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DOTTORATO DI RICERCA IN FARMACOLOGIA, TOSSICOLOGIA E TERAPIA

XXI CICLO

Pharmacogenetics of cytochromes in breast cancer treatment

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CONTENTS

RIASSUNTO

La farmacogenetica studia l'impatto di polimorfismi genetici nella variabilità interindividuale dell'effetto terapeutico, in termini di risposta e tossicità, al trattamento farmacologico.
Nella definizione del trattamento per il tumore della mammella si tiene conto di numerosi fattori tra cui la condizione ormonale delle pazienti stesse: la presenza di recettori per gli estrogeni a livello tumorale prevede il trattamento con farmaci anti-estrogenici (exemestane) mentre la malattia localmente avanzata o metastatica viene trattata con chemioterapia citotossica (ciclofosfamide). In entrambi i casi, la presenza di alterazioni genetiche a carico dei citocromi P450 (CYP) potrebbe influenzare la terapia in termini di risposta o di sviluppo di tossicità. Lo studio ha previsto l'arruolamento di due gruppi di pazienti sottoposte a terapia farmacologica (ciclofosfamide in regime CMF) o a terapia ormonale (exemestane) per le quali è stato fatto il profilo genetico di alcune particolari isoforme di CYP. Lo scopo dello studio è stato quello di valutare l'effetto dei polimorfismi individuati sull'outcome clinico delle pazienti in modo da fornire delle indicazioni su una possibile "personalizzazione" della terapia in relazione al profilo genetico della paziente. Lo studio si può definire completo per il gruppo di pazienti in trattamento farmacologico in quanto è stato possibile raccogliere i dati farmacodinamici di tossicità e sopravvivenza. Al contrario, per il gruppo in trattamento ormonale l'arruolamento non è stato completato quindi è stato possibile solo avere dei dati preliminari di frequenza dei polimorfismi.
Lo scopo principale dello studio è stato quindi quello di valutare l'impatto di polimorfismi a carico dei CYP (siano essi coinvolti nel metabolismo della ciclofosfamide o rappresentino l'enzima target per exemestane) sulla sopravvivenza e sullo sviluppo di tossicità al trattamento. Inoltre, è stato possibile effettuare anche delle correlazioni di tipo caso/controllo per la valutazione del rischio relativo di sviluppo del tumore in relazione alla presenza dei polimorfismi studiati. Sono state quindi individuate le varianti genetiche a carico delle isoforme CYP2B6, CYP2C9, CYP2C19, CYP3A4 e CYP3A5 coinvolte nella bioattivazione della ciclofosfamide e dell'isoforma CYP19 enzima target di exemestane. La genotipizzazione delle pazienti (195 con trattamento farmacologico e 121 con trattamento anti-estrogenico) si è basata su tecniche che utilizzano l'amplificazione del DNA tramite PCR, quali il Restriction Fragment Length Polymorphism (RFLP), l'analisi automatizzata dei frammenti, la tecnologia del Pyrosequencing e la tecnologia TaqMan. I dati clinici di tossicità sono stati valutati da un oncologo medico secondo la classificazione NCI-CTC mentre il dato di sopravvivenza è stato considerato l'Overall Survival.

Dati interessanti e significativi sono emersi per il gruppo di pazienti in trattamento farmacologico con ciclofosfamide in quanto è stato possibile valutare le relazioni tra profilo genetico e profilo farmacodinamico. Lo studio ha infatti evidenziato un ruolo significativo dei polimorfismi dell'isoforma CYP2C9 coinvolti nella bioattivazione epatica della ciclofosfamide sia per quanto riguarda la sopravvivenza che la tossicità. La presenza di almeno un allele mutato per il polimorfismo CYP2C9*2 (C430T) è correlata con un maggior rischio di sviluppo di tossicità epatica alla fine del primo ciclo di trattamento. La riduzione di attività enzimatica conseguente alla variazione genica potrebbe determinare una minore bioattivazione del profarmaco con un suo accumulo a livello epatico. Inoltre anche il polimorfismo CYP2C9*3 (A1075C) ha evidenziato un impatto non solo sulla tossicità epatica ma anche sulla sopravvivenza. La presenza di almeno un allele mutato riduce la sopravvivenza probabilmente in relazione alla minore disponibilità di farmaco attivo. Inoltre è emersa una correlazione significativa anche tra la presenza di un polimorfismo a carico di CYP2C19 (G681A) e lo sviluppo di tossicità ematologica severa (grado 3-4) alla fine della terapia. La presenza del polimorfismo è associata ad una riduzione nell'attività metabolica dell'enzima quindi potrebbe spiegare una riduzione nella bioattivazione della ciclofosfamide con sviluppo di tossicità.

In conclusione, durante questo lavoro di tesi, sono stati evidenziati interessanti marcatori molecolari che potrebbero avere un valore prognostico nel trattamento del carcinoma della mammella con ciclofosfamide se confermati in una casistica più ampia. L'applicazione di questi parametri nella pratica clinica potrebbe essere utile per progettare una personalizzazione del trattamento basato su specifiche caratteristiche genetiche del paziente con carcinoma della mammella.

ABSTRACT

Pharmacogenetic focuses on intersubject variations in therapeutic drug effects and toxicity depending on genetic polymorphisms. In defining the treatment strategies for breast cancer several factors are considered: patients with estrogen-positive breast cancer are treated with anti-estrogens (exemestane) whereas locally advanced or metastatic disease is treated with cytotoxic agents, including cyclophosphamide. In both cases, the presence of genetic alterations of cytochrome P450 (CYP) could influence the absorption, distribution, bioactivation, metabolism, elimination and drug action and thus have consequences in the efficacy and toxicity of treatment. This study is focused on the analysis of genetic polymorphisms of several CYP isoforms involved in cyclophosphamide bioactivation or as exemestane target.

The aim of this research is to point out a predictive role of genetic alterations in terms of toxicity and of overall survival after pharmacological treatment in breast cancer patients. Since the difficulty of the enrollment of patients treated with exemestane did not allow us to drawn the same pharmacogenetic/pharmacodynamic correlations. For this subgroup we have assessed only the relative risk to develop breast cancer for all the polymorphisms analysed.

One hundred ninty-five patients treated with cyclophosphamide in association with methotrexate and 5-fluorouracil (CMF regimen) were genotypized for a set of genetic polymorphisms of CYP2B6, CYP2C9, CYP2C19, CYP3A4 and CYP3A5 involved in bioactivation of the drug. In the group of 121 patients treated with exemestane the analysis were performed for aromatase (CYP19) gene, which is also the target of the drug. Genotyping was performed using PCR based methods, such as Restriction Fragment Length Polymorphism (RFLP), fragment analysis, Pyrosequencing technology and TaqMan technology. Clinical parameters of toxicity (according with NCI-CTC scale) and Overall Survival were monitored by an oncologist.

Interesting results were obtained for the correlations between the genotypic and the pharmacodynamic profiles of subgroup of patients treated with cyclophosphamide. The study underlined the significant role of CYP2C9 isoforms involved in bioactivation of cyclophosphamide both in overall survival and in the development of toxicity. Patients carrying at least one mutant allele of CYP2C9*2 (C430T) polymorphism showed an higher risk to develop hepatic toxicity with respect to wild type patients after the 1st cycle of chemotherapy. Similar results were obtained with CYP2C9*3 (A1075C): the presence of at least one mutated allele was found significantly correlated with the development of hepatic

toxicity (grade 1-3) after the entire course of treatment. A reduction of enzyme activity due by polymorphism could determine an impaired bioactivation of the prodrug with a consequent accumulation in the liver. Moreover, CYP2C9*3 polymorphism has showed an impact in the overall survival: the presence of at least one mutated allele is related to a reduced survival. This is probably due to a lesser bioavailability of the active molecule.

The presence of CYP2C19 G681A polymorphism was correlated with an higher risk to develop severe (grade 3-4) hematological toxicity at the end of the therapy. This variant characterizes the poor metaboliser (PM) allele and thus could be responsible for a reduction in the bioactivation of cyclophosphamide.

In conclusion, interesting molecular markers with a predictive value on pharmacodynamics of cyclophosphamide were identified, but their role has to be confirmed in a larger group of patients. A possible application of these parameters in the clinical practice, could be useful to design a tailored treatment based on the peculiar genetic characteristics of each breast cancer patient.

ABBREVIATIONS

SNP: Single Nucleotide Polymorphism

CMF: Cyclophosphamide, Methotrexate, 5-Fluorouracil

ER: Estrogen Receptor

CYP: cytochrome p450

EM: Extensive Metabolizer

PM: Poor Metabolizer

SERM: Selective Estrogen Receptor Modulator

AI: Aromatase Inhibitor

PCR: Polymerase Chain Reaction

RFLP: Restriction Fragment Length Polymorphism

LD: Linkage Disequilibrium

NCI-CTC: National Cancer Institute – Common Toxicity Criteria

OR: Odd Ratio

HR: Hazard Ratio

CI: Confidence Interval

1. INTRODUCTION

1.1 Pharmacogenetics

A significant variability in drug response may occur in cancer patients treated with the same medications. The pharmacodynamics of current anticancer drugs is in effect often unpredictable and may reflect the existence of complex interactions between genetic factors, that result in variable therapeutic outcomes. Pharmacogenetics, the study of the role of inheritance in interindividual variability in drug response, has the potential to contribute to the development of more rational pharmacological therapies for various type of cancers. The pharmacogenetics of breast cancer therapy is also an area of intensive research, and the role of interindividual differences in drug metabolism in relation to breast cancer outcomes remains to be explained. Actually, pharmacogenetics and pharmacogenomics are often used interchangeably to describe the effects of genetic variation on patients responses to drugs. Pharmacogenetics can be defined as the study of single gene identified on the basis of the pharmacokinetic characteristics of a specific drug, on the basis of variability in drug response, or both. These genetic variants may influence the absorption, distribution, metabolism and elimination of drugs and have a consequence in its efficacy and toxicity. In contrast, pharmacogenomics encompasses the study of gene expression, for example in tumour tissue to identify potential cancer therapy targets and genome wide analysis to identify the entire set of genes relevant to drug response and drug targets.

The identification of gene alterations affecting drug toxicity and response represents a novel area of interest in the pharmacology of antitumoral drugs. Mutations within the coding region of a gene result in a structurally aberrant protein whereas quantitative variations of normal proteins are the consequence of mutations affecting the transcriptional or translational machinery, the regulatory regions of gene promoter and RNA splicing. A nucleotide diversity in DNA sequence that is stable and found at a frequency of more than 1% in a given population is called polymorphism. The simplest type of polymorphism implies a single base diversity between genome sequences (Single Nucleotide Polymorphism, SNP). Other common types of polymorphisms are minisatellites which are characterized by a variable number of tandem repeats of multiple copies of repeated DNA sequences (0.1-10kb), and microsatellites in which a sequence of up to four nucleotides is repeated many times.

In the light of this latest information, there are three different approaches for the analysis of drug-related phenotypes: a single gene approach, a candidate pathway gene approach

and a genome wide approach (Fig. 1). Treatment response can be altered by polymorphisms of single genes encoding enzymes that metabolize chemotherapeutic drugs; the clear disadvantage in considering a single gene approach is the possility of losing significant associations. Differently, the main advantages of the gene candidate pathway strategy are to combine information of several genes that are common in a metabolic pathway and to minimize the "noise" of a non-targeted genome wide approach, although it probably excludes some genes of importance. The last approach is based on global gene expression profiling study, using DNA microarray that could identify genes with levels of expression that are related to drug response. The advantage of this strategy is the inclusion of all genes of potential importance and the identification of new targets or molecular markers; the disadvantage is the expression signals of irrelevant genes (defined as "noise") that could increase the number of false positive. The identification of genes through the expression profiles is complementary to the identification of polymorphisms in genes that alter protein function and drug response. A significant challenge in this field is represented by a polygenic studies to fully elucidate the genetic determinants of drug response[1]. Interactions between genes, SNP or both will probably provide more information than the single gene approach.

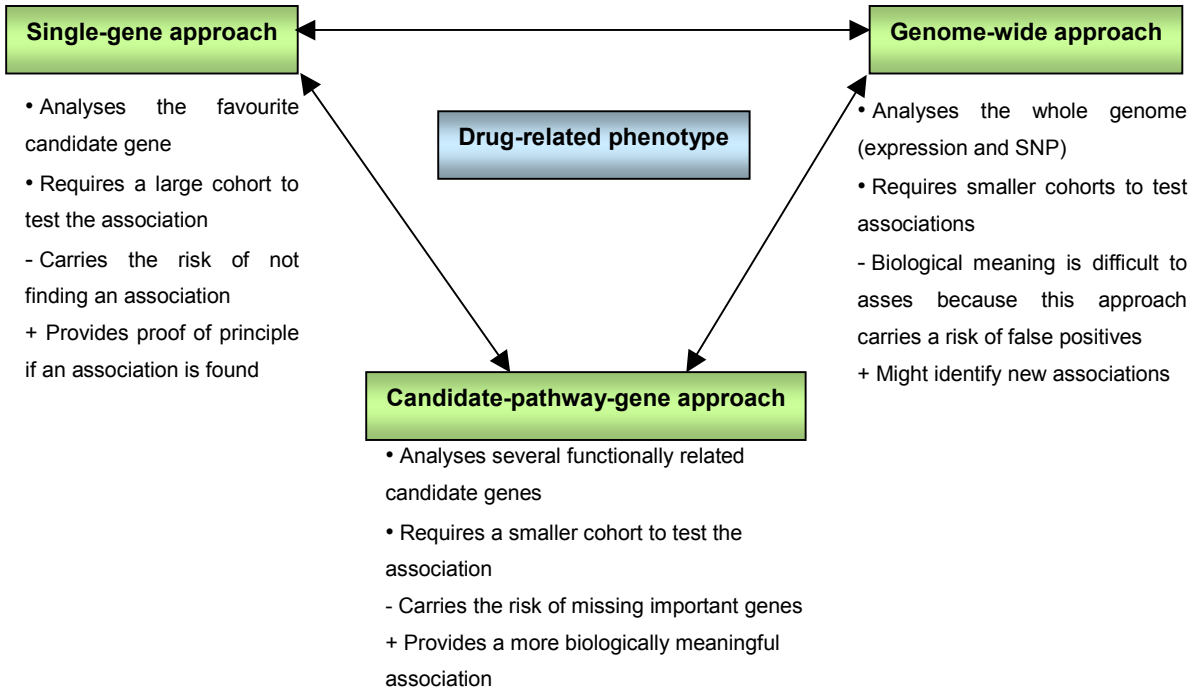

Fig. 1: Differences among single gene, candidate pathway gene and genome wide approaches: main characteristics (•), advantage (+) and disadvantage (-) are indicated.

