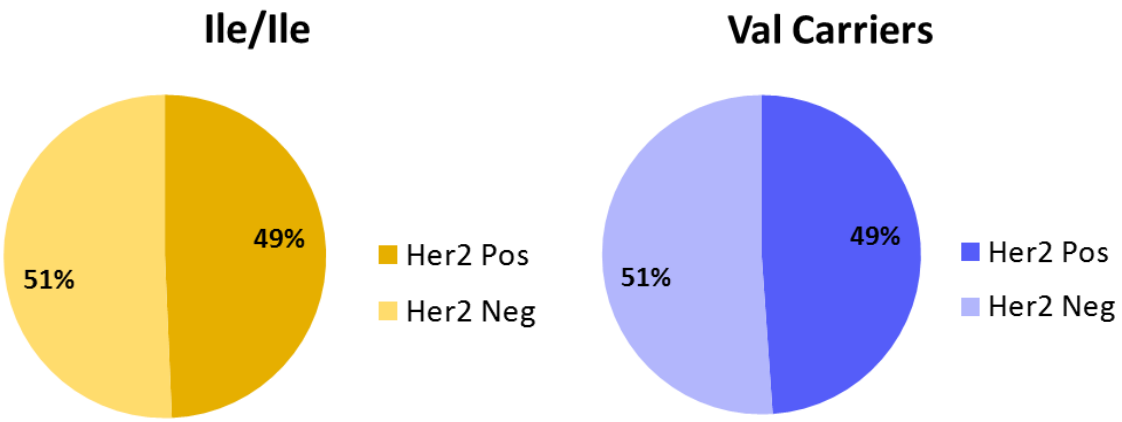

Figure 4.10: Distribution of HER2 status among Ala1170Pro Pro carriers and among Ala/Ala patients

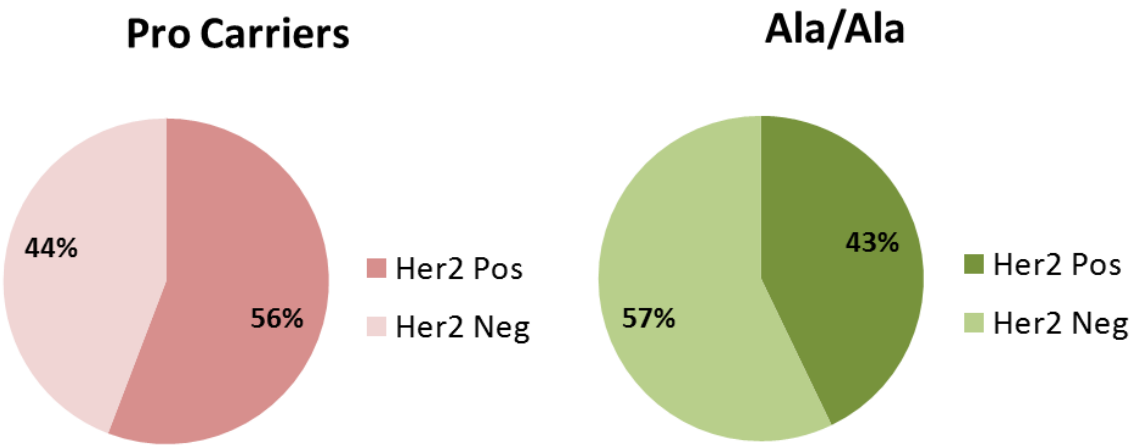

Figure 4.9: Distribution of HER2 status among Ile655Val Ile/Ile patients and among carriers of Val allele

The same statistical tests were repeated considering the other subsets of patients defined in Table 4.6 and the results are shown in Table 4.12.

As detailed in Table 4.6, for the HER2 status category 1, a patient was considered HER2-positive if she had at least one HER2-positive determination and was otherwise negative. For the HER2 status category 3, only patients with single or concordant determinations were included in the analysis and patients with missing HER2 in one or more primary tumours were excluded.

		HER2 status 1			HER2 status 2			HER2 status 3		
		Neg	**Pos**	**Total**	**Neg**	**Pos**	**Total**	**Neg**	**Pos**	**Total**
Total sample	no.	180	179	359	180	174	354	173	168	341
	%	50	50	100	51	49	100	51	49	100
Ile655Val										
Ile/Ile	no.	111	112	223	111	108	219	105	104	209
	%	50	50	100	51	49	100	50	50	100
Val	no.	69	67	136	69	66	135	68	64	132
	%	51	49	100	51	49	100	51	49	100
χ^2 significance - p		0.860			0.938			0.818		
Odds Ratio (95% CI)		0.962 (0.628 – 1.474)			0.938 (0.640 - 1.510)			0.950 (0.614 - 1.469)		
Ala1170Pro										
Ala/Ala	no.	104	78	182	104	78	182	100	76	176
	%	57	43	100	57	43	100	57	43	100
Pro	no.	76	101	177	76	96	172	73	92	165
	%	43	57	100	44	56	100	44	56	100
χ^2 significance - p		0.007			0.015			0.020		
Odds Ratio (95% CI)		1.772 (1.167 - 2.2692)			1.684 (1.106 - 2.564)			1.658 (1.081 - 2.545)		

Table 4.12: HER2 status in the whole population and stratified according to HER2 SNPs
HER2 status 1, 2 and 3 describe different, although widely overlapping, subsets of patients. This grouping takes into account the presence of discordant determinations, the lack of HER2 determination in some samples and the sites of HER2 determination (as explained in Section 4.3.1 and Table 4.6).

These data suggest that the Proline allele of the Ala1170Pro SNP in the HER2 gene (either in homozygous or heterozygous genotype) is associated with over-expression of HER2 in breast tumours.

Haplotype analysis

The distribution of the haplotypes within the HER2-positive and the HER2-negative subgroups was then estimated using Gaunt's model (2007). Although a global χ^2 test comparing the two distributions did not reach statistical significance, the 655Ile/1170Pro haplotype was more common in HER2-positive patients, whereas the 655Ile/1170Ala haplotype was more common in HER2-negative patients (Table 4.13).

	HER2 Status 2		
	Pos (%)	**Neg (%)**	**p***
655Ile/1170Ala	52.3	59.9	*0.039*
655Ile/1170Pro	26.2	19.6	*0.028*
655Val/1170Ala	15.5	15.1	0.638
655Val/1170Pro	6	5.4	0.575
		p** = 0.128	

Table 4.13: Distribution of haplotypes within HER2-positive and HER2-negative subgroups.
The presence of a possible unbalance in the distribution was tested with two χ^2 tests. The first test compared the prevalence of individual haplotypes within HER2-positive and HER2-negative groups (p*). The second test compares the overall distribution of the haplotypes within HER2-positive and HER2-negative groups (p**).

4.6.2 Other tumour characteristics and HER2 SNPs

The evaluation of other tumour characteristics (expression of hormone receptors and stage of the cancer at the date of consent) and their potential association with the HER2 SNPs are presented in the next three tables (4.14, 4.15 and 4.16). It must be highlighted that the total numbers of patients included in these analyses are variable and slightly lower compared to the analysis presented in the previous section. This is explained by the fact that patients with discordant hormone receptor assessment or 'weak' score (Table 4.3) were excluded, as well as patients with regional recurrence (but not distant recurrence) and patients with suspicion (but not definite diagnosis) of distant metastases.

Firstly the expression of hormone receptor was evaluated among HER2-negative and HER2-positive subgroups. The proportion of HR-positive patients among the HER2-positive subset was significantly lower (63.9%) than it was among HER2-negative patients (76.6%)(Table 4.14). This observation is in accordance with literature data (Lal *et al.*, 2005).

			Hormone Receptors *		
			Neg	**Pos**	**Total**
HER2 status 2	**Neg**	no.	41	134	175
		%	23.4	76.6	100
	Pos	no.	57	101	158
		%	36.1	63.9	100
Total		no.	98	235	333
χ^2 significance – p			0.011		
Odds Ratio (95% CI)			0.542 (0.336 - 1.874)		

Table 4.14: Hormone Receptor status stratified according to HER2 status.
* after exclusion of patients with discordant HR determinations and weak HR score

Secondly the tumour stage (early or advanced) of patients at date of consent was evaluated according to hormone receptor status and HER2 status (Table 4.15).

			Metastatic at date of consent **		
			No	**Yes**	**Total**
Hormone Receptors *	**Neg**	no.	89	5	94
		%	94.7	5.3	100
	Pos	no.	189	43	232
		%	81.5	18.5	100
Total		no.	278	48	326
χ^2 significance – p			0.002		
Odds Ratio (95% CI)			4.050 (1.551 - 10.574)		
HER2 status 2	**Neg**	no.	159	22	181
		%	87.8	12.2	100
	Pos	no.	138	26	164
		%	84.1	15.9	100
Total		no.	297	48	345
χ^2 significance – p			0.321		
Odds Ratio (95% CI)			1.326 (0.738 - 2.511)		

Table 4.15: Stage at date of consent stratified according to hormone receptor status and HER2 status.
* after exclusion of patients with discordant HR determinations and weak HR score
** after exclusion of patients with regional recurrence and suspicion of distant metastases

While advanced and early stage patients were well balanced for HER2 status, the proportion of advanced patients among HR-negative patients was significantly lower than it was among HR-positive patients. The relatively low proportion of HR-negative advanced stage patients may be at least in part explained by the fact that the study screening strategy included not only newly-diagnosed patients but also all patients with known HER2 status on follow-up in the breast clinic. This implies that the HR-negative advanced stage patients, who are known to have much worse prognosis compared with the HR-positive advanced stage patients, had much fewer follow-up visits, hence fewer opportunities to be offered study participation.
When hormone receptor status and stage at date of consent were stratified according to the two HER2 SNPs, no significant association emerged (Table 4.16).

			Hormone Receptors *		**Metastatic at date of consent ****		
			Neg	Pos	Total	No	Yes	Total
Total sample		no.	98	233	331	296	50	346
		%	29.6	70.4	100	85.5	14.5	100
Ile655Val	**Ile/Ile**	no.	54	146	200	182	33	215
		%	27	73	100	84.7	15.3	100
	Val	no.	44	87	131	114	17	131
		%	33.6	66.4	100	87	13	100
χ^2 significance – p			0.199			0.543		
Odds Ratio (95% CI)			0.731 (0.453 - 1.180)			0.822 (0.438 - 1.545)		
Ala1170Pro	**Ala/Ala**	no.	49	118	167	144	25	169
		%	29.3	70.7	100	85.2	14.8	100
	Pro	no.	49	115	164	152	25	177
		%	29.9	70.1	100	85.9	14.1	100
χ^2 significance – p			0.915			0.860		
Odds Ratio (95% CI)			0.975 (0.608 - 1.562)			0.947 (0.520 - 1.725)		

Table 4.16: Hormone Receptor status and stage at date of consent stratified according to the HER2 SNPs
* after exclusion of patients with discordant HR determinations and weak HR score
** after exclusion of patients with regional recurrence and suspicion of distant metastases

4.7 Discussion

The aim of this investigation was to evaluate the potential influence of two common coding SNPs on HER2 expression in breast tumours. The target sample size of 500 - 700 patients, calculated on the basis of an expected HER2-positivity rate of 20% (Section 2.11), was not achieved. However, the observed significant enrichment in HER2-positive cases made possible the planned analyses in a substantially less numerous population (367 patients) with a similar statistical power.

While several studies (summarised in Section 1.2.4) reported conflicting data on the possible role of the Ile655Val SNP, one publication (Han *et al.*, 2005) showed that in Korean women carrying the 655Ile/1170Pro haplotype (the most common combination in that population) the likelihood of HER2 overexpression was 1.5 to 1.8 times higher than it was in the rest of the patients. These authors, taking into account the results of the previous publications, suggested that polymorphisms other than Ile655Val (including Ala1170Pro) "may be associated with HER2 protein expression and breast cancer progression". Our analysis in a population composed of almost only Caucasian individuals seems to be in accordance with these observations, showing that carriers of the Pro allele in the Ala1170Pro SNP have $\sim$ 1.7 times higher likelihood of having a HER2-positive tumour.

Our haplotype analysis showed that the two SNPs are not likely to be in linkage disequilibrium, consistent with previous studies (Benusiglio *et al.*, 2005; Han *et al.*, 2005) and confirms a possible role for the Pro allele of the Ala1170Pro SNP or for other variants not considered in the study which may be associated with this SNP.

These results are reinforced by the fact that the SNP genotypes were well balanced for stage at date of consent and hormone receptor status of patients, suggesting that the potential effect of the SNPs on HER2 expression was not confounded by these two fundamental clinical/biological factors.

However, our study, does present several limitations. Firstly, our study population has a relatively low proportion of HR-negative advanced patients (Section 4.6.2) and this might limit the generalizability of the results to all breast cancer patients.

Another caveat come from the absence of a matched control population of women without breast cancer. However, the distribution of genotypes for the Ile655Val SNP was comparable with published data in the NCBI database and with the abundant

literature data in Caucasian populations (NCBI_a, 2009; Tao *et al.*, 2009; Lu *et al.*, 2010; Dahabreh and Murray, 2011; Ma *et al.*, 2011). For the Ala1170Pro SNP a more limited amount of data is available (Benusiglio *et al.*, 2005; Benz *et al.*, 2006; Tommasi *et al.*, 2007; NCBI_b, 2009), but the published genotype frequencies appear similar to the ones observed in our population (Table 4.17).

			%		no.	From
		Pro/Pro	Ala/Pro	Ala/Ala		
HER2PG study	Whole pop of breast cancer patients	8	40	51	361	UK (Caucasian 97.5%)
	HER2 neg	8	34	58	180	
	HER2 pos	9	46	45	174	
Benusiglio et al. (2005)	Cases (breast cancer pts)	12	43	45	2014	UK
	Controls	11	45	44	2181	
Benz et al. (2006)	Cases (breast cancer pts) (HER2 neg?)	11	42	47	625	Italy
	Cases (breast cancer pts) (HER2 pos)	14	47	39	48	USA (Caucasian)
	Controls	17	48	35	46	USA (Caucasian)
	Controls	6	22	72	30	USA (African-American)
Tommasi et al. (2007)	Cases (breast cancer pts)	11	42	47	628	Italy
	Controls	13	48	39	169	
NCBI	Pop 1	8	54	38	24	European descent
	Pop 2	5	48	47	60	European descent

Table 4.17: HER2 Ala1170Pro SNP genotype frequencies in the current study (HER2 PG) compared with published available data

Finally, as mentioned in Section 4.3.1, the cut-offs for ISH and IHC determination of HER2 status in tumours were not always the ones adopted in the current guidelines

and possibly varied over the time-course of the study. Moreover, HER2 status was determined by a number of different pathologists over time, the details of both IHC and ISH assessments were not always available in the pathology reports and the determinations of HER2 amplification status in tumours was performed using different methods over time (FISH and SISH). This lack of uniformity may diminish the validity of our analysis, although there was no obvious indication that it might have biased the genotype effects observed.

The potential effects of SNPs on the pathogenesis of breast cancer have been extensively evaluated by many cooperative research groups using both candidate gene approaches and genome-wide association studies (GWAS). The most recent investigations, however, aim to ascertain the differential effect of genetic variants on the risk of developing particular types of breast cancer, such as the 'triple-negative' subset or the HR-positive and the HR-negative subsets (Broeks *et al.*, 2011; Figueroa *et al.*, 2011; Stevens *et al.*, 2011). Our findings seem to be consistent with the heterogeneous biological origin of the different breast cancer subtypes.

The biological explanation of the effect of the Ala1170Pro SNP observed in this population remains unclear. One possible mechanism which could explain the different distribution of the genotypes within HER2-positive and HER2-negative tumours is the selection of one of the two alleles which may bear a higher oncogenic potential ('allele specific amplification') (LaFramboise *et al.*, 2005). This issue, together with the hypotheses on the potential role of the 1170 residue in the HER2 protein, will be discussed in Chapter Five.

Chapter 5. Results III: Evaluation of breast tumour samples

5.1 Overview

The observation that the Pro allele of the HER2 Ala1170Pro SNP might be associated with HER2 amplification/overexpression in breast cancer (Chapter 4) prompted us to evaluate the HER2 SNPs in tumour DNA. After describing the rationale behind this investigation, this chapter presents the results of the tumour collection process, including the pathological features of the samples collected. The findings of Laser Capture Microdissection (LCM) and DNA extraction are described in the following sections. The final part of the chapter illustrates the results and possible limitations of the tumour DNA genotyping, followed by the interpretation of these findings in the context of the relevant literature.

5.2 Hypothesis

In order to investigate the potential role of the Ala1170Pro SNP in the development of HER2-positive breast cancers, the role of 'preferential allelic amplification' (or 'allele-specific amplification') was evaluated.

This phenomenon, consisting in the amplification in the tumour of only one of two possible alleles of a particular locus present in the germ-line DNA, has been described in the context of several cancers. It is proposed that one allele (the 'pathogenic' variant) can undergo a positive selection through the copy number gain process associated with tumour evolution (LaFramboise *et al.*, 2010).

Therefore, in a setting of patients with HER2-amplified breast cancer who are heterozygous for one HER2 SNP we could hypothesize that if the SNP has no influence on the development of the tumour, the two alleles will have the same chance of being amplified, so that 50% of the tumours will have one allele amplified and 50% of the tumours will have the other allele (Figure 5.1a). On the contrary, if there is a selective pressure on one of the two alleles, the majority of the tumours will have this allele amplified (Figure 5.1b).

In order to test this initial hypothesis a set of experiments aiming at the assessment of the HER2 SNPs in tumour samples was planned.

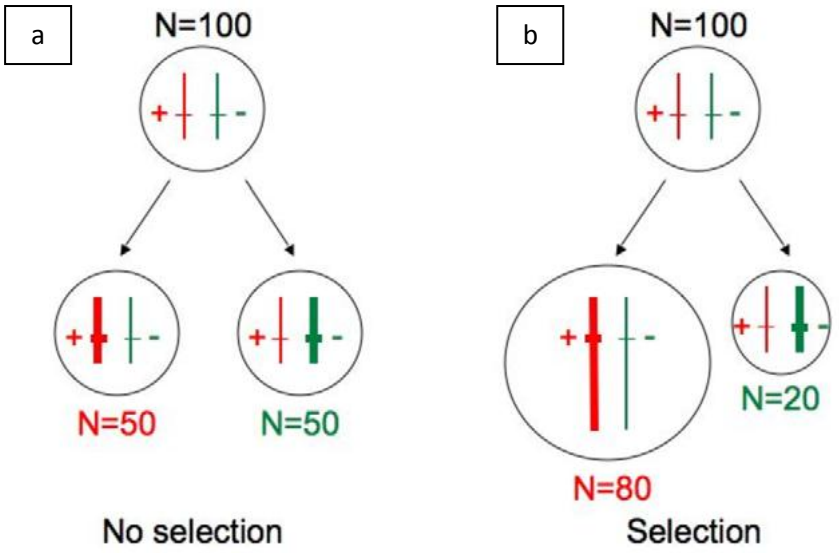

Figure 5.1: Rationale for allele-specific amplification in tumours.
a) When there is no selection the two alleles are equally represented among the tumours; b) when one of the alleles ('+', in red) is pathogenic it undergoes selective pressure and will be preferentially amplified in the tumours. Modified from LaFramboise *et al.* (2010).

5.3 Tumour samples collected

All the 367 patients enrolled in the study consented to the genetic analysis of the breast cancer samples taken at the time of their diagnosis or surgery. These specimens, in the form of FFPE blocks, were therefore requested from the Hospital Pathology Department of the Newcastle upon Tyne Hospitals NHS Foundation Trust. Two hundred forty nine tumour samples from 241 patients (2/3 of total) were made available to our laboratory (in 8 cases two blocks per patient were received). In the remaining one third of cases the tumour block was not available either because the patient had surgery in a different hospital or the block was prepared more than 10 years previously and/or was not easily accessible in the Pathology archive (Table 5.1). The pathological characteristics of the collected tumour samples from which it was possible to extract DNA (see Section 5.4) are also shown in Table 5.1. They were very comparable with the characteristics of the tumours in the whole population of 367

patients (presented in Section 4.3) in terms of HER2-status, hormone receptor expression, histologic grade, histologic subtype and lymph node involvement.

Breast cancer samples	**Number**	**%**
Total number of blocks received	249	
Number of patients	241	
Double Blocks	8	
Inadequate tumour material on histological evaluation	7	
Poorly processed blocks (LCM not performed or unsuccessful)	11	
Microdissected invasive tumour samples available for DNA extraction	**223**	**100**
Block from primary tumour	206	92
Block from lymph node metastasis	18 *	8
HER2-positive	109	49
HER2-negative	114	51
Hormone Receptors Positive	147	66
Hormone Receptors Negative	66	30
Hormone Receptors Low	10	4
Ductal Invasive	181	81
Lobular Invasive	14	6
Mixed or others	28	13
Lymph node negative	93	42
Lymph node positive	121	54
Distant metastases	9	4
Histologic grade 1	7	3
Histologic grade 2	80	36
Histologic grade 3	131	59
Not assessable	5	2
Microdissected normal breast tissue samples	**23**	
matched tumour sample	23	
HER2-positive	13	
HER2-negative	10	
Homozygous for both HER2 SNPs in germ-line DNA	4	
Heterozygous for at least one HER2 SNP in germ-line DNA	19	
Microdissected In Situ tumour samples	**9**	
matched tumour sample	9	
matched normal and tumour sample	4	
HER2-positive	4	
HER2-negative	5	
Homozygous for both HER2 SNPs in germ-line DNA	2 **	
Heterozygous for at least one HER2 SNP in germ-line DNA	6 **	

Table 5.1: Characteristics of breast cancer samples received from the Pathology Department
*: for one patient, two samples were isolated, one from primary and one from metastatic tumour
**: for one sample germ-line DNA was not available

5.4 Laser Capture Microdissection

In order to isolate only tumour cells for the genetic analysis the Laser Capture Microdissection (LCM) method was used (Section 2.7). An initial histological evaluation on Hematoxylin and Eosin (H&E) stained sections prepared from the available blocks was performed as described in Section 2.7.1 in order to exclude those samples with inadequate material which were unsuitable for further processing.

Out of the 241 blocks, 234 cases were considered to have adequate material for tumour sample isolation. Seven blocks were excluded due to inadequate invasive tumour area (number of tumour cells on examined section < 300) (Table 5.1).

LCM was then conducted according to the procedures described in Sections 2.7.2 and 2.7.3. The tumour cell density in captured fragments was > 90% in most samples. Inflammatory cells, collagen and other admixtures (i.e. mucin), were avoided wherever possible (Figures 5.2, 5.3 and 5.4).

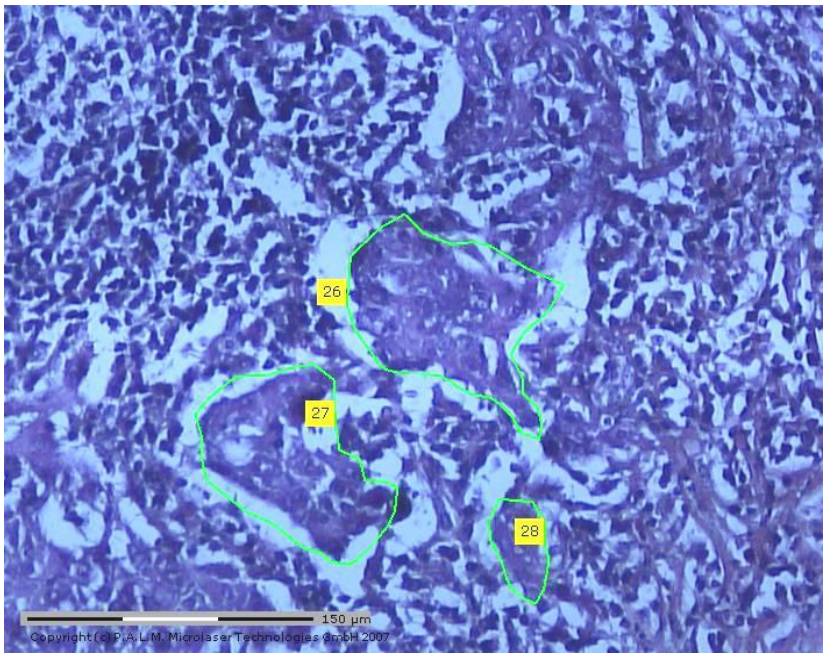

Figure 5.2: Ductal invasive carcinoma with heavy lymphocytic infiltrations
(Marked areas: captured elements)

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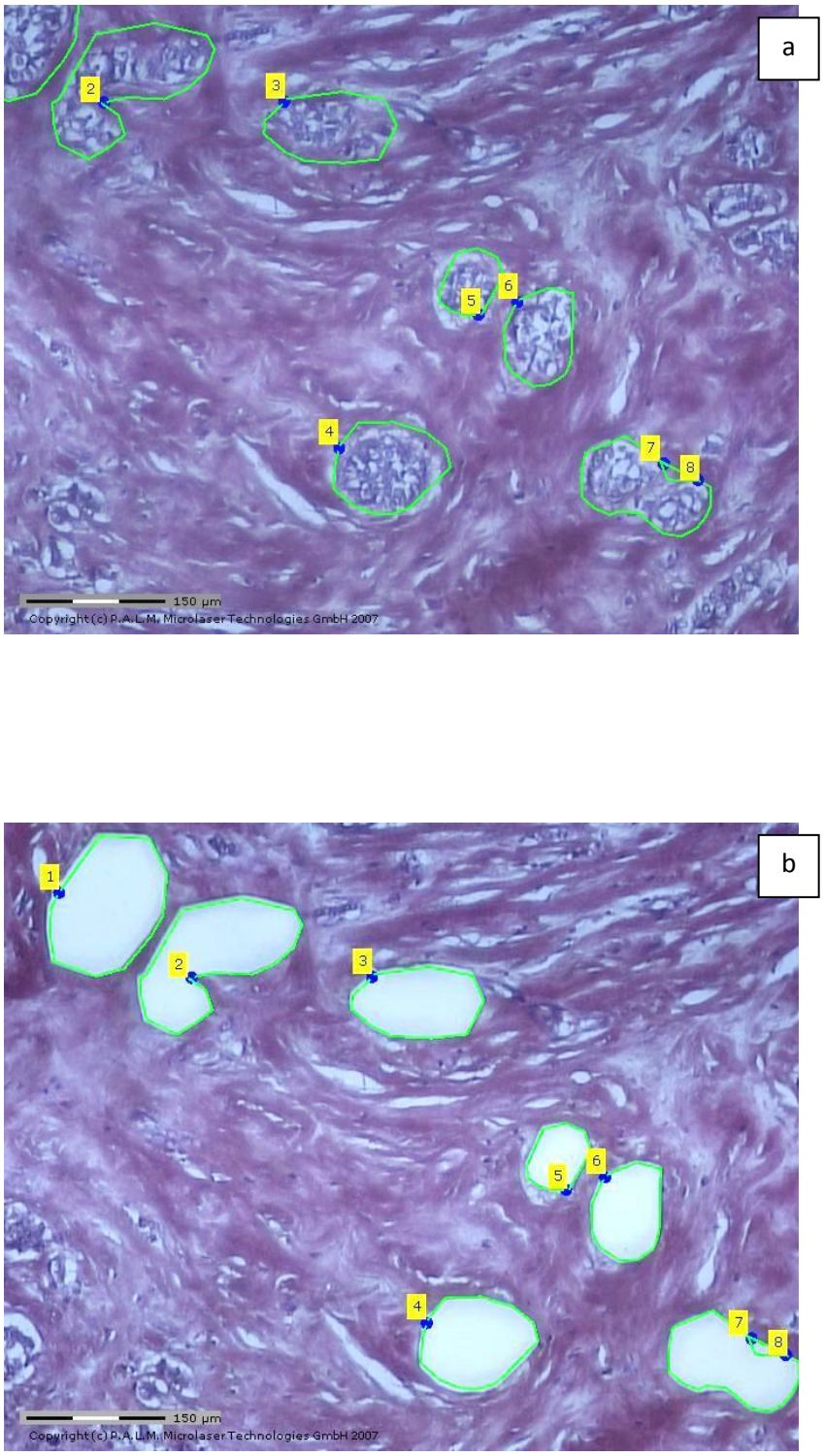

Figure 5.3: Ductal invasive carcinoma with abundant collagen.
Collagen could be avoided by LCM (green circles: captured elements). a): before LCM; b): after LCM

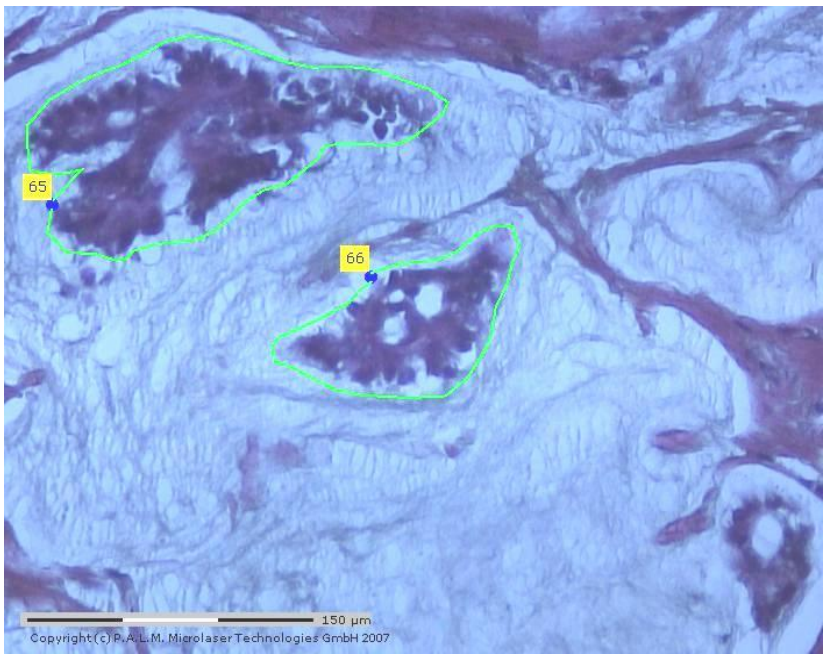

Figure 5.4: Well differentiated mucinous adenocarcinoma.
Mucus was not included in the extracted sample.