A major goal in the clinical management of breast cancer is to minimise side effects from potentially toxic therapy and maximise efficacy. In cancer pharmacogenetics, drug responses may be due to variants in the individual's germline DNA or somatic changes in the tumour. The former are more likely to be relevant in the prediction of adverse reactions whereas the latter may be pertinent in the aggressiveness of the tumour. Any drug must be effective against the condition for which it is taken and must have minimal short- or long-term side effects.

1.2 Pharmacogenetics in breast cancer treatment

Breast cancer is a heterogeneous disease with several classifications including those dependent upon histological subtype, estrogen receptor status and more recently tumor gene expression profile.

The introduction of whole genome profiling technologies has expanded the information of genes and genetic pathways involved in breast cancer development. More recently, commercialized multigene prognostic and predictive tests have entered the complex and expanding landscape of breast cancer diagnostics[2]. There are two major breast cancer multigene test platforms: onco*type* DX™ and Mammaprint®. The pathways studied in both these tests are three: cell proliferation, ER and HER-2. The main differences are: the number of genes analysed (21 versus 70, respectively) and a predictive and prognostic value versus an only prognostic value. Onco*type* DX™ determines the 10-years risk for disease recurrence (RS, recurrence score) and indicates that patients with a low score benefit from tamoxifen treatment, whereas patients with an high score benefit from chemotherapy with CMF (cyclophosphamide, methotrexate, 5-fluorouracil) regimen. Differently, Mammaprint® has a wider indication than onco*type* DX™ by including both ER-positive and ER-negative patients[3].

The increasing interest in personalized medicine has led to an in-depth research into genetic pathways of drug metabolism and into the role of biomarkers to optimize therapeutic decision for each individual patients.

In recent years there have been several advances in the treatment of breast cancer. However, significant heterogeneity in the efficacy and toxicity of chemotherapeutic agents is consistently observed. Although many clinical (age, gender, diet, organ function) and environmental variables have been associated with drug response, genetic differences in drug metabolism and drug targets can have a great effect on treatment outcome.

Current drug treatment of breast cancer falls into in three categories: conventional chemotherapy (alkylating agents such as Cyclophosphamide, anthracycline, taxanes), hormonal therapies (tamoxifen, aromatase inhibitors such as Exemestane) and novel molecular targeted therapies (monoclonal antibody as Herceptin). With multiple treatment choice available, there now needs to be a method to prospectively screen patients prior to treatment selection. This would help to lower patients morbidity from treatment. A genetic screening could be useful to personalize therapy thus minimizing side effects.

At present, several studies report a possible role of genetic variants of cytochrome, an important family of metabolic enzymes, both in cyclophosphamide metabolism and in exemestane activity.

1.2.1 Pharmacological treatment with Cyclophosphamide

The alkylating agents react with many electronrich atoms in cells to form covalent bonds. The most important reactions with regard to their antitumoral activities are interactions with DNA bases. The alkylating agents could be monofunctional (reaction with only one strand of DNA) or bifunctional (reaction with atoms of both strands of DNA forming “cross-link”). Unless repaired, this lesion will prevent the cell with replicating effectively. The lethality of the monofunctional alkylating agents results from the recognition of the DNA lesion by the cell and the response of the cell to that lesion. The preferential site of DNA alkylation is the guanine in position N7.

The most frequently used alkylating agent for breast cancer treatment is cyclophosphamide. This is a prodrug that undergoes both phase I and phase II metabolism, with bioactivation through CYP enzymes to 4-hydroxycyclophosphamide. This is catalyzed by several isoforms of CYP: CYP2B6 (45%), CYP2C9 (12%), CYP3A4 (25%) and, with a minor contributions by CYP3A5 (1%) and CYP2C19 (0.6%) (Fig. 2). The 4-hydroxycyclophosphamide is in equilibrium with its open-ring tautomer aldophosphamide, which undergoes chemical decomposition to form phosphoramide mustard, a bifunctional DNA alkylator and acrolein. The mustard derivative is also a cardiotoxic compound whereas the acrolein is associated with urologic toxicity. For cyclophosphamide the metabolism is separated in 10% dechloroethylation to the cytotoxic chloroacetaldehyde and 90% activation by 4-hydroxylation. CYP3A4 and CYP3A5 was shown to be responsible for the 95% of the formation of the dechloro-components.

The 4-hydroxycyclophosphamide and aldophosphamide are detoxified to carboxycylophosphamide by glutathione-S-transferase (GST) and by aldehyde dehydrogenase (ALDH), respectively. The main side effects associated to cyclophosphamide administration were: neurotoxicity (by dechloroethyl-chloroacetaldehyde) and bladder toxicity (by acrolein) in addition to the common hepatic and hematological toxicities.

The drug-metabolizing CYP enzymes are known to be highly polymorphic and have variant alleles with decreased or absent metabolic activity, resulting in large interindividual variability in the plasma concentrations of drugs. The CYP enzymes can be divided into

two classes: *class I* composed of CYP1A1, CYP1A2, CYP2E1 and CYP3A4 which are well conserved and are active in the metabolism of precarcinogens and drug; and *class II* composed of CYP2B6, CYP2C9, CYP2C19 and CYP2D6 which are highly polymorphic and active in metabolism of drugs, but not of precarcinogenes.

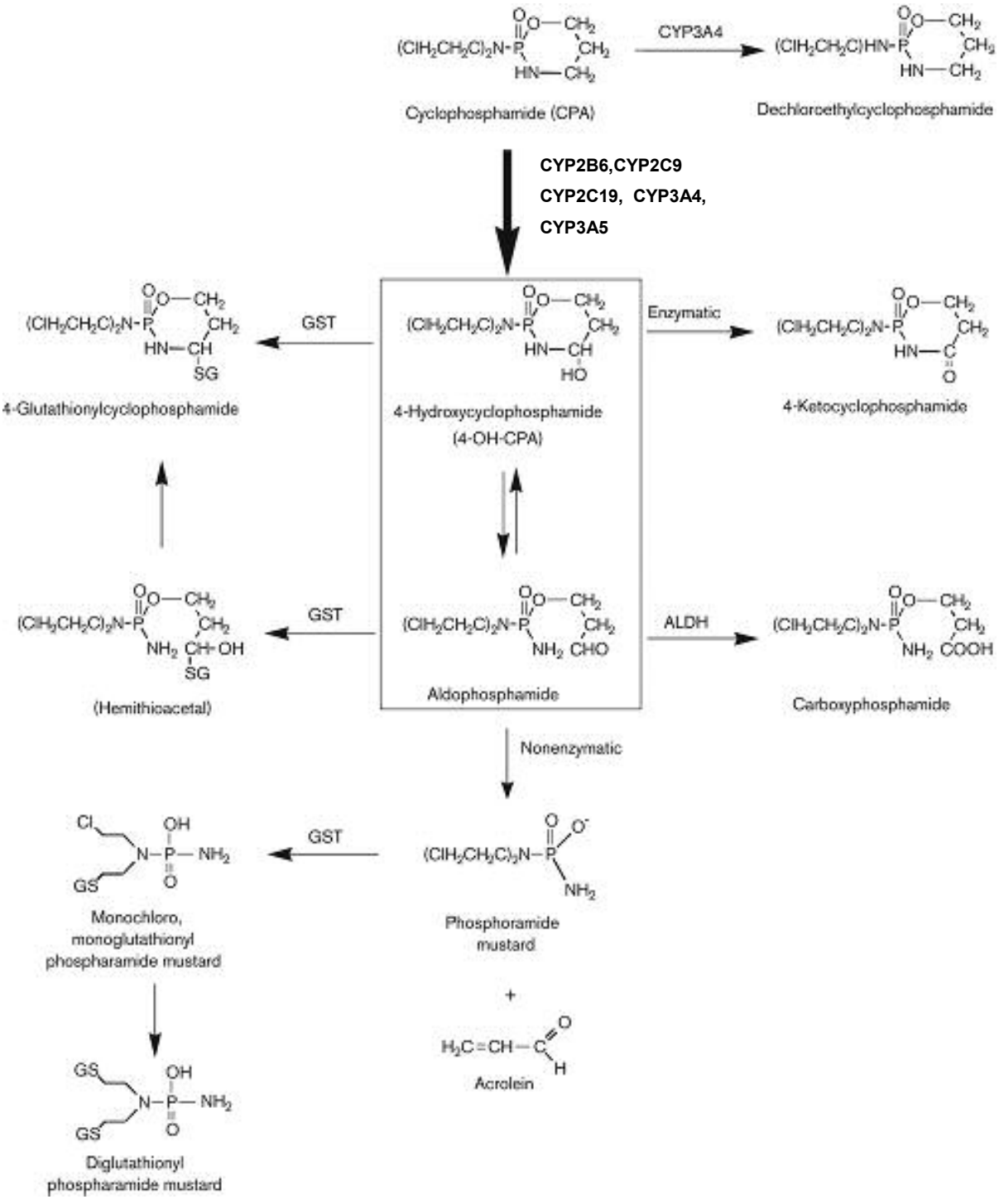

Fig. 2: Metabolic pathway of cyclophosphamide.

1.2.2 CYP2B6 polymorphisms

CYP2B6 constitutes approximately 5% of total CYP in the liver, but is also detected at lower levels in extrahepatic tissues, like intestine, kidney, lung, brain and skin. CYP2B6 activity in the liver microsomes varies more than 100-fold: a broad of interindividual variability of *in vivo* pharmacokinetic could be explained with genetic alteration in CYP2B6 isoform.

In 2001, nine SNPs of this gene were described; five resulted to define an amino acid substitutions: Ex1+C64T (Arg22Cys, rs8192709), Ex4+G516T (Gln172His, rs3745274) Ex5+C777A (Ser259Arg, rs45482602), Ex5+A785G (Lys262Arg, rs2279343), Ex9+1459C>T (Arg487Cys, rs3211369)[4]. The combination of these polymorphisms could describe six different alleles: CYP2B6*2 (Arg22Cys), CYP2B6*3 (Ser259Arg), CYP2B6*4 (Lys262Arg), CYP2B6*5 (Arg487Cys), CYP2B6*6 (Gln172His and Lys262Arg) and CYP2B6*7 (Gln172His, Lys262Arg, and Arg487Cys) (Fig. 3). Currently, variant alleles count up to CYP2B6*29[5] but the most investigated variants in the Caucasian population were: C1459T (alleles CYP2B6*5 and CYP2B6*7); G516T (alleles CYP2B6*6, CYP2B6*7, CYP2B6*9, CYP2B6*13, CYP2B6*19 and CYP2B6*20) and A785G (alleles CYP2B6*4, CYP2B6*6, CYP2B6*7, CYP2B6*16, CYP2B6*19 and CYP2B6*20)[6,7].

All these polymorphisms are associated with a decrease expression of CYP2B6.

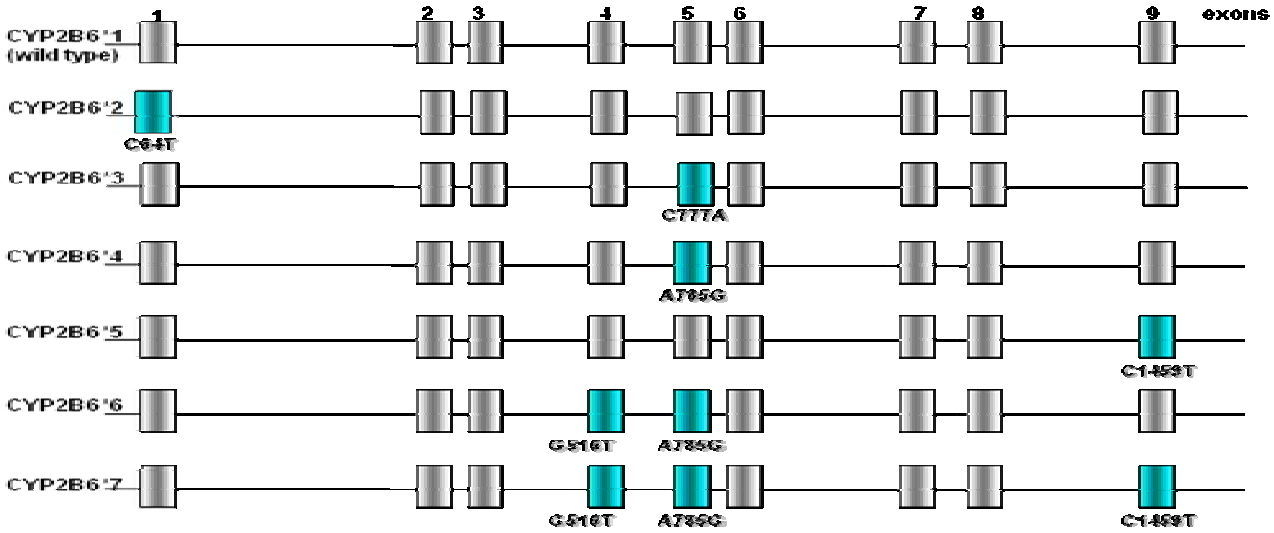

Fig. 3: Allelic variants of the human CYP2B6 gene. Exons containing mutations resulting in aminoacid changes are shown as light blue boxes, exons resulting in the wild type aminoacid sequences are shown as grey boxes.

Few studies available have been investigate the relationship between the genetic polymorphisms of CYP2B6 and pharmacokinetics of cyclophosphamide. Xie et al.[8,9] have reported that only the variant G516T was associated with the increase of cyclophosphamide 4-hydroxylation. In particular, this variant determines an alternative splicing that lacks the region including exons 4 to 6. This results in severely reduced levels of functional full-length mRNA transcript, protein and activity[10]. The A785G variant has low frequency in Caucasian population (2-6%) because it is often linked with G516T variant. *In vitro* studies indicate that A785G variant was expressed at a lower level compared with the wild type allele[7].

The C1459T polymorphism (alleles CYP2B6*5 and CYP2B6*7) was correlated with decreased protein levels[11] whereas the association with its activity was found in females but not in males[12]. This results are not confirmed in a Japanese study, probably because of ethnic differences[13]. Moreover, a study of Takada[14] demonstrated that patients carrying the CYP2B6*5 allele and treated with pulse cyclophosphamide for systemic lupus erythematosus had significantly higher risk to develop nefrotoxicity with respect to wild type patients.

CYP2B6 polymorphisms are often studied in relation to other different substrates such as bupropion, coumarin or efavirenz; the results underlined that the effect of SNPs in the enzymatic activity is substrate-dependent[15].

1.2.3 CYP2C9 polymorphisms

CYP2C9 is the main isoform of CYP2C subfamily expressed in the liver[16] and is involved in the metabolism of 10-20% of commonly prescribed drugs.

Approximately 34 variant alleles have currently been codified for CYP2C9[5] but the main important in the Caucasian population are CYP2C9*2 (C430T, Arg144Cys, rs1799853) and CYP2C9*3 (A1075C, Ile359Leu, rs1057910). Both these variants encode for altered CYP2C9 activity, but the most profound decrease of activity was emerged for CYP2C9*3[17]. The 1075C allele seems to reside within the CYP2C9 active site and thus it would be expected to produce changes in substrate metabolism: the homozygous variant allele has been associated with the poor metabolizer phenotype for several drugs. The variant 430T allele is located in the active site of the enzyme and could influence the interactions of substrates with CYP[18].

However, the different pharmacokinetic effects in most substrates indicated a substrate specificity[19]. Few studies are conduced to define the role *in vivo* of genetic variant of CYP2C9 in cyclophosphamide pharmacokinetic[8,20].

1.2.4 CYP2C19 polymorphisms

Among the CYP2C enzymes, the isoform CYP2C19 takes part in the activation of cyclophosphamide[21,22]. Interindividual differences in CYP2C19 activity divide the population into extensive metabolizer (EM) and poor metabolizer (PM)[23]. PMs may not gain therapeutic benefit from prodrugs activated by CYP2C19. Seven alleles have been described in this gene that produce an inactive CYP2C19 enzyme; however only two alleles account for the majority of the PM phenotypes. CYP2C19*2 is the most prevalent PM allele and is characterized by a G681A substitution in the exon 5 (rs4244285) that produces an aberrant splice site. The CYP2C19*3 polymorphism (G636A, rs4986893) is found mainly in Asian population and determines a premature stop codon[24].

A recent study demonstrates that only the variant CYP2C19*2 had a role in pharmacokinetics of cyclophosphamide: carriers of at least one variant allele had lower drug metabolism with respect to the other genotypes[22]. However, data in this field are still controversial[20] and need further investigations and clarifications.

1.2.5 CYP3A polymorphisms

Oxidative metabolism of cyclophosphamide involves also the CYP3A subfamily, the most abundant CYPs in human liver and small intestine. Four isozymes with very similar substrate specificity, whose genes are located on chromosome 7, are included in the CYP3A subfamily (CYP3A4, CYP3A5, CYP3A7 and CYP3A43). Among them, CYP3A4 and CYP3A5 are involved in cyclophosphamide metabolism both in the bioactivation and in the dechloroethylation pathway. CYP3A4 is the most expressed CYP in the liver, comprising 25% to 40% of total hepatic CYP content and representing the most abundant CYP isoform in the gastrointestinal tract. CYP3A5 is commonly considered the second most important CYP3A protein in the liver, although its expression is largely inter-individually variable (10-97% of human livers)[15].

It has been reported that enzymes belonging to the CYP3A subfamily exhibit a large inter-subject variability in hepatic and intestinal activity. The biochemical bases of such differences are not completely understood at the moment, but they have a deep influence

on drug efficacy and toxicity. It has been assumed that an important role could be related to polymorphic genetic variants. Actually, at least 20 mutations have been detected in the CYP3A4 gene sequence, but none of them has a strong prevalence in the population. CYP3A4*1B (A-392G; rs2740574) is a point mutation in a regulatory element of the 5'-flanking region of the gene, that is thought to be associated with a reduced level of expression of the protein.

Concerning CYP3A5, a single nucleotide polymorphism, CYP3A5*3 (G6986A; rs776746) encodes aberrant splicing and causes the absence of protein and thus CYP3A5 activity[25]. The presence of this mutation in at least one of the alleles ends up in an increase of the expression levels of the protein[26]. At the moment, other 11 mutations have been described for CYP3A5 [5] but with a minor effect in enzyme expression than CYP3A5*3. A study performed by Petros et al.[27] reported that the CYP3A5 variant in breast cancer patients treated with high dosage of cyclophosphamide was correlated with lower survival rates.

1.2.6 Anti-estrogenic treatment with Exemestane

The treatment of patients with hormone-sensitive breast cancer consists of adjuvant setting with endocrine therapy. In malignant breast tissue, the action of estrogen is deregulated, resulting in a shift to proliferation without differentiation nor apoptosis. The percentage of epithelial cells expressing ER is significantly increased under these conditions[28]. The intent of antihormonal treatment of ER-positive breast cancer is based upon blocking the ER signal transduction pathway or inhibiting the synthesis of estrogen (Fig. 4) leading to a reduction of tumor proliferation. There are two classes of drugs mainly used in the clinical practice: the Selective Estrogen Receptor Modulator (SERM) and the Aromatase Inhibitor (AI). The first class acts blocking the ER as selective antagonist of estrogen in several tissue, including breast, heart and bone. The treatment with tamoxifen, the prototypical SERM, is associated with an increased risk of endometrial cancer by promoting cancer cell proliferation[29]. The AI are a new class of endocrine therapy drugs that inhibit the action of aromatase (CYP19), an enzyme necessary for the conversion of androgens to estrogens.

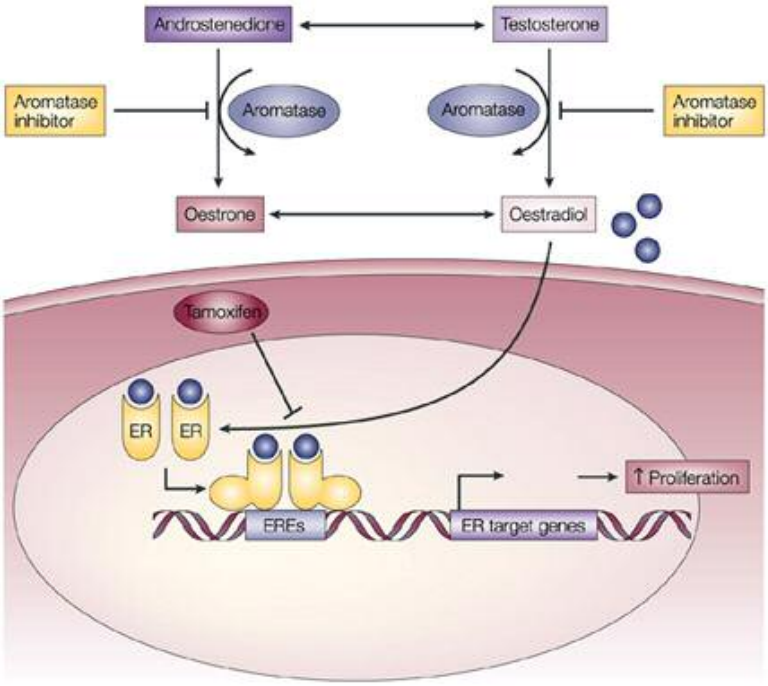

Fig. 4: Differences in the mechanism of action of aromatase inhibitors (AI) and tamoxifen (SERM).

Aromatase Inhibitors are classified in a number of different ways, including steroidal irreversible inhibitors (formestane, exemestane) and non-steroidal reversible inhibitors (aminoglutethimide, rogletimide, fadrozole, anastrozole, letrozole and vorozole).

Recent studies demonstrated that the treatment with a third-generation steroidal AI (such as exemestane) was found superior to traditional tamoxifen treatment for postmenopausal women in increasing clinical outcome[30-33]. The AI are not recommended for treatment of premenopausal women because ovarian estrogen production may resume through feedback mechanisms[34].

The characteristic side effects of exemestane were: arthralgia, diarrhea, thromboembolic events, gynecologic symptoms, vaginal bleeding and muscle cramps. Moreover, these compounds have been associated with an increase in urine and plasma bone resorption markers, indicating that bone loss might be a significant problem during AI use in post-menopausal women[35].

Differences in the exemestane response rate and in toxicity within a patient population may be associated to an impaired activity of aromatase target enzyme (CYP19).

1.2.7 CYP19 polymorphisms

Human aromatase is encoded by CYP19, a single-copy gene on chromosome 15q21.2. CYP19 comprises 20 exons, with exons II through IXX encoding the open reading frame of

aromatase. At least nine different first exons are known to encode the unique 5´-untranslated regions of aromatase mRNA. Each first exon has its own upstream promoter region. First exons and corresponding promoters are used alternatively in a tissue- or cell-specific manner, enabling tissue- or cell-specific regulation of aromatase. The most proximal promoters, PII and I.3, are used predominantly for the gonads, whereas promoter I.4 is used for adipose tissue and skin (Fig. 5). The most distal promoter (I.1) is used almost exclusively for the placenta. These regulatory regions might participate in the pathogenesis of malignant breast transformation because of the different control of aromatase expression due to the different exon I in normal breast cancer (exon I.4) and in malignant transformation (exon I.3 and promoter II)[36].

Recently, a resequencing of the gene encoding CYP19 has been performed[37]. A total of 88 genetic polymorphisms, including SNPs that altered the encoded aminoacid sequence, were identified.

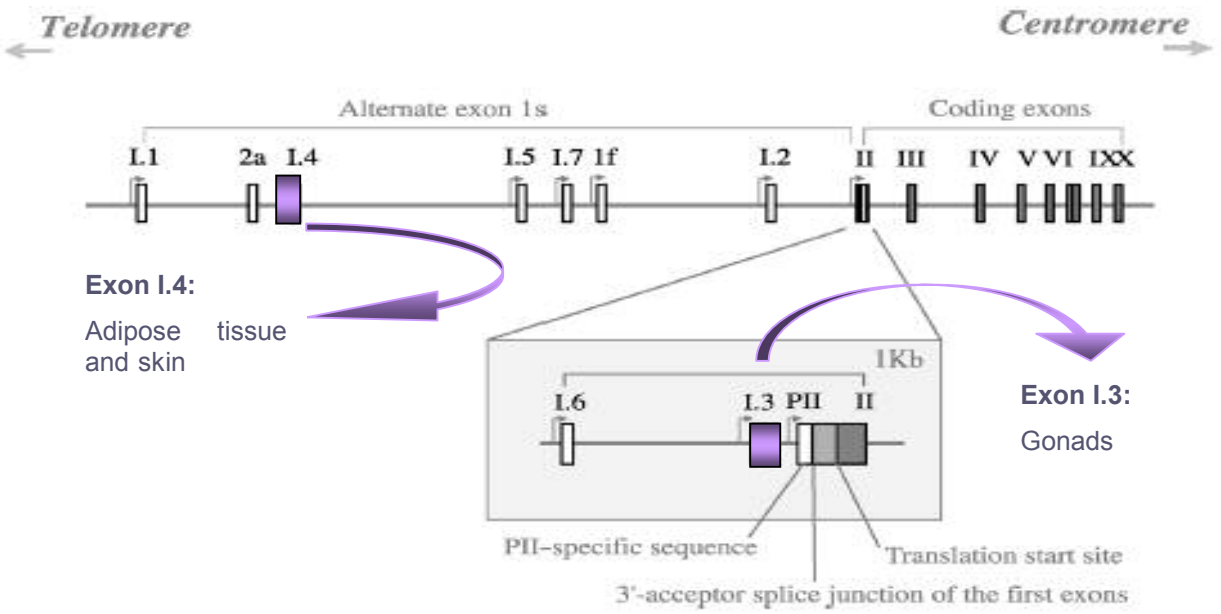

Fig. 5: Structure of human aromatase gene (CYP19).

Differences in the exemestane response rate within a patient population may be associated to an impaired activity of aromatase target enzyme (CYP19) or to an inter-patient difference in the metabolic elimination of exemestane, leading to variations in the pharmacokinetic profile of the drug. Moreover, individual differences in the biosynthesis and catabolic pathways of estrogens, as well as differences in the expression of their cellular receptors and transporters, may directly influence the pharmacodynamics (toxicity and response) of exemestane.

Genetic polymorphisms, potentially involved in exemestane efficacy, have been described. In particular, CYP19, is encoded by a polymorphic gene and this could be linked to a different ability to interact with the drug. Among the CYP19 polymorphisms, the Ex11+G410T in the 3' UTR region (rs4646) has been recently associated to an increased progression free survival in patients treated with aromatase inhibitors[38].

Another CPY19 polymorphism that could alter the activity of AI is a $(TTTA)_n$ tetranucleotide repeat located in intron 4. In literature the seven repeats are considered the cutoff to distinguish the long allele (>7 repeats) and the short allele (≤7 repeats)[39,40]. However, several studies indicate that the presence of a higher number of repeats (10 or 12) was associated with breast cancer risk[39,41-44].