When it was difficult to completely avoid inflammatory cells due to heavy infiltrations, this was stated and taken into account (8 cases) (Figure 5.5).

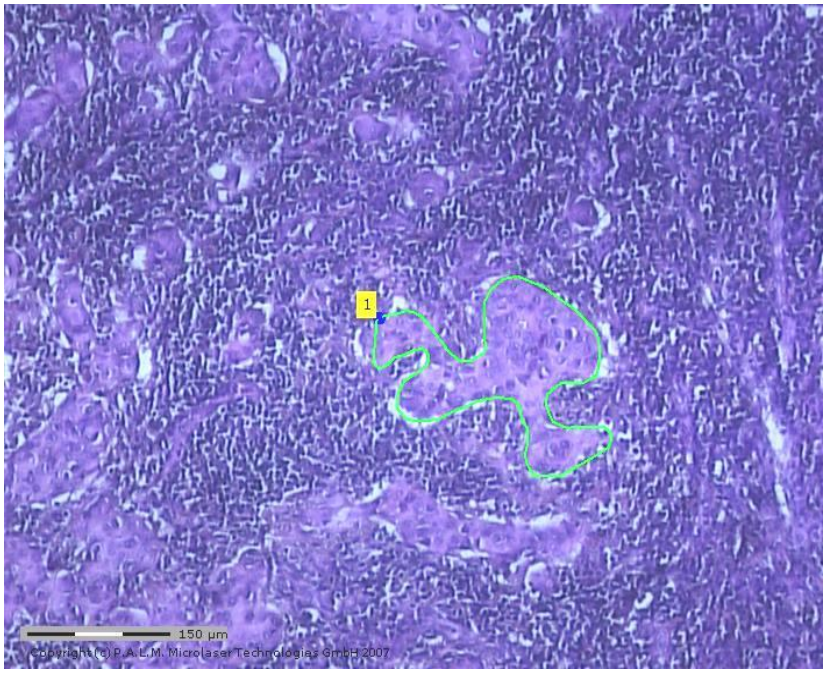

Figure 5.5: Ductal invasive carcinoma, metastatic to lymph node.
In this case, lymphocytes could not be completely avoided (marked area).

Since DNA samples were generated from a limited number of cells in each tumour, every effort was made to obtain captured elements as representative of the whole tumour sections as possible on a morphological level, in order to minimize the effect of tumour heterogeneity. The selection of elements was based on the initial histological evaluation of the available blocks and on whole section scanning during the LCM procedure.

In lobular carcinomas the dissection was conducted when there were areas that included at least 40 - 50% of tumour cells or when cell aggregates of considerable size (> 100 μm^2) were recognized (Figures 5.6 and 5.7). Collagen was avoided wherever possible. The percentage of tumour cells in the captured area was documented for each case.

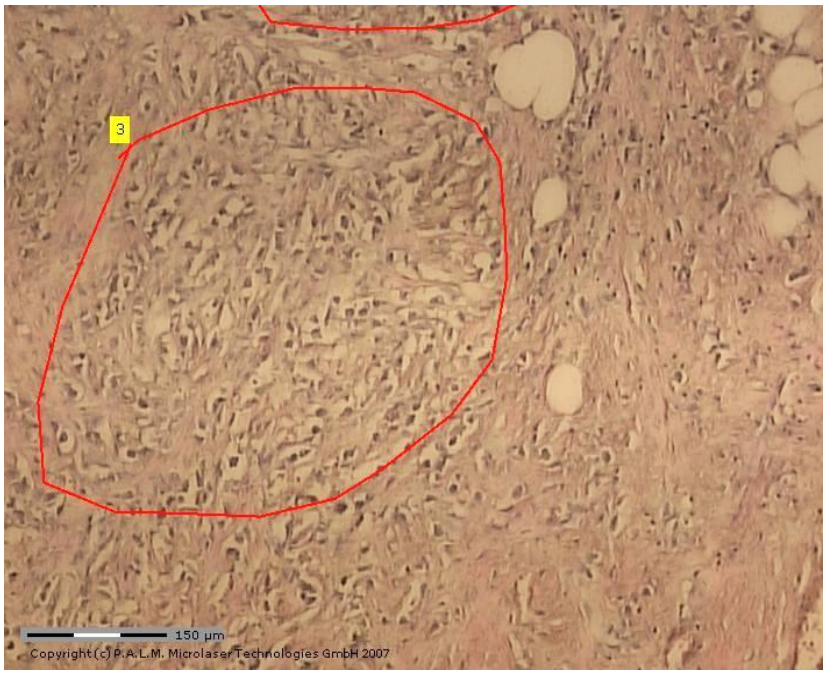

Figure 5.6: Lobular invasive carcinoma.
Areas with relatively high tumour cell density (in this case 50-60%) were selected for dissection.

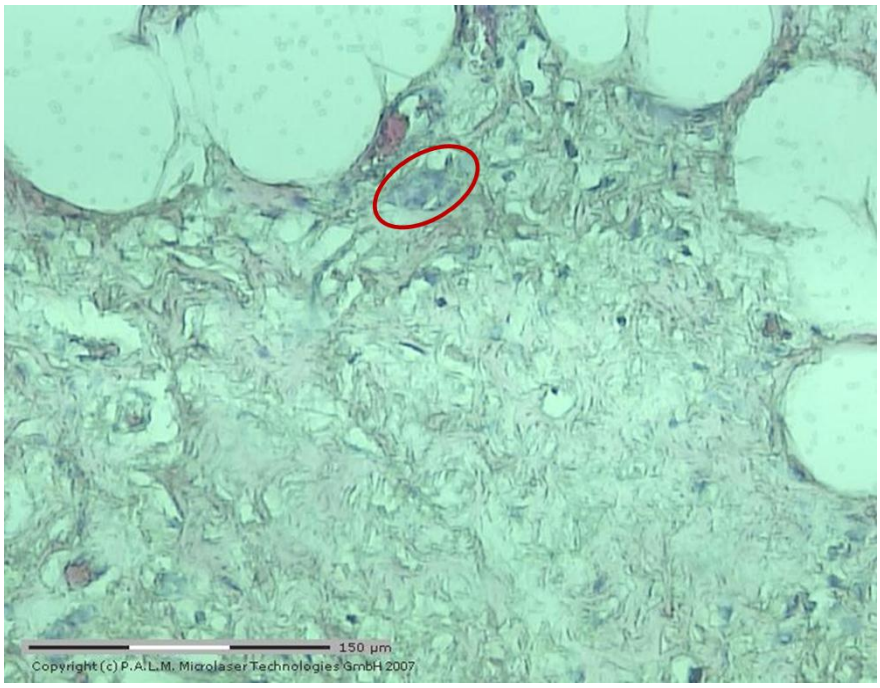

Figure 5.7: Lobular carcinoma with very scarce tumour cells (circle), inadequate for LCM.

In addition to invasive tumour samples, 23 normal samples were isolated from areas adjacent to the invasive component. These included whole breast lobules (epithelial cells of acini and terminal ducts, myoepithelial cells) and epithelial cells of larger ducts, wherever possible (Figure 5.8). Lesions with epithelial hyperplasia were not included in normal samples.

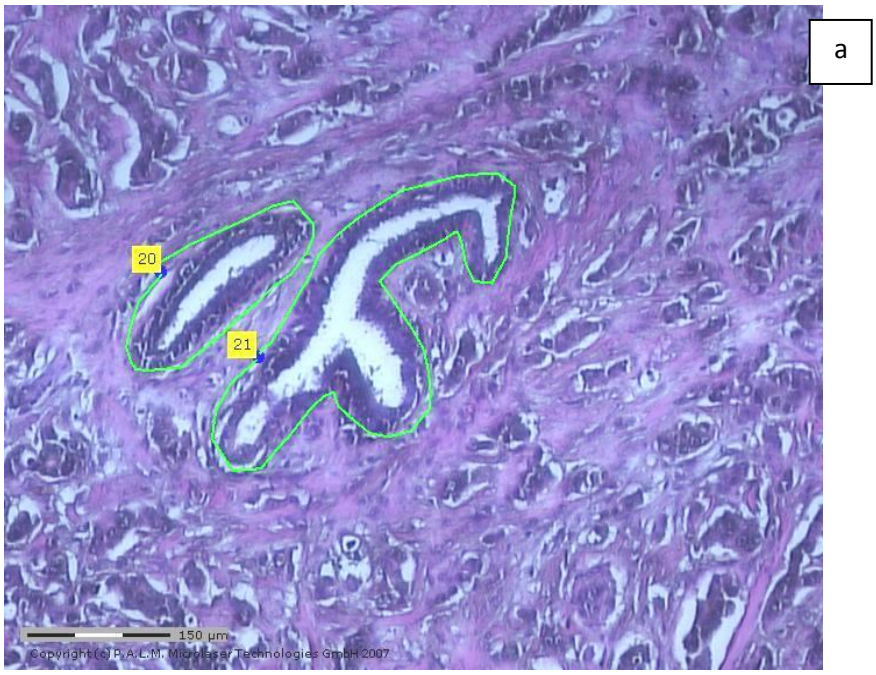

Figure 5.8: a) Normal ducts, surrounded by invasive ductal carcinoma. Normal elements were collected in a separate sample.

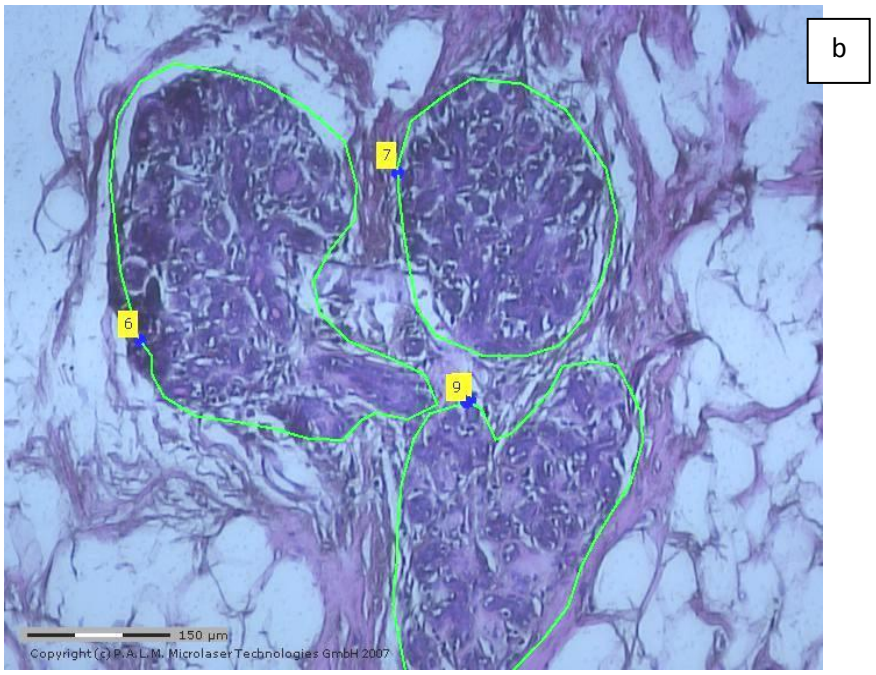

Figure 5.8: b) Normal breast lobules.

Nine separate In Situ samples were also isolated. In all cases they were Ductal Carcinomas In Situ (DCIS) of low, intermediate or high grade and included exclusively

epithelial cells. Necrosis, in case of high-grade DCIS, was dissected and removed from the section prior to the capture of the desired area (Figure 5.9).

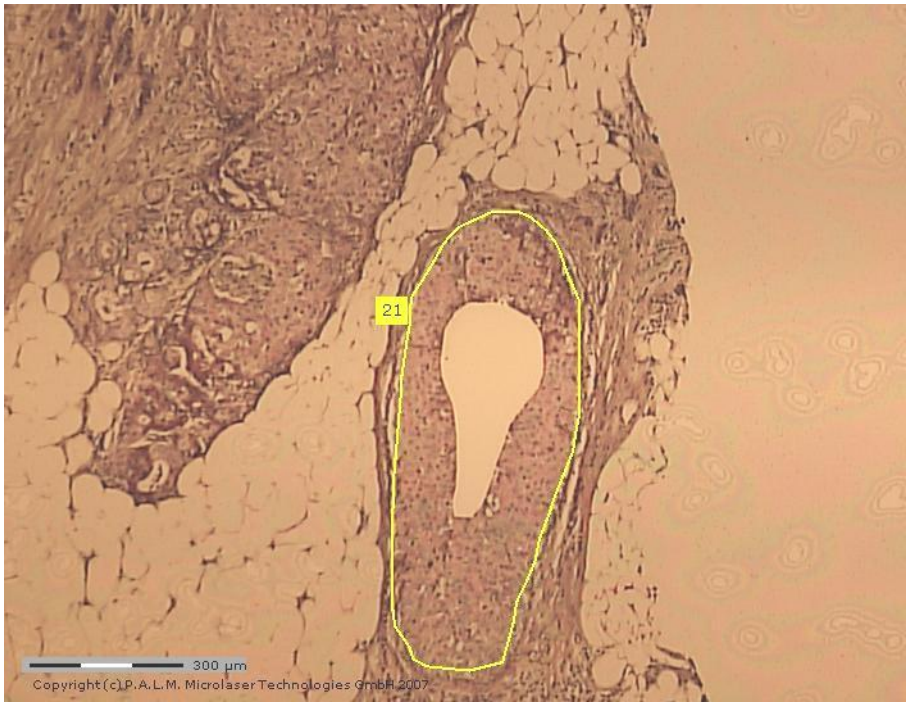

Figure 5.9: High grade Ductal Carcinoma In Situ.
The area to be captured is marked in yellow. Central necrotic area has been removed in advance.

Normal and In Situ samples were selected preferentially in patients that were heterozygous for one or both SNPs investigated (Table 5.1).
Furthermore, one lymph node metastasis sample was extracted separately from the primary tumour.
Eleven of all the submitted blocks with available material were poorly processed during slide preparation (tumour area missing from slide) and/or the LCM procedure (tumour area detached) and a DNA sample could not be extracted.
The numbers of invasive tumour, normal and In Situ samples dissected are summarized in Table 5.1.

5.5 DNA extraction

The extraction of DNA from microdissected tissue was performed as described in Section 2.8. The median DNA concentration (21.9 ng/µl) was, as expected, significantly

lower than the concentrations obtained from peripheral nucleated blood cells (Section 4.4), reflecting the lower number of cells in the samples (Table 5.2). This fact, together with the small elution volume (20 - 25 μl) obtained, is an obvious limitation to the number of genetic analyses that can be performed with the same DNA sample.

Total DNA samples (number)		**223**
Concentration (ng/μl)	Median	21.9
	Range	1.2 - 82.7
260/280 ratio	Median	3.18
	Range	0.67 - 6.27
260/230 ratio	Median	1.01
	Range	0.18 - 2.69

Table 5.2: Characteristics of DNA samples extracted from microdissected tissue

The individual Nanodrop measurements, however, were interpreted with caution, as the DNA purity data (260/280 and 260/230 ratios median and range values shown in Table 5.2) suggested poorer quality compared with the extracted germ-line DNA (Section 4.4).

The concentration and purity measures did not seem to correlate with the numbers of dissected cells in each sample. These data might be attributed, at least in part, to interference from the PEN membrane that is captured together with the tumour fragments during the LCM procedure.

5.6 Genotyping

Similarly to the genotyping of the germ-line DNA samples, the genotyping of the HER2 Ile655Val and Ala1170Pro SNPs in DNA extracted from microdissected tissue was performed using the TaqMan assay described in Section 2.9. Samples were pre-diluted to the concentration of 10 ng/μl in order to achieve maximum uniformity. Samples at concentrations below 10 ng/μl were used undiluted.

Unlike the genomic DNA genotyping, the allocation of the tumour DNA samples to their distinct genotype by visual assessment was not always immediately feasible (Figure 5.10). This was due to a combination of different causes.

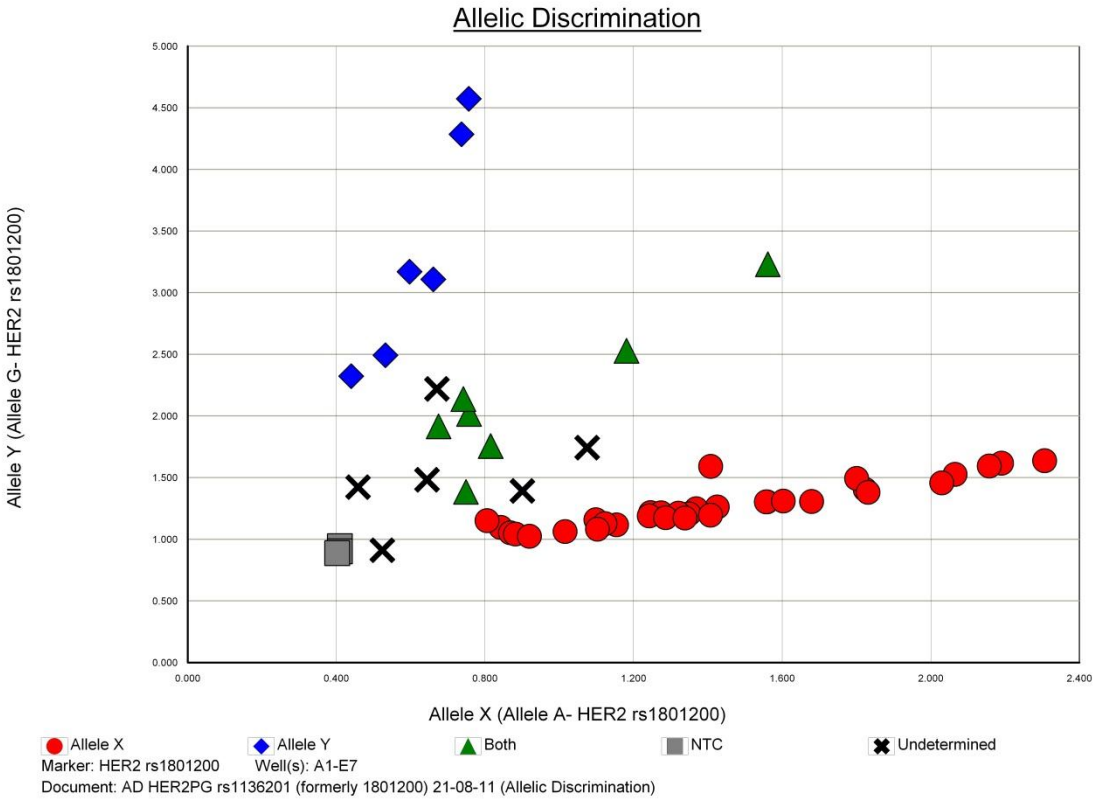

Figure 5.10: Allelic discrimination plot from one representative genotyping experiment for the HER2 Ile655Val SNP.
A=Ile, G=Val.

Firstly, a peculiar amplification pattern was expected in the case of HER2-amplified tumours arising in a heterozygous germ-line background. Given that one of the two alleles is present in a higher copy number in these samples, it was expected to observe a higher fluorescence signal for the amplified allele and a lower signal generated by the non-amplified allele. This situation would in turn result in a point in the allelic discrimination plot which would be closer to the homozygous X or Y clusters the higher the copy number of the amplified HER2 allele (black spots in Figure 5.11). It was therefore necessary to establish an appropriate cut-off which would allow definition of the sample as homozygous or heterozygous.

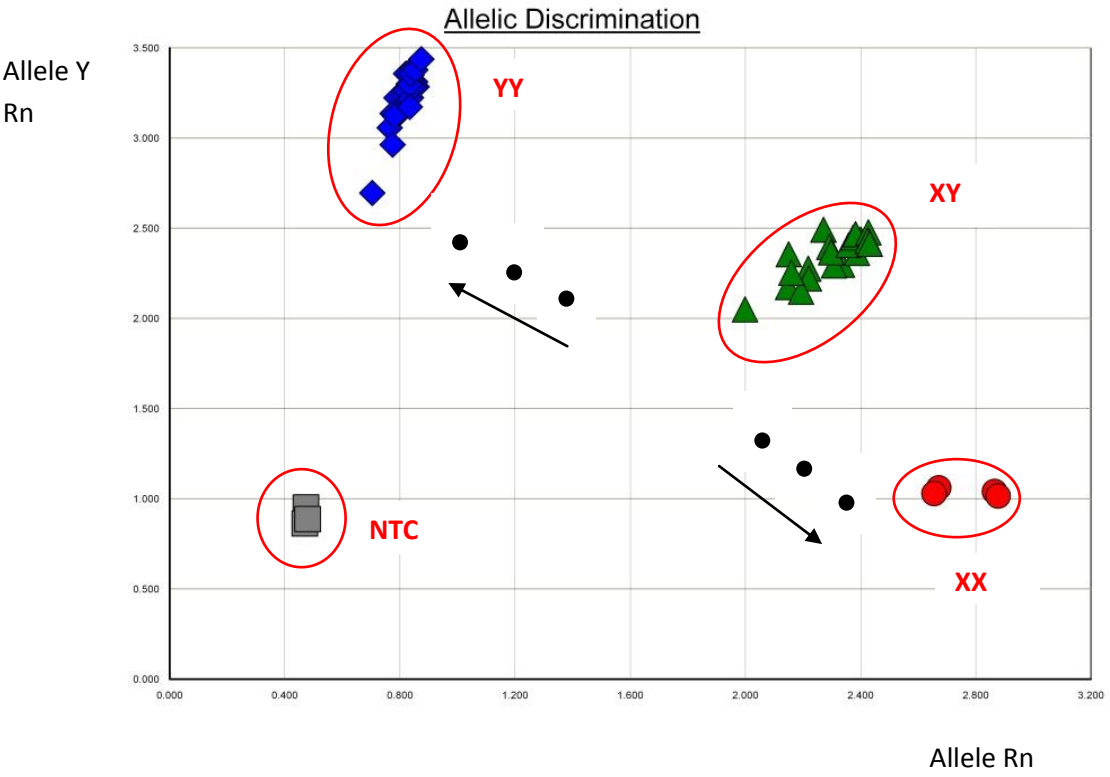

Figure 5.11: Example of Allelic Discrimination Plot.
The black spots represent hypothetical DNA samples from HER2-amplified tumours arising in a heterozygous germ-line background. The spots will be closer to the homozygous clusters the higher the copy number of the amplified HER2 allele.

Additionally, technical issues could affect the results of the PCR reaction. In particular, the DNA purity values of these samples were overall significantly lower and more variable than they were among the genomic samples, leading to a lower reliability of the concentration measurements and, in turn, to varying amounts of DNA in the individual PCR reactions. This resulted in lower and more variable fluorescent signal intensity at the end of the reaction.

Furthermore, a contamination of the invasive tumour components by infiltrating lymphocytes and the effect of intra-tumour genetic heterogeneity could not be excluded in all cases, as previously mentioned.

In order to overcome these obstacles a combination of two solutions was adopted.

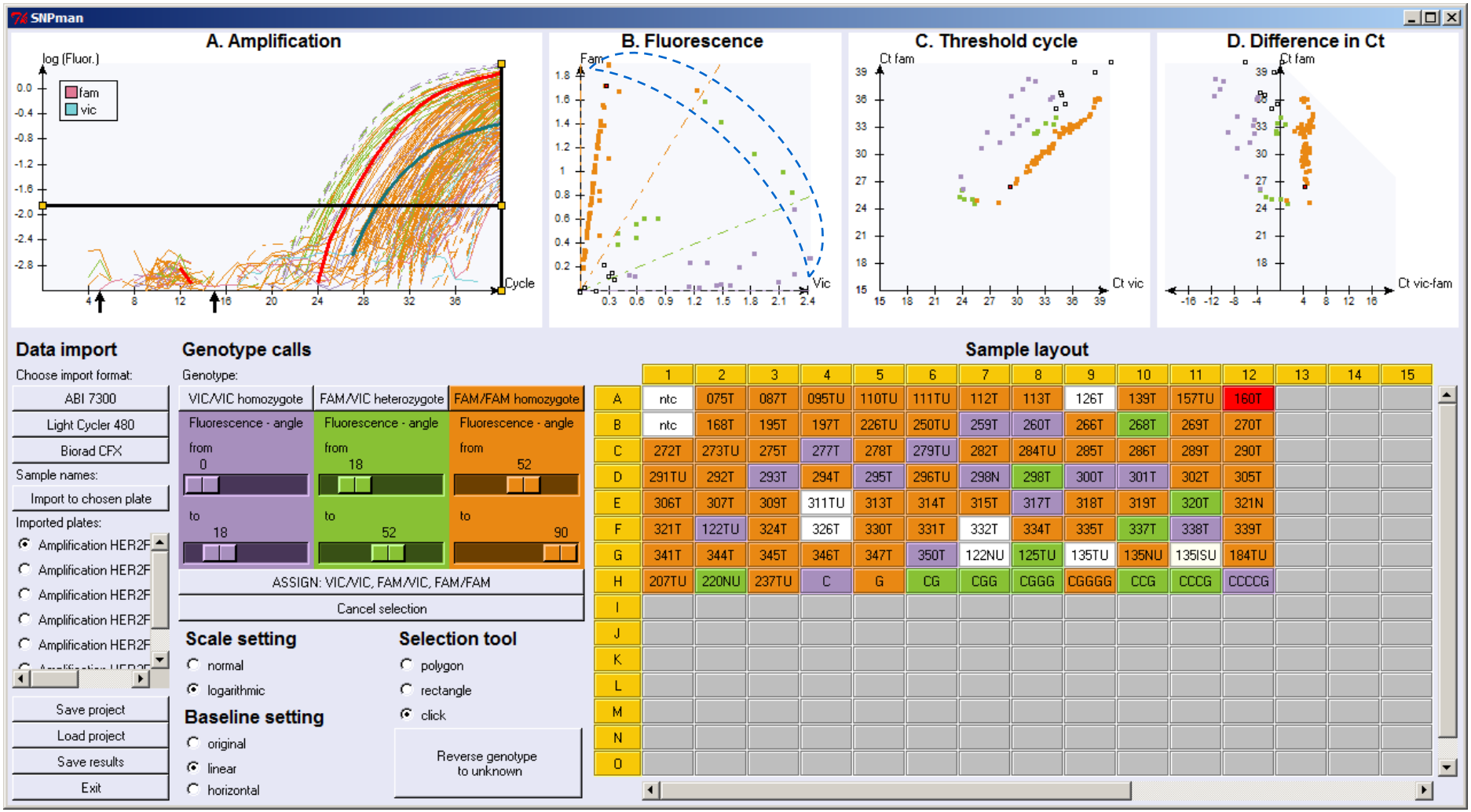

Figure 5.12: Representative screenshot from the SNPman program.
Details are provided in the text as well as in Konopac *et al.* (2011) and SNPman (2011).

The first was the use of the 'SNPman' program (Konopac *et al.*, 2011; SNPman, 2011), which utilizes the fluorescence data collected over the whole PCR run, rather than relying on the end-point fluorescence measurements only, as in the TaqMan SDS software. The SNPman software significantly facilitated genotype calls by the inspection of run data. The amplification graph, the fluorescence graph (allelic discrimination plot) and the threshold cycle graph were displayed simultaneously, and it was possible to visualize the amplification signal of each sample and allele at any given cycle and to set the most appropriate fluorescence threshold.

Figure 5.12 is a screenshot representative of a single PCR run. The disposition of DNA samples in the 96-well plate is shown in the 'sample layout' panel. Panel A shows the amplification curve of each individual sample (sample in well A12 is highlighted in the figure) in the context of all other samples in the plate. The amplification curve can be represented in either normal or logarithmic scale. The vertical black bar can be moved along the X-axis in order to look at the amplification signal at each amplification cycle, whereas the horizontal bar can be shifted to select the most appropriate amplification threshold. Panel B shows the allelic discrimination plot (see Section 2.9) of each individual sample at a given amplification cycle (selected through panel A). Panels C and D show the threshold cycle for both alleles (C) and their difference (D) at a given amplification cycle and threshold (selected through panel A).

The second solution aimed at identifying appropriate cut-offs which would discriminate heterozygous from homozygous samples in case of uncertain 'visual' allocation. Two sets of DNA mixtures were prepared using the genomic DNA of patients homozygous for the two alleles of the HER2 SNPs, according to the following proportions: 1:0, 4:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:4 and 0:1. An example of the results of this 'semi-quantitative' approach is shown in Figure 5.12, Graph B (blue dotted line). The use of these control samples and the SNPman software allowed the setting of three angle sectors, one for the heterozygous call and two for the homozygous calls. The cut-offs were arbitrarily set between the 4:1 and 3:1 mixtures and between the 1:3 and 1:4 mixtures.

5.6.1 Invasive component

The results of the genotyping of the DNA samples extracted from the invasive components of the tumours are summarized in Table 5.3.

	Ile655Val				Ala1170Pro			
	Genomic DNA		Tumour DNA		Genomic DNA		Tumour DNA	
HER2-negative Tumours	Ile/Ile	65	Ile	62	Ala/Ala	65	Ala	65
			undet	3				
	Ile/Val	44	Ile	11	Ala/Pro	39	Ala	15
			Ile/Val	19			Ala/Pro	16
			Val	11			Pro	6
			undet	3			undet	2
	Val/Val	3	Val	3	Pro/Pro	8	Pro	8
	NA	2	Ile	2	NA	2	Ala	1
							Pro	1
HER2-positive Tumours	Ile/Ile	69	Ile	67	Ala/Ala	48	Ala	48
			undet	2				
	Ile/Val	34	Ile	10	Ala/Pro	49	Ala	21
			Ile/Val	5			Ala/Pro	9
			Val	19			Pro	19
	Val/Val	5	Val	5	Pro/Pro	11	Pro	11
	NA	1	Val	1	NA	1	Pro	1
Tot	223				223			

Table 5.3: Genotyping results of DNA samples from invasive tumour components compared with results from genomic DNA.
NA = genomic DNA sample not available. Undet = genotyping of tumour DNA sample unsuccessful. Figures represent numbers of samples. Tumour genotype is presented with one allele only instead of a couple of alleles in case of homozygousity. This indicates that the allele detected by the PCR is not necessarily present in two copies but it might be the only one allele present in the tumour (in case of loss of the other allele) or the 'predominant' allele in the tumour (in case of mono-allelic amplification), as explained in Section 5.6.

There was complete correspondence between the genotyping of homozygous samples in germ-line DNA and in tumour DNA for both the SNPs, except for only 5 cases in which the genotyping of tumour DNA was unsuccessful. This is reassuring in terms of reliability of both the genomic and tumour DNA genotyping results. The genotyping calls of the tumour DNA samples from a germ-line heterozygous background are the most informative and are also shown in Figure 5.13, after exclusion of the unsuccessful genotyping cases.

These figures show that in HER2-positive tumours heterozygosity was maintained in a lower proportion of cases (15% and 18% for the Ile655Val and the Ala1170Pro SNPs, respectively) compared to HER2-negative tumours (46% and 43%). An exploratory statistical analysis (χ^2 test) comparing the allelic ratio in the apparent homozygous tumour samples against the null hypothesis of equal representation, did not show any significant difference in the distribution of the alleles in tumours, except for a possible imbalance of the Pro and Ala allele (15 vs 6) of the Ala1170Pro SNP in the HER2-negative group (p = 0.05) (Table 5.3 and Figure 5.13).

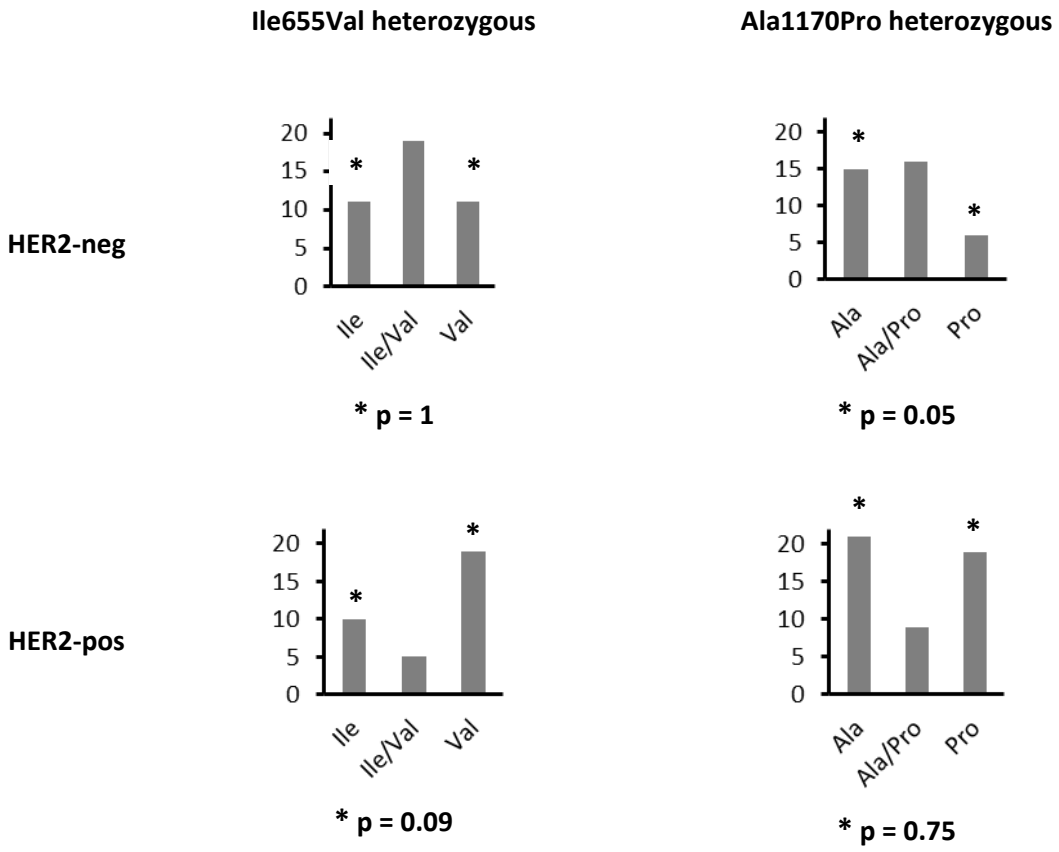

Figure 5.13: Genotyping results of tumour DNA samples from a germ-line heterozygous background. P-values represent level of significance of χ^2 tests comparing the allelic ratio in the apparent homozygous tumour samples against the null hypothesis of equal representation)

In 27 patients with germ-line genotype heterozygous for both SNPs the genotyping of tumour DNA showed that heterozygosity was maintained or lost concordantly in the two SNPs, except for one case only (4% of total) (highlighted in yellow in Table 5.4). This finding can be regarded as a good internal confirmation of the validity of the genotyping calling method adopted. The evaluation of apparent homozygous cases also shows that all the four possible haplotype combinations are represented in this limited group, thus confirming the very low likelihood of linkage disequilibrium between the SNPs, as discussed in Section 4.5.

HER2 Status	HER2 Ile655Val	HER2 Ala1170Pro
Negative	Ile	Ala
Negative	Ile	Ala
Negative	Ile	Ala
Positive	Ile	Ala
Positive	Ile	Ala
Negative	Val	Ala
Negative	Val	Ala
Positive	Val	Ala
Positive	Val	Ala
Negative	Ile	Pro
Negative	Ile	Pro
Negative	Ile	Pro
Positive	Ile	Pro
Positive	Ile	Pro
Positive	Ile	Pro
Positive	Ile	Pro
Positive	Ile	Pro
Negative	Val	Pro
Negative	Val	Pro
Positive	Val	Pro
Negative	Ile/Val	Ala/Pro
Negative	Ile/Val	Ala/Pro
Negative	Ile/Val	Ala/Pro
Negative	Ile/Val	Ala/Pro
Negative	Ile/Val	Ala/Pro
Positive	Ile/Val	Ala/Pro
Positive	Ile/Val	Ala

Table 5.4: Genotyping results of tumour DNA samples in 27 cases with germ-line 'double heterozygous' background
(genomic DNA heterozygous for both SNPs)

5.6.2 In Situ component and normal breast tissue

DNA extracted from the microdissected normal breast tissue was genotyped in 23 cases (Table 5.5). In only one case there was a lack of correspondence between the breast tissue and the genomic DNA (highlighted in pink in Table 5.5). This was a sample from a germ-line heterozygous Ala1170Pro patient where the genotyping of the normal breast DNA by visual estimation was uncertain (between Ala/Pro and Ala) and the genotype allocation according to the criteria described above assigned the Ala genotype. These data provide a further estimation of the reliability of the genotyping process.

	Ile655Val	**Ala1170Pro**
concordant with genomic DNA	20	20
discordant with genomic DNA	0	1
undetermined	2	1
genomic DNA not available	1	1
Tot	23	23

Table 5.5: Genotyping results of DNA samples extracted from normal breast tissue

The genotyping of DNA from the In Situ component was performed in 9 cases (Table 5.6). In all occasions in which the genotyping was successful the genotype of the In Situ component was the same as the one of the invasive component.

	Ile655Val		**Ala1170Pro**	
	genomic DNA homozygous	genomic DNA heterozygous	genomic DNA homozygous	genomic DNA heterozygous
concordant with invasive component	6	2	2	4
discordant with invasive component	0	0	0	0
undetermined	0	0	0	2
genomic DNA not available	1		1	
Tot	9		9	

Table 5.6: Genotyping results of DNA samples extracted from In Situ component

5.7 Discussion

The experiments presented in this chapter were conducted in order to further investigate the role of the two HER2 SNPs in over-expression/amplification of HER2 in breast cancers. These investigations were prompted by the observation, discussed in Chapter 4, of an association between the Pro allele of the HER2 Ala1170Pro SNP and HER2 amplification/overexpression in our population.

Two publications investigated the differences in the two SNPs' genotype between genomic DNA and tumour DNA. Puputti *et al.* (2006) using an automated sequencing method, observed a switch in the genotype for the Ile655Val SNP from Ile/Val in normal tissue to the Val/Val genotype in cancer tissue in three cases out of four HER2-positive ductal invasive carcinomas. Conversely, no loss of heterozygosity was observed among five HER2-negative tumours. The authors hypothesised that a loss of the Ile allele in the HER2-positive cancers might be due to a variety of different mechanisms (chromosomal non-disjunction, mitotic recombination, gene conversion, physical deletions or chromosome breakage) and speculated that the Val allele could give a proliferative advantage to cancer cells.

A second paper (Benz *et al.*, 2006), adopting a single base primer extension method, investigated a series of 29 patients with the Ala1170Pro heterozygous genotype (assessed in DNA from peripheral nucleated blood cells). One allele only (Ala allele in 7 cases and Pro allele in 5 cases) was detectable in all the 12 HER2-positive tumours, whereas in all the 17 HER2-negative cancers the heterozygosity was maintained. The authors speculated that the apparent loss of heterozygosity in the HER2-positive tumours was due to mono-allelic amplification and confirmed this hypothesis by showing that all the informative HER2-positive cases displayed transcriptional overexpression of their amplified allele compared to the non-amplified allele.

Our data regarding both SNPs in a larger group of informative (heterozygous) women did not confirm the initial hypothesis of allele-specific amplification for the Ala1170Pro SNP, neither was there any clear indication of preferential amplification of one of the Ile655Val alleles (Table 5.3 and Figure 5.13). A modest proportion (15 and 18%) of the HER2-positive cases in our population retained their heterozygosity for the two variants. This is in accordance with the observation of Puputti *et al.* (2006) regarding the Ile655Val SNP, but in contrast with the findings of Benz *et al.* (2006) who did not

observe any Ala1170Pro heterozygous HER2-positive tumours. Such a discrepancy might be explained by a number of potential factors. Firstly, the genotyping methods were different between Benz's study and ours, as was the choice of cut-offs for discriminating homozygous and heterozygous samples. Moreover, in our population the HER2 status was assessed by IHC in the vast majority of the cases, hence one might hypothesise that a limited proportion of the HER2 overexpressing tumours had low or no amplification of the gene, as described in the literature (Ellis *et al.*, 2005), leading in turn to the detection of both alleles with the TaqMan method. Finally, a minor contamination by infiltrating normal lymphocytes could not be excluded in all cases, as mentioned earlier.

With regard to the HER2-negative cancers, our observation of an apparent loss of heterozygosity (LOH) for the two variants in a significant proportion of cases (54% and 57%) seems to contrast with both Benz's and Puputti's works. However, a vast amount of literature data confirms that LOH in chromosome 17 is among the most common genetic alterations in breast cancer (Niederacher *et al.*, 1997; Orsetti *et al.*, 2004). In particular, Niederacher *et al.* (1997) showed a LOH in two microsatellites polymorphisms located on 17q close to the HER2 gene in over 40% of cases (among 49 and 87 informative samples), consistent with our findings.

The conventional view of LOH in cancer has been as a mechanism of inactivation of tumour suppressor genes. This view is not in keeping with our observation of the apparent preferential loss of the Ala1170Pro Pro allele of the HER2 oncogene among HER2-negative cancers. In order to account for this observation, it might be hypothesised that other known or putative tumour suppressor genes targeted by genetic rearrangements of 17q play a causative role in the development of HER2-negative tumours.

From a mechanistic point of view, while a published computational model (Fleishman *et al.*, 2002) suggested an explanation for the potential role of the Ile655Val SNP in carcinogenesis, the Ala1170Pro SNP has not been evaluated for its potential biologic function so far (Section 1.2.4). However, the position of the latter variant within the carboxyl-terminal tail, which is the main substrate of activation-dependent tyrosine phosphorylation and subsequent recruitment of adapter proteins, could suggest that the SNP may be involved in a phosphorylation regulatory function.

Several bioinformatics tools have been developed aiming at predicting the effect of coding SNPs on protein function and/or structure. Table 5.7 shows the predicted effect of the two HER2 SNPs according to five different published algorithms which are available online. It must be acknowledged that these tools use different combinations of methods based on sequence homology, protein structure information and physicochemical properties of the amino acids, and this might explain the apparently contradictory results shown in the table.

SNP	Software	Direction	Predicted effect of SNP
Ile655Val	SIFT (2011)	Ile→Val & Val→Ile	Tolerated
	Align-GVGD (2011)	Ile→Val & Val→Ile	Intermediate-low likelihood of being deleterious
	PolyPhen (2011)	Ile→Val & Val→Ile	Benign
	PMut (2011)	Ile→Val & Val→Ile	Neutral
	SNPs3D (2011)	only Ile→Val assessable	Non-deleterious
Ala117Pro	SIFT (2011)	Ala→Pro & Pro→Ala	Tolerated
	Align-GVGD (2011)	Ala→Pro & Pro→Ala	Intermediate-low likelihood of being deleterious
	PolyPhen (2011)	Ala→Pro & Pro→Ala	***Probably damaging***
	PMut (2011)	Ala→Pro & Pro→Ala	***Pathological***
	SNPs3D (2011)	only Pro→Ala assessable	***Slightly deleterious***

Table 5.7: Predicted of effect of HER2 Ile655Val and Ala1170Pro SNPs on protein structure/function according to five different bioinformatics programs

With this caveat, the Ala1170Pro SNP is predicted to affect the structure or function of the HER2 protein by three of the five methodologies, whereas the Ile655Val SNP seems to be neutral according to all the programs.

In conclusion, this investigation could not support the potential causative effect of the HER2 Ala1170 Pro SNP in the development of HER2-positive breast cancers. The role of the two HER2 SNPs in over/expression amplification of HER2 remains to be elucidated.

Chapter 6. Results IV: Patients treated with trastuzumab

6.1 Overview

This chapter focuses on a subgroup of the whole study population, which is composed of the patients who were treated with trastuzumab, as either palliative treatment of advanced stage disease or as adjuvant treatment of early stage disease. The results of the genotyping of the HER2 and Fc Gamma Receptor SNPs in the genomic DNA of these patients and the pharmacokinetic and biomarker data obtained through the measurement of trastuzumab and circulating HER2 in their plasma samples (according to the methods described in Chapter 3) constitute the main object of the first sections. The second part of the chapter illustrates the clinical and biological characteristics of these patients, the analysis of disease outcome as well as toxicity and the potential role of the SNPs considered. All the results of this chapter are then critically discussed in the context of the relevant scientific literature.

6.2 Patients recruited

A total of 167 patients treated with trastuzumab in the Northern Centre for Cancer Care (NCCC) were recruited. One hundred thirty nine patients received this treatment with the aim of reducing their risk of recurrence (adjuvant trastuzumab). Within this latter group, in 129 cases trastuzumab was administered for primary early breast cancer (adjuvant treatment *stricto sensu*), after surgery and adjuvant chemotherapy. In the remaining 10 patients trastuzumab was given either after a recurrence or in cases when imaging could neither confirm nor exclude the presence of metastases at first diagnosis. While all 139 patients were included in the toxicity analysis, only the first group of 129 patients was considered in the analysis of disease outcome (Figure 6.1). Twenty eight women who received trastuzumab for the first time as treatment of advanced stage disease were enrolled in the study. A further six patients received palliative trastuzumab after having been previously exposed to the drug as adjuvant treatment. These latter patients contributed to the analysis of the early stage group, but were excluded from the analysis of the advanced stage patients (Figure 6.1).

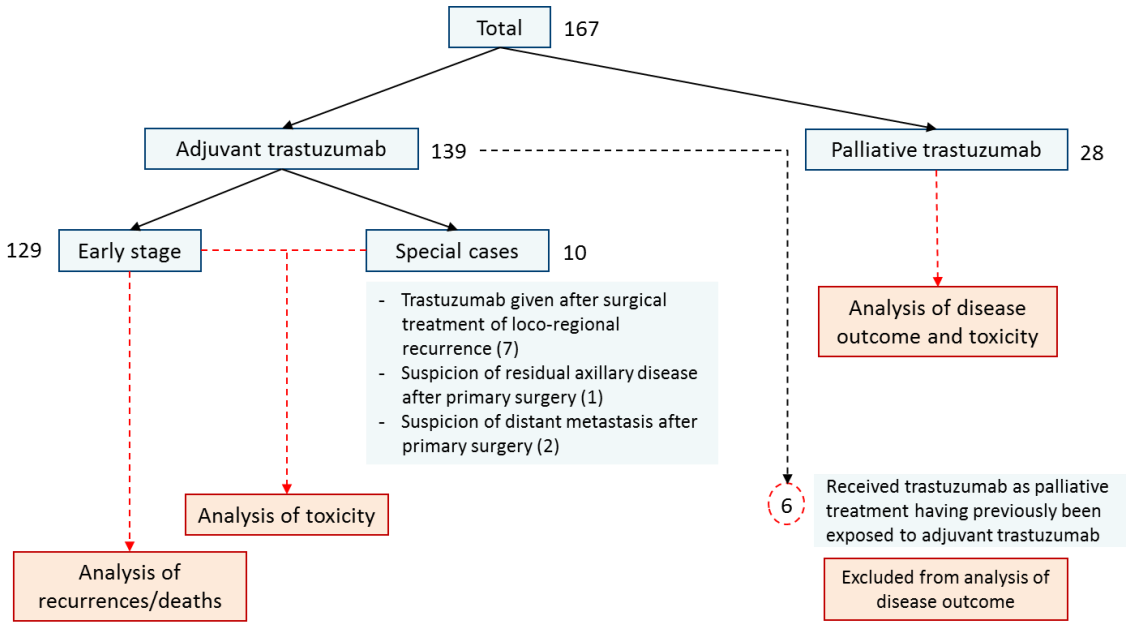

Figure 6.1: Number of patients treated with trastuzumab included in the study

These figures are comparable with the results of a recent analysis performed by Garrison and Veenstra (2009) on the current trend in numbers of patients treated with trastuzumab for early breast cancer and metastatic breast cancer. Although that study refers to trastuzumab prescriptions in the United States of America, it is reasonable to assume that the authors' projection is applicable to most Western countries.

6.3 Genotyping of the four SNPs in genomic DNA

The results of the genotyping of the two SNPs in the HER2 gene in the trastuzumab treated patients are a subset of the genotyping results in the whole population, which have already been described in Chapter 4. The genotyping of the two SNPs in the Fc Gamma Receptor Genes, namely FCGR2A His131Arg (rs1801274) and FCGR3A Phe158Val (rs396991), was performed according to the same method in all 167 patients treated with trastuzumab. As with the HER2 SNPs, it was always possible to allocate each individual patient sample to its distinct genotype by visual assessment. Two examples of allele discrimination plots from two representative experiments are shown in Figure 6.2.

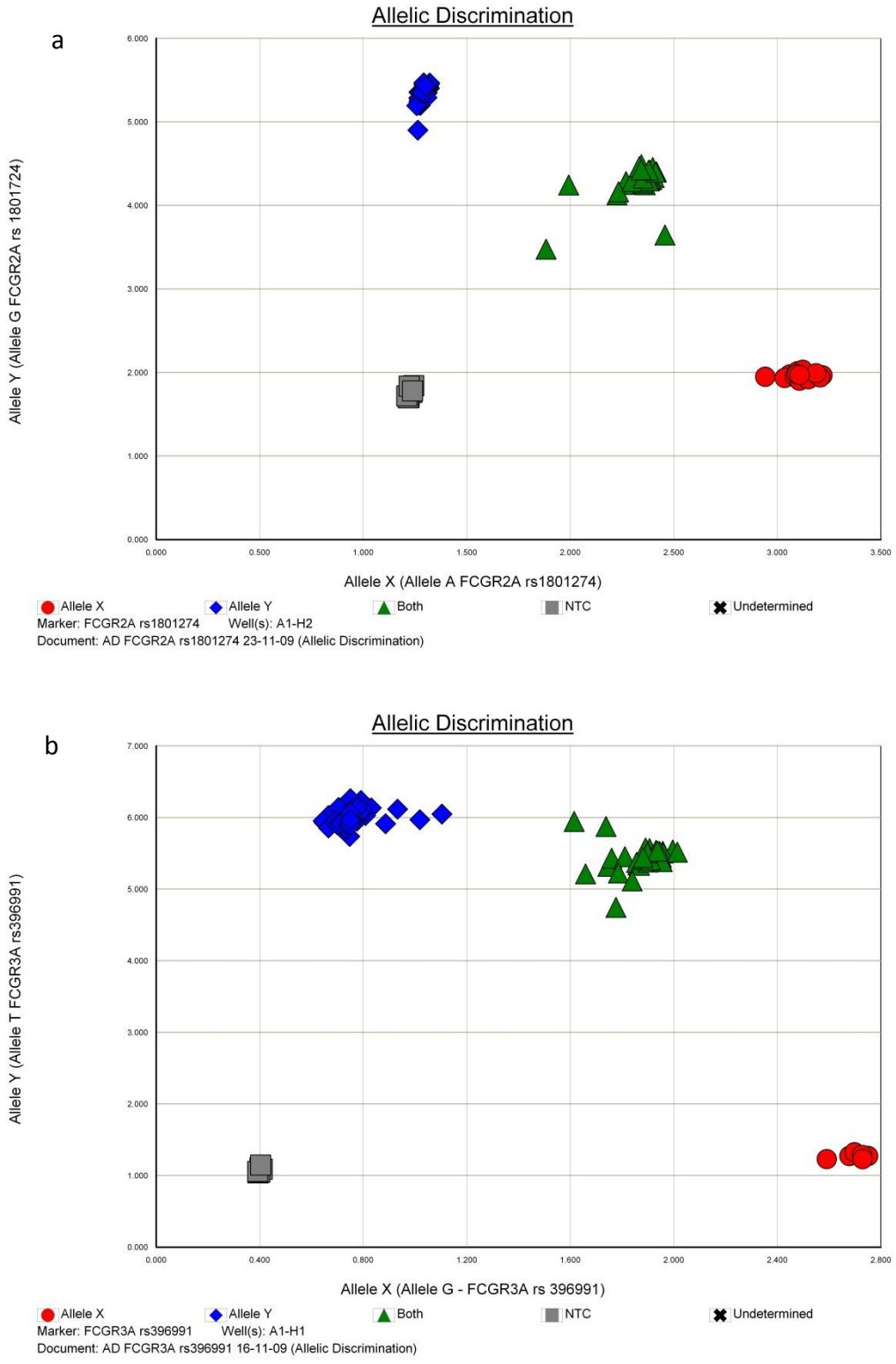

Figure 6.2: Allelic discrimination plots from two representative genotyping experiments.
a) FCGR2A His131Arg: A=His, G=Arg; b) FCGR3A Phe158Val: T=Phe, G=Val

The same four cell lines (CCRF-CEM, K562, SKBR3 and MCF7) mentioned in Chapter 4 were used as controls for the genotyping experiments. A published genotype for the

FCGR2A SNP was available only for K562 (Warmerdam *et al.*, 1990). This was heterozygous (His/Arg) and is in accordance with our determination.

The results of all the genotyping experiments for the two Fc Gamma Receptor SNPs were pooled after normalization to the ntc values and are shown in Figure 6.3.

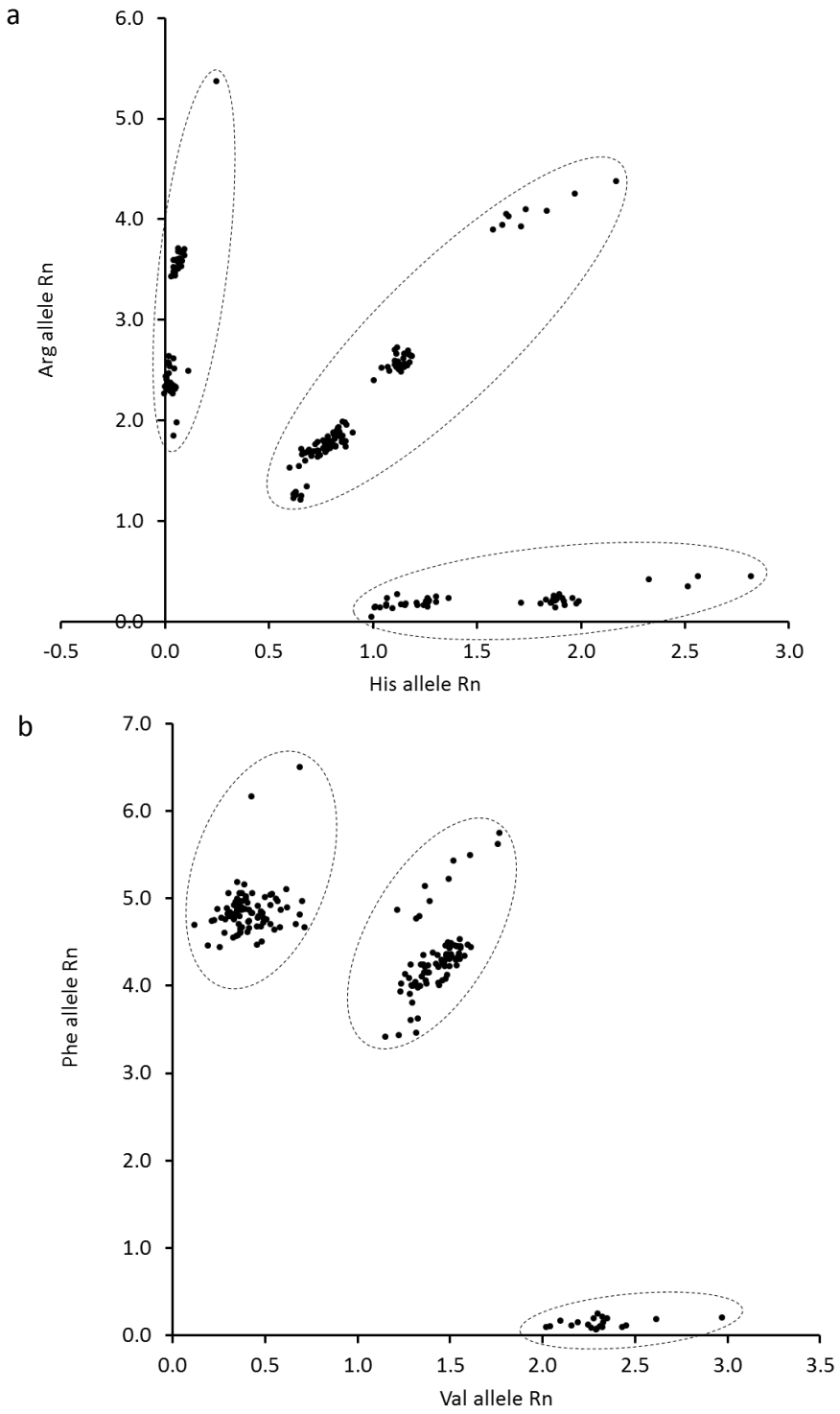

Figure 6.3: Pooled results of FCGR2A His131Arg and FCGR3A Phe158Val genotyping experiments after normalization to ntc.
a) FCGR2A His131Val; b) FCGR3A Phe158Val

The distribution of genotypes for the four SNPs in the trastuzumab treated population is shown in Table 6.1.