Moreover, a significant association with survival in Caucasian women was observed for long allele[45] whereas the short allele was found associated with a lower estrogen levels[46].

A decreased expression of CYP19 was also associated with two other polymorphisms: Ex8+C47T (Arg264Cys, rs700519) and Ex10+C1558T (rs10046), possibly affecting drug efficacy. These polymorphisms have been associated to a variation in the bone mineral density of post-menopausal women, underlining their phenotypic impact, and a possible further involvement in the individual attitude to develop side effects to the drug[47]. A strong linkage disequilibrium was found among $(TTTA)_n$ tetranucleotide repeats and CYP19+C1558T[48,49].

1.2.8 CYP17 polymorphisms

CYP17 encodes for cytochrome p450c17α, which catalyzes steroid 17α-hydroxylase and 17-20 lyase activities at key points in estrogen (estradiol) biosynthesis. The 5'-untranslated region of the gene contains a single-base polymorphism (T27C; rs743572) that creates a Sp1-type (CCACC box) promoter[50]. Since the number of 5' promoter elements correlates with the promoter activity, the 27C allele may increase CYP17 expression and thus increase estradiol biosynthesis. An elevated level of circulating estrogens could be related to markedly increased risk of breast cancer. Several studies focused on the impact of this common germline polymorphism in breast cancer development[51,52]. Moreover, this SNP has been associated with a lower bone mass density[47,53], as well as CYP19 polymorphisms, could have a role in the exemestane efficacy and toxicity and could be associated to estrogens-dependent exemestane side effects.

2. AIM OF THE STUDY

Pharmacogenetics could represent a useful and innovative tool for optimization of schedule and dosage of anticancer chemotherapy. In this work of thesis we have studied the role of genetic variants of several CYP isoforms in breast cancer therapy.
This study has been conduced in two group of patients: the first ones were treated with chemotherapy cyclophosphamide-based (195 patients) while the second ones were treated with an anti-estrogenic treatment with exemestane (121 patients).
For both these groups we evaluated the relative risk to develop breast cancer for all the polymorphisms investigated:

- CYP2B6 (G516T, A785G, C1459T)
- CYP2C9 (C430T, A1075C)
- CYP2C19 (G681A)
- CYP3A (A-392G)
- CYP3A5 (G6986A)

} Cyclophosphamide-based regimen

- CYP19 (G410T, $TTTA_n$, C47T, C1558T)
- CYP17 (T27C)

} Exemestane treatment

In addition to the relative risk to develop the disease, for the group of patients treated with cyclophosphamide we could also investigate the possible role of the above cited polymorphisms in the clinical response (evaluated as Overall Survival) or the development of toxicity after the first cycle of therapy and after the entire course of treatment with cyclophosphamide in an adjuvant setting.

3. MATERIALS AND METHODS

3.1 Patients enrollment and drug administration

Two groups of patients were enrolled for this study and were characterized by their treatment: adjuvant chemotherapeutic regimen or anti-estrogenic therapy.

3.1.1 Cyclophosphamide treatment

One hundred and ninty-five breast cancer patients were enrolled between 1999 and 2005 at the CRO-National Cancer Institute of Aviano, Italy. The subjects were treated with adjuvant setting based on CMF regimen (Cyclophosphamide 100 mg/m^2/p.o., day 1 and day 14; Methotrexate 40 mg/m^2/i.v. bolus, day 1 and day 8; 5-Fluorouracil 600 mg/m^2/i.v. bolus, day 1 and day 8) every twenty eight days. Eligible cases were patients with diagnosis of breast cancer, aged between 18 and 75 years, and of Caucasian ethnicity. All cases recruited in the study signed a written informed consent approved by the local Ethical Committee.

3.1.2 Exemestane treatment

The study must include almost three hundred patients with metastatic or locally-advanced inoperable ER-positive breast cancer. At the moment of the enrollment, all the cases presented measurable lesions or non target lesions as defined by RECIST criteria[54]. Signed informed consent was obtained from all cases recruited in the study. Elegibily criteria for the enrollment was: age ≥55 years (post-menopausal women); performance status (ECOG) 0 to 2; life expectancy >3 months; absolute neutrophil count (ANC) ≥1,500 µl, platelets ≥100,000 µl and hemoglobin ≥9.0 g/dl. Patients was treated with exemestane, 25-mg/p.o./single daily dose. The planned duration of treatment was at least 8 weeks.

3.2 Molecular analysis

- ✓ *Samples storing*. Whole blood samples were collected from the patients, catalogued in proper data base and stored in freezer at –20°C.

- ✓ *Genomic DNA extraction*. Genomic DNA was extracted from 200 µl of whole blood by the EZ1 DNA Kit Reagent Cartridge (Blood 200 µl) QUIAGEN® using the automatized extractor BioRobot EZ1.

- ✓ *Polymerase Chain Reaction (PCR).* PCR is an in vitro reaction exploiting DNA polymerase catalytic activity to dramatically amplify a fragment of DNA starting from small quantity of template DNA in the presence of deoxynucleotides triphosphate (dNTPs), PCR oligonucleotides used as primers and a DNA polymerase in a proper reaction buffer. Three distinct events must occur during a PCR cycle: denaturation of the template, primer annealing and DNA synthesis by a thermostable polymerase (Fig. 6). For this work of thesis a Thermofilus Aquaticus (Taq) polymerase has been used. DNA denaturation occurs when the reaction is heated to 92-96°C. The time required to denature the DNA depends on its complexity, the geometry of the tube, the thermal cycler and the volume of the reaction, usually a 30 seconds denaturation time is used. For DNA sequences that have a high G/C content, longer denaturation times have been used to improve the yield of PCR. After denaturation, the oligonucleotide primers hybridize to their complementary single-stranded target sequences. The temperature of this step varies from 37°C to 65°C, depending on the homology of the primers for the target sequences as well as the base composition of the oligonucleotides. Primers are used at a higher concentration than the target DNA, and are shorter in length; as a result, they hybridize to their complementary sequences at an annealing rate several order of magnitude faster than the target duplex DNA can re-anneal. The last step is the extension of the oligonucleotide primer by a thermostable polymerase. This portion of the cycle is usually carried out at 72°C. The time required to copy the template, fully depends on the length of the PCR products.

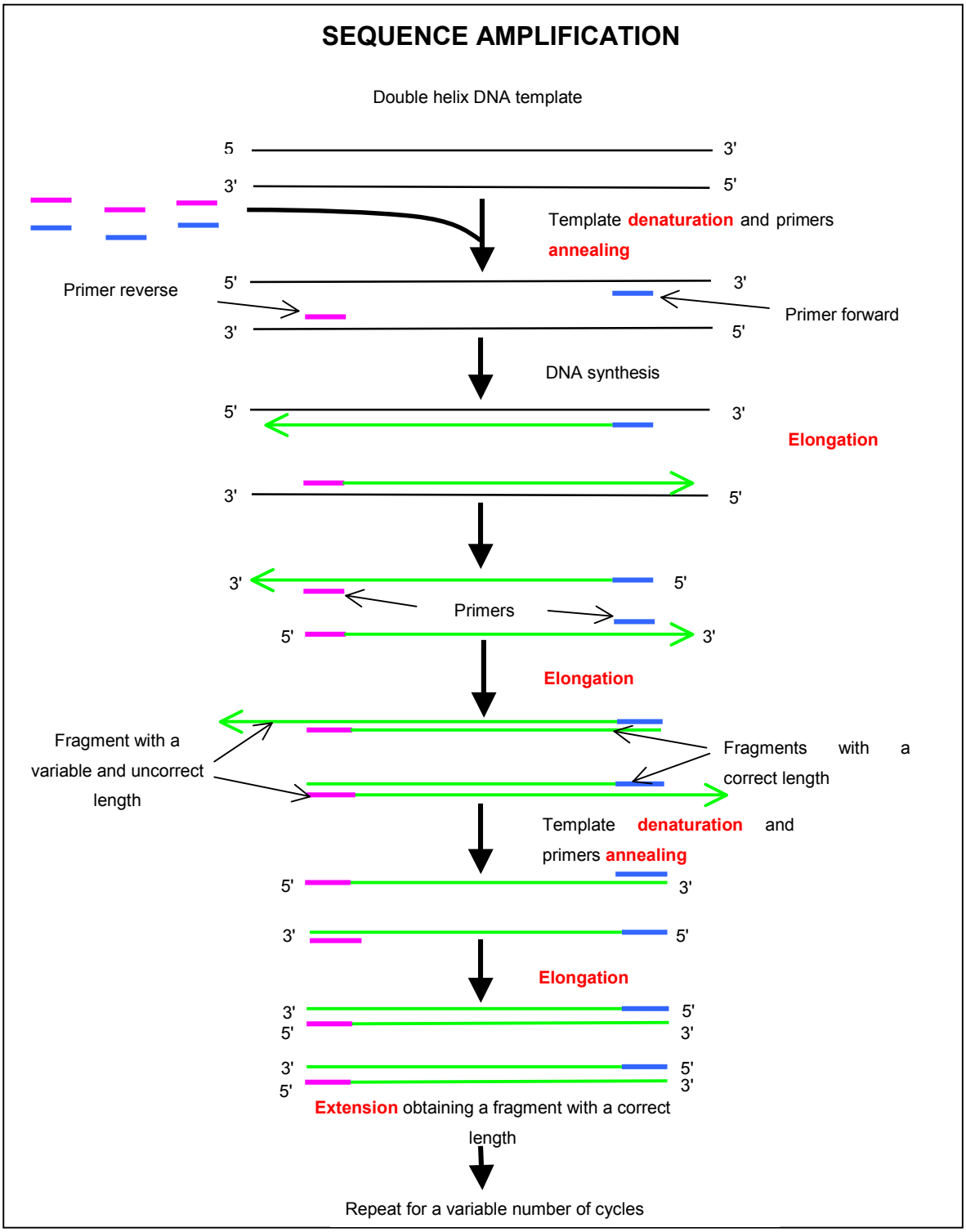

Fig. 6: Representation of PCR reaction.

Amplification strategy for each polymorphism-containing genomic fragment, was optimized based on the following parameters set up:

- ✓ Primer design: a specific software, Pyrosequencing Assay Design Software, has been used for primer design. The aim of primer design is to obtain a balance between two goals: efficiency and specificity of amplification. Given a target DNA sequence, primer analysis software attempts to strike a balance between these two goals by using pre-selected default values for each of the primer design available. In particular optimal primer pairs should be closely matched in melting temperature and not able to form loops and primer dimers. Primer length (about 20-base pairs), sequence and GC contents are taken into account to select proper primers sequences.
- ✓ Mg^{2+} concentration: the presence of divalent cations is critical, and it has been shown that magnesium ions are superior to manganese, and that calcium ions are ineffective. The optimal Mg^{2+} concentration for Taq polymerase efficiency must be set up to match dNTPs and primer concentration/ sequence. dNTPs are the major source of phosphate groups in the reaction, and any change in their concentration affects the concentration of available Mg^{2+}.
- ✓ Number of cycles: the number of cycles to be performed for each PCR protocol was set up checking the accumulation of target sequence by gel electrophoresis after each amplification cycle. The correct number of cycles to be used should guarantees a sufficient balance between efficiency and specificity of amplification. Thirty–five cycles of amplification resulted correct for most of the protocol employed in this work.
- ✓ Annealing temperature: the temperature of annealing was set up performing a temperature gradient PCR (Mastercycler Gradient Eppendorf, Hamburg, Germany). Annealing temperatures could be calculated by several methods considering the melting temperature (Tm) of the primer-template pairs. However, in practice, because the Tm is variously affected by the individual buffer components and even the primer and template concentrations, any calculated Tm value should be considered just as a first approximation. A range of 60±5°C was tested by 1 degree increments to find the optimal reaction conditions.
- ✓ Enhancing agents: various additives such as DMSO (1-10%), PEG-6000 (5-15%), glycerol (5-20%), nonionic detergents, formamide (1.25-10%) and bovine serum

albumin (10-100 µg/ml) were considered for incorporation into the reaction to increase specificity and yield.

3.2.1. Specific PCR conditions for the polymorphisms analyzed

NCBI (National Center for Biotechnology Information) database and tools were used to develop the polymorphisms analyses. The NCBI presents a web site showing links to all kinds of important protein and nucleotide database, literature (PubMed), and search and analysis tools. Important database included in the web site and consulted for assay design (genetic sequences, polymorphisms description, primer design), during this work of thesis, were the GenBank sequence database, OMIM (Online Mendelian Inheritance in Man), UniGene (Unique Human Gene Sequence Collection) and SNP500 (Single Nucleotide Polymorphisms database).

Each polymorphism analyzed required specific PCR conditions for amplification of the gene fragment containing the variant of interest. PCRs were carried out in a reaction volume of 50 µl with 10 mM Tris-HCl (pH 8.3) and KCl 50 mM, each dNTP at 200 µM, forward and reverse primers at 1 µM and one unit of DNA polymerase AmpliTaq Gold (Roche for Applied Biosystems, Branchburg, New Jersey). One hundred ngs of genomic DNA were introduced in the reaction tube as template. Thermal cycling program consisted of a denaturation/ Taq polymerase activation step at 95°C for 10 minutes. Subsequently, 35 cycles were performed consisting of a denaturation step (95°C, 30 seconds), an annealing step (specific annealing temperature, 30 seconds) and an extension step (72°C, 30 seconds). A final extension step of 10 minutes at 72°C was added at the end of the 35 cycles. Specific condition of Mg^{2+} concentration, annealing temperature and primer sequences are reported in Table 1.

Table 1: PCR primers sequences and specific condition for each polymorphism.