		No.	%	MAF (%)	p for H-W
HER2 Ile655Val	Ile/Ile	103	61.7	22	0.64
	Ile/Val	55	32.9		
	Val/Val	9	5.4		
HER2 Ala1170Pro	Ala/Ala	73	43.7	33	0.33
	Ala/Pro	79	47.3		
	Pro/Pro	15	9.0		
FCGR2A His131Arg	His/His	37	22.2	46	0.64
	His/Arg	80	47.9		
	Arg/Arg	50	29.9		
FCGR3A Phe158Val	Phe/Phe	83	49.7	31	0.38
	Phe/Val	66	39.5		
	Val/Val	18	10.8		
Total		167	100		

Table 6.1: Distribution of genotypes for all four SNPs in the trastuzumab treated population.
H-W = Hardy-Weinberg equilibrium (p value for χ^2 test); MAF = minor allele frequency

There was no significant deviation from Hardy-Weinberg equilibrium for all SNPs. The minor allele frequencies for FCGR2A His131Arg and FCGR3A Phe158Val (respectively 46% and 31%) were comparable with published data in Caucasian populations (NCBI_c, 2009; NCBI_d, 2009).

The estimated haplotype frequencies and the linkage disequilibrium measures of the two HER2 SNPs have already been shown in Chapter 4. The same analysis for the Fc Gamma Receptor SNPs, limited to the trastuzumab-treated group, is shown in Table 6.2. The |D'| and r^2 values displayed in the table, together with the results of a classical χ^2 statistics comparing the observed and expected diplotype frequencies (Table 6.3), suggest that a non-random associations of the alleles at the two loci is likely, but the strength of this association is moderate. In particular, the minor allele of the FCGR2A

His131Arg SNP (His) seems to be weakly associated with the minor allele of the FCGR3A Phe158Val SNP (Val).

LD statistics according to MIDAS and CubeX

Haplotypes estimated frequency (%)		**|D'|**	**r^2**	**χ^2**	**p**
FCGR2A_Arg/FCGR3A_Phe	42	0.296	0.0449	7.5	<0.01
FCGR2A_His/FCGR3A_Phe	27				
FCGR2A_His/FCGR3A_Val	19				
FCGR2A_Arg/FCGR3A_Val	12				

Table 6.2: Linkage disequilibrium statistics for the FCGR2A His131Arg and FCGR3A Phe158Val SNPs in 167 patients.
|D'| = Linkage disequilibrium coefficient; r^2 = correlation coefficient. Values were calculated using MIDAS (Gaunt *et al.*, 2006) and Cubex (Gaunt *et al.*, 2007).

			FCGR3A Phe158Val			Total
			Val/Val	Phe/Val	Phe/Phe	
FCGR2A His131Arg	His/His	Count	10	13	14	37
		Expected Count	4	14.6	18.4	
	His/Arg	Count	3	38	39	80
		Expected Count	8.6	31.6	39.8	
	Arg/Arg	Count	5	15	30	50
		Expected Count	5.4	19.8	24.9	
Total			18	66	83	167

p = 0.002

Table 6.3: χ^2 statistics comparing FCGR2A and FCGR3A observed diplotype frequencies with expected frequencies calculated according to the observed genotypes in the trastuzumab treated patients.

6.4 Trastuzumab and HER2 in plasma

A total of 163 plasma samples from 138 patients were collected with the aim of obtaining pharmacodynamic and pharmacokinetic information from the trastuzumab-treated population. In 29 patients (out of 167) plasma was not collected because these women had already completed their treatment at the date of consent.

Paired plasma samples (pre-trastuzumab and on-trastuzumab) were collected in 24 cases, as a result of the approval of a specific amendment to the protocol (Section 2.2) (Figure 6.4).

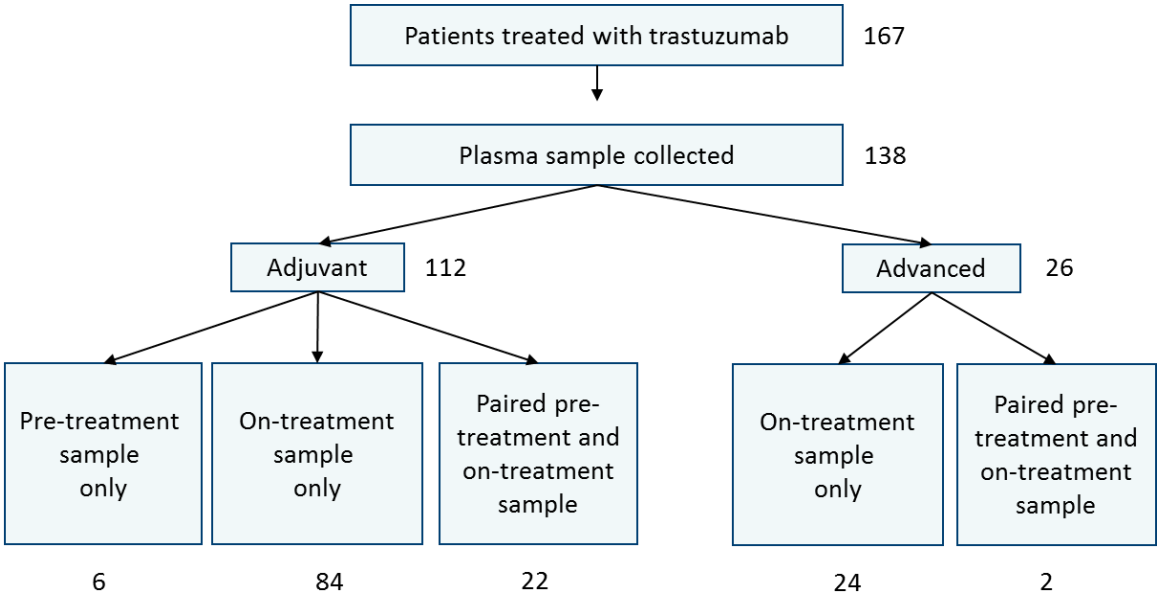

Figure 6.4: Plasma samples collected in trastuzumab-treated patients (numbers refer to number of patients)

Trastuzumab

Trastuzumab concentration was measured in all available 'on-treatment' samples. The results of the cell-based ELISA in the advanced patients are shown in Figure 6.5. In this limited (26 patients) and heterogeneous subgroup (see Section 6.5.2) mean and median trough values were 53 µg/ml and 71 µg/ml respectively, with a high coefficient of variation (124%).

The patients treated in the adjuvant setting formed a more homogenous group in terms of treatment schedule and constitute the majority of the treated population. Figure 6.6 and 6.7 show the results of the trastuzumab ELISA in 106 patients treated in the adjuvant setting. The on-treatment samples were collected, as explained in Section 2.2, immediately before drug administration in order to obtain 'trough' trastuzumab concentration. This meant 3 weeks after previous administration in the vast majority of cases. In the remaining nine cases the administration was delayed by one to three weeks so that the samples were drawn after 4 to 6 weeks since the previous cycle.

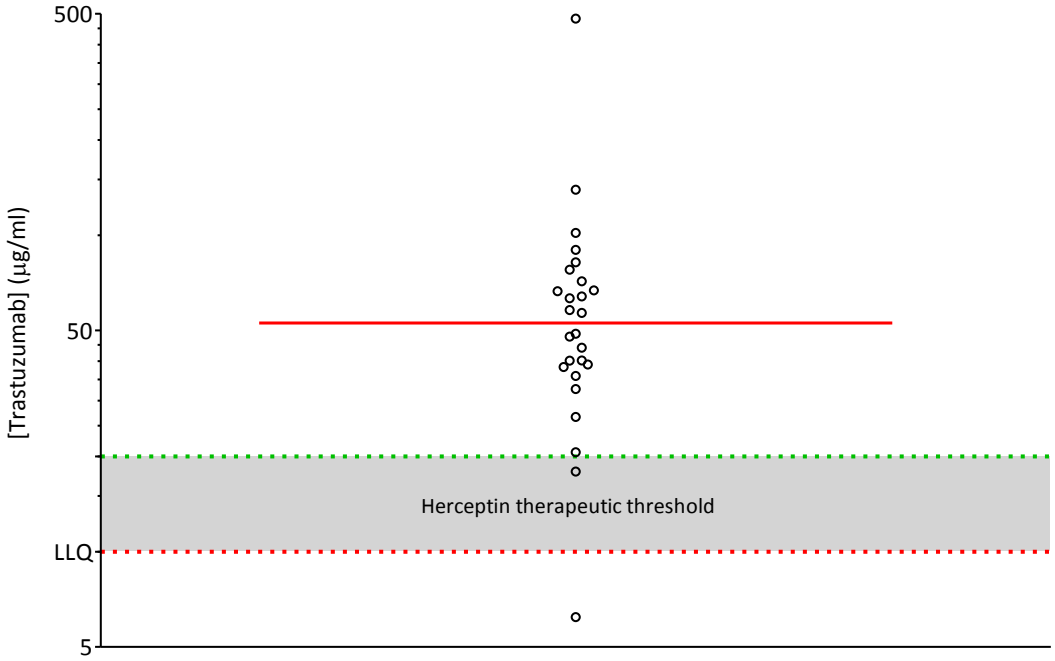

Figure 6.5: Trastuzumab trough concentrations in 26 patients in the palliative setting.
Red line refers to median value. LLQ = lower limit of quantification

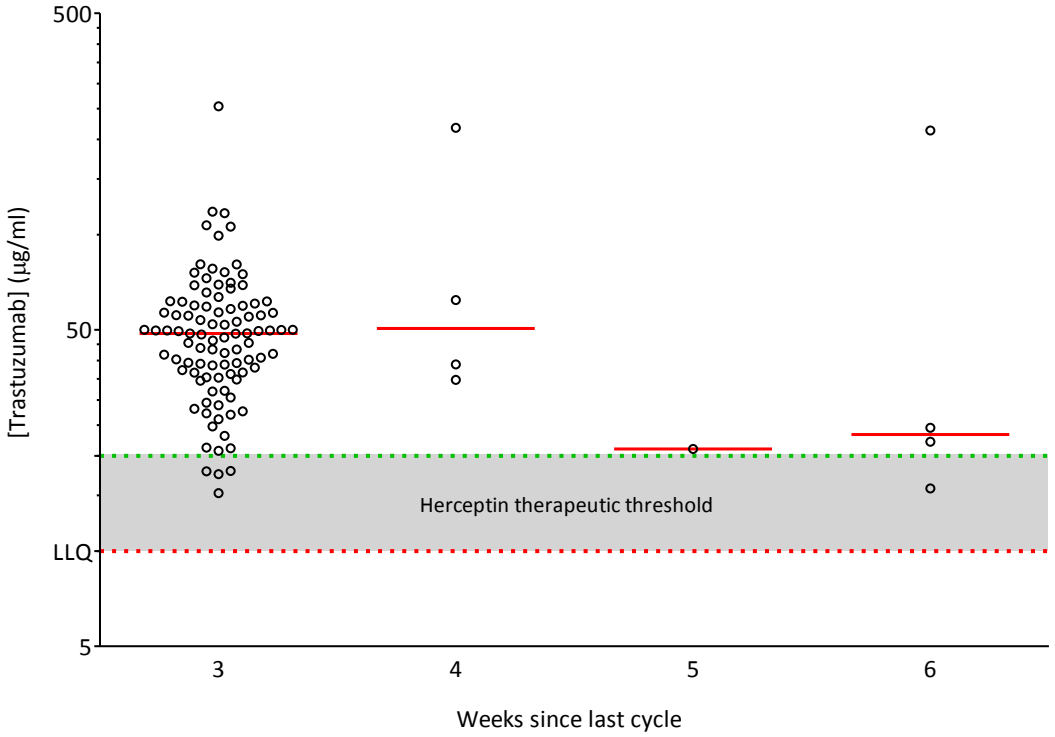

Figure 6.6: Trastuzumab trough concentrations in 106 patients in the adjuvant setting plotted against number of weeks since previous administration.
Red lines refer to median values. LLQ = lower limit of quantification

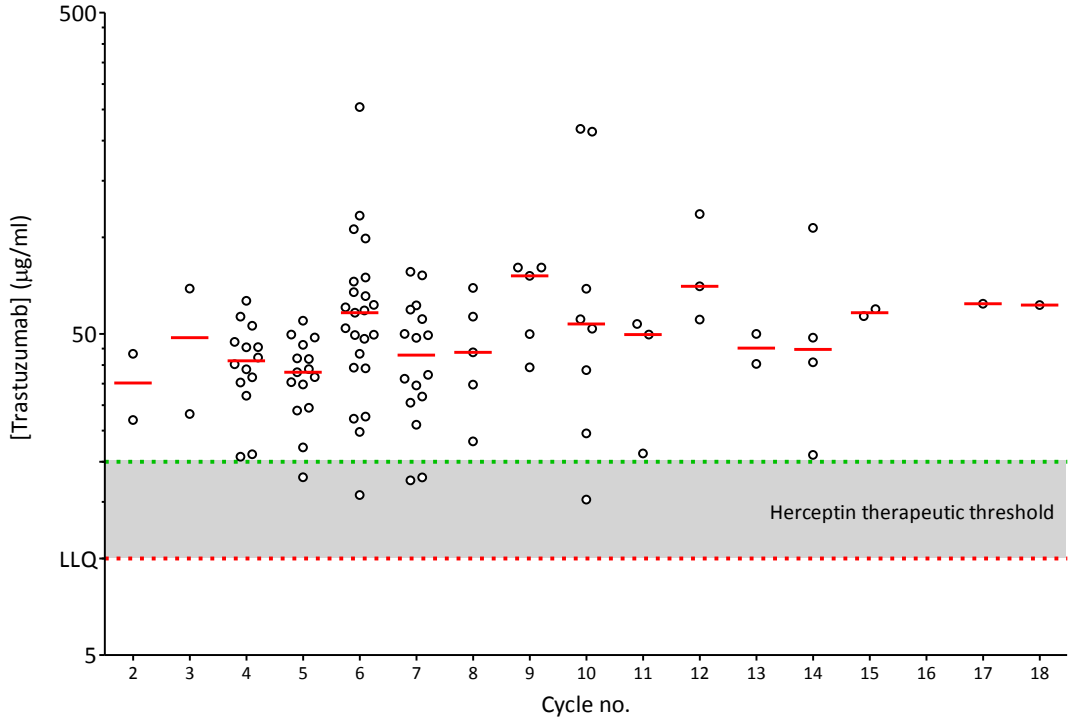

Figure 6.7: Trastuzumab trough concentrations in 106 patients in the adjuvant setting plotted against cycle number.
Red lines refer to median values. LLQ = lower limit of quantification

Due to its prolonged terminal half-life, trastuzumab concentrations tended to remain above the assumed therapeutic threshold (10-20 µg/ml) for up to 6 weeks (Figure 6.6). Mean and median trough concentrations (54 µg/ml and 49 µg/ml, respectively) were consistent with previously-published studies, with a coefficient of variation of 68%. Only three patients had very high (> 200 µg/ml) plasma concentrations (Figures 6.6 and 6.7). The majority of samples were collected at cycles four to seven (Figure 6.8). The distribution of trastuzumab plasma concentrations against cycle number illustrated in Figure 6.6 looks consistent with the reported 'time to steady-state' of approximately 20 weeks (Bruno *et al.*, 2005).

Circulating HER2

Circulating HER2 Extra-Cellular Domain (ECD) was measured in all available plasma samples and the results are shown in Figure 6.8. Sixty four per cent of the measurements were below the lower limit of quantification for the assay. Only in a limited number of cases was HER2 concentration above 15 ng/ml, the value identified

by previous studies as the cut-off discriminating 'positive' from 'negative' determinations. An exploratory statistical analysis (Mann-Whitney U test) comparing pre-treatment versus post-treatment values in the adjuvant setting and on-treatment values in adjuvant versus advanced setting did not show any significant difference in either case.

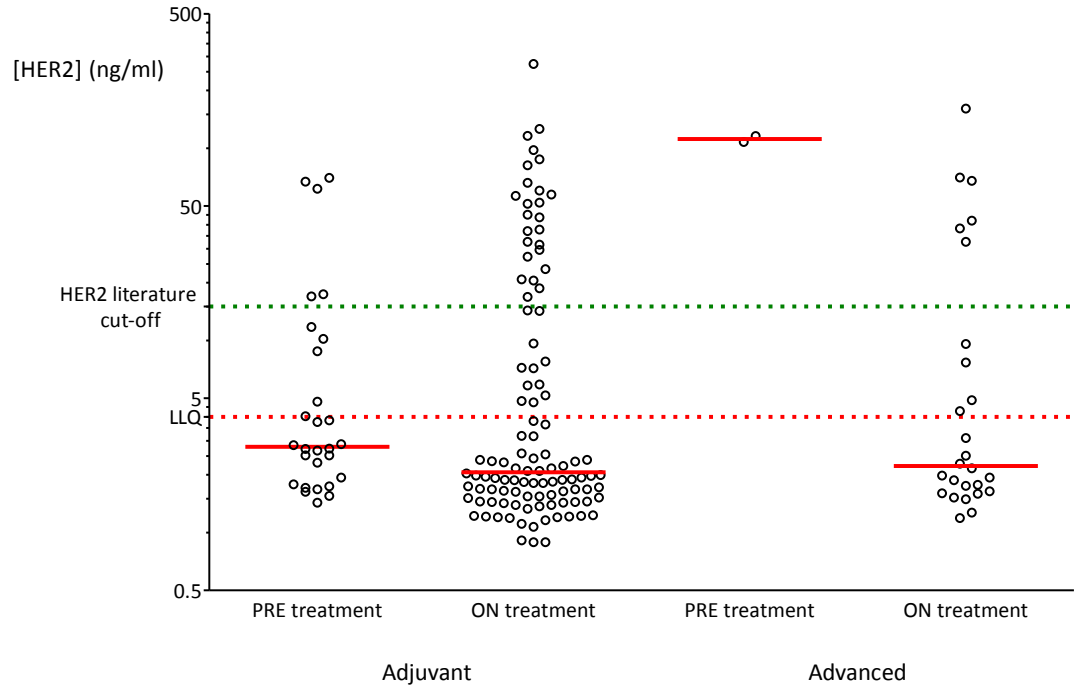

Figure 6.8: Circulating HER2 in the trastuzumab-treated population.
Red lines refer to median values. LLQ = lower limit of quantification. 'HER2 literature cut-off' (15 ng/ml) refers to the conventional level which discriminates 'positive' and 'negative' values in published studies

The potential effect of the SNPs in the HER2 gene on the level of circulating HER2 was also assessed (Figure 6.9). In order to achieve a better uniformity of the data only on-treatment samples were included. Analysis of variance showed no significant association between the genotypes and circulating HER2. This was also the case when samples from advanced and early stage patients were considered separately.

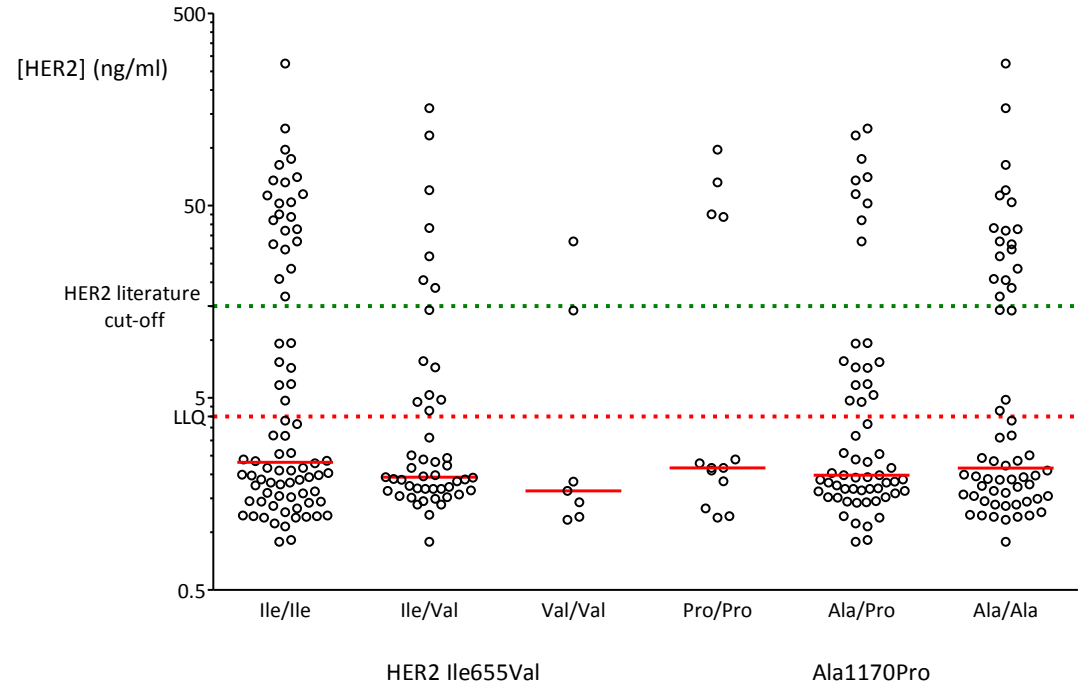

Figure 6.9: Circulating HER2 in the trastuzumab-treated population grouped according to the HER2 Ile655Val and Ala1170Pro genotypes.
Red lines refer to median values

Biomarker analysis

When circulating HER2 was plotted against trastuzumab concentration in the whole population (Figure 6.10a) and in the adjuvant (Figure 6.10b) and advanced setting (Figure 6.10c) no significant correlation between the two parameters emerged.
The results of the biomarker analysis in the limited group of patients in which paired plasma samples (pre-trastuzumab and on-trastuzumab) were available are shown in Figure 6.11.

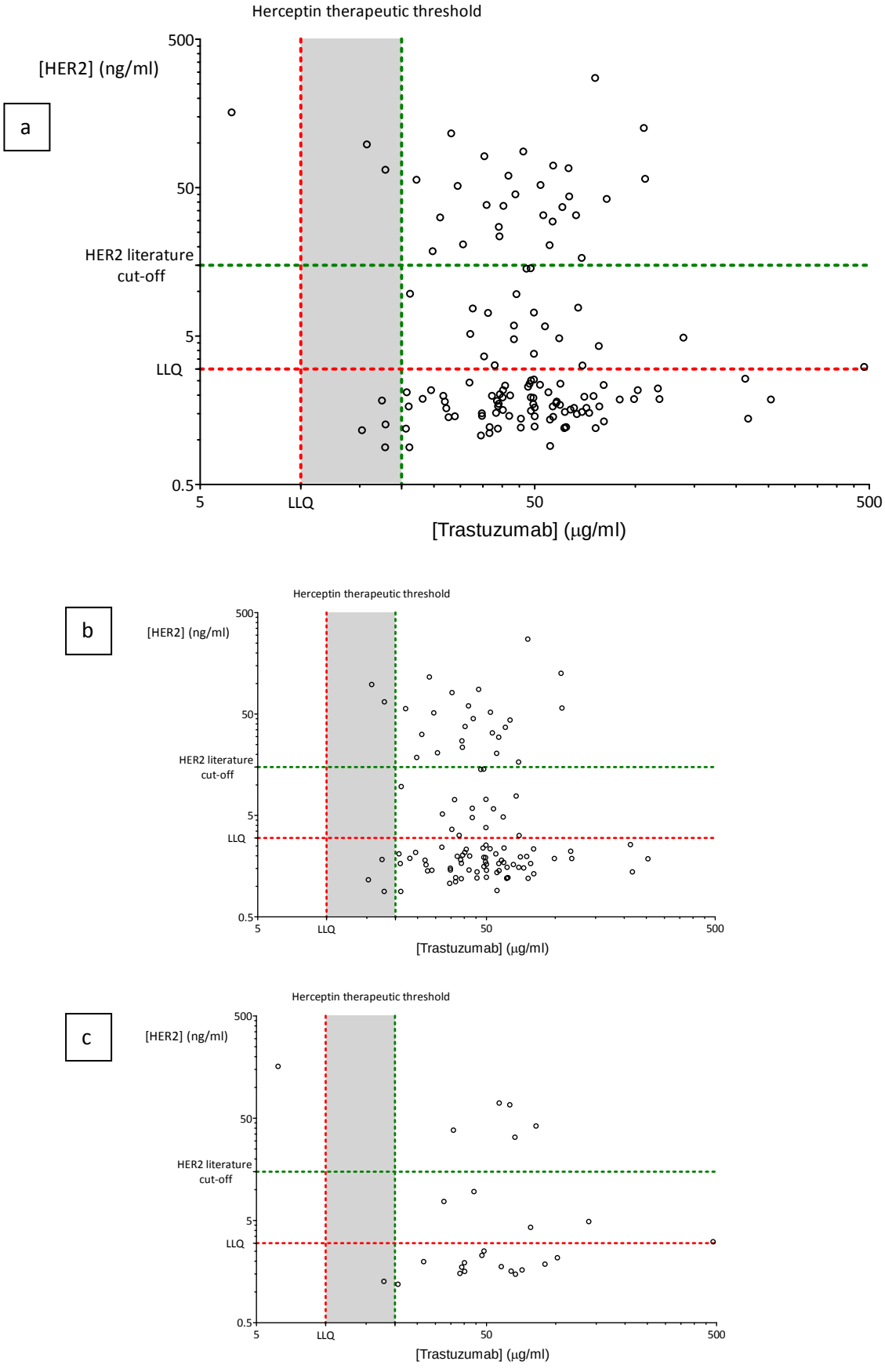

Figure 6.10: Circulating HER2 plotted against trastuzumab concentration in the whole population (a) and in adjuvant (b) and advanced (c) settings

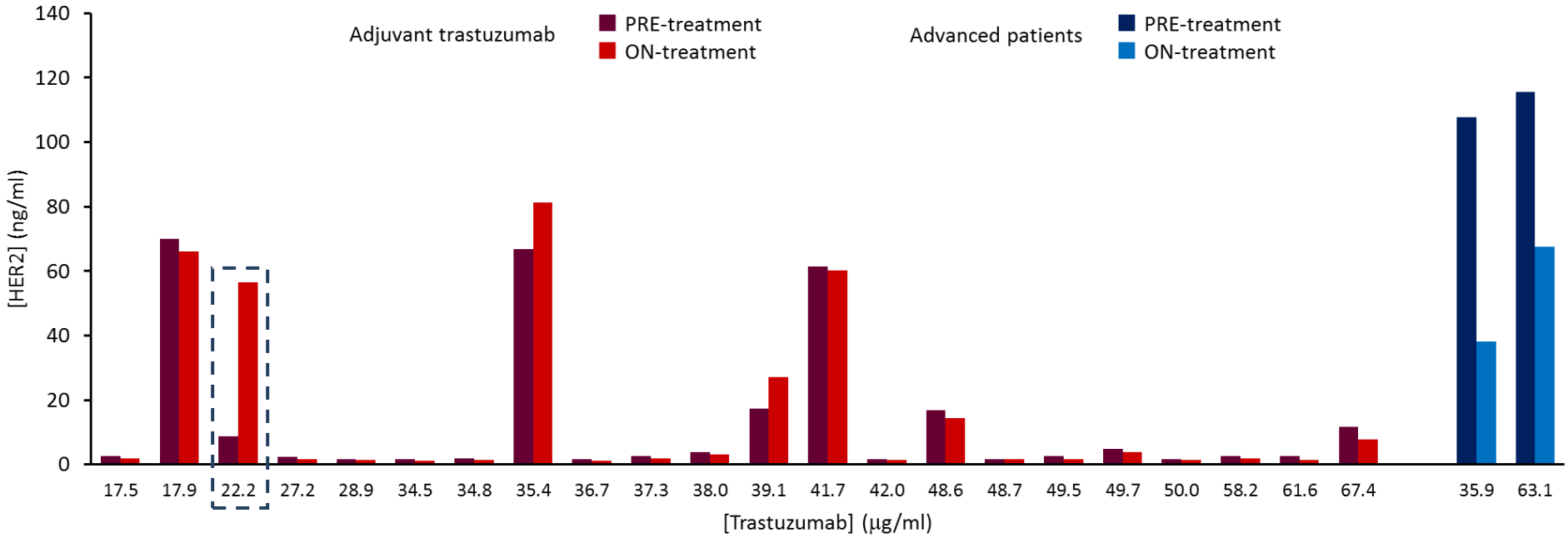

Figure 6.11: Circulating HER2 in paired (pre-treatment and on-treatment) plasma samples from 22 early stage and two advanced stage patients treated with trastuzumab. Each pair of columns represents one patient. Patients have been sorted according to the trastuzumab concentration (from lowest to highest).