SNP		PCR primers	Annealing Temperature (°C)	Mg^{2+} concentration (mM)
CYP2B6	G516T	5'-GAC AGA AGG ATG AGG GAG GAA-3' #5'-CTC CCT CTG TCT TTC ATT CTG T-3'	58	2.5
	A785G	5'-TGA GAA TCA GTG GAA GCC ATA GA-3' 5'-TAA TTT TCG ATA ATC TCA CTC CTG C-3'	56	1.5
	C1459T	5'-GGT CTG CCC ATC TAT AAA C-3' 5'-CTG ATT CTT CAC ATG TCT GCG-3'	60	1.5
CYP2C9	C430T	5'-GTA TTT TGG CCT GAA ACC CAT A-3' #5'-CAC CCT TGG TTT TTC TCA ACT C-3'	61	2.5
	A1075C	#5'-TGC ACG AGG TCC AGA GAT-3' 5'-GAT ACT ATG AAT TTG GGA CTT C-3'	60	2.5
CYP2C19	G681A	#5'- CAG AGC TTG GCA TAT TGT ATC-3' 5'- GTA GTA AAC ACA AAA CTA GTC AAT G-3'	61	2.5
CYP3A4	A-392G	5'- ATC TGT AGG TGT GGC TTG T-3' #5'-GGG TTC TGG GTT CTT ATC A-3'	57	2.5
CYP3A5	G6986A	5'- ACC ACC CAG CTT AAC GAA TG-3' #5'- TGA CAC ACA GCA AGA GTC TCA-3'	65	2.5
CYP19	C47T	#5'- TTG AAA GAT GCC ATA GAA GT-3' 5'- AAC TCA GTG GCA AAG TCC-3'	57	2
	$(TTTA)_n$	5'- TTG TCT ATG AAT GTG CCT TTT-3' *5'- CTG GGT GAT AGA GTC AGA GC-3'	55	1.5

*5'-HEX fluorofore labeled primer #5'-biotinylated primer

Some polymorphisms (CYP19 G410T, CYP19 C1558T and CYP17 T27C) were analyzed using the allelic discrimination by TaqMan Assay.

For this assay the PCR was performed in a reaction volume of 5 µl using the Genotyping Master mix and the specific TaqMan probes for each polymorphism. The PCR conditions for amplification were standardized according to the manufacturer's instructions: a denaturation/ Taq polymerase activation step at 95°C for 10 minutes, subsequentely, 40 cycles were performed consisting of a melting step (92°C for 15 seconds), an annealing step and an extension step (60°C for 60 seconds).

3.2.2 Polymorphism analysis

The method of detection of different type of genetic changes varies depending on the type of mutation to be detected. All the methods described below have been employed to detect the polymorphisms investigated in this work of thesis and are based on PCR technique to produce the amplified fragment on which the analysis will be performed.

3.2.2.1 Restriction Fragment Length Polymorphism (RFLP)

Certain genetic changes may alter a restriction enzyme recognition site, either by creating a new site or destroying an existing site. In these cases, a molecular diagnostic assay can be designed in which a region containing the potential mutation site is amplified via PCR and the product is digested with the appropriate restriction enzyme to determine if the restriction digest pattern is altered due to the presence of the mutation (Fig. 7).

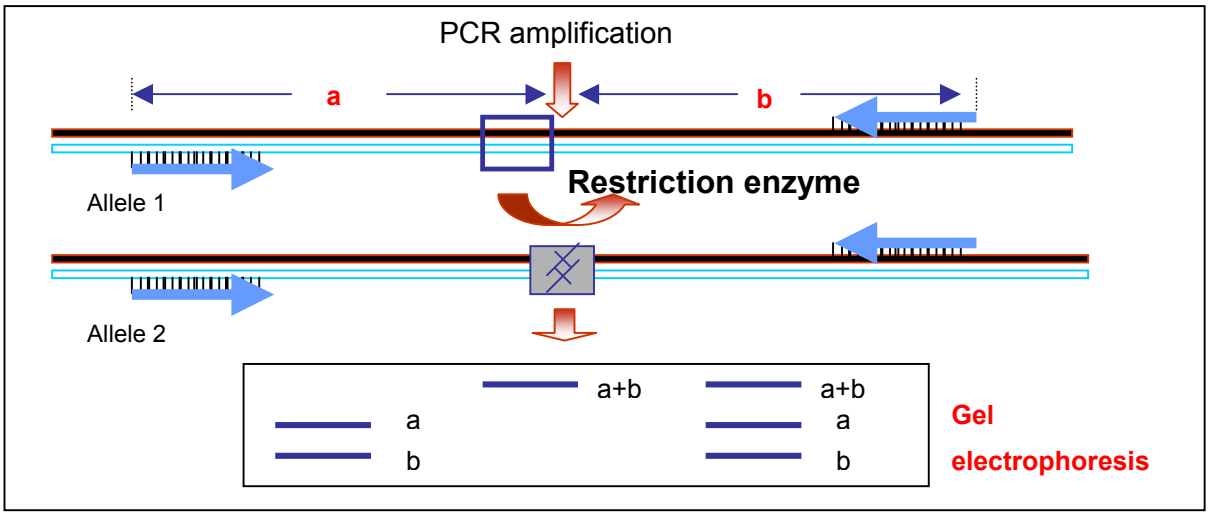

Fig. 7: Scheme of the RFLP technique for the SNPs analysis.

CYP2B6 A785G and CYP2B6 C1459T have been analyzed using RFLP: the amplified PCR fragments contain a StyI and a BglII restriction site, respectively.
The PCR product of CYP2B6 A785G is incubated overnight at 37°C with 5 units of restriction enzyme (New England Biolabs, Inc., Beverly, MA) and the digestion product analyzed on a 3% agarose gel stained with ethydium bromide. Wild type allele results in four fragments (56, 117, 171 and 297 bp), while the polymorphic allele corresponds to three fragments (56, 117 and 467 bp) (Fig. 8).

GACAGAAGGATGAGGGAGGAAGATGCAGAAAGAGGTAAATGTGAGATAGATCAAAGGAGATATAGAG
TCAGTGAGTGAGGGGTTCAGAGGCAGAGGGGAGTGGGGAAGTGGGGTTC***C/CATGG***AGGGATTGGG
GCCCAGGAGGCGCTCTCTCCCTGTGACCTGCTAGCTCAGC***C/CTAGG***CAAACCTCACCACCCCTTCT
TTCTTGCAGCTGTTTGAGCTCTTCTCTGGCTTCTTGAAATACTTTCCTGGGGCACACAGGCAAGTTTAC
AAAAACCTGCAGGAAATCAATGCTTACATTGGCCACAGTGTGGAGAAGCACCGTGAAACCCTGGACC
CCAGCGCCC***C/CAA<u>G</u>GG***ACCTCATCGACACCTACCTGCTCCACATGGAAAAAGTGGGGTCTGGGAGA
GGAAAAAGGGAAGGGAGGGGAGGGAGGGCAAGATGGAGAGGTGAGAAGAGGGAGGGAAAAGGGG
TAGGGAAGGGGAAGATGGGGAGGGAAGAAGAAAGACTAGGGAGGGGAGAATAGGGAAAGGGAGGA
GAGAACATGAGGAAGGAAAGAAAGATGAGGTGAAAGGAGGGAGAAAATAGGGAGGAGGAACTGAGA
CAGGGAGAGAGGGGAGGTGGGAAGACAGAATGAAAGACAGAGGGAG

<u>Fig. 8:</u> CYP2B6 A785G (GenBank N° AC023172). The primer annealing sequences are evidenced in gray. The restriction site is bold italic. The polymorphic base is underlined bold italic. The slash indicates where the enzyme cuts the fragment.

For the polymorphisms CYP2B6 C1459T the digestion with 5 units of the enzyme (New England Biolabs, Inc., Beverly, MA) was performed overnight at 37°C. The digested products were analyzed on a 3% agarose gel stained with ethydium bromide. Wild type allele results in one uncut fragment of 1400 bp, while the polymorphic allele contains a BglII restriction site generating two distinct fragments (214 and 1186 bp) (Fig. 9).

TGAGAATCAGTGGAAGCCATAGACCCTCCCTGTCTCATACGCATCAGTCCCATTCACAATTTATATAC
AATTGGGCTTTTCCCCCCTTGAGCTCTTGAAGCCTGTGGATCTCAGGCTGAGACTAGAAGACAGCCT
AGAGACACAGACACAGACACATGGCCAGAAACACATACCCCACACATACCCCAGAAACAAACACAAG
GCAGTAACCAAACCTTAAGCCTCCTTAGCACAAGCCAAGTGCTATTCTAAGAATTTTTACAATTGTCTC
CTTTAACCTCACAAGAGCCCTTTGAGGGAGGTGCCATTCTCCCCATATGAGAAGTGAAGAACACTAAG
AATGTTGTCATAAATGTGCTCACACTCACACATGTACAGGTACACACACACACACACACACACAGAAC
CAGAAATGGGTCATTATACTCTGTGGGTTTCACCAACCCTTTGGAACTGTAACAGAAGCTAGAAACTC
TCTTATATAAAAATGAACATGGCGCCGGGTGCAGTGGCTCATGCCTGTAATCCCACTTTGGGAGGCC
AAGGCAGGTGGATTATCTGAGGTCAGGAGCTCGAGGCTAGCCTGGCCAATATGATGATACCCCATGT
CTACTAAAAATAGAAGAAATTAGCCAGGCGTGGTGGCAGGTGCCTGTAATCCCAGCTACTTGGTAGG
CTGAGGCACAAGAATCATTTGAACCACCTGGGAGGTGGAGGTTGCAGTGAGCCAAGATGGTGCCAC
TGTGCTCCAGTCTGGGTGACAAAGAGAGACTCCATCTCAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAGAGAGAGAGAGAAATGAACGTGGCACATCCACTCAAAGATTTGCATCTGGTTTCAGAGCAGC
TTCCTAAAAGTCCACCCTGAATTGTAGGTTAAAGGCCAGTCTTATGCAAATCTGTTGCAGTGGACATTT
GTGTCTGGGCTTAGGGACATGGCAGAGCGAAGTGTATGCACCTGCCCTGTGCCCACACTGGTGACC
TTCTGTGTCCACAGGGAAGCGGATTTGTCTTGGTGAAGGCATCGCCCGTGCGGAATTGTTCCTCTTC
TTCACCACCATCCTCCAGAACTTCTCCATGGCCAGCCCCGTGGCCCCAGAAGACATCGATCTGACAC
CCCAGGAGTGTGGTGTGGGCAAAATACCCCCAACATACC***A/GATCCT***GCTTCCTGCCCCGCTGAAGG
GGCTGAGGGAAGGGGGTCAAAGGATTCCAGGGTCATTCAGTGTCCCCGCCTCTGTAGACAATGGCT
CTGACTCCCCGCAACTTCCTGCCTCTGAGAGACCTGCTACAAGCCAGCTTCCTTCCCCTCCATGGCA
CCAGTTGTCTGAGGTCACATTGCAAGTGAGTGCAGGAGTGAGATTATCGAAAATTA

Fig. 9: CYP2B6 C1459T (GenBank N° AC023172). The primer annealing sequences are evidenced in gray. The restriction site is bold italic. The polymorphic base is underlined bold italic. The slash indicates where the enzymes cuts the fragment.

Quality control for this type of assay requires that appropriate positive and negative control samples be present in each assay in order to confirm that the enzyme is properly active.

3.2.2.2 Automated fragment analysis

Automated fragment analysis is performed to detect small variation in the length of a DNA fragment. It is based on the technique of capillary electrophoresis coupled with fluorescence detection. It occurs when an electric field is applied to an electrolyte (DNA fragment) solution within a capillary, causing ions migration. DNA fragments, having a negative charge, move toward the anode (+) and are separated by size. Polyacrylamide gels are used as the electrolyte solution to provide the sieving medium for the separations. DNA fragment are produced by PCR employing one 5' fluorescence labeled primer. These dye-labeled fragments are detected by fluorescence and in turn rendered into a sequence or sized fragment (Fig. 10).

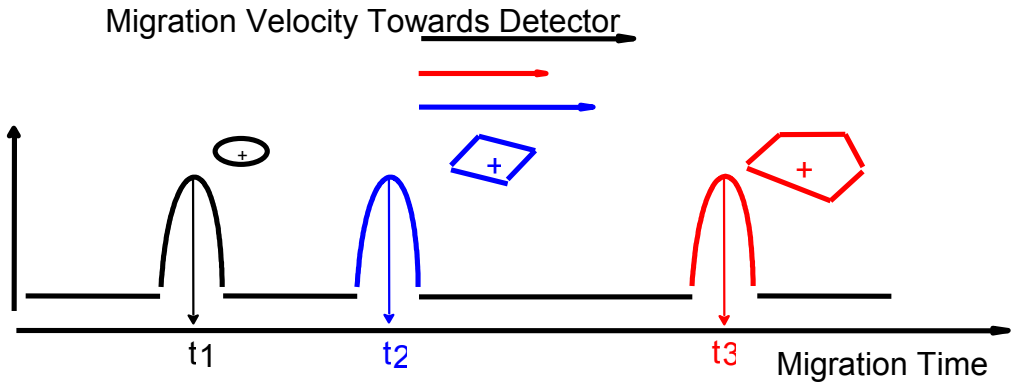

Fig. 10: Scheme of the separation of different sized DNA fragments labeled with different fluorofores by capillary gel electrophoresis.

This technique has been used to detect CYP19 $(TTTA)_n$ repeats, a polymorphism characterized by a variable number of tandem repeats (VNTR) of a TTTA micro-satellite in intron 4. The different number of repeats (from 7 to 13) generates fragments with a different molecular weight (Table 2). Forward PCR primer is 5' labeled with HEX (isomer-free succinimidyl ester of 6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein, excitation and emission maxima of 535 and 556 nm, respectively) fluorofore. A small fraction of PCR product is dissolved in formamide and mixed with a DNA size marker labeled with a different fluorofore (ROX, 5-carboxy-X-rhodamine, succinimidyl ester) (Gene Scan 400HD Size Standard, Applied Biosystems, Foster City, CA) that will be recognized by the instrument software and will allow the fragment size detection.

Table 2: Fragment weight associated with the number of repeats.

REPEATS	MOLECULAR WEIGHT (bp)
7	97
8	101
9	105
10	109
11	113
12	117
13	121

The samples are analyzed in the Genetic Analyzer ABI Prism 3100 instrument (Applied Biosystems). Gene Scan analysis software (Applied Biosystems) allows data extraction and elaboration.

TTGTCTATGAATGTGCCTTTTTTGAAATCATATTTTTAAAATAT***TTTATTTATTTATTTATTTATTTATTTA A(TTTA/TTTA/TTTA/TTTA/TTTA)***TTGAGACAGGCTCTGACTCTATCACCCAG

Fig. 11: CYP19 $(TTTA)_n$ repeats (GenBank N° AY957953). The primer annealing sequences are evidenced in gray. The micro-satellite $(TTTA)_n$ is in brackets underlined bold italic.

3.2.2.3 Pyrosequencing analysis

Pyrosequencing is a relatively recently described method for SNP identification consisting of a real time pyrophosphate detection method[55,56].

This technique is based on indirect bioluminometric assay of the pyrophosphate (Ppi) that is released from each dNTP upon DNA chain elongation. Following Klenow polymerase-mediated base incorporation, Ppi is released and used as substrate, together with adenosine 5'-phosphosulfate, for ATP sulfurylase, which results in the formation of ATP. Subsequently, the ATP accomplishes the conversion of luciferin to its oxi-derivative by the action of luciferase. The ensuing light output becomes proportional to the number of added bases, up to about 4 bases. To allow processivity of the method, dNTP excess is degraded by apyrase, which is also present in the starting reaction mixture, so that only dNTPs are added to the template during sequencing procedure. The process is fully

automated and adapted to a 96-well format, which allows rapid screening of large panel of samples.

Pyrosequencing analysis is performed on PCR- amplified DNA. One of the PCR primers must be biotin-labeled for immobilization to streptavidin coated Sepharose beads. This allows the separation of the two DNA strands produced by PCR, since the assay must be carried out on single stranded DNA. If reverse primer is biotynilated we have the forward assay, otherwise, if the forward primer is biotin labeled, the assay is called reverse (Fig. 12).

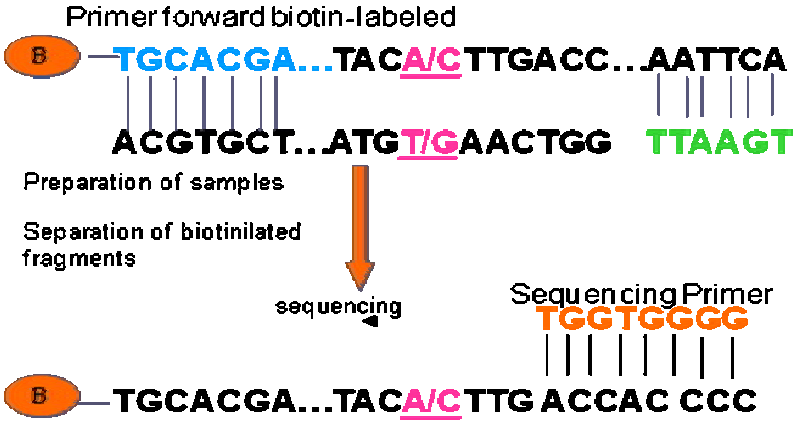

Fig.12: PCR schematic representation of Pyrosequencing method. SNP position is indicated by the slash (/). “B” stands for biotin.

PCR reaction product is mixed with streptavidin coated High Performance Sepharose beads (Amersham Biosciences, Uppsala, Sweden) in the presence of a binding buffer (Tris 10mM, Sodium Chloride 2M, EDTA 1mM and Tween 20 0.1%, pH 7.6). The mixture is allowed to shake for 10 minutes at room temperature. The samples are subsequently transferred to a 96-well filter plate and vacuum (vacuum manifold for 96 well filter plate, Millipore) applied to remove all liquid. Denaturation solution (Sodium Hydroxide 0.2 M) is added to denature double stranded PCR product DNA. After 1 minute incubation vacuum is applied to remove the solution and non immobilized DNA. The beads are washed twice with a washing buffer (Tris 10 mM, pH 7.6) in the presence of the vacuum. The beads with the immobilized template are resuspended by adding 45 µl annealing buffer (Tris 20 mM, Magnesium Acetate Tetra-Hydrate 2 mM, pH 7.6) and sequencing primer (2µM) is added to each sample. Design of sequencing primers for Pyrosequencing follows the same criteria as for the PCR primers, except that the melting temperature of this primer may, if necessary, be lowered. The sequencing primer could thus be shorter than the PCR

primers, typically 15 bp. The position of the primer is flexible within 5 bases from the SNP and can be designed on both the positive (reverse assay) or on the negative (forward assay) strand. Thirty-five µl of this mixture is transferred to a Pyrosequencing 96 wells plate (PSQ 96 Plate Low).

The plate is incubated for 5 minutes at 60°C to allow complete sequencing primer annealing on the template DNA. After samples cooling, the plate is transferred on the Pyrosequencing instrument.

The biotin labeled DNA template, annealed to the sequencing primer, is incubated with enzymes (DNA polymerase, ATP sulfurylase, luciferase and apyrase) and the substrates (adenosine 5'phosphosulfate and luciferin).

The first of four dNTPs is added to the reaction. DNA polymerase catalyzes the incorporation of the dNTP into the DNA strand, if it is complementary to the base in the template strand. Each incorporation event is accompanied by release of Ppi in a quantity equimolar to the amount of incorporated nucleotide (Fig. 13a). ATP sulfurylase converts PPi to ATP in the presence of adenosine 5'phosphosulfate. This ATP drives the luciferase mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by a charge coupled device (CCD) camera and seen as peak in a pyrogram. The height of each peak (light signal) is proportional to the number of nucleotides incorporated (Fig. 13b).

Apyrase, a nucleotide degrading enzyme, continuously degrades ATP and unincorporated dNTPs (Fig. 13c). This switches off the light and regenerates the reaction solution. The next dNTP is then added. Addition of dNTPs is performed one at a time. It should be noted that deoxyadenosine alfa-thio triphosphate is used as a substitute for the natural dATP since it is efficiently used by the DNA polymerase, but not recognized by the luciferase. As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined from the signal peaks in the pyrogram (Fig. 13d).

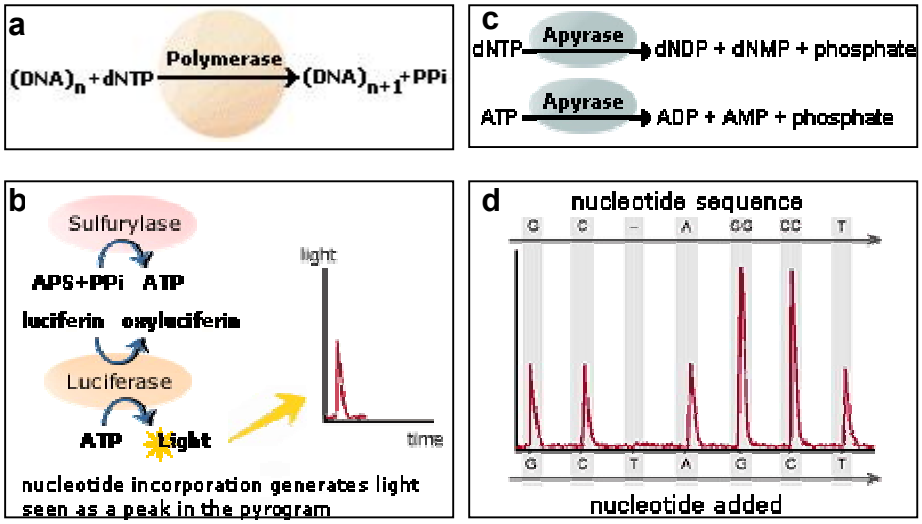

Fig.13: Scheme of the successive steps leading from sequence primer elongation to the analysis of the nucleotide sequence by Pyrosequencing technology.

Table 3: Sequences of sequencing primers and kind of Pyrosequencing test employed.

SNP		Sequencing primer	Kind of test	Sequence to be analyzed
CYP2B6	G516T	5'- CCC CAC CTT CCT CCT -3'	forward	CCA**G/T**TCCATT
CYP2C9	C430T	5'- GGG AAG AGG AGC ATT GAG GAC -3'	forward	**C/T**GTGTTCAA
	A1075C	5'- TGG TGG GGA GAA GGT C -3'	reverse	AA**T/G**GTATCTCTGGACC
CYP2C19	G681A	5'- TTA AGT AAT TTG TTA TGG GT -3'	reverse	TCC**C/T**GGGAAATAA
CYP3A4	A-392G	5'- CCA TAG AGA CAA GGG CA- 3'	forward	**A/G**GAGAGAGG
CYP3A5	G6986A	5'- AGA GCT CTT TTG TCT TTC A- 3'	forward	**G/A**TATCTCTTCC
CYP19	C47T	5'- CTT CTG TGG AAA TCC TG -3'	reverse	C**A/G**TCTTTTTTC

The figures reported below (Fig.s 14-20), represent Pyrosequencing assays for each polymorphism analyzed. PCR primers annealing regions are in gray, sequencing primers annealing region are bold italic, the SNP sites are underlined red bold italic.

CCTCATGGA***CCCCACCTTCCTCTT***CCA**G/T**TCCATTACCGCCAACATCATCTGCTCCATCGT-*BIOTIN

Fig.14: CYP2B6 G516T (GenBank N° AC023172).

GTATTTTGGCCTGAAACCCATAGTGGTGCTGCATGGATATGAAGCAGTGAAGGAAGCCCTGATTGATC
TTGGAGAGGAGTTTTCTGGAAGAGGCATTTTCCCACTGGCTGAAAGAGCTAACAGAGGATTTGGTAGG
TGTGCATGTGCCTGTTTCAGCATCTGTCTTGGGGATGGGGAGGATGGAAAACAGAGACTTACAGAGCT
CCTCGGGCAGAGCTTGGCCCATCCACATGGCTGCCCAGTGTCAGCTTCCTCTTTCTTGCCTGGGATCT
CCCTCCTAGTTTCGTTTCTCTTCCTGTTAGGAATTGTTTTCAGCAATGGAAAGAAATGGAAGGAGATCC
GGCGTTTCTCCCTCATGACGCTGCGGAATTTTGGGATG***GGGAAGAGGAGCATTGAGGAC*****C/T**GTGTT
CAAGAGGAAGCCCGCTGCCTTGTGGAGGAGTTGAGAAAAACCAAGGGTG-*BIOTIN

Fig.15: CYP2C9 C430T (GenBank N° AY341248).

*BIOTINTGCACGAGGTCCAGAGATCTT***GACCTTCTCCCCACCA**GCCTGCCCCATGCAGTGACCTGTGA
CATTAAATTCAGAAACTATCTCATTCCCAAGGTAAGTTTGTTTCTCCTACACTGCAACTCCATGTTTTCG
AAGTCCCCAAATTCATAGTATC

Fig.16: CYP2C9 A1075C (GenBank N° AY341248).

*BIOTINCAGAGCTTGGCATATTGTATCTATACCTTTATTAAATGCTTTTAATTTAATAAATTATTGTTTTCT
CTTAGATATGCAATAATTTTCCCACTATCATTGATTATTTCCC**A/G**GGA***ACCCATAACAAATTACTTAA***A
AACCTTGCTTTTATGGAAAGTGATATTTTGGAGAAAGTAAAAGAACACCAAGAATCGATGGACATCAAC
AACCCTCGGGACTTTATTGATTGCTTCCTGATCAAAATGGAGAAGGTAAAATGTTAACAAAAGCTTAGT
TATGTGACTGCTTGCGTATTTGTGATTCATTGACTAGTTTTGTGTTTACTAC

Fig.17: CYP2C19 G681A (GenBank N° AL133513).

ATCTGTAGGTGTGGCTTGTTGGGATGAATTTCAAGTATTTTGGAATGAGGACAG***CCATAGAGACAAGG***
GCA**A/G**GAGAGAGGCGATTTAATAGATTTTATGCCAATGGCTCCACTTGAGTTTCTGATAAGAACCCAG
AACCC-*BIOTIN

Fig.18: CYP3A4 A-392G (GenBank N° AF280107).

ACCACCCAGCTTAACGAATGCTCTACTGTCATTTCTAACCATAATCTCTTTAA***AGAGCTCTTTTGTCTTT***
CA**G/A**TATCTCTTCCCTGTTTGGACCACATTACCCTTCATCATATGAAGCCTTGGGTGGCTCCTGTGTG
AGACTCTTGCTGTGTGTC-*BIOTIN

Fig.19: CYP3A5 G6986A (GenBank N° AF280107).

*BIOTINTTGAAAGATGCCATAGAAGTTCTGATAGCAGAAAAAAGAC/TG***CAGGATTTCCACAGAAG**AGA
AACTGGAAGAATGTATGGACTTTGCCACTGAGTT

Fig.20: CYP19 C47T (GenBank N° NM000103).

3.2.2.4 Taqman assay

The allelic discrimination assay is a multiplexed (more than one primer/probe pair per reaction) end-point assay that detects variants of a single nucleic acid sequence. The presence of two primer/probe pairs in each reaction allows genotyping of the two possible variants at the single nucleotide polymorphism (SNP) site in a target template sequence. The actual quantity of target sequence is not determined. For each sample in an allelic discrimination assay, a unique pair of TaqMan® Reporter Dye (VIC and FAM) probes that target an SNP site is used. One fluorescent dye detector is a perfect match to the wild type (allele 1) and the other fluorescent dye detector is a perfect match to the mutation (allele 2). The allelic discrimination assay classifies unknown samples as: homozygotes (samples having only allele 1 or allele 2) and heterozygotes (samples having both allele 1 and allele 2). The allelic discrimination assay measures the change in fluorescence of the dyes associated with the probes.