As can be seen in the graph, in all patients with very low circulating HER2 there was an apparent decrease in HER2 concentration when trastuzumab was administered. This effect is likely to be artifactual due to the interference of trastuzumab on the HER2 ELISA, which has been observed and described during the validation process (Section 3.3.2). In patients with higher HER2 concentration the administration of trastuzumab led to non-significant variations of HER2 levels in 'adjuvant' patients, with exception of one case. This patient (highlighted in Figure 6.10) received trastuzumab as 'adjuvant' treatment after excision of loco-regional recurrence, but post-surgical residual disease could not be ruled out. Her pre-trastuzumab HER2 level was below the literature cut-off and rose to 55 ng/ml after four trastuzumab administrations. Two months after the 'on-treatment' HER2 measurement the patient was diagnosed with distant metastases. In only two advanced stage patients were paired plasma samples available. In both cases there was a drop in circulating HER2, although not below the normal literature cut-off. The two patients had a partial remission and disease stabilization and were both still on treatment at the date of the analysis. With this limited number of patients no obvious interaction of trastuzumab trough concentration with circulating HER2 was apparent.

6.5 Characteristics of patients

6.5.1 Patients treated with adjuvant trastuzumab

The demographic and clinical characteristics of patients treated with adjuvant trastuzumab are shown in Table 6.4, whereas Table 6.5 illustrates the characteristics of their tumours. All patients received a loading dose of 8 mg/m^2 followed by seventeen 3-weekly 6 mg/m^2 doses for a total treatment duration of one year, as per the 'HERA' trial protocol (Piccart-Gebhart *et al.*, 2005). Trastuzumab was administered after adjuvant chemotherapy, with the exception of seven cases in which it was started concomitantly with the second part of the chemotherapy, composed of taxanes in 6 cases and vinorelbine in one case. In one patient trastuzumab was given concomitantly with lapatinib as part of the 'ALTTO' trial (ALTTO, 2011). In no case was trastuzumab administered concomitantly with anthracyclines.

Total (number)			**139**
Ethnicity		Caucasian (%)	95
		Non Caucasian (%)	5
Gender		Female (%)	100
		Male (%)	0
Age at date of start trastuzumab		Median (years)	54
		Range (years)	27 - 77
Year start trastuzumab		2006-2008 (%)	45
		2009-2011 (%)	55
Pre-existing cardiovascular risk factors *		Yes (%)	26
		No (%)	74
Pre-trastuzumab Left Ventricle Ejection Fraction (LVEF)**		Median (%)	57
		Range (%)	49 - 81
LVEF variation with chemotherapy ***		Median (%)	- 3.5
		Range (%)	- 22 ↔ +13
Previous anthracyclines		Yes (%)	98.5
		No (%)	1.5
Previous anthracyclines cumulative dose (mg/m^2)	Doxorubicin (48 pts)	Median (mg/m^2)	240
		Range (mg/m^2)	210 - 375
	Epirubicin (89 pts)	Median (mg/m^2)	300
		Range (mg/m^2)	90 - 625

Table 6.4: Characteristics of 139 patients who received adjuvant trastuzumab

* including hypertension, diabetes, coronary disease, valvular heart disease, atrial fibrillation, idiopathic cardiomyopathy

** pre-trastuzumab LVEF measured with MUGA scan was available in 111 patients

*** variation of LVEF between pre-chemotherapy and post-chemotherapy (pre-trastuzumab) measurements was available in 76 patients

		No.	%
Total		*139*	
Trastuzumab administered after diagnosis of primary early breast cancer		**129**	**100**
Histologic subtype	Ductal NST	114	88.4
	others	15	11.6
Focality	Unifocal	101	78.3
	Multifocal	24	18.6
	Occult	4	3.1
Maximum tumour diameter	< 1 cm	12	9.3
	1 - 2 cm	41	31.8
	2 - 5 cm	68	52.7
	> 5 cm	8	6.2
Number of positive lymph nodes	0	59	45.7
	1 - 3	35	27.1
	≥ 4	35	27.1
Histologic grade	1	1	0.8
	2	41	31.8
	3	83	64.3
	Not applicable	4	3.1
Hormone Receptors	Negative	47	36.4
	Weak	73	56.6
	Positive	9	6.9
HER2 (IHC)	1 *	1	0.8
	2 *	31	24.0
	3	97	75.2

Table 6.5: Characteristics of tumours in patients who received adjuvant trastuzumab
% is relative to the patients treated after diagnosis of primary early breast cancer)
* all patients with HER2 IHC status = 1 or 2 had positive ISH determination

6.5.2 Advanced stage patients

Twenty eight advanced stage patients were treated with trastuzumab with the aims of disease control, symptom palliation and potential prolongation of survival. As shown in

Table 6.6, this limited group of patients was heterogeneous with regard to several aspects, including previous treatments and drugs co-administered with trastuzumab.

Total (number)			**28**
Ethnicity/Gender		Caucasian/Female (%)	100
Age at date of start trastuzumab		Median (years)	60
		Range (years)	38 - 73
Pre-existing cardiovascular risk factors		Yes (%)	32
		No (%)	68
Previous anthracyclines		Yes (%)	61
		No (%)	39
Previous anthracyclines cumulative dose (mg/m^2)	Doxorubicin (11 pts)	Median (mg/m^2)	240
		Range (mg/m^2)	60 - 400
	Epirubicin (6 pts)	Median (mg/m^2)	360
		Range (mg/m^2)	210 - 690
Previous chemotherapy treatments for advanced disease		0 (%)	68
		1 (%)	32
Previous hormonal treatments for advanced disease		0 (%)	57
		1 - 3 (%)	43
Sites of disease		Bone only (%)	11
		Breast/soft tissues (%) *	18
		Viscera (%) **	71
Drugs associated with trastuzumab		Taxanes (%) ***	64
		Vinorelbine/capecitabine (%)	18
		Hormonal treatments (%)	7
		None (%)	11
Trastuzumab schedule		Weekly (%) ****	11
		3-weekly (%)	89

Table 6.6: Characteristics of 28 advanced stage patients treated with trastuzumab
* with or without bone metastases
** with or without bone metastases and breast/soft tissues
*** in one case paclitaxel was replaced by vinorelbine due to neurotoxicity
**** in all three cases schedule was changed to 3-weekly after, respectively, 62, 4 and 1 months

6.6 Disease outcome

The analysis of recurrences and deaths in the 'adjuvant' group was limited to the 129 patients who received trastuzumab after surgery and adjuvant chemotherapy for a primary early breast cancer. In this population the median follow-up (calculated from the date of first trastuzumab administration to the date of last contact or death) was 26 months (range, 1 - 67). Only seven patients (5% of the total) experienced a recurrence of their disease and three of them (2% of the total) died from advanced disease. These small numbers do not currently permit any statistical analysis.
The numbers of breast cancer-related events were expectedly very different in the palliative setting. With a median follow-up of 23 months (range 4 - 106), 25 out 28 patients (89% of total) experienced a progression of their disease and twelve patients (43% of total) died from advanced disease. The outcomes of patients in terms of best objective response according to RECIST criteria (Eisenhauer *et al.*, 2009) are reported in Table 6.7.

	Number	**%**
Complete response	2	7
Partial response	14	50
Stable disease	5	18
Progression of disease	1	4
Not evaluable	6	21
Total	**28**	**100**

Table 6.7: Objective response in 28 advanced stage patients treated with trastuzumab

Notably, a significant proportion (20%) of patients had no evaluable disease and only one patient had progressive disease as best response. As mentioned in Section 6.2, six further advanced-stage patients who received trastuzumab having previously been exposed to the drug in the adjuvant setting were not included in this analysis.
With the caveats of the small sample size and the heterogeneity of the patient characteristics described earlier, an exploratory analysis was conducted in order to evaluate the potential association of the four SNPs with disease outcome. Besides time to disease progression (TTP, calculated from the date of first trastuzumab dose to the

date of disease progression) and overall survival (OS, calculated from the date of first trastuzumab dose to patient's death) a third parameter of treatment outcome was introduced. This was 'time on trastuzumab' (TOT, calculated from the date of first trastuzumab dose to the date of trastuzumab definitive discontinuation) and was introduced in order to account for those cases in which a slight radiological progression was observed, but the clinical benefit of trastuzumab (in terms of symptom relief or long-term overall disease control) led to the decision of continuing the treatment beyond progression. When the TTP and TOT survival curves of the advanced stage patients grouped according to the different genotypes were compared, the FCGR3A Phe158Val SNP gave a statistically significant result (Figure 6.12a and 6.12b), in that the Val allele was associated with longer TTP and TOT, but not OS. The other three SNPs were not associated with these two survival parameters and the analysis of best objective responses did not show any association with the four SNPs considered (Table 6.8).

		No.	**Median TTP (m)**	**p ***	**Median TOT (m)**	**p ***	**No. *****	**ORR (%)**	**p ****
HER2 Ile655Val	Ile/Ile	16	11	0.606	14	0.441	11	64	0.318
	Val carriers	12	10		13		11	82	
	Ile carriers	26	11	0.283	15	0.051	20	70	0.519
	Val/Val	2	9		5		2	100	
HER2 Ala1170Pro	Ala/Ala	14	11	0.876	14	0.949	11	82	0.318
	Pro carrier	14	10		12		11	64	
	Ala carriers	26	10	0.616	14	0.830	20	70	0.519
	Pro/Pro	2	9		9		2	100	
FCGR2A His131Arg	His/His	5	15	0.361	17	0.570	5	80	0.581
	Arg carriers	23	10		13		17	71	
	His carriers	20	10	0.610	16	0.384	19	74	0.636
	Arg/Arg	8	10		9		3	67	
FCGR3A Phe158Val	Val/Val	5	15	0.228	17	0.414	4	75	0.708
	Phe carriers	23	10		13		18	72	
	Val carriers	10	15	0.030	31	0.011	8	75	0.631
	Phe/Phe	18	10		9		14	71	

Table 6.8: Parameters of disease outcome according to the four SNPs in 26 advanced patients.
No. = number of patients; TTP = Time to progression; TOT = Time on trastuzumab; ORR = objective response rate

* p = significance level of log-rank test

** p = significance level of Fisher's exact test

*** in 6 patients objective response was not evaluable (no measurable lesions)

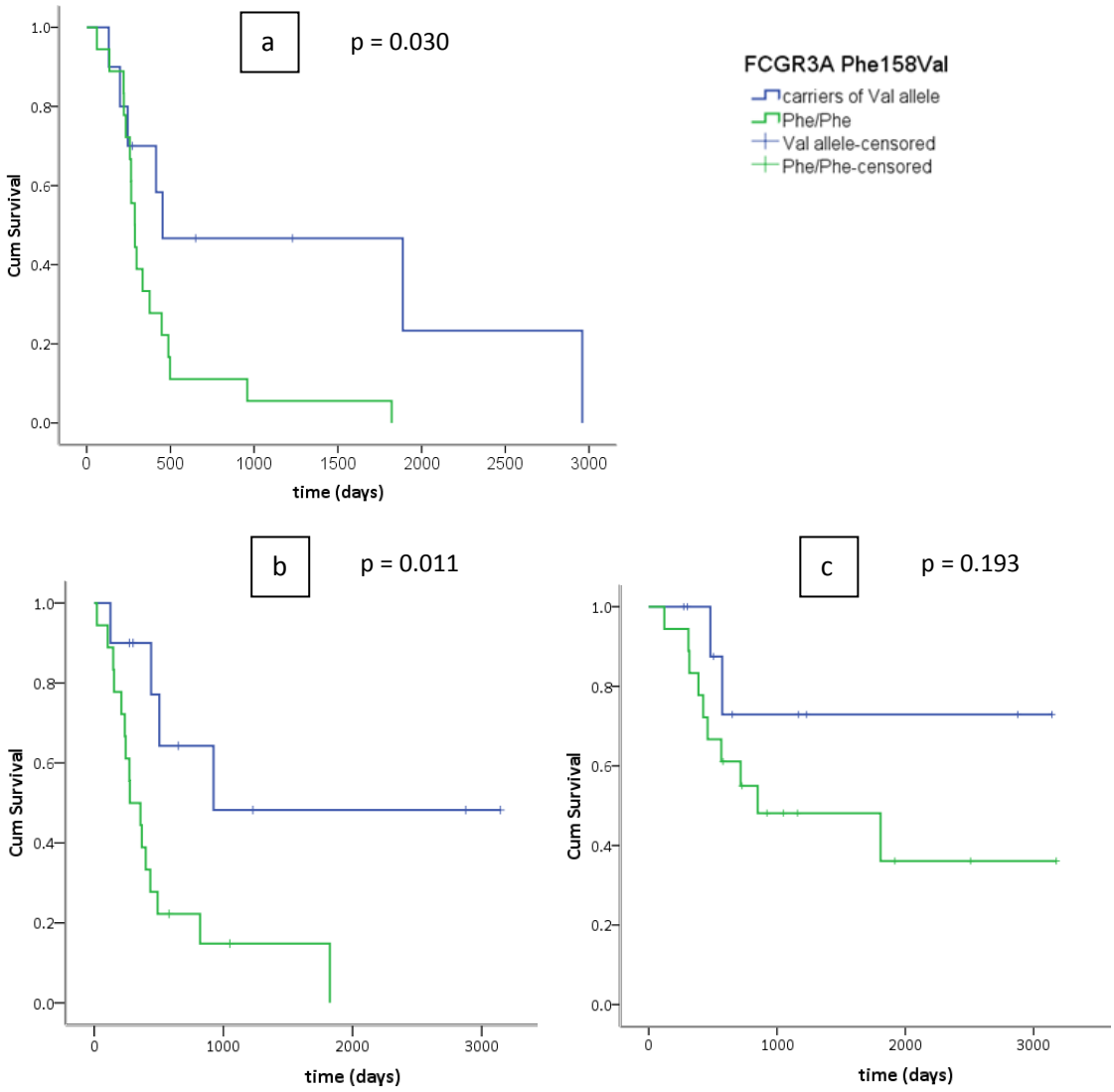

Figure 6.12: Influence of FCGR3A SNP on parameters of treatment outcome in advanced patients treated with trastuzumab.
a) time to progression (TTP); b) time on trastuzumab (TOT); c) overall survival (OS). P-values refer to significance levels of log-rank tests.

6.7 Toxicity

As described in Section 1.3.3, cardiotoxicity is the most clinically significant among the side effects attributed to trastuzumab. It was decided to limit the analysis of cardiotoxicity to the 'adjuvant' population (Table 6.4), since this group was more homogenous than the 'advanced' group in terms of previous treatments, concomitant anti-cancer medications and other potentially confounding factors including disease-

related symptoms. Such analysis, in the context of an observational study, entailed a series of potential obstacles which are described in the next sections.

6.7.1 Choice of cardiac end-points

Potential symptoms of cardiac dysfunction (including fatigue, breathlessness, and peripheral oedema) were collected at every visit, but were not included in the analysis for the following reasons:

- they were in the vast majority of cases mild or moderate;
- it was often difficult to discriminate whether they were due to trastuzumab, to other concomitant medications or to the previous chemotherapy treatment;
- they were not always uniformly reported and graded in the clinical notes.

An objective measure of cardiac function is the ejection fraction of the left ventricle (LVEF), which is the fraction of blood pumped out of the left ventricle with each heart beat and is measured either with a radioisotope scan (MUGA) or with echocardiography. The normal values in the general adult population have been reported to range widely between 50 and 80%. An absolute LVEF drop of more than 10% and an LVEF drop to below 45% have often been included in the evaluation of cardiotoxicity in trastuzumab-treated patients (Suter *et al.*, 2007).

In the 'adjuvant' population LVEF was generally measured before adjuvant chemotherapy, after adjuvant chemotherapy (before start of trastuzumab) and then regularly (every 3 or 4 months) during trastuzumab treatment. These variations were included as meaningful end-points in the analysis, although some caveats must be taken into account:

- Pre-adjuvant chemotherapy LVEF was not always available;
- The same timing of LVEF measurement was not always followed in this population, so that some patients had more scans than others;
- LVEF measured by MUGA and by echocardiography were in some cases not easily comparable;
- The timing of LVEF measurements was also affected by the implementation of new guidelines (Jones *et al.*, 2009), recommending less frequent monitoring compared to the previous guidelines (Suter *et al.*, 2007) derived from the HERA clinical trial protocol (Piccart-Gebhart *et al.*, 2005).

When a significant drop in LVEF was observed (accompanied or not by symptoms) one or more of the following actions was taken:

- Trastuzumab was temporarily suspended;
- Cardiac medication was introduced;
- Trastuzumab was definitively suspended.

These medical decisions were included as additional end-points of cardiac toxicity, taking into consideration that they can be influenced by the following elements:

- Severity of the LVEF drop;
- Presence/absence of symptoms;
- Pre-existing cardiovascular risk;
- Age of the patient;
- Risk of recurrence of the tumour;
- Implementation of the aforementioned cardiac monitoring guidelines (Jones *et al.*, 2009), which recommended simplified criteria regarding trastuzumab interruption, restarting and introduction of cardiac medications.

All end-points (summarized in Table 6.9) were included in the analysis, with the general caveat that there is a partial overlap among them and they can reflect slightly different clinical aspects of the trastuzumab-induced cardiotoxicity.

End-points		**Cases (no.)**	**Event (no.)**	**Censored (no.)**
A	LVEF drop ≥ 10%	127	37	90
B	LVEF drop below 45%	137	24	113
C	A and B	134	17	117
D	Cardiac medication introduced	138	44	94
E	Trastuzumab temporarily or definitively stopped for cardiac reasons	139	31	108
F	Trastuzumab definitively stopped for cardiac reasons	139	18	121
G	A and B and F	139	12	127

Table 6.9: Cardiotoxicity end-points

6.7.2 Choice of factors potentially impacting cardiotoxicity

A list of potential factors which might influence the occurrence of trastuzumab-induced cardiotoxicity was considered (Table 6.10).

Factors		Cases (no.)	1 (no.)	2 (no.)	3 (no.)
1	Age groups (years) (1= <50, 2 = 50-60, 3 = >60)	139	49	42	48
2	Pre-existing cardio-vascular risk factors (1 = yes, 2 = no)	139	36	103	
3	Adjuvant radiotherapy to left chest wall (1 = yes, 2 = no)	139	32	107	
4	BMI groups (kg/m^2) (1 = <25, 2 = 25-30, 3 = >30)	137	41	60	36
5	Baseline LVEF (1 = above median, 2 = below median)	106	51	55	
6	LVEF drop ≥ 7% due to adjuvant chemo (1 = yes, 2 = no)	76	28	48	
7	Cardiac medication before start trastuzumab (1 = yes, 2 = no)	139	22	117	
8	Trough [trastuzumab] (1 = above median, 2 = below median)	106	53	53	
9	Year start trastuzumab (1 = 2006-2008, 2 = 2009-2011)	139	62	77	
10	HER2 Ile655Val (1 = Ile/Ile, 2 = Val carriers)	139	87	52	
11	HER2 Ile655Val (1 = Ile carriers, 2 =Val/Val)	139	132	7	
12	HER2 Ala1170Pro (1 = Ala/Ala, 2 = Pro carriers)	139	59	80	
13	HER2 Ala1170Pro (1 = Ala carriers, 2 = Pro/Pro)	139	126	13	
14	FCGR2A His131Arg (1 = His/His, 2 = Arg carriers)	139	32	107	
15	FCGR2A His131Arg (1 = His carriers, 2 = Arg/Arg)	139	97	42	
16	FCGR3A Phe158Val (1 = Val/Val, 2 = Phe carriers)	139	13	126	
17	FCGR3A Phe158Val (1 = Phe/Phe, 2 = Val carriers)	139	65	79	

Table 6.10: Parameters evaluated as potentially influencing the occurrence of trastuzumab-induced cardiotoxicity.
'1', '2' and '3' refer to groups defined in the table for each parameter

This included several elements which had previously been described in the literature as potential risk factors, such as age, pre-existing cardio-vascular risk factors (including hypertension, diabetes, coronary disease, valvular heart disease, atrial fibrillation, idiopathic cardiomyopathy) and adjuvant radiotherapy to the left chest wall. The prescription of cardiac medications before the start of trastuzumab was regarded as an additional indicator of cardiac risk assessed by the physician. Pre-trastuzumab LVEF and a decrease in LVEF between pre-chemotherapy and post-chemotherapy (pre-trastuzumab) measurements were considered as parameters potentially influencing the LVEF variation during trastuzumab treatment.

Further factors included body mass index, trastuzumab trough plasma concentration and the year in which trastuzumab was commenced (grouped in 2006 - 2008 and 2009 - 2011), which was able to discriminate with good approximation between patients who were affected by the introduction of the new cardiac monitoring guidelines and those who were not.

The effect of the four SNPs (in HER2 and FCGR genes) was accounted for according to both the recessive (i.e. only having two copies of the causal allele affects the phenotype) and dominant model (i.e. having one or two copies of the causal allele has the same effect).

Similarly to the cardiac end-points, it should be considered that some of the putative factors are inter-related. For instance, a potential cumulative effect can be hypothesized for some factors (e.g. age and pre-existing cardiovascular risk), as well as a potential causal effect of some factors on others (e.g. baseline LVEF, LVEF drop with chemotherapy, cardiac medication prescribed before start of trastuzumab).

6.7.3 Analysis

Since 26 of the 139 patients (19%) were still on treatment at the time of the analysis, it was decided to treat the end-points listed in Table 6.9 as time variables, with 'event time' being the time from the day of first trastuzumab administration to the day of the event and 'censoring time' being the time from the day of first trastuzumab administration to the day of last trastuzumab administration.

An exploratory series of log-rank tests were then performed in order to compare these variables in patients grouped according to the factors listed in Table 6.10.

As can be seen in Tables 6.9 and 6.10, end-points and factors were not always available in all of the 139 treated patients. The significance levels (p-values) of these tests are summarized in Table 6.11. Given the high number (119) of statistical tests performed, and the consequent high likelihood of getting a significant result by chance, these figures must be interpreted with extreme caution and with a descriptive intent.

Firstly, the year of start of trastuzumab (row 9 in Table 6.11) showed a trend towards an association with several cardiac end-points. In particular, the patients who started trastuzumab in years 2006 - 2008 had a higher likelihood of having their treatment temporarily or definitively interrupted due to heart-related side effects compared with patients who started in years 2009 - 2011 (Figure 6.13). The p-value here (< 0.0005) was close to the threshold defined using the Bonferroni correction for multiple comparisons (0.00042 for a target alpha level of 0.05). This effect is plausible and

might be explained by the implementation of the aforementioned new cardiac monitoring guidelines.

	A	B	C	D	E	F	G
1	0.178	0.162	*0.046*	0.770	*0.045*	0.063	0.121
2	0.500	0.232	0.470	0.500	0.656	0.411	0.524
3	0.256	0.143	0.145	0.386	0.978	0.478	0.296
4	0.870	0.415	0.626	0.801	0.835	0.984	0.917
5	**0.000**	0.438	0.563	0.618	0.952	0.726	0.712
6	0.052	0.137	*0.044*	0.308	0.676	0.095	*0.026*
7	0.938	**0.003**	**0.003**	0.622	*0.019*	0.143	*0.012*
8	0.573	0.206	0.489	0.916	0.733	0.272	0.254
9	0.102	0.061	0.082	**0.002**	**0.000**	**0.008**	0.152
10	0.898	0.855	0.989	0.745	0.782	0.164	0.360
11	0.723	0.248	0.483	0.736	0.603	0.311	0.419
12	0.062	0.307	0.905	0.791	0.377	0.814	0.533
13	0.110	0.248	0.067	0.830	0.581	*0.047*	**0.004**
14	0.557	0.870	0.659	0.728	0.163	0.105	0.392
15	0.162	0.718	0.971	0.512	0.702	0.892	0.735
16	0.521	0.350	0.527	0.995	0.862	0.291	0.888
17	0.072	0.086	0.069	0.235	0.658	0.108	0.133

Table 6.11: Significance levels (p-values) of multiple logrank tests comparing the time variables listed in Table 6.9 in patients grouped according to the factors listed in Table 6.10.
Letters (A to G) and numbers (1 to 17) refer, respectively, to end-points listed in Table 6.9 and factors listed in Table 6.10.

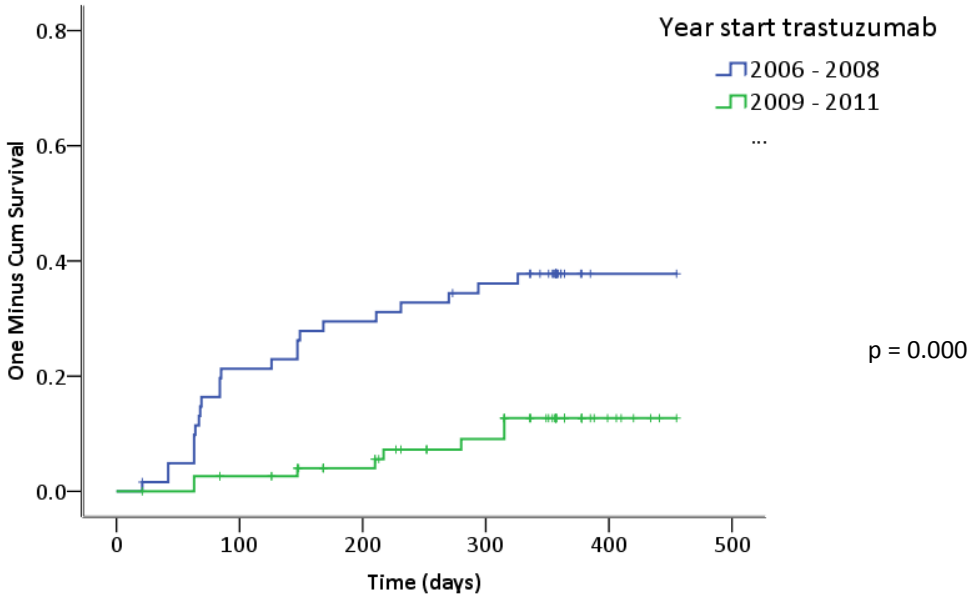

Figure 6.13: Influence of year of trastuzumab start on time to trastuzumab temporary or definitive suspension for cardiotoxicity

A second significant association is shown between baseline LVEF (row 5 in Table 6.11) and time to LVEF drop ≥ 10%. The logrank test, conducted only in a proportion of patients (76 out 139) in which baseline LVEF measurements were comparable (because all performed by MUGA scan), showed that patients with higher baseline LVEF had a higher likelihood of experiencing a LVEF drop ≥ 10% (Figure 6.14). This might be at least in part explained by the statistical phenomenon of the 'regression to the mean', which describes the natural variation in repeated data due to random measurement errors (Barnett *et al.*, 2005).

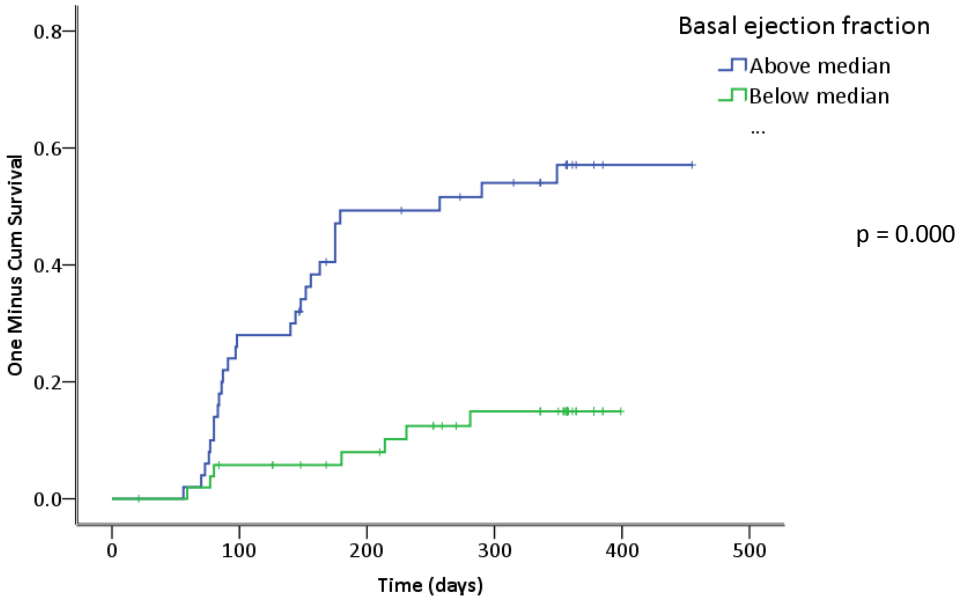

Figure 6.14: Influence of basal LVEF on time to absolute LVEF drop ≥ 10%

A third consideration concerns the potential effect of the prescription of cardiac medications before the start of trastuzumab (row 7 in Table 6.11). Although the p-values are not near the adjusted threshold, the presence of a trend towards an association with different cardiac end-points suggests that this parameter, which reflects an overall pre-existing cardiovascular risk assessed by the physician, might be associated with the occurrence of trastuzumab-induced heart toxicity.