TaqMan probe-based chemistry uses a fluorogenic probe to detect specific PCR product as it accumulates during PCR cycles. In Fig. 21 are reported a schematic representation of a TaqMan Assay. During the denaturation step, the reporter (R) and the quencher (Q) are attached to the 5' and 3' ends of a TaqMan probe. When both dyes are attached to the probe, reporter dye emission is quenched. During each extension cycle, the hot-start DNA polymerase system cleaves the reporter dye from the probe. After being separated from the quencher, the reporter dye emits its characteristic fluorescence.

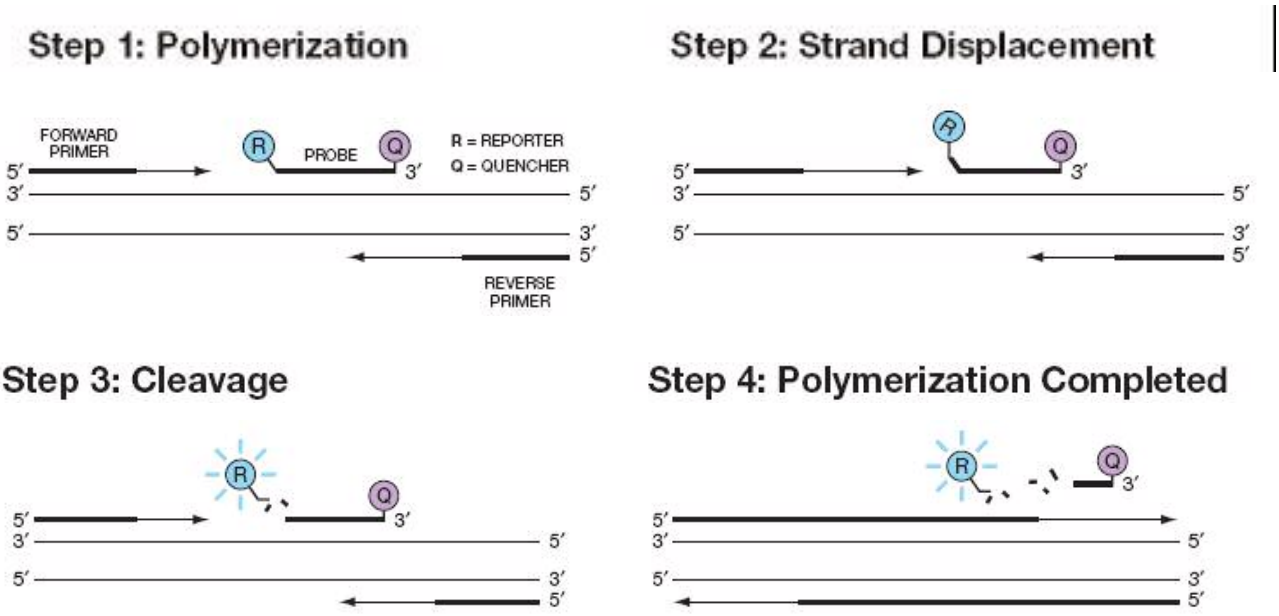

Fig. 21: Schematic representation of TaqMan® technology.

The samples are analyzed using the Applied Biosystem 7900 HT Fast Real-Time PCR System instrument. The allelic discrimination was performed with the SDS software 2.3 (Applied Biosystems). In Table 4 are reported the sequence analyzed for each polymorphisms and their coupled Reporter Dye.

Table 4: PCR sequences and Reporter Dyes.

SNP		Sequences	Reporter Dye (VIC)	Reporter Dye (FAM)
CYP19	G410T	CTAGGTGCTATT**[G/T]**GTCATCTGCTCC	Allele T	Allele G
	C1558T	AATGCTCCAGAGT**[A/G]**GGTACTGACCA	Allele T	Allele C
CYP17	T27C	TCTACTCCAC**[C/T]**GCTGTCTATCTTGC	Allele T	Allele C

3.2.2.5 Linkage Disequilibrium

In population genetics, linkage disequilibrium (LD) is the non-random association of alleles at two or more loci, not necessarily on the same chromosome. Linkage disequilibrium describes a situation in which some combinations of alleles or genetic markers occur more or less frequently in a population than would be expected from a random formation of haplotypes from alleles based on their frequencies. Non-random associations between polymorphisms at different loci are measured by the degree of linkage disequilibrium.

The level of linkage disequilibrium is influenced by a number of factors including genetic linkage, the rate of recombination, the rate of mutation, random drift, non-random mating, and population structure.

The deviation of the observed frequency of a haplotype from the expected is a quantity commonly denoted by a capital D': its value could be included between 0 and 1 where 0 indicates a linkage equilibrium and 1 indicates a linkage disequilibrium. Another measure of LD which is an alternative to D' is the correlation coefficient between pairs of loci, denoted as r^2. This is also adjusted to the loci having different allele frequencies.

A perfect LD was defined when the allele frequencies are identical and thus only two haplotype could exist (D'=1, r^2=1); a complete LD was present when the allele frequencies are not indentical and thus almost three haplotype exist (D'=1, r^2<1)[57].
The definition of linkage disequilibrium among the polymoprhisms studied could be useful to define the haplotypes (the combination of several SNPs on a single chromosomes) that could be more informative than the single polymorphisms. In this work of thesis, analyses of linkage disequilibrium among SNPs were performed.

3.3 Response and Toxicity evaluation

3.3.1 Cyclophosphamide treatment

Overall survival was calculated from the time of diagnosis until death or last follow-up.
Toxicity data were registered after the first cycle of chemotherapy and after the entire course of the treatment; the classification was in according to NCI-CTC scale version 2.0. The toxicities were grouped in hematological, hepatic and non-hematological (besides the hepatic toxicity) for the correlations with the genetic data.

3.3.2 Exemestane treatment

The enrollment of the patients is still in progress. However, the response to chemotherapy treatment will be evaluated for target and no-target lesions according to RECIST criteria. Patients will be assessed for tumor response every 8 weeks for at least 24 weeks.
Safety will be assessed every 8 weeks for at least 24 weeks, through physical evaluation, laboratory tests, and ECG according to NCI-CTC version 2.0.

3.4 Statistical methods

The distribution of individual characteristics was evaluated by simple descriptive statistics and variable frequency, between the groups, was compared using Fisher's exact tests.
To assess the relative excess risk of toxicity between patients with different polymorphic status and to control for confounding factors, unconditional logistic regression models (including all available prognostic factors: age, histological type of tumor, grading, prior

chemotherapy) were fitted by computing odds ratios (OR) and the corresponding 95% confidence intervals (95% CI).

Categorical data were associated with the pharmacodynamic and clinico-pathological features of the patients, using the two tailed Fisher's exact test or the Chi-squared test.

Overall survival was estimated using the Kaplan and Meier product limit method. Differences were tested using through the log-rank test. To assess the relative excess risk of death in patients with different genotypes and to control for confounding factors, proportional hazards models (including age, histologic type of tumor) were fitted computing hazard ratios (HRs) and the corresponding 95% CIs. The proportional assumption was examined with log-log survival plots or by adding time-dependent interaction terms to the model.

4. RESULTS

4.1 Patient's characteristics and treatment

For patients treated with conventional chemotherapy cyclophosphamide-based, the study was retrospective, thus the enrollment of patients was concluded on December 2005. This group comprises 195 Caucasian patients from CRO- National Cancer Institute of Aviano. Patient's characteristics before treatment are reported in Table 5. The median age of patients was 53 years (range 25 to 75 years). All these patients received an adjuvant tretatment with cyclophosphamide after surgical treatment and were all checked on a regular follow-up basis (median 100 months; range 1 to 160) from the time of diagnosis until last follow-up or death.

For the group of patients treated with exemestane the study is prospective and the enrollment is still in progress. It is not possible, at the moment, to define their characteristics.

Two hundred and fifty healthy Caucasian control women were randomly selected from a pool of healthy volunteers (age range: 18-60 years), who had signed the informed consent to use their biologic material for genetic analysis. This group of healthy controls, matched for age with the cases, was used for the definition of the Relative Risk to develop breast cancer.

Table 5: Patient's characteristics.

Patient's characteristics		N (%)
Age at diagnosis	Median (range)	53 (25 – 75 yrs)
Chemotherapy schedule	CMF (6 cycles) + RT	85 (43.6%)
	CMF (6 cycles)	31 (15.9%)
	EC (4 cycles) + CMF (3 cycles)	79 (40.5%)
Stage	I	12 (6.1%)
	II	68 (34.9%)
	III	64 (32.8%)
	IV	51 (26.2%)
Grading	1	7 (3.6%)
	2	56 (28.7%)
	3	131 (67.2%)
	4	1 (0.5%)
Histologic type	Invasive ductal	158 (81.0%)
	Invasive lobular	25 (12.8%)
	Medullary	6 (3.1%)
	Other	6 (3.1%)
Prior chemotherapy	Yes	89 (45.6%)
	No	86 (44.1%)
	Not available	20 (10.2%)
Prior chemotherapy regimen	CMF	10 (1.2%)
	EC	79 (88.8%)

RT = radiotherapy

EC = epirubicin + cyclophosphamide

4.2 Patients genotyping and Relative Risk of breast cancer

4.2.1 Polymorphisms analyzed by RFLP

- ✓ CYP2B6 A785G

This polymorphism was tested on 195 breast cancer patients. Enzymatic digestion of the amplified product allowed to distinguish among patients who were wild type, heterozygous and homozygous for the polymorphism considered. In Fig. 22 it is shown how the digestion products appeared at the UV lighter after separation on a 3% agarose gel.

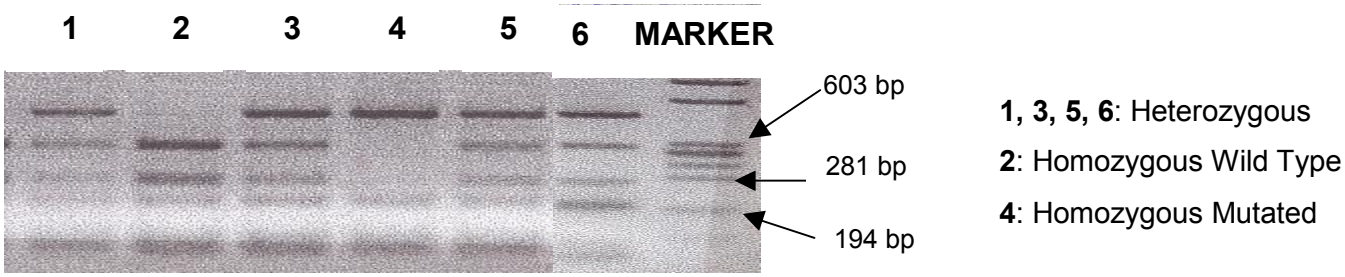

Fig. 22: Example of RFLP analysis with a gel electrophoresis after digestion with StyI.

- ✓ CYP2B6 C1459T

After enzymatic digestion, the fragments produced were visualized at the UV lighter after separation on a 3% agarose gel. The polymorphism was tested on 195 breast cancer patients. In Fig. 23 it is shown how the digestion products appeared for wild type, heterozygous and homozygous mutated patients.

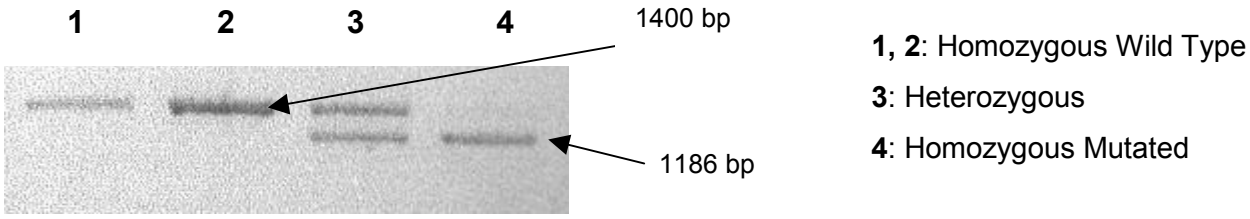

Fig. 23: Example of RFLP analysis with a gel electrophoresis after digestion with BglII.

The frequencies of both these polymorphisms in the Caucasian population at the homozygous status were: 22 out of 195 patients (11.3%) and of 8 out of 195 (4.1%) for the A785G and the C1459T, respectively. The heterozygous status was found in 77 out of 195 (39.5%), and in 30 out of 195 (15.4%), respectively. Ninty-six patients out of 195 (49.2%), resulted wild type for A785G polymorphism and 157 out of 195 (80.5%) for C1459T. The comparison with the frequencies of a 250 healthy Caucasian volunteeres was reported in Table 6. No significant associations were observed.

Table 6: Distribution of CYP2B6 A785G and CYP2B6 C1459T polymorphisms in controls and breast cancer patients.

SNP			Breast cancer N. (%)	Controls N. (%)	OR (95% CI)	p-value *
CYP2B6	A785G	AA	96 (49.2%)	121 (48.4%)	1#	
		AG	77 (39.5%)	113 (45.2%)	0.86 (0.58-1.27)	0.48
		GG	22 (11.3%)	16 (6.4%)	1.73 (0.86-3.48)	0.16
	C1459T	CC	157 (80.5%)	196 (78.4%)	1#	
		CT	30 (15.4%)	39 (15.6%)	0.96 (0.57-1.61)	0.89
		TT	8 (4.1%)	15 (6%)	0.66 (0.27-1.61)	0.39

OR, odds ratios; CI, confidence intervals

reference category

* Fisher's exact test

4.2.2 Polymorphisms analyzed by automated Fragment Analysis

- ✓ CYP19 - $(TTTA)_n$ repeats

The microsatellite was analyzed by automated fragment analysis based on capillary gel electrophoresis comparing the amplified product weight with an internal standard added to each sample. In Fig. 24 an example of the electropherogram corresponding to the most frequent number of repeats (7/7) is displayed; the associations between molecular weight and number of repeats are reported in “Materials and Methods” section (Table 2, pag. 36).