It is impossible to draw any conclusion with regard to the possible effect of the four SNPs and trastuzumab trough concentration in plasma on cardiotoxicity from these data (rows 8 and 10 - 17 in Table 6.11). Even the association of the HER2 Ala1170Pro

Pro/Pro genotype with end-point E (LVEF drop ≥ 10% and below 45% and trastuzumab definitively stopped), with a p-value of 0.004 and not accompanied by significant hits for other cardiac end-points (row 13 in Table 6.11), is not sufficient to generate any hypothesis on a potential causative role of this SNP.

Of note, in only two cases was an extreme decrease in LVEF (down to 15% and 10%) observed in this population. This was accompanied by symptoms of severe congestive heart failure which required admission and intensive treatment in the Cardiology Department. In both cases trastuzumab was definitively discontinued and the patients recovered in terms of performance status and LVEF, although a return to the baseline values was obtained only after 6 and 10 months. One of the two patients experienced disease recurrence after 17 months since the start of trastuzumab treatment. Table 6.12 describes the clinical characteristics of these two cases, including their germ-line genotype for all the four SNPs considered. The very low number of congestive heart failure events does not allow any further statistical analysis.

	Patient # 172	Patient # 232
Ethnic Group	Caucasian	Caucasian
follow-up (months)	29	26
Year start trastuzumab	2008	2009
Age start trastuzumab (years)	68	51
Tot number of cycles received	7	9
BMI (kg/m^2)	26.7	24.8
Cardiovascular co-morbidities	hypertension	No
LVEF pre-chemotherapy (%)	55	47
LVEF pre-trastuzumab (%)	62	52
Minimum LVEF during trastuzumab (%)	10-15	<10
Cardiac medication prescribed before start of trastuzumab	No	Yes (ACE-inhibitor)
Type of adjuvant chemotherapy	Epirubicin x 4 → Capecitabine x 4	FEC x 3 → Docetaxel x 3
Epirubicin cumulative dose (mg)	400	300
Adjuvant radiotherapy	Yes	Yes
Dose and site of radiotherapty	45 Gy to left chest wall, axilla	50 Gy to right axilla + supraclavicular fossa; 40 Gy to right chest wall
HER2 Ile655Val	Ile/Ile	Ile/Ile
HER2 Ala1170Pro	Pro/Pro	Pro/Pro
FCGR2A His131Arg	His/His	His/His
FCGR3A Val158Phe	Phe/Phe	Phe/Val
[trastuzumab] above/below median	Below	Below
Tumour histologic type	Ductal	Ductal
Diameter (mm)	29	25
Focality	Unifocal	Unifocal
Lymph nodes involved	5 out of 15	10 out of 10
Grade	3	3
Hormone Receptors	Positive	Negative
HER2 IHC score	2 +	3 +
ISH performed	Yes (FISH: 6.2 HER2 copies/cell)	No
Recurrence	No	yes
Time to recurrence (months)	29 +	17
Death	No	no

Table 6.12: Characteristics of two patients who experienced severe congestive heart failure during trastuzumab

6.8 Discussion

The original design of this section of the project was mainly oriented towards the evaluation of advanced stage patients treated with trastuzumab and the role of pharmacogenetic, pharmacokinetic, and biomarker factors in this context. Indeed, part of the initial power calculations (Section 2.11) for the study was based on this subgroup of patients. However, the rapidly increasing use of the drug in the 'adjuvant' setting made the early stage population the major subgroup in the study (Figure 6.1), offering both the opportunities and the challenges of examining different aspects of trastuzumab treatment.

In the limited group of advanced stage patients we observed a possible positive effect of the FCGR3A Phe158Val Val allele (in the dominant model) in delaying tumour progression. This observation, limited by the small number and heterogeneity of patients as well as the exploratory nature of the simple logrank test, must be put in the context of several papers published after this project was initiated.

Table 6.13 shows the findings of a list of studies which explored the potential effect of the FCGR SNPs on trastuzumab treatment. Although these studies are not directly comparable in terms of samples size, disease setting and methodology, their results appear inconsistent, with two works (not published *in extenso)* (Foster *et al.*, 2002; Hurvitz *et al.*, 2009a) showing no significant association with the SNPs and two full publications (Musolino *et al.*, 2008; Tamura *et al.*, 2011) suggesting a role for the FCGR variations. Of note, the only study which had the opportunity of evaluating this issue as part of a randomized controlled trial (Hurvitz *et al.*, 2009a) did not report any data for the control arm and, maybe more importantly, did not explore the possible ability of the two SNPs to predict a differential benefit of trastuzumab (for instance in terms of disease-free survival hazard ratio).

Ref	SNP analysed	Genotyping method	H-W equilibrium	LD	Setting	Number of patients	Regimen	Control Arm	Variables	Associations
Foster *et al.* (2002)	FCG3A 158 F/V	PCR	No (p=0.016)	NA	Metastatic, 2nd or 3rd line *	63	trastuzumab single agent weekly	No	ORR	No association
Musolino *et al.* (2008)	FCGR3A 158 F/V, FCGR2A 131 H/R, FCGR2B 232 I/T	PCR & direct sequencing	Yes, except for FCGR2B in trastuzumab treated patients	No LD	Metastatic, 1st line	54	trastuzumab weekly plus paclitaxel or docetaxel	Concomitant, no randomization, 34 Her2-neg metastatic treated with taxanes	ORR, PFS, *ex vivo* ADCC	FCGR3A 158 V/V: higher ORR, longer PFS, better ADCC. FCGR2A 131 H/H: '*trend*' for higher ORR, longer PFS and better ADCC. Patients with 158 V/V and/or 131 H/H: higher ORR and longer PFS (only independent predictive factor) and better ADCC. No association for FCGR2B. No association in control arm
Hurvitz *et al.* (2009a)	FCGR3A 158 F/V, FCGR2A 131 H/R	Sanger sequencing and Sequenom mass spectrometry	NR	NR	Adjuvant **	~ 1200 (including controls)	ACTH or TCH	Randomized, ~ 400 Her2-pos patients treated with ACT	DFS	No association of SNPs with DFS of trastuzumab treated patients. *Data not reported for control arm. Not explored benefit derived from trastuzumab (DFS HR) stratified by SNP genotype*
					Metastatic, 1st or 2nd line	43	"trastuzumab -based"	No	TTP	No association of SNPs with TTP
Tamura *et al.* (2011)	384 SNPs of different FCGR loci	GOLDENGATE beads array (illumina Co.)	NR	NR	Neoadjuvant	15	FEC followed by weekly trastuzumab	No	pCR	FCGR2A 131H/H: higher pCR rate
					Metastatic (1st line?)	36	weekly trastuzumab	No	ORR and PFS	FCGR2A 131H/H: higher ORR and longer PFS. FCGR3A 158V/V: 'trend' for higher ORR

Table 6.13: Trastuzumab and FCGR SNPs in clinical trials
* = subset of H0649G trial (Cobleigh *et al.*, 1999); ** = subset of BCIRG 006 trial (Slamon *et al.*, 2011); PCR = polymerase chain reaction; ORR = Overall Response Rate; DFS = Disease-Free Survival; PFS = Progression-Free Survival; TTP = Time To Progression; LD = Linkage Disequilibrium analysis; ADCC = Antibody-dependent cell-mediated cytotoxicity; pCR = pathological Complete Response; NA = not applicable; NR = not reported; FEC = fluorouracil, epirubicin, cyclophosphamide; ACTH = Adriamycin + Cyclophosphamide followed by paclitaxel: trastuzumab weekly with T, then 3-weekly, total 1 year; TCH = Docetaxel plus carboplatin: trastuzumab weekly with TC, then 3-weekly, total 1 year, ACT = Adriamycin + Cyclophosphamide followed by paclitaxel; HR = hazard ratio

Another important issue closely connected with the putative role of the two SNPs is the potential influence of linkage disequilibrium on the analysis. Both FCGR2A and FCGR3A genes (like the rest of the classical low-affinity FCGR genes) are located on chromosome 1 long arm (1q23) and FCGR2A His131Arg and FCGR3A Phe158Val are separated by approximately 35 kb. Our observation of a moderate LD between the FCGR SNPs is in accordance with the majority of literature data (van der Pol *et al.*, 2003; Lejeune *et al.*, 2008), although some early papers presented different results (Lehrnbecher *et al.*, 1999). A non-random association of alleles at these two loci might in fact potentially lead to the attribution of a causative role to a SNP which is only associated with the 'true' causative genetic variation (Lejeune *et al.*, 2008). In terms of mechanism it is important to highlight that the affinity profiles of the various Fc Gamma Receptor genes for the different Ig subclasses are different, as is the effect of the SNPs on differential binding affinity (as described in Section 1.4.4). In particular, while a different affinity of the two FCGR3A allelic forms for human IgG1 (like trastuzumab) has been described (Wu *et al.*, 1997), for the FCGR2A SNP a difference was initially demonstrated only for human IgG2 (Parren *et al.*, 1992). However, as mentioned in Section 1.4.4, a recent paper (Shashidharamurthy *et al.*, 2009) suggested that the His variant might also have higher binding affinity for human IgG1 and IgG3. Taking all of the available data together, the role of the FCGR SNPs on the outcome of patients treated with trastuzumab still needs to be elucidated.

This is the first study, to the best of our knowledge, looking at the PK and biomarker aspects of a population mainly composed of early stage patients treated with three-weekly adjuvant trastuzumab. While the PK data seem in accordance with the previous literature, the inverse relationship between trastuzumab trough concentration and circulating HER2 concentration reported in the setting of metastatic disease (Pegram *et al.*, 1998) was not observed in our 'adjuvant' setting. The vast majority of the HER2 measurements were below the lower limit of quantification of the assay and this is plausible in patients with no macroscopic evidence of disease. Whether high circulating HER2 levels in these patients might predict a recurrence of the disease or a rise between pre-trastuzumab and on-trastuzumab determination might suggest lack of trastuzumab benefit will be assessed with longer follow-up and more disease-progression events. The use of circulating HER2 testing in the management of advanced patients has been a matter of discussion for a long time, as discussed in

Section 1.2.3 (Leyland-Jones and Smith, 2011). Our very limited data in the advanced stage group cannot provide any significant contribution to this debate.

The analysis of cardiotoxicity in the 'adjuvant' group entailed many challenges and limitations, as detailed in Section 6.7. They included, in the first place, the general effect of changes in clinical practice on the choice of the appropriate end-points in an observational trial. Other specific limitations were given by the intrinsic value of the ejection fraction variation as an objective measure of the heart-related effects of trastuzumab. In fact, asymptomatic or mildly symptomatic LVEF drops (the majority of the events in our population) might not be the optimal clinical end-point. Furthermore, this parameter has been shown to be influenced by many factors (including pre-existing cardiac risk, previous anthracyclines administration and baseline LVEF value) in several publications (as discussed in Section 1.3.3) and some of these effects seem to be confirmed by our observations. It might be argued that the potential effect of genetic variations can be masked by these clinical parameters in a sample of limited size.

Congestive heart failure is a very rare event in this setting and this was confirmed by our data, with only two events observed. The evaluation of the influence of genetic determinants on this clinically significant side effect would require larger cohorts of patients and/or different research approaches, such as genome-wide association studies.

With the limitations mentioned above, our study in a homogenous and adequately characterized group of patients does not confirm the role of the HER2 Ille655Val SNP in the risk of trastuzumab-related cardiotoxicity, as suggested by Beauclair *et al.* (2007). It must be taken into account, however, that Beauclair's publication was based on a smaller and more heterogeneous sample of advanced stage patients.

The FCGR2A receptor is expressed in human ventricular cardiomyocytes, where it might contribute to the development of dilated cardiomyopathy (Staudt *et al.*, 2007). Moreover, a recent paper suggested that the FCGR2A His131Arg SNP might influence the efficacy of immuno-modulatory therapy involving immunoadsorption and subsequent IgG substitution in patients with this disease (Staudt *et al.*, 2010). This prompted us to investigate the role of the FCGR SNPs in the adjuvant trastuzumab setting, but an analogous effect of these SNPs on trastuzumab-induced cardiotoxicity could not be demonstrated. However, it is interesting to notice that both patients who

experienced severe congestive heart failure were His homozygous for the FCGR2A His131Arg SNP.

In conclusion, our data contribute to the published and currently active trials which aim to better elucidate the pharmacogenetics of trastuzumab treatment in breast cancer. This research area, given the emergence of other effective HER2-targeted drugs (such as lapatinib, pertuzumab and trastuzumab-DM1) which offer diversified therapeutic options to patients and clinicians, will become more and more important if we are to achieve a better individualization of the treatment of patients with HER2-positive tumours.

Chapter 7. Conclusion

The evaluation of biological and clinical diversity in breast cancer has emerged as one of the most promising areas in oncology research over the last decades, as described in Sections 1.1.2 through 1.2.4 (Bertos and Park, 2011). While the biology of the tumour (including the study of oncogenes and tumour-suppressor genes, signalling pathways, epigenetic changes etc.) has been the most extensively studied aspect of this diversity, the host's genetic background has also been regarded as a potential factor influencing the natural history of the disease and the impact of different therapeutic approaches. The main aim of the study described in this thesis was in fact to investigate different aspects of the possible role of germ-line genetic variations in this complex disease. Single nucleotide polymorphisms (SNPs) have been investigated as potential risk factors for the development of breast cancer using different research strategies, including candidate gene approach and genome-wide association studies (GWAS) (Peng *et al.*, 2011; Zhang *et al.*, 2011). However, given the biological heterogeneity of this neoplasm, one might hypothesize that different SNPs can play a role in the biology of different subtypes of breast cancer, rather than in the development of the disease as a whole.

The HER2-positive tumours, which account for up to 20% of all cases, certainly form one of the most widely studied breast cancer subtypes and constitute the main focus of this thesis. Our study showed that the less frequent variant (proline) of a SNP (Ala1170Pro) occurring in the HER2 gene itself is associated with HER2 over-expression/amplification in a population of over 360 breast cancer patients (Chapter 4). This coding SNP is related to an amino-acid located in the C-terminal intracellular regulatory domain of the receptor, but its potential mechanistic role has not been examined. Several bioinformatics tools (described in Section 5.7) predict that Ala1170Pro might affect the structure or function of the HER2 protein.

In contrast, another extensively studied SNP affecting a residue located in the trans-membrane domain of the receptor (Ile655Val) was not associated with HER2 over-expression/amplification in our cohort (Chapter 4).

In order to investigate further the role of these SNPs, the same genetic variants were explored in the context of DNA extracted from the patients' primary tumours. This

analysis was performed in a significant fraction of the whole breast cancer population (2/3 of total) which was also representative of the total sample in terms of tumour characteristics (Section 5.3). In particular, we hypothesized that the proline allele of the Ala1170Pro could be more 'pathogenic' than the alanine allele and, therefore, undergo a positive selection through allele-specific amplification during the development of HER2-positive tumours (Section 5.2). This hypothesis, however, was not confirmed by our observation on the distribution of the alleles. In conclusion, although the observation of an association of the proline allele of Ala1170Pro with HER2 positivity is intriguing, the role of the two HER2 SNPs in HER2 over-expression/amplification remains to be elucidated.

The natural history and prognosis of HER2-positive breast cancer patients has radically changed since trastuzumab, the first HER2-targeted treatment, was developed, approved and introduced in clinical practice (Sections 1.3.1 through 1.3.5) (Dawood *et al.*, 2010). However, resistance to treatment (primary and acquired) and the toxicity profile of the drug can limit the effectiveness of trastuzumab-based therapy. The second main object of this project was the investigation of a group of trastuzumab-treated patients in terms of disease outcome and side effects. The analysis of the influence of pharmacogenetic (PG), biomarker and pharmacokinetic (PK) parameters on these clinical aspects was presented in Chapter 6.

Of note, while the evaluation of circulating HER2 was conducted using a commercially available ELISA kit, the measurement of trastuzumab in plasma samples (PK) was made possible by the development and validation of a novel cell-based ELISA (Chapter 3).

The evaluation of patients with advanced disease treated with trastuzumab was limited by the small available patient population and the heterogeneity of the combination treatments (Section 6.5.2 and 6.6). However, the observation of a possible association of the valine allele of the FCGR3A Phe158Val SNP with a better outcome is in accordance with two studies published while this project was in progress (Musolino *et al.*, 2008; Tamura *et al.*, 2011) (discussed in Section 6.8).

A particular section of the thesis was dedicated to the evaluation of cardiac toxicity in a group of 139 patients treated with adjuvant trastuzumab (Sections 6.7.1 through 6.8). Although a role of germ-line genetic variants (either in the HER2 gene or in Fc Gamma Receptor genes) could not be demonstrated, this analysis highlighted the challenges and limitations which can be encountered in the conduct of an

observational pharmacogenetic study. As discussed in Sections 6.7.3 and 6.8, these included the number of clinical parameters potentially influencing trastuzumab-related cardiotoxicity, the choice of the appropriate end-points, the intrinsic value of the ejection fraction variations and the rarity of severe congestive heart failure events.
Further to the results presented in this thesis and summarized above, this project made possible the construction of an archive composed of germ-line DNA samples, tumour DNA samples, plasma samples and tumour FFPE blocks from a population of over 360 breast cancer patients (enriched in HER2-positive cases, as discussed in Chapter 4). These biological samples, collected, transferred, stored and analysed according to the Human Tissue Act (HTA, 2004), are matched with individual patients' anonymised data (including tumour pathology, medical history, outcome and side effects of treatments).
All these samples and data are available for the exploration of further potential factors which might influence the biology of the disease and/or its response to treatment. Current plans include the identification and evaluation of other relevant SNPs and the construction of tissue microarrays (TMAs) which would allow multiple IHC analyses. This future work will hopefully contribute to the increasing amount of research aimed at a better understanding of the biology of breast tumours and at a better individualization of anti-cancer treatments.

Appendix 1. Trastuzumab review

This article was prepared and published in 2010 and constitutes part of the literature review which was conducted for this research project.

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REVIEW

The Pharmacology of Trastuzumab and Its Use in Breast Cancer

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Abstract: Trastuzumab is a therapeutic humanised monoclonal antibody targeting the Her2 receptor tyrosine kinase. Her2 is over-expressed, due to gene amplification, in approximately 30% of breast cancers and confers a worse prognosis. Trastuzumab has been used clinically for over a decade and is now used routinely in the treatment of Her2-positive breast cancer in the metastatic and adjuvant setting. Its mechanisms of action and resistance, as well as its pharmacokinetics and efficacy in different clinical scenarios have been explored and increasingly clarified during its pre-marketing and post-marketing development. Nonetheless, numerous scientific and clinical issues remain not fully elucidated. This review discusses the pharmacodynamic and pharmacokinetic properties of trastuzumab and its current use in the treatment of breast cancer.

Keywords: trastuzumab, Her2, breast cancer

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Introduction

Trastuzumab is a humanised monoclonal antibody (mAB) used therapeutically in the treatment of Epidermal Growth Factor Receptor 2 (ErbB2, Her2, Neu) positive breast cancer. The efficacious use of antibodies in the treatment of cancer has been a goal of biologists for over 100 years.[1] That it has recently come to fruition is due to numerous technical advances over the last 40 years, not least the development of monoclonal antibody technology. In the 1970s Kholer and Milstein developed a process whereby the fusion of antigen-exposed mouse spleen cells with mouse myeloma cells, followed by clonal selection, generated unlimited supplies of highly specific and clonally-derived antibodies.[2] They immediately recognised and commented on the therapeutic potential of this technology. However, substantial barriers to the clinical exploitation of mABs remained, not least immunogenicity of murine antibodies in humans.[3] Subsequent developments, using the nascent recombinant DNA technology of the 1970s and 80s, saw the splicing of human and rodent antibody sequences[4,5] to the point where hypervariable regions of human light and heavy immunoglobulin (Ig) chains could be substituted with antigen specific hypervariable regions of rodent origin prior to transfection into melanoma cell lines and clonal selection.[6] At the time of writing there are 31 FDA-approved monoclonal antibodies, 12 of which are used in oncology, either therapeutically or for imaging.[7]

The period between the end of the last century and the beginning of the current one has seen great improvements in the management of breast cancer, to the extent that an increased incidence in Europe has been accompanied by a decrease in mortality.[8] The decrease in mortality is attributable to the introduction of screening programs in a number of nations and improvements in therapy resulting from improved understanding of the molecular etiology of the disease. However, breast cancer is the most commonly occurring cancer in women, remains the second most common cause of death from cancer overall and is the most common cause of death from cancer in women aged between 20 and 59.[9] It is a heterogeneous disease and elements of this heterogeneity have been exploited to develop therapeutic options. For example ~70% of breast cancers are estrogen and/or progesterone receptor positive. Many of these tumours are dependent on estradiol for survival and proliferation, and therefore susceptible to inhibition of proliferation by small molecule estrogen signalling antagonists.[10] Her2 is over-expressed, due to gene amplification, in approximately 25% to 30% of breast cancers and is associated, in the absence of targeted therapy, with a poorer prognosis than Her2-negative tumours.[11] A 185 kDa receptor tyrosine kinase (RTK), Her2 in common with other RTKs has an extracellular domain, a transmembrane domain and a cytoplasmic kinase domain.[12,13] In contrast to other RTKs no extracellular ligand for Her2 has been demonstrated. Despite the lack of identified ligand, Her2 is still capable of mediating intracellular proliferative and pro-survival signalling via heterodimerisation with other members of the ErbB family of receptors and is believed to be the preferred binding partner of EGFR (Her1), Her3 and Her4.[14,15] This may be of particular importance for the facilitation of ligand driven Her3 signalling, as Her3 has limited intrinsic tyrosine kinase activity and requires the activity supplied by the heterodimeric partner.[16,17] Of the potential combinations of ErbB dimers the Her2/Her3 heterodimer is believed to be the main oncogenic dimer.[18] It has also been hypothesised that ligand-independent homodimerisation is sufficient to drive proliferative signalling.[19] This review will discuss the metabolism and pharmacology of trastuzumab and the use of trastuzumab in the clinic.

Metabolism

The study of the catabolism of therapeutic antibodies presents a challenge to pharmacologists. Generically the degradative metabolism of small molecule drugs can be categorised as a process of enzymatic transformation of a compound to more hydrophilic product (Phase I), more amenable to direct excretion or conjugation with an endogenous molecule (Phase II) prior to excretion.[20] The metabolism of small molecule drugs predominantly, but not exclusively, occurs in the liver and excretion generally occurs via urine and faeces. Therapeutic proteins generally, and therapeutic antibodies specifically, are not typically substrates for the characterised enzymes of drug metabolism. While the mechanism of trastuzumab metabolism has not been extensively studied, it is assumed that the pathway of degradation accurately reflects that of endogenous IgG antibodies, the endpoint of catabolism being the

release of the constituent amino acids by intracellular proteolytic degradation. The mechanisms that influence the metabolism of antibodies can be classified into three broad categories (Fig. 1).

FcγR

The FcγRs—may have a role in the clearance of antibodies,[21] but they also serve as the bridge between the Ab:Ag interaction and the effector mechanisms of the Ab-mediated immune response. By analogy, the FcγR molecules are essential for some of the proposed mechanisms of action of trastuzumab and will be discussed in the context of pharmacodynamics.[22]

FcRn

In the mid 1960s two apparently disparate observations of the behaviour of IgG *in vivo* were elegantly synthesised to generate a readily testable hypothesis.[23] These observations were:

- That ingested maternal IgG was transmitted across the intestinal epithelium of neonatal mice by pinocytosis and that this transmission was saturable, with functional antibody re-entering the circulation or being degraded in the epithelium.
- That the rate of IgG catabolism increased in proportion to concentration.

Brambell and colleagues postulated that both phenomena could readily be explained by the presence of a receptor that protected IgG from proteolysis in lysosomes. This receptor, termed FcRn for neonatal Fc receptor, was initially identified in the intestine of neonatal rats[24] and subsequently in the syncytiotrophoblasts of humans[25] and is the receptor that mediates the transfer of IgG from maternal to infant circulation in rats and humans respectively. Structurally the FcRn is a membrane bound heterodimer comprised of an alpha chain, which is a member of the MHC class I family, and a β2 microglobulin chain (β2M).[24] That the FcRn is also the IgG protective receptor postulated in the Brambell hypothesis has been demonstrated definitively, at least in mice, in gene knockout experiments. Mice deficient in either β2M or the FcRn alpha chain have both a lower concentration of endogenous IgG and a more rapid clearance of radio-labelled IgG.[26–30] In humans, FcRn is expressed in placenta, heart, lung, liver, kidney, pancreas, breast, endothelial cells and dendritic cells.[25,31–33] The Fc portion of antibodies interacts with the FcRn receptor at pH6, but not at physiological pH[25] and it is likely that plasma IgG is taken into endothelial cells by fluid phase endocytosis rather than receptor-mediated transfer. Once in the early endosome the FcRn bound IgG is protected from the lysosomal proteolysis and may potentially be transferred into the tissue or returned to the circulation. While the former scenario has not been disproved the latter has been demonstrated in a Tie 2 Cre/Lox mouse model engineered to have no expression of the FcRn alpha chain in endothelial or haematopoietic cells.[30] Phenotypically this mouse model resembles both the FcRn and β2M knock-out mice with a lower concentration of endogenous IgG and increased rate of clearance of human IgG, indicating that the expression of FcRn in these tissues alone

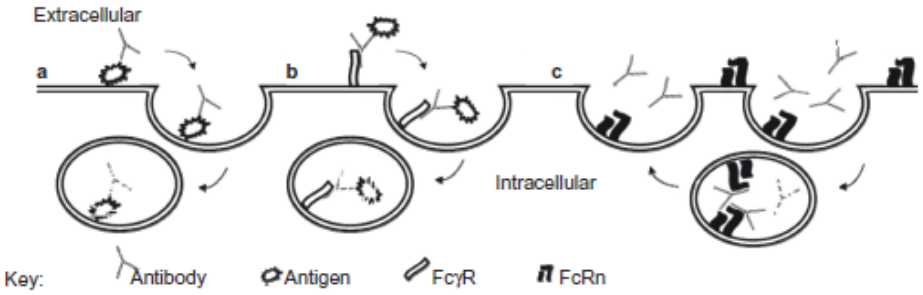

Figure 1. Receptor mediation of IgG catabolism. a) Antigen mediated endocytosis. Antigen on cell membrane is recognised by the antibody and the complex is then amenable to endocytosis and subsequent proteolysis in the lysosome. b) FcR mediated endocytosis of Ab:Ag complex. Fc Receptors on the surface of phagocytic cells interact with the antibody Fc region and may trigger endocytosis. c) FcRn mediated protection of IgG. Following fluid phase endocytosis and intra vesicular acidification IgG in the vesicle can interact with the FcRn receptor and is protected from proteolysis and available for recycling back to the interstitial compartment.

is sufficient to maintain the stability of IgG in blood.[30] Whether FcRn functions predominantly as a stabiliser rather than a transporter in all other adult tissues is not known. It is hypothesised that expression of FcRn in the proximal tubular cells of the kidney contributes to re-absorption of IgG from the urine[31] and it has been observed that IgG concentrations in the bile are very low despite the presence of FcRn expression on hepatocytes,[34] both phenomena that will result in reduced plasma IgG clearance. While FcRn function increases the stability of IgG and is the saturable molecule that results in nonlinear clearance of IgG at high concentrations, it is not envisaged that endothelial FcRn expression will influence the blood concentration of trastuzumab, which at maximum measured concentrations is ~5% of the IgG serum concentration, and at steady state is ~0.5%.[35,36] However, it is of interest that FcRn is expressed in normal breast and breast tumour tissue,[33] and may protect local concentrations of drug at the intended target. While FcRn should not influence the blood concentration of trastuzumab at therapeutic concentrations, under physiological conditions it is worth noting that high-dose corticosteroids do result in increased catabolism of IgG in both mice and humans[37,38] and it has been demonstrated that corticosteroids lead to a decrease in FcRn expression.[39]

Antigen-mediated catabolism

The interactions of antibody with antigen can influence the catabolism of antibodies, both by receptor-mediated endocytosis and the endocytosis of immune complexes following interaction of the antibody with soluble Ag.[21,40] This process will be saturable and may contribute to a non linear aspect of the pharmacokinetics of trastuzumab at low concentrations.[41,42] However, in contrast to the relationship between FcRn and IgG, the phenomenon of generic Ab:Ag-mediated catabolism cannot be extrapolated to the influence of membrane or soluble Her2 on the catabolism of trastuzumab. While the interaction between trastuzumab and the Her2 antigen may influence catabolism, it is also essential for the pharmacodynamics of trastuzumab and will be discussed below.

Pharmacokinetics

Trastuzumab is currently administered clinically as a 90 minute IV infusion of a 4 mg/kg loading dose followed by 2 mg/kg every week, or by a three weekly regimen of 8 mg/kg as a loading dose followed by 6 mg/kg every three weeks. A population PK analysis of data from 476 patients extracted from previous studies, the majority of whom were treated with the weekly regimen, revealed a two compartment linear model as the most appropriate fit of the data. The model predicted a steady-state trough concentration of 66 µg/ml with clearance of 0.225 l/d, volume of distribution (Vd) of 2.95 l and a terminal half-life (t½) of 28.5 days.[43] This compares with a t½ of 23 days for endogenous IgG and the Vd corresponds to plasma volume. High levels of circulating Her2 extracellular domain (ECD), more than 4 sites of metastases and high body weight were all associated with a lower predicted plasma concentration. While these covariates were statistically significant the magnitude of the effect was small compared to the overall variability of exposure and they are unlikely to be of clinical or predictive benefit.[43] The estimated steady-state trough concentration is comparable to that seen in the three weekly schedule.[36,44,45]

The population PK analysis was in contrast to earlier observations. Although the three initial phase one studies of trastuzumab have not been published *in extenso*, the available data show that half-life tended to increase with increasing dose, from approximately 1 day in the lowest dose group (10 mg) to 2 weeks in the highest dose group (500 mg).[46] Early phase II studies with a PK element reported a mean half life of between 1.1 and 11 days, with a high level of ECD being associated with a shorter half life and lower trough concentration.[41,47,48] The first published study using the weekly weight-adjusted regimen[48] reported PK parameters that, other than the mean t½ value of 6.5 days, were consistent with the subsequent population PK model[43] with Vd of 2.7 litres and a mean trough concentration at steady state of approximately 70 µg/ml. The first dose escalation study of trastuzumab to be published included concentration-time curves over a three week period from patients administered 1, 2, 4 or 8 mg/kg. The dose concentration-time curve at the highest dose appeared to show biphasic elimination with a fast initial decrease in trastuzumab concentration over 4 days followed by a slower decrease over the subsequent 11 days. Mean estimates of half life were short compared to those predicted from the two compartment linear model developed by Bruno et al,

ranging from 2.7 days following 1 mg/kg to 10.4 days following administration of the 8 mg/kg dose. A similar non-linear mechanism was seen, with clearance which decreased with increasing dose, suggesting that the pharmacokinetics of trastuzumab were non-linear and saturable at clinically relevant doses.[42] However, when plotted against dose the clearance and t½ values appeared to plateau at 4 mg/kg, indicating trastuzumab pharmacokinetics are linear and non-saturated at higher doses, suggesting that two distinct mechanisms of distribution or elimination influence clearance.

The initial assumption that trastuzumab pharmacokinetics were non-linear and the observation that accumulation can occur for up to 32 weeks before steady-state is reached, led to the hypothesis that a longer duration dosing schedule, more convenient for the patient, could be administered without lowering exposure to the drug. The three-weekly schedule results in a higher peak and lower trough concentration than seen with the weekly schedule, but overall exposure to drug and efficacy are comparable.[36,44] The effectiveness of the three-weekly schedule is more likely to be due to the t½ of 28.5 days than non-linear PK.

There are no apparent pharmacokinetic drug interactions between trastuzumab and cyclophosphamide, cisplatin, gemcitibine, paclitaxel or lapatinib.[43,44,46,49–51] With regard to the potential interaction with other monoclonal antibodies, a phase I trial of trastuzumab in combination with the anti-Vascular Endothelial Growth Factor (VEGF) monoclonal antibody bevacizumab reported that the pharmacokinetics of either agent were unaltered by co-administration.[52]

Mechanism of Action

The mechanism by which trastuzumab exerts an anti tumour activity is complex and likely to be multifactorial. The question of the clinical relevance of proposed mechanisms of action is also complicated by differences in the response of tumour cells to trastuzumab *in vivo* and *in vitro*. For example, while trastuzumab-induced apoptosis has been observed in clinical tissue and xenografts,[53,54] it is not commonly observed in tissue culture models. All proposed mechanisms are predicated on the interaction of the complementary determining region (CDR) of the antibody with the antigen, and the development of trastuzumab as an antibody specifically targeting Her2 can be viewed as a success. However, the complexity of trastuzumab's mechanism of action lies in the downstream consequences of the Ab:Ag interaction, and can be categorised as those that are immunologically mediated and those that are antagonistic of signalling pathways.

Immunological mechanisms

Antibody-dependentcell-mediatedcytotoxicity(ADCC) is an immunological process whereby the cytolytic activity of a subset of lymphocytes, natural killer (NK) cells, is targeted to the destruction of host tissues by target-specific antibodies (Fig. 2). The process is

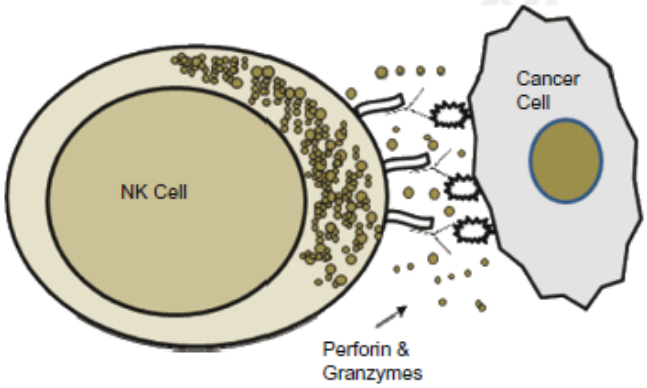

Figure 2. ADCC mechanism: Antibodies of the IgG isotype (Trastuzumab) bind the antigens (Her2) on the surface of target cell. Fc gamma Receptors (FcγR) on the NK cell recognizes the Fc region of the bound antibody on the target cell. Cross-linking of the FcγR on the NK cells promotes the release of Perforin and Granzymes, which induce apoptosis in the target cell.

mediated by the interaction of the antibody CDR with the antigen on the target cell surface and the interaction of the Fc portion of the antibody with the FcγRIII receptor on the surface of the NK cell. The formation of this interaction then triggers the release of cytotoxic effector proteins from the NK cell and the death of the target cell.[55] The role of ADCC in the efficacy of trastuzumab was first suggested *in vitro* as part of the initial characterisation of the drug[56] and has been subsequently demonstrated in murine models, *ex vivo* and in clinical samples. For example, trastuzumab prevents the growth of Her2-positive BT474M1 explants in wildtype nude mice, but this inhibition is abrogated in FcγR –/– animals of the same background.[57] Administration of trastuzumab enhances *ex vivo* ADCC activity in the neoadjuvant[58,59] and metastatic setting[60] and the induction of ADCC is associated with improved response. An increase in tumour-associated NK cells has also been observed in Her2-positive primary breast tumours in trastuzumab treated patients compared to controls not treated with trastuzumab.[22] The FcγRIIIa gene contains a single nucleotide polymorphism that results in a substitution of a valine for a phenylalanine at residue 158. There are suggestions that breast cancer patients who are homozygous for the valine genotype have increased ADCC activity and prolonged progression free survival when treated with trastuzumab.[59,61]

The clinical relevance of other antibody mediated immune effector mechanisms is uncertain. Low affinity anti-Her2 CD8+ T cells have been identified and can be clonally expanded *ex-vivo*. There is *in vitro* evidence that trastuzumab enhances the cytolytic activity of these cells, via internalisation of Her2 and increased expression of Her2 epitopes in the context of Class I MHC, but the clinical significance of this is unknown.[62] It has also been demonstrated that while trastuzumab can recruit complement to the Her2-positive cell surface this has no effect on cell death.[63]

Cell signalling mechanisms

The growth of Her2-positive cells is inhibited *in vitro* by trastuzumab and this growth inhibition is concomitant with inhibition of the pro-survival and proliferative PI3K/AKT and MAPK signalling pathways.[64-66] Of these two pathways, *in vitro* data suggest that the PI3 K/AKT axis is the most important. It has been demonstrated that introduction of constitutive AKT activity by transfection is sufficient to abrogate trastuzumab-mediated growth inhibition,[67] cell-cycle arrest and differential gene expression.[68] There is also evidence that functional PTEN, a phosphatase that antagonises PI3 K signalling, is essential for trastuzumab-mediated growth inhibition.[69,70] The lack of trastuzumab-induced apoptosis in cell line models is surprising in light of the observed inhibition of PI3 K signalling. In contrast, increased levels of apoptosis are observed in clinical tumour samples from patients treated with trastuzumab, but paradoxically this increase is seen without an increase in AKT phosphorylation in the same samples.[53] Another study has revealed an association between poor survival outcome in patients treated with trastuzumab and a potential high PI3 K activity phenotype, characterised by low PTEN expression or PI3 K mutation conferring constitutive activation.[70]

Anti-angiogenesis

Angiogenesis is the formation of new blood vasculature in response to low oxygen tension or angiogenic signalling pathways. It is considered a hallmark of cancer and is necessary for the growth of solid tumours beyond the size dictated by the diffusion of oxygen through the tissue.[71] Both low oxygen and the PI3 K/AKT signalling pathway can mediate an increase in the activity of the Hypoxia Inducible Factor-1 transcription factor and the subsequent induction and down-regulation of pro and anti angiogenic factors respectively.[72,73] These include VEGF which is a potent inducer of endothelial proliferation, and the anti angiogenic factor thrombospondin-1. Her2 expression has been shown to be correlated with VEGF expression in breast tumours.[74] This association appears to be mediated via the PI3 K/AKT signalling pathway[74,75] and induction of VEGF can be abrogated by trastuzumab *in vitro*.[76] Treatment with trastuzumab of mice bearing established tumours from xenografts of Her2-positive cells results in normalisation and decreased density of tumour vasculature.[77]

Inhibition of Her2 shedding

Her2 is cleaved by a metalloproteinase, ADAM10, to yield the released extra-cellular domain found in

plasma and a 95 kDa fragment that is the membrane-bound internal kinase domain.[78,79] In the pre-trastuzumab era expression of the p95 fragment was associated with poor prognosis.[80] Trastuzumab inhibits the cleavage of Her2 in SKBR3 and BT-474 cells *in vitro*.[81]

Early Clinical Studies

The early clinical development of trastuzumab, unlike the vast majority of anti-cancer agents, has been characterized by the exclusive focus on a particular subset of patients, i.e. the Her2-positive breast cancer patients. Since the first three phase I trials, patients have been pre-selected on the basis of Her2 expression in tumours. In these initial studies the authors failed to reach a maximum tolerated dose (dose range from 10 to 500 mg IV weekly) and none of the four reported serious adverse events was considered to be related to trastuzumab treatment.[46] Four subsequent phase II trials evaluated the activity of weekly administration, both as monotherapy and in combination. In particular, a first study of trastuzumab given as single-agent at a flat dose of 100 mg weekly with a loading dose of 250 mg showed an overall response rate (ORR) of 11.6% in 46 patients with pre-treated (median of 2 prior regimens) metastatic tumours expressing Her2 in more than 25% of cells (more than 50% of cells in 85% of cases).[36] A second combination study of trastuzumab (same schedule as above) and cisplatin (75 mg/m^2 every 28 days) showed an interesting ORR of 24% in a series of 37 heavily pre-treated patients. In this trial tumours were required to have evidence of over-expression (2+ or 3+) of Her2 as determined by immuno-histochemistry.[41] A third, much larger, multicentre trial evaluated the activity of a single-agent, weight-based weekly regimen (loading dose 4 mg/kg followed by 2 mg/kg) in 222 pre-treated patients. The ORR assessed by an independent response evaluation committee was 15% in the intent-to-treat population and the most clinically significant adverse event was cardiac dysfunction, occurring in 4.7% of cases.[48] Finally, a randomized trial conducted in previously untreated patients demonstrated the equivalence in terms of activity and safety of two different weekly schedules (2 mg/kg or 4 mg/kg). Most interestingly, a retrospective correlative analysis of Her2 gene amplification by fluorescent *in situ* hybridization (FISH) showed that objective remissions (26%) were almost entirely confined to FISH-amplified cases: ORR was in fact 34% in 79 FISH-positive cases and only 7% in 29 FISH-negative patients.[82] Taken together, the results of these initial clinical studies prompted the manufacturer and the researchers to broaden their evaluation within controlled settings.

Efficacy

Metastatic setting

In 2001 a pivotal randomized phase III trial provided evidence of trastuzumab efficacy in combination with chemotherapy in patients with previously untreated, Her2-positive, metastatic breast cancer.[83] Patients received chemotherapy (doxorubicin/cyclophosphamide or paclitaxel) either alone or in combination with the antibody. The addition of weekly trastuzumab conferred a clinically-significant increase in time to disease progression (4.6 vs. 7.4 months), ORR (50% vs. 32%), duration of response (9.1 vs. 6.1 months) and median survival (25.1 vs. 20.3 months). A second randomized trial of docetaxel alone or with trastuzumab achieved similar results.[84] Concomitantly with these large controlled trials two important phase II studies showed that a three-weekly schedule (8 mg/kg loading dose followed by 6 mg/kg), in combination with paclitaxel or as monotherapy, was able to achieve the same results in terms of response rates and plasma trough levels as the standard weekly regimen.[36,44] Beside the taxanes, other chemotherapeutic agents, such as vinorelbine, capecitabine and carboplatin have been tested in combination with trastuzumab in various non-randomized and randomized trials. The combination with vinorelbine, in particular, has been compared in a small randomized phase II trial with a weekly regimen of trastuzumab plus a taxane (either paclitaxel or docetaxel) and the results of this comparison revealed equivalence between the two arms in terms of efficacy and safety.[85]

A recent review of a large mono-institutional cohort of patients has shown that, despite the well-documented negative prognostic influence of Her2 over-expression, Her2-positive metastatic breast cancer patients treated with trastuzumab can achieve a better overall outcome than Her2-negative patients.[86]

Adjuvant setting

When used as adjuvant treatment for early stage Her2-positive breast cancers, trastuzumab has been consistently demonstrated to reduce the risk of recurrence by 30 to 50%. Table 1 shows the major findings of four large and two smaller randomized trials in terms of efficacy and toxicity, along with some notable differences with regard to various aspects, including the size of patient population, duration of treatment, type of associated adjuvant chemotherapy, trastuzumab schedule and timing of administration.

In particular, the large HERA trial[87–89] adopted a rather pragmatic approach, encompassing a wide range of adjuvant chemotherapy regimens in its inclusion criteria. This feature makes the study highly representative of clinical practice and confers a wide applicability to the results. Trastuzumab every three weeks was administered for one year after the completion of chemotherapy in this setting and a third randomization arm (data not released yet) evaluated a two-year treatment duration. The NSABP B-31 and the NCCTG-N9831 trials[90,91] reflect the popularity of the AC → P schedule (Doxorubicin/Cyclophosphamide followed by Paclitaxel). They both focused on the concomitant administration of paclitaxel and weekly trastuzumab, which had been shown to be synergistic, but the NCCTG-N9831 researchers also considered a comparison with a sequential arm. The BCIRG 006 study[92,93] aimed to specifically evaluate the efficacy of trastuzumab in association with an anthracycline-free regimen (Docetaxel/Carboplatin) or with a more standard regimen (Doxorubicin/Cyclophosphamide followed by Docetaxel).

As can be seen from a cross-comparison of the efficacy results reported in Table 1, the concomitant administration of trastuzumab (NSABP B-31/NCCTG-N9831 joint analysis and BCIRG 006) with chemotherapy seems to confer a slightly larger benefit in terms of efficacy, compared with the sequential administration, although these data could be biased by different durations of follow-up and different rates of treatment cross-over among the studies. On the other hand, incidence of cardiotoxicity appears slightly higher in the trials of concomitant trastuzumab.

Table 1. Reported trastuzumab adjuvant trials.

Study	Most recent update	Pts no.	Stage	Design	Trastuzumab administration	Pts with LVEF drop (%)
HERA	Gianni et al,[89]	5102	Node-pos and node-neg (tumour size > 1 cm)	Ch	–	0.8*
				Ch → H	3W; total 1 year	3.7*
				Ch → H	3W; total 2 years	NR
NSABP B-31	Perez et al,[91]	2006	Node-pos	AC → P	–	NR
				AC → PH	W; total 1 year	NR
NCCTG N9831	Perez et al,[91]	3505	Node-pos or high risk Node-neg	AC → P	–	NR
				AC → PH	W; total 1 year	NR
				AC → P →H	W; total 1 year	
BCIRG 006	Slamon et al,[93]	3222	Node-pos or high risk Node-neg	AC → T	–	11**
				AC → TH	W with T, then 3W; total 1 year	19**
				TCaH	W with TCa, then 3W; total 1 year	9**
FinHER	Joensuu et al,[95]	232	Node-pos or high risk Node-neg	T-FEC or V-FEC	–	10.5***
				TH-FEC or VH-FEC	W with T or V; total 9 weeks	6.8***
FNCLCC-PACS 04	Spielmann et al,[96]	528	Node-pos	FEC or ET	–	2.6****
				FEC or ET → H	3W; total 1 year	11.1****

The small FinHer trial[94,95] provided interesting findings on the potential efficacy of weekly trastuzumab administered with chemotherapy (vinorelbine or docetaxel) for a very limited period (9 weeks).

It is noteworthy that a recent medium-sized study of adjuvant trastuzumab failed to demonstrate a clear advantage in disease-free survival and overall survival.[96] The authors identified a potential explanation for this result in the rather high rate of treatment discontinuation, mainly due to cardiac toxicity.

Neo-adjuvant setting

Many non-randomized studies have demonstrated the extreme activity of trastuzumab in association with chemotherapy in the pre-operative setting.[97–99] A very promising randomized phase II trial confirmed the efficacy of this approach,[100] achieving an unprecedented rate of pathological Complete Remissions (pCR) (66.7% for chemotherapy plus trastuzumab versus 25% for chemotherapy alone) in a limited series of patients with operable primary breast cancer. Recently, a large randomized phase III trial has evaluated the combination of trastuzumab with a poly-chemotherapy regimen in the setting of locally-advanced breast cancer, achieving a benefit in terms of pCR rate and a substantial prolongation in event-free survival, with an acceptable cardiac safety.[101] In this study, which included non-inflammatory T4, inflammatory and N2 or ipsilateral tumours, trastuzumab was administered both pre-operatively (in association with doxorubicin, paclitaxel and cyclophosphamide/methotrexate/fluorouracil) and post-operatively, to complete one year of treatment. Importantly, 19% of patients in the control arm crossed over to adjuvant trastuzumab after the results of the major randomized trials became available. Despite the exciting results in terms of pCRs many clinical questions, including choice of chemotherapeutic drugs, combination with anthracyclines and duration of treatment, remain unsolved and use of pre-operative trastuzumab in clinical practice is still not widely established.

Grade III–IV cardiotoxicity + deaths (%)	Cardiac deaths (no.)	Median follow-up (months)		DFS (HR)	P	OS (HR)	P	Cross-over (%)
0.1	1			ref	–	ref	–	
0.8	0		48	0.76	<0.0001	0.85	0.1087	52
NR	NR			NR	–	NR	–	
0.9	1	JOINT ANALYSIS AC → P		ref	–	ref	–	
3.8	0							
0.3	1		35					21
2.5	0	AC → PH		0.48	<0.00001	0.65	0.0007	
0.7	0			ref	–	ref	–	
2	0		65	0.64	<0.001	0.63	<0.001	2.1
0.4	0			0.75	0.04	0.77	0.038	
1.7	0			ref	–	ref	–	
0.9	0		62	0.65 (DDFS)	0.12	0.55	0.094	0
0.4	0			ref	–	ref	–	
1.5	0		47	0.86	0.41	1.27	NR	0

Abbreviations: Ch, any chemotherapy; A, adriamycin; C, cyclophosphamide; H, herceptin; P, paditaxel; T, docetaxel; Ca, carboplatin; F, fluorouracil; E, epirubicin; V, vinorelbine; W, weekly; 3W, 3-weekly; NR, not reported; LVEF, left ventricle ejection fraction; DFS, Disease Free Survival; HR, Hazard Ratio; OS, Overall Survival; DDFS, Distant Disease-Free Survival; Cross-Over, Proportion of patients in the observation arm who crossed-over to the trastuzumab arm after 1st release of efficacy data.
Note: *significant LVEF drop, **LVEF drop > 10%, ***LVEF drop > 20%, ****LVEF < 45% and/or 45%–49% + ≥15% decrease.

Predictors of efficacy

Trastuzumab was originally developed and is routinely used only in Her2 over-expressing and/or amplified breast tumours and the implementation of the recent American Society of Clinical Oncology/College of American Pathologists guidelines on Her2 testing is contributing to improve patient selection for this targeted treatment.[102] A retrospective analysis of Slamon's pivotal phase III trial of trastuzumab in advanced patients confirmed that the benefit was limited to patients with Her2-amplified tumours.[83,103] A recent exploratory analysis from the NSABP group suggested that the advantage of adjuvant trastuzumab may not be limited to Her2-positive cases,[104] but it must be highlighted that these results emerge from a central re-testing of samples that were considered Her2-positive in peripheral laboratories. Many randomized trials have been retrospectively evaluated for the potential role of estrogen receptor (ER) and progesterone receptor (PR) expression in trastuzumab efficacy. All reports consistently show that the benefit derived from use of trastuzumab is similar in hormone-receptor positive and in hormone-receptor negative patients, in advanced,[105] adjuvant[87,90] and neo-adjuvant setting.[101,106] Likewise, the co-expression of EGFR and Her2, evaluated in a limited series of advanced patients, does not seem to predict any additional effect.[107] Other biologic markers potentially implicated in response to therapy will be discussed in the context of trastuzumab resistance.

Safety

Trastuzumab is generally well tolerated and the most frequent acute adverse event is a hypersensitivity-like infusion reaction.[108] However, the occurrence of cardiac dysfunction can be a major concern in a minority of patients and definitive trastuzumab discontinuation following cardiotoxicity might favour recurrence or progression of the disease.

The potential mechanisms of this significant side effect have been studied recently. There is abundant laboratory evidence that Her2 has an important role in cardiomyocyte development and function. Her2 in fact functions as a co-receptor for Her3 and Her4 and their peptide ligands, the neuregulins, all of which are expressed in cardiac tissue. According to one of the most accepted models, the inhibition of Her2 signalling leads to mitochondrial dysfunction and ATP depletion and, in turn, to reduced contractility of the cardiomyocyte.[109,110]

Clinically, trastuzumab-related cardiotoxicity can span a range of various clinical situations, from asymptomatic variations in the heart contractility (measured as Left Ventricular Ejection Fraction—LVEF) to severe and sometimes fatal cardiac failure, and has some peculiar features such as the absence of ultrastructural changes in the heart muscle and a general tendency to reversibility.

In the pivotal registration trial,[83] congestive heart failure (CHF) was reported in an unexpectedly high proportion of patient: its incidence in the paclitaxel plus trastuzumab and the anthracycline plus trastuzumab arms was respectively 13% and 27%. In the four large adjuvant trials,[89,91,93,111] where cardiac eligibility criteria were stringent and trastuzumab was interrupted or discontinued in response to the development of cardiac dysfunction, the incidence of grade III-IV CHF ranged from 0.4 to 3.8% (Table 1). Exhaustive analyses of clinical data in both the advanced and early settings have shown that older age, pre-existing cardiac diseases or risk factors and use of anthracyclines can all contribute to an increased likelihood of developing trastuzumab-related cardiotoxicity.[112]

In clinical practice, a widely accepted approach to minimize this potentially life-threatening side-effect includes a baseline assessment of LVEF followed by regular on-treatment monitoring, possible interruption/discontinuation of trastuzumab and the introduction of appropriate cardiac medication (e.g. Angiotensin-Converting Enzyme Inhibitors).[113]

Controversial Clinical Issues

Monotherapy or combination

Trastuzumab has been shown to be effective both as a single agent and in combination with chemotherapy. In order to define the best treatment strategy, two similar randomized studies have recently compared a 'sequential' therapy with the combination of trastuzumab plus docetaxel as first-line treatment in two series of patients with advanced breast cancer.[114,115] In the first trial the sequential arm consisted of trastuzumab three-weekly, followed by docetaxel alone (100 mg/m^2) at progression, whereas in the latter trastuzumab monotherapy was given weekly and docetaxel (60 mg/m^2) was introduced at progression without suspending the

antibody. It should be noted that in the first paper the vast majority of patients had visceral metastases and in the second trial patients with only bony disease were excluded from the trial. In terms of safety, no substantial difference emerged between the sequence and the combination, whereas ORR and progression-free survival (PFS) were substantially higher in the combination arm compared to the monotherapy stage of the sequential arm in both studies. The subsequent introduction of docetaxel did not seem to completely fill the gap in terms of efficacy, since the hazard ratio for overall survival (OS) was 2.72 ($P = 0.04$) in favour of the combination in Inoue's work[115] and median OS were 20.2 months in the sequential arm and 30.5 months for the combination in Bontenbal's trial,[114] although this difference did not achieve statistical significance. Taken together, these results seem to favour the combination regimen, at least in this subset of advanced patients with mostly visceral disease.

Combination with hormone treatments

A recent study has shown that the addition of trastuzumab to anastrozole in the treatment of metastatic patients with both Her2-positive and Hormone Receptor-positive tumours confers a significant benefit in terms of ORR (6.8% vs. 20.3%) and PFS (2.4 months vs. 4.8 months), without an evident impact on safety.[116] As a result, the European Medicines Agency approved the use of the combination of trastuzumab with an aromatase inhibitor in this setting. Although many oncologists argue that a traditional combination of trastuzumab and chemotherapy would better control this subset of tumours, the hormonal/trastuzumab approach could be useful where chemotherapy is not feasible or in patients with minimal bony metastatic disease, in order to delay the introduction of traditional cytotoxic drugs.

Combination with anthracyclines

The combination of trastuzumab with doxorubicin was initially found to cause an unacceptable rate of heart failure[83] and was never introduced in clinical practice, although its synergism was proved in '*in vitro*' and clinical studies. In order to overcome this drawback, many authors have evaluated the use of alternative, less cardiotoxic anthracyclines. In particular, liposomal doxorubicin, both in the pegylated and in the non-pegylated form, has been shown to be active and relatively safe in association with trastuzumab in several phase II studies.[117–119] On the other hand, conflicting results have been reported with regard to cardiac safety of trastuzumab plus epirubicin.[120,121] Although promising early evaluations have been reported, the simultaneous use of anthracyclines and trastuzumab is currently limited to clinical trials.

Trastuzumab and CNS metastases

Her2-positive breast cancer patients have a higher risk of developing metastases to the central nervous system (CNS) and treatment with trastuzumab does not seem to alter this risk.[122,123] Trastuzumab has been shown to penetrate the blood-brain barrier (BBB) to a very limited extent, with cerebrospinal fluid (CSF) concentrations approximately two orders of magnitude lower than serum concentration, despite a possibly impaired BBB.[124,125] Nonetheless, several retrospective studies have shown that trastuzumab is able to prolong survival in patients who develop CNS metastases,[126,127] compared with control groups of Her-2 negative patients and Her2-positive patients who did not receive trastuzumab.[123] Whole brain radiation treatment (WBRT) is generally considered to be the standard of care for this subset of patients and is able to improve the CSF:serum trastuzumab concentration ratio by approximately five-fold.[124] Since different agents can achieve objective remissions in the CNS, both alone and in combination with WBRT (i.e. temozolomide, capecitabine, lapatinib, topotecan),[128] many oncologists believe that a reasonable strategy could be to continue trastuzumab in order to control metastases outside the brain and to administer active therapies to control CNS disease. New approaches in order to deliver trastuzumab directly to the CNS are under investigation.

Use in Imaging

Trastuzumab has been evaluated as an imaging tool in the context of nuclear medicine techniques. In particular, the development of indium-111 (^{111}In)—labelled trastuzumab and its use in Single Photon Emission Computed Tomography (SPECT) have shown potential value for clinical staging of Her2-positive breast cancers in terms of identification of new tumour lesions, but when the myocardial distribution of the drug was evaluated, the authors

where not able to demonstrate any predicting ability with regard to trastuzumab-related cardiotoxicity.[129] In order to overcome the limited spatial resolution of ^{111}In-DTPA-trastuzumab SPECT imaging, trastuzumab has been successfully radiolabelled with Zirconium-89 (^{89}Zr) and its uptake has been detected by Positron Emission Tomography (PET), achieving a higher spatial resolution and a better signal-to-noise ratio.[130]

In another approach the Fragment antigen-binding F(ab')2 of trastuzumab was labelled with Gallium-68 (^{68}Ga), and detected with PET.[131] Both the whole antibodies and the antibody fragments, which are characterized by a faster clearance, have allowed non-invasive imaging of the pharmacodynamics of anti-cancer agents as part of pre-clinical studies in animal models[132,133] but they are not currently used in clinical practice.

Resistance

Only 15 to 30% of Her2-positive metastatic breast cancers respond to trastuzumab administered as a single agent, whereas the response rate when used in combination with taxanes is approximately 60 to 70%. This suggests that a proportion of patients are primarily resistant to trastuzumab. Moreover, virtually all patients with advanced disease treated with trastuzumab will have recurrent disease, indicating an acquired resistance. Furthermore, in the adjuvant setting there are a significant proportion of patients who suffer from recurrent disease despite having received trastuzumab.

These clinical observations have prompted several researchers to examine in depth the biological mechanisms of trastuzumab resistance.[134]

Two Her2 isoforms, the first lacking the extracellular domain (p95HER2) and the second lacking a small portion of the juxta-membrane extracellular domain (HER2Δ16), were described to harbour enhanced transforming activity[135–137] and recent *in vitro* and clinical works show that these variants are involved in trastuzumab resistance.[138,139] Increased signalling from members of the ErbB family of receptors and from Insulin-like Growth Factor Type 1 Receptor (IGF-1R) has been observed by Motoyama et al[66] and Natha et al[140] in the context of trastuzumab-resistant cells and these initial data have been confirmed by the recent demonstration of a heterotrimeric (Her2/Her3/IGF-1R) complex which would disrupt effective binding of trastuzumab to Her2 and activate unique down-stream signalling.[141] Activation of the PI3K pathway has been shown to be important in trastuzumab downstream effects and can be regulated by Phosphatase and Tensin homolog (PTEN). *In vitro* and clinical studies suggest that activation of this pathway and loss of PTEN function may contribute to trastuzumab unresponsiveness.[142] The membrane-associated glycoprotein MUC4, which may hinder trastuzumab binding to its epitope on Her2, has also been proposed as an additional marker of resistance.[143] Some of these pathways and mechanisms are currently being evaluated as potential therapeutic targets in pre-clinical and clinical studies.[144]

A large trial compared the use of capecitabine monotherapy with the combination of capecitabine and lapatinib, a Her2 and EGFR associated tyrosine kinase-inhibiting small molecule, in trastuzumab-resistant patients. With this combination the authors obtained an advantage in terms of ORR and PFS[145] and lapatinib is now approved for the treatment of Her2-positive breast cancer.

Use of trastuzumab beyond progression of disease

Since limited second-line options are currently available in the clinic for patients who experience disease progression after first-line trastuzumab-containing therapies, many oncologists have been using trastuzumab following disease progression for several years, mostly in combination with various chemotherapeutic agents. This choice was based on trastuzumab's known chemo-sensitizing property and supported by data from a number of retrospective series.[146–148] Recently, a randomized study has assessed the efficacy of continuation of trastuzumab in association with capecitabine[149] and has confirmed the clinical benefit of this option, at least in terms of PFS. A second controlled study, which assessed the efficacy of continued trastuzumab in association with lapatinib versus lapatinib alone, demonstrated an advantage for the combined anti-Her2 therapy.[150]

These observations, along with several *in vitro* experiments,[151] seem to suggest that in patients who lose clinical responsiveness, the anti-tumour effect of trastuzumab is in part retained and can be exploited in the context of different combination regimens.

Place in Therapy

Trastuzumab has a widely-established role in the routine treatment of early and advanced Her2-positive breast cancer patients. In the adjuvant setting, trastuzumab is commonly administered for a total duration of one year, with variations regarding the timing and schedule of administration (Table 1). In metastatic patients, a combination therapy with chemotherapeutic agents is usually preferred: in particular, taxanes are the most extensively-studied partner drugs in this context. However, combination with different agents, including aromatase inhibitors, is feasible and effective. The pharmacodynamic and pharmacokinetic equivalence of the weekly and 3-weekly regimen allows clinicians to choose the most convenient schedule, taking into account patients' preference, associated chemotherapy regimen and hospital facilities. Furthermore, the safety and tolerability of this treatment allow a prolonged treatment in the vast majority of cases. Besides the introduction of lapatinib in trastuzumab-resistant patients, the development of new drugs and combinations which can potentially refine the treatment of Her2-positive cancers is a wide and exciting field of investigation.

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Appendix 2. Trastuzumab cell-based ELISA paper

This article was submitted and published in 2009 and presents one of the methods employed in this research project (more details are presented in Chapter 3).

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Technical note

Development and validation of cell-based ELISA for the quantification of trastuzumab in human plasma

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ABSTRACT

Trastuzumab is a therapeutic monoclonal antibody against the Her2 oncoprotein, which is over-expressed in approximately 30% of breast cancers, and is now used routinely in the management of early and metastatic Her2+ disease. However, not all Her2+ breast cancer patients respond to trastuzumab and the pharmacodynamic and pharmacokinetic parameters behind this variation in response are unknown. Pharmacological investigations into variable response to trastuzumab have been hampered by the lack of a published feasible method to determine trastuzumab concentration in plasma. Here we describe the development and validation of a cell-based ELISA to measure trastuzumab in human plasma. The assay specifically measures the interaction between trastuzumab and Her2 and has a dynamic range of between 10 and 120 μg/ml. The mean intra-assay and inter-assay variability of the ELISA was 9%. Trastuzumab in plasma was stable for at least 10 weeks at −20 °C and 72 h at 4 °C, and was unaffected by 5 freeze/thaw cycles. Having validated the assay, the trough plasma trastuzumab concentrations of 30 patients being treated for metastatic or early disease were measured. The median trough concentration was 62 (range 21 to 441) μg/ml.
This cell-based ELISA method has undergone appropriate validation and is suitable for quantification of trastuzumab in the plasma of patients treated with Herceptin.

1. Introduction

Breast cancer is the most commonly diagnosed cancer and, despite improvements in treatment, remains the leading cause of cancer-related mortality in women in Europe (Ferlay et al., 2007). Incidence of breast cancer has been rising over the last 30 years, however improvements in early detection and therapeutic management of the disease have led to a concomitant decrease in mortality over the last 20 years in many countries (Botha et al., 2003). The improvements in therapy have been largely due to an increased understanding of the molecular aetiology of the disease. Breast cancer is a heterogeneous disease, and bio-molecular elements responsible for this heterogeneity have been exploited in attempts to specifically target cancer cells. For example ~70% of breast cancers are estrogen and/or progesterone receptor positive. Many of these tumours are initially dependent on estradiol for proliferation and estrogen antagonism is central to the management of hormone receptor positive tumours (EBCTCG, 1998). More recently the over-expression, due to gene amplification, of the receptor tyrosine kinase human epidermal growth factor receptor 2 (cErb2, Her2) has been exploited in cancer chemotherapy (Cobleigh et al., 1999).

Her2 gene amplification occurs in upto 30% of breast cancers (Slamon et al., 1987) and is an oncogenic event, driving ligand-independent proliferation (Worthylake et al., 1999) and survival via aberrant PI3K/AKT signalling (Yakes et al., 2002). The oncogenic overexpression of Her2 has provided a tumour specific target for the therapeutic management of breast cancer.

Trastuzumab (Herceptin) is a humanised monoclonal antibody targeting an epitope resulting from the tertiary structure of Her2 (Cho et al., 2003). It is currently used

Abbreviations: ELISA, Enzyme-Linked Immunosorbent Assay; TBS, Tris Buffered Saline; HRP, Horseradish peroxidise.

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clinically as an adjuvant therapy in early breast cancer and in the treatment of metastatic disease (Baselga et al., 2006). The exact mechanism of action of trastuzumab is uncertain, but appears to be multifactorial and includes the direct inhibition of proliferative signalling and induction of antibody-dependent cell-mediated cytotoxicity (ADCC) (Cooley et al., 1999). Four major trials have shown that trastuzumab reduces the risk of recurrence of early Her2 positive breast cancer by approximately 50% (Piccart-Gebhart et al., 2005; Romond et al., 2005; Slamon et al., 2005). When coadministered with docetaxel in the treatment of metastatic disease, trastuzumab improves overall survival from 23 to 31 months (Marty et al., 2005). However only 38% of patients with Her2-positive metastases respond to single agent trastuzumab therapy (Vogel et al., 2002), and cancer recurs in the majority of patients with metastatic disease (Slamon et al., 2001).

There are few published clinical investigations of biomarkers that may be predictive of response or susceptibility to resistance to trastuzumab. Several studies have reported pharmacokinetic data on trastuzumab and interindividual variability in trough concentrations of between 10-fold and 1000-fold have been observed, with low concentrations being associated with high circulating Her2 extracellular domain (ECD) in plasma (Baselga et al., 2005). It has also been observed that response is associated with a 1.6-fold higher mean trough trastuzumab concentration than that seen in non-responders (Cobleigh et al., 1999).

A barrier to investigating potential predictive pharmacokinetic parameters and pharmacodynamic biomarkers is the lack of a published assay to measure trastuzumab concentration in plasma. Pharmacokinetic studies published so far have failed to provide sufficient detail to allow the assay to be carried out independently (Baselga et al., 1996), (Cobleigh et al., 1999), (Tokuda et al., 1999), or have used purified Her2 protein that is not commercially available (Pegram et al., 1998). Although a very detailed method for quantification of trastuzumab has been published (Maple et al., 2004) the method described requires a capture antigen that is supplied only as a standard in a commercially-available Her2 ELISA kit. The quantities required mean that only a limited number of samples could be analysed for each kit purchased, in addition to the cost of capture antibodies and routine ELISA reagents.

We are currently undertaking clinical trials in breast cancer where measurement of trastuzumab pharmacokinetics would be advantageous. Given the lack of commercially-available purified trastuzumab antigens, we have developed and validated a cell-based ELISA to quantify trastuzumab in diluted human plasma. The assay uses a high density of formaldehyde-fixed SKBR3 Her2-positive breast cancer cells as the capture antigen in what is otherwise a conventional direct ELISA.

2. Materials and methods

Trastuzumab (Herceptin) was purchased from Roche (Welwyn Garden City, UK). TMB (3,3′,5,5′-tetramethylbenzidine) substrate kit was purchased from Pierce (Cramlington, UK). Fetal calf serum (FCS), HRP conjugated goat anti-human antibody and Alexa fluor488 conjugated goat anti human antibody were purchased from Invitrogen (Paisley, UK). Goat serum was purchased from Millipore (Herts, UK). Human plasma from nine individuals was purchased from the Blood Transfusion Service (Newcastle upon Tyne, UK). Phosphate buffered saline, Tris base, sodium chloride, concentrated hydrochloric acid, Tween 20, sodium azide, poly-D-lysine, Hoescht 33342, RPMI 1640, Formalin and Costar 96 well tissue culture plates were all purchased from Sigma (Poole, Dorset, UK). Sterile μ-clear black-walled optical 96 well plates were purchased from Greiner (Gloustershire, UK). SKBR3 breast adenocarcinoma cells and MDA MB 231 breast adenocarcinoma cells were gifts from Dr. Felicity May at the NICR, UK.

2.1. Patient samples

Plasma samples from 30 patients with Her2-positive breast cancer were collected during 2008. All the patients received trastuzumab every three weeks (8 mg/kg loading dose followed by 6 mg/kg) as part of their treatment of metastatic or non metastatic disease at the Newcastle General Hospital, Newcastle, UK. Samples were withdrawn immediately before the administration of the next dose of trastuzumab, after a minimum of 6 weeks of treatment. Patient samples were collected under appropriate ethical approval and with full informed patient consent.

2.2. Immunocytochemistry

SKBR3 and MDA MB 231 cells were plated at 5000 cells per well in optical 96 well plates and allowed to adhere for 24 h at 37 °C and 5% CO_2. After washing with PBS cells were fixed with 10% formalin for 20 min. The cells were blocked for 1 h with 20% goat serum in Tris buffered saline (TBS) with 0.1% Tween 20 for 1 h at room temperature and subsequently incubated with human plasma spiked with trastuzumab and diluted 1/4000 in wash buffer (TBS with 0.1% Tween 20 and 1% goat serum). Each well was washed 3 times with wash buffer, then incubated for 1 h at room temperature with an Alexafluor 488 tagged goat anti-human antibody diluted 1/500 in wash buffer with 10 μg/ml Hoescht 33342. Wells were washed 3 times with PBS and imaged with a ×20 objective on a Pathway HT Inverted fluorescent microscope (BD, Oxford, UK).

2.3. Trastuzumab ELISA

SKBR3 cells were propagated in RPMI 1640 with 10% FCS at 37 °C and 5% CO_2. To prepare single-use aliquots, SKBR3 cells were maintained and trypsinised at approximately 90% confluence, and split 1 in 2 until thirty two 125 cm^2 tissue culture flasks with cells at approximately 90% confluence were attained. The cells were trypsinised, pooled and counted on a Coulter Z1 cell counter (BD, Oxford, UK). The resuspended cells were centrifuged at 1000 rpm in a Centaur 2 bench top centrifuge (MSE, London, UK) for 5 min, the medium was aspirated and the pellet resuspended in RPMI 1640 with 10% FCS and 10% DMSO at a concentration of 2.6×10^7 ml^{-1}. This suspension was stored at − 80 °C in 0.5 ml aliquots until needed for the assay.

On day one of the assay the inner 60 wells of two 96 well tissue culture plates were incubated with 100 μl per well of 100 μg/ml 70,000–150,000 kDa poly-D-lysine for 5 min at room temperature under sterile conditions. The poly-D-lysine was aspirated and each well was washed with sterile deionised

water and the plates were allowed to dry under sterile conditions. Once dry, a single-use aliquot of SKRB3 cells was thawed and re-suspended to a total volume of 13 ml in RPMI 1640 with 20% FCS and a concentration of 1×10^6 ml^{-1}. This cell suspension was distributed into the poly-D-lysine coated wells of the previously-coated plates at a density of 1×10^5 cells per well in 100 µl volumes. The plates were incubated for exactly 24 h at 37 °C and 5% CO_2 to allow the cells to recover and adhere to the poly-D-lysine coated substrate. On day two of the assay the medium was gently aspirated by vacuum and the cells were washed with cold PBS and fixed with 100 µl per well 10% formalin for 20 min. The formalin was aspirated and the cells washed 3 times with 100 µl PBS per well. Following washing, the fixed cells were incubated overnight at 4 °C in 100 µl blocking buffer (Tris buffered saline with 0.1% Tween 20, 20% goat serum and 0.2% sodium azide).

On day 3 of the assay all samples, standards and QCs were diluted 1/4000 in wash buffer (TBS with 0.1% Tween 20 and 1% goat serum). The block buffer was aspirated from the plate, replaced with 100 µl per well of the diluted samples, standards and QCs in triplicate (or replicates of 15 to determine intra assay variability) and incubated for 1 h at room temperature on a Gyro rocker STR9 (Bibby Scientific, Stone, UK) at 20 rpm. Following the primary incubation the samples were removed by aspiration and each well was washed 3 times with 100 µl wash buffer. The final wash was replaced with 100 µl per well HRP goat anti-human antibody diluted 1/1000 in wash buffer and the plate was incubated for 1 h at room temperature on a Gyro rocker at 20 rpm. Each well was then washed 5 times with 300 µl wash buffer. HRP activity remaining was detected with TMB Substrate Kit as per manufacturer's instruction. Briefly 100 µl of the combined TMB substrate and H_2O_2 solution was added to each well and was incubated in darkness at room temperature for 20 min on a Titertek plate shaker on setting 6. After 20 min the reaction was stopped with the addition of 100 µl per well 180 mM sulphuric acid and agitation for 10 s on a Titertek plate shaker on setting 6. The absorbance at 450 nm of each well was read on a Spectramax plate reader. Sample and QC values were interpolated from the standard curve using a Power equation in Excel ($y = cx^b$).

2.4. Validation

The robustness of the assay was characterised by a set of validation experiments designed to determine dynamic range, inter and intra assay variability, lower limit of detection, dilutional linearity, parallelism, stability at different storage temperatures and stability over five freeze–thaw cycles. A 150 mg vial of trastuzumab was dissolved in 2 ml sterile deionised water and aliquots stored at −80 °C. The 75 mg/ml solution was diluted to 1 mg/ml solutions in human plasma from one individual. For the initial determination of a dynamic range, trastuzumab at 1 mg/ml was diluted in human plasma by serial 1 in 2 dilutions to give a range of concentrations from 1 mg/ml to 60 ng/ml. These solutions were further diluted 1/4000 in wash buffer. Subsequent validation experiments and patient determinations used a standard curve with concentrations of 120, 100, 80, 60, 40, 20 and 10 µg/ml and concentrations of 100 and 25 µg/ml for quality control samples. Standard curves were made up fresh for each experiment. Quality control samples were made as

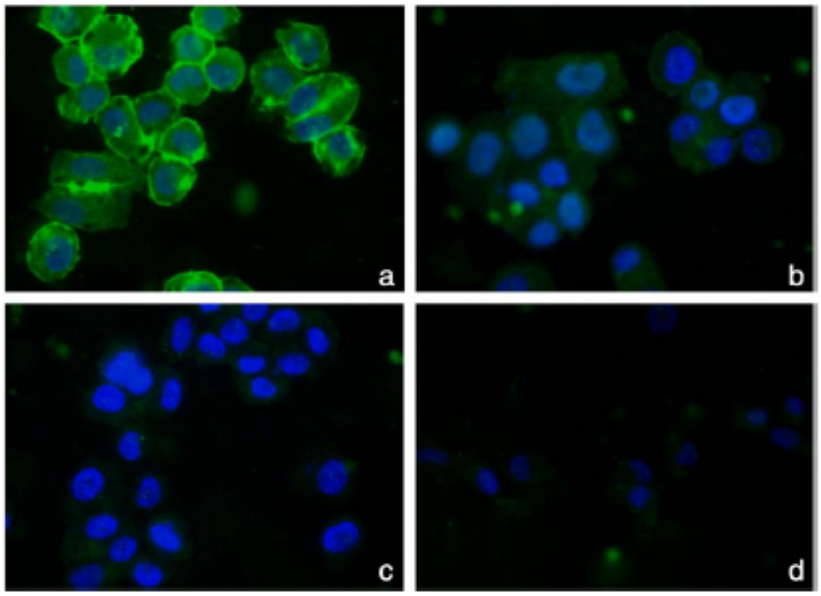

Fig. 1. Immunocytochemistry detection of trastuzumab in human plasma. Trastuzumab in human plasma was used as the primary antibody at 120 µg/ml (a and d), 10 µg/ml (b) or 0 (unspiked human plasma, c) with formalin fixed Her 2+ SKBR3 cells (a, b, c) or Her2 − MDA MB 231 cells (d) as the capture antigen. All primary antibodies were detected with an Alexafluor 488 tagged goat anti-human antibody. Signal above background was detected with 120 and 10 µg/ml trastuzumab on the SKBR3 cells but not by 120 µg/ml trastuzumab on MDA MB 231 cells.

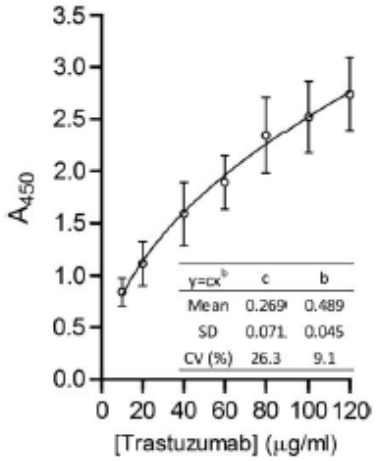

Fig. 2. Standard curve of human plasma spiked with trastuzumab as determined by cell-based ELISA. The inset table shows the mean, standard deviation and CV of the constants from a power equation ($y = cx^b$). Figure represents mean and SD of 13 independent experiments undertaken as part of the assay validation.

single use aliquots and were stored at −80 °C. These samples were used to validate the assay as described below.

3. Results

3.1. Specificity of interaction between trastuzumab and Her2

We first wanted to know if the selective binding of trastuzumab at pharmacological concentrations in a plasma matrix could be observed by immunocytochemistry. Using as the antigen SKBR3 breast carcinoma cells, which over-express Her2, a strong fluorescent signal was observed when the cells were incubated with 120 μg/ml trastuzumab in human plasma diluted 1/4000 in wash buffer (Fig. 1a), and a signal above background detected when 10 μg/ml trastuzumab in human plasma diluted 1/4000 in wash buffer was used as the primary antibody (Fig. 1b). No signal was detected when SKBR3 cells were incubated with unspiked human plasma diluted 1/4000 (Fig. 1c). When MDA MB 231 cells, which do not over-express Her2, were incubated with 120 μg/ml trastuzumab no fluorescence above background was observed (Fig. 1d).

3.2. Validation of ELISA for quantification of trastuzumab

Having determined the specificity of the interaction between trastuzumab and Her2 in the SKBR3 cells we went on to develop a cell-based ELISA that would allow the quantification of trastuzumab in plasma from breast cancer patients. Initial experiments identified a dynamic range for the assay of between 8 and 125 μg/ml when samples had been diluted 1/4000 in wash buffer. Seven trastuzumab concentrations between these values were used to generate standards and these concentrations were used in all subsequent validation experiments and analysis of patient samples. The plot of signal versus concentration was best fitted by a power curve ($y = cx^b$) with an R^2 value of 0.996 and a p value of 0.714 for deviation from the model (Fig. 2). Intra assay variability was determined by running 15 replicates of the low and high QC, and this was repeated twice, for a total of 3 independent experiments, and the mean values were used to contribute towards the acquisition of data to determine inter assay variability. The mean intra assay variability was 9% and on one occasion only was above 15%. (Table 1). Inter assay variability was assessed over 11 independent experiments performed as part of the validation. Mean inter-assay variability was 12% for the low QC and 11% for the high QC. The variability of the back-calculated concentrations of trastuzumab from the standard curve were between 5 and 12% and all calculated values of the standards were within ±8% of the intended value (Table 1). The mean percentage recovery for the low and high QC samples was 98% and 95% respectively.

3.3. Stability of Herceptin QC samples in different storage conditions

The stability of trastuzumab under numerous storage conditions was determined. Both the high and low QCs were stable over 72 h at 4 °C (CV = 4 and 5% respectively) and

Table 1
Intra- and inter-assay variability (n = intra-assay replicates in individual experiments for the intra-assay variability determination and number of independent experiments for the inter-assay experiments).

Intra-assay variability								
	Low QC 1	Low QC 2	Low QC 3	Mean low QC	High QC 1	High QC 2	High QC 3	Mean high QC
Mean trastuzumab (μg/ml)	23	28	29	27	84	96	106	95
n	15	15	15		14	15	15	
SD (μg/ml)	4	2	2		8	6	10	
CV (%)	16	8	6		9	6	10	

Inter-assay variability									
	Low	High	std 10	std 20	std 40	std 60	std 80	std 100	std 120
Mean trastuzumab (μg/ml)	25	95	11	19	39	56	87	101	121
n	11	11	13	13	13	13	13	13	13
SD (μg/ml)	3	11	1	2	5	4	7	5	7
CV (%)	12	11	11	12	12	8	8	5	6
Recovery (%)	98	95							

10 weeks at −20 °C (CV = 4 and 9% respectively) with no downward trend. QC samples were also stable for at least 6 months at −80 °C. There was a slight downward trend in the absorbance values of the QC samples over the period of the validation but this had no effect on the calculated QC concentrations and was attributed to a decrease in the HRP activity of the secondary antibody. Absorbance values returned to those seen at the beginning of the method development when new secondary antibody was purchased. There was no loss of signal over 5 freeze/thaw cycles from −80 °C to RT.

3.4. Limit of quantification

The lower limit of quantification was determined by analysing the plasma of eight individuals who had not received trastuzumab. The apparent plasma concentration was 5.6 ± 1.1 µg/ml (Mean and SD). Therefore a concentration of 9 µg/ml trastuzumab (Mean + 3SD) was established as the lower limit of detection and at this value it is predicted that less than 1 in 200 positive values will be false.

3.5. Dilutional linearity and parallelism

Dilutional linearity was demonstrated by spiking matrix with 1 mg/ml trastuzumab and serially diluting the spiked sample 1 in 2 followed by a 1/4000 dilution in wash buffer. No hook effect was observed up to 1 mg/ml. Dilutions between 1/16 and 1/64 had predicted and measured results within the dynamic range of the assay and the trastuzumab concentrations corrected for dilution of these samples were within 20% of the predicted concentration. Three patient samples with measured concentrations above the dynamic range of the assay were used to determine parallelism. The samples were serially diluted in matrix 1 in 2 followed by a 1/4000 dilution in wash buffer. For each sample the lowest dilution that resulted in a measured concentration within the dynamic range of the assay and a calculated concentration less than 20% different from the preceding concentration was reported as the determined concentration. In 2 of the patients the minimum required dilution was 1/4 and in 1 patient a 1/16 dilution was required.

3.6. Clinical characteristics of patients and determination of patient samples

The trough trastuzumab concentrations of plasma samples from 30 patients being treated for metastatic and non metastatic breast cancer were measured using the cell-based ELISA method described. The median age of the patients was 55. The majority (77%) of patients were being treated for non metastatic disease and 61% of these patients had node-negative disease. Of the 30 patients, 1 was receiving concomitant lapatinib, 2 paclitaxel, 2 docetaxel, 2 vinorelbine and 1 anastrozole. The remaining 22 patients were receiving trastuzumab as a single agent. The median trastuzumab trough concentration was 62 µg/ml (range 21 to 441 µg/ml) (Fig. 3). Trastuzumab concentration was not normally distributed due to the presence of three samples from three individuals with high concentrations. No demographic or disease parameters explained the high trastuzumab concentration in these three patients. Excluding the three high outliers from analysis revealed a mean trastuzumab concentration of 63 ± 23 µg/ml (mean and SD).

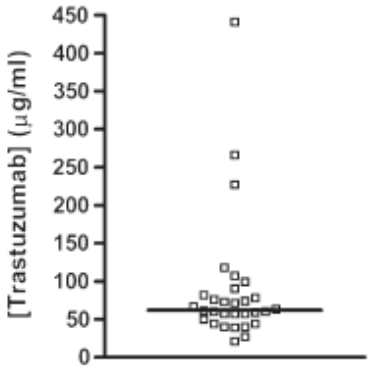

Fig. 3. Distribution of trough trastuzumab concentrations in plasma from 30 patients being treated for early or metastatic breast cancer. All samples were diluted 1/4000 in TBS with 0.1% Tween 20 and 1% goat serum and trastuzumab concentration determined by cell-based ELISA. Samples with concentrations above the highest standard were serially diluted 1/2 in blank human plasma prior to 1/4000 dilution in TBS with 0.1% Tween 20 and 1% goat serum. The lowest dilution that yielded a linear dilution response was reported. Median value was 62 µg/ml (range 21 to 441 µg/ml).

4. Discussion

Herceptin is now routinely used clinically as adjuvant therapy in the treatment of Her2-positive breast cancer. However both *de novo* and acquired resistance remains a substantial barrier to prolonged efficacy. The pharmacodynamic and pharmacokinetic parameters that contribute to the variability in response to trastuzumab remain to be fully elucidated. The lack of a freely-available assay to quantify trastuzumab has precluded pharmacokinetic investigations independent of the manufacturers of Herceptin. This paper describes a robust and reliable method to quantify trastuzumab concentrations in human plasma. It is not possible to directly compare the performance of this assay with other trastuzumab assays used in clinical investigations, due to the lack of detail in the literature. However, the Genentech assay reports a sensitivity of 156 ng/ml (Baselga et al., 1996; Pegram et al., 1998). This is approximately two orders of magnitude greater than sensitivity of the assay described here (9 µg/ml). This is expected and explicable by the difference in purity of the capture antigen. The Genentech assay uses a purified human recombinant Her2 protein that is not available commercially, compared with the multiple antigens exposed on the non-permeabilized formalin-fixed SKBR3 cells. The sensitivity of the assay described here is sufficient to discriminate plasma concentrations less than the pharmacological target of a trough trastuzumab concentration of 20 µg/ml (Leyland-Jones et al., 2003). The assay is specific for the interaction between trastuzumab and Her2 as illustrated by the immunofluorescence experiments. No fluorescence signal was observed when a 1 in 4000 dilution of human plasma without trastuzumab was used as the primary antibody on the Her2 positive SKBR3 cells, nor when a 1 in 4000 dilution of human plasma spiked with

120 μg/ml trastuzumab was used on the Her2 negative cell line MDA MB 231.

The assay described here is more readily comparable with the sandwich-type ELISA described in detail by Maple et al. (2004). The method is comparable in terms of performance to that assay, with similar precision, dynamic range and accuracy, but is simpler to execute and is not limited by access to reagents of restricted availability.

The dynamic range of the assay of 10 to 120 μg/ml encompasses the expected mean trough trastuzumab concentration, as determined by previously-published trastuzumab pharmacokinetic studies. The mean and CV (63 μg/ml, 37% CV) of the patient samples measured was also comparable to previously published pharmacokinetic papers (Cobleigh et al., 1999; Baselga et al., 2005).

The use of formalin-fixed cells as capture antigens for the detection and quantification of therapeutic antibodies in plasma is not limited to trastuzumab. A similar assay to quantify the binding activity of an anti-IL-2/IL-15 receptor β antibody has been described, but this method did not quantify the antibody in a plasma matrix (Yang et al., 2006). Other therapeutic antibodies that may be quantifiable by this approach include rituximab and cetuximab. The described assay for the measurement of trastuzumab in human plasma is cheap, robust, reliable and will allow future studies to be undertaken into the effect of trastuzumab pharmacokinetics on variability of efficacy.

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