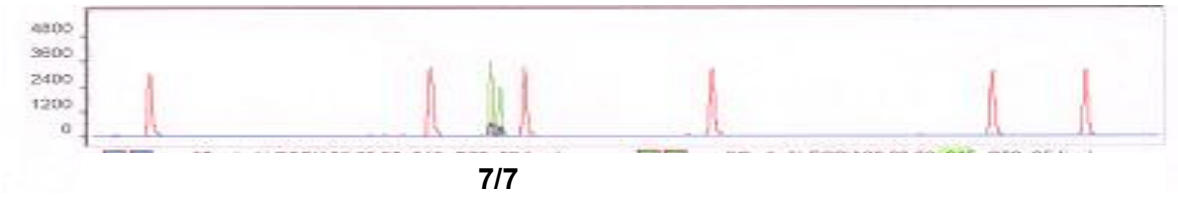

Fig.24: Example of electropherogram of CYP19 – $(TTTA)_{7/7}$ repeats.

The frequencies of the allelic repeats of the tetranucleotide TTTA are reported in Table 7; no significant differences have been observed among cases and controls.

Table 7: Allelic distribution of CYP19 – $(TTTA)_n$ repeats.

SNP		Breast cancer N. (%)	Controls N. (%)	OR (95% CI)	p-value *
CYP19 - repeats	**7**	130 (53.7%)	276 (55.2%)	1#	
	8	21 (8.7%)	42 (8.4%)	1.06 (0.60-1.87)	0.88
	9	1 (0.4%)	2 (0.4%)	1.06 (0.09-11.82)	1.00
	10	6 (2.5%)	5 (1.0%)	2.55 (0.76-8.50)	0.19
	11	81 (33.5%)	167 (33.4%)	1.03 (0.73-1.44)	0.86
	12	3 (1.2%)	7 (1.4%)	0.91 (0.23-3.58)	1.00
	13	0 (0%)	1 (0.2%)	0.71 (0.03-17.47)	1.00

OR, odds ratios; CI, confidence intervals

reference category

* Fisher’s exact test

As reported in literature, a different impact in breast cancer development could be related to the presence of long (> 7 repeats) or short (≤ 7 repeats) allele. However, comparing the two group, no significant association where found in our cases (Table 8).

Table 8: Allelic distribution of long and short CYP19 – (TTTA) allele.

SNP		Breast cancer N. (%)	Controls N. (%)	OR (95% CI)	p-value *
CYP19 - repeats	≤ 7	130 (53.7%)	276 (55.2%)	1#	
	> 7	112 (46.3%)	224 (44.8%)	1.06 (0.78-1.44)	0.75

OR, odds ratios; CI, confidence intervals

reference category

* Fisher's exact test

4.2.3 Polymorphisms analyzed by Pyrosequencing

- ✓ CYP2B6 G516T

This polymorphism was analyzed by Pyrosequencing method. In the Attachment 1, the "pyrograms" corresponding to wild type, heterozygous and homozygous variant genotype are reported. The frequencies of genotypes in breast cancer patients and in controls group were reported in Table 9.

- ✓ CYP2C9 C430T and A1075C

Both these polymorphisms were genotypized using Pyrosequencing technology and their relative frequencies were reported in Table 9. These polymorphisms appeared to be quite rare in the Caucasian population at the homozygous status with a frequency of 2 out of 195 patients (1%) for both CYP2C9 variants. An example of "pyrograms" for wild type, heterozygous and homozygous variant subjects was reported in Attachment 2 and 3.

- ✓ CYP2C19 G681A

The G681A variant of CYP2C19 gene was analyzed with Pyrosequencing; the frequency of wild type, heterozygous and homozygous variant was reported in Table 9. In Attachment 4 an example of "pyrograms" characteristic for this polymorphism was reported.

- ✓ CYP3A4 A392G and CYP3A5 G6986A

CYP3A4*1B and CYP3A5*3 polymorphisms were analyzed by Pyrosequencing technology. The CYP3A5 polymorphism analysis required a particular modulation of the PCR conditions since the polymorphic site was situated in a quite homologous region of the gene.

To selectively amplify only the locus corresponding to CYP3A5*3 polymorphism, the annealing temperature was increased and DMSO was added to the reaction mix. Actually, the PCR primers used were partially homologous to the other CYP3A genes isoforms, whereas they were 100% homologous to the gene isoform of interest. PCR conditions were verified directly during Pyrosequencing analysis, observing a greater specificity by increasing the annealing temperature and the DMSO percentage (Attachment 5). The PCR conditions selected are reported in the "Materials and methods" section (Table 2, pag. 29). In the Attachments 6 and 7, the "pyrograms" corresponding to wild type and heterozygous CYP3A4 and CYP3A5 genotypes, respectively, are represented.

The polymorphisms considered for the CYP3A4 and CYP3A5 genes were quite rare. No homozygous variant was found in the population study and only few heterozygous patients were detected (Table 9).

- ✓ CYP19 C47T

For the group of patients treated with exemestane only the CYP19 C47T polymorphism was analyzed by Pyrosequencing. The relative frequencies of genotypes were reported in Table 9. A representation of “pyrograms” for this variant was available in the Attachment 8.

None of these polymorphisms have highlighted a significant association with the relative risk of developing breast cancer (indicated by the Odd Ratio value).

Table 9: Distribution of CYP2B6, CYP2C9, CYP2C19 and CYP19 polymorphisms in controls and breast cancer patients.

SNP			Breast cancer N. (%)	Controls N. (%)	OR (95% CI)	p-value *
CYP2B6	G516T	GG	98 (50.2%)	133 (53.2%)	1#	
		GT	82 (42.1%)	104 (41.6%)	1.07 (0.72-1.58)	0.76
		TT	15 (7.7%)	13 (5.2%)	1.57 (0.71-3.44)	0.31
CYP2C9	C430T	CC	148 (75.9%)	182 (72.8%)	1#	
		CT	45 (23.1%)	63 (25.2%)	0.88 (0.56-1.36)	0.58
		TT	2 (1.0%)	5 (2%)	0.49 (0.09-2.57)	0.47
	C1075T	CC	166 (85.1%)	201 (80.4%)	1#	
		CT	27 (13.8)	47 (18.8%)	0.69 (0.41-1.16)	0.20
		TT	2 (1.1%)	2 (0.8%)	1.21 (0.17-8.69)	1.00
CYP2C19	G681A	GG	145 (74.4%)	176 (70.4%)	1#	
		GA	43 (22.0%)	67 (26.8%)	0.78 (0.50-1.21)	0.32
		AA	7 (3.6%)	7 (2.8%)	1.21 (0.42-3.54)	0.79
CYP3A4	A-392G	AA	183 (93.8%)	236 (94.4%)	1#	
		AG	12 (6.2%)	13 (5.2%)	1.19 (0.53-2.67)	0.68
		GG	0 (0%)	0 (0%)	-	-
CYP3A5	G6986A	GG	172 (88.2%)	223 (89.2%)	1#	
		GA	23 (11.8%)	27 (10.8%)	1.10 (0.61-1.99)	0.76
		AA	0 (0%)	0 (0%)	-	-
CYP19	C47T	CC	115 (95%)	232 (92.8%)		
		CT	6 (5%)	18 (7.2%)	0.67 (0.26-1.74)	0.50
		TT	0 (0%)	0 (0%)	-	-

OR, odds ratios; CI, confidence intervals # reference category * Fisher's exact test

4.2.4 Polymorphisms analyzed by Taqman Assay

- ✓ CYP19 G410T and C1558T

These two polymorphisms were analyzed using a TaqMan Validated Assay. With the high specificity of this technology it is possible to obtain an allelic discrimination in a very low volume of reaction. In attachments 9 and 10 an example of the results after an allelic discrimination assay for these two polymorphisms was reported: in abscissa the fluorescence of one Reporter Dye was reported, whereas the fluorescence of the other Reporter Dye was in ordinate. There are three distinct groups: samples that had only one fluorescence (homozygotes for only one allele; blue or red signals) and samples that had both the fluorescence (heterozygotes; green signals).

In Table 10 the genotype frequencies in cases and in controls were compared; no significant association were found between CYP19 polymorphisms and the development of breast cancer.

- ✓ CYP17 T27C

The isoform CYP17 was genotypized with a TaqMan Validated Assay and the results were indicated in Table 10. No significant difference was found between genotype frequencies among cases and controls. An example of allelic discrimination of CYP17 was reported in Attachment 11.

Table 10: Distribution of CYP19 and CYP17 polymorphisms in controls and breast cancer patients.

SNP			Breast cancer N. (%)	Controls N. (%)	OR (95% CI)	p-value *
CYP19	G410T	GG	58 (48%)	130 (52%)	1#	
		TG	50 (41.3%)	103 (41.2%)	1.09 (0.69-1.72)	0.73
		TT	13 (10.7%)	17 (6.8%)	1.71 (0.78-3.76)	0.21
	C1558T	CC	31 (26.6%)	62 (24.8%)	1#	
		CT	45 (37.2%)	132 (52.8%)	0.68 (0.39-1.18)	0.20
		TT	45 (37.2%)	56 (22.4%)	1.61 (0.90-2.88)	0.14
CYP17	T27C	TT	43 (36%)	82 (32.8%)	1#	
		TC	59 (50%)	127 (50.8%)	0.88 (0.55-1.43)	0.62
		CC	17 (14%)	41 (16.4%)	0.79 (0.40-1.55)	0.61

OR, odds ratios; CI, confidence intervals

reference category * Fisher's exact test

4.2.5 Linkage Disequilibrium

Among the polymorphisms of the CYP2B6 isoform evaluated in this study, there was a complete LD between G516T and A785G (Fig. 25): these variants in the homozygous status were always found linked together. As reported in literature, the contemporaneous presence of these two variants characterizes the alleles CYP2B6*6 and CYP2B6*7.

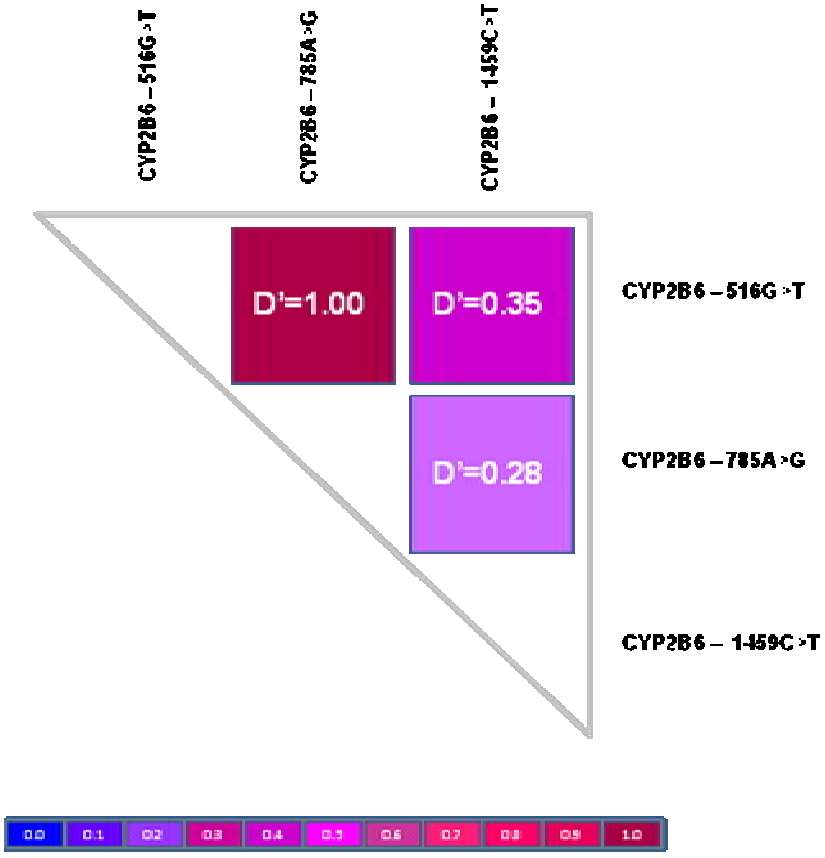

Fig. 25: Linkage Disequilibrium analysis for CYP2B6.

The combination of these three polymorphisms generates four different alleles: CYP2B6*4, CYP2B6*5, CYP2B6*6 and CYP2B6*7 (Fig. 3, pag. 16): their frequencies are reported in Table 11. The *1 represents the wild type allele, the *4 (or *5, *6, *7) represents the variant allele. None of these alleles had shown a significant correlation with the development of breast cancer, indicating that, in this case, the haplotype analysis is no more indicative of the analysis of the single genotypes.

Table 11: Distribution of CYP2B6 alleles in controls and breast cancer patients.

SNP			Breast cancer N. (%)	Controls N. (%)	OR (95% CI)	p-value *
CYP2B6	*4	*1/*1	182 (97.8%)	238 (95.2%)	1#	
		*1/*4	4 (2.2%)	12 (4.8%)	0.44 (0.14-1.37)	0.20
		*4/*4	0 (0%)	0 (0%)	-	-
	*5	*1/*1	155 (86.1%)	204 (81.6%)	1#	
		*1/*5	25 (13.9%)	41 (16.4%)	0.80 (0.47-1.38)	0.80
		*5/*5	0 (0%)	5 (2.0%)	0.12 (0.01-2.18)	0.07
	*6	*1/*1	102 (54.8%)	138 (55.2%)	1#	
		*1/*6	69 (37.1%)	100 (40.0%)	0.93 (0.63-1.39)	0.76
		*6/*6	15 (8.1%)	12 (4.8%)	1.69 (0.76-3.77)	0.22
	*7	*1/*1	174 (96.6%)	235 (94.0%)	1#	
		*1/*7	6 (3.4%)	15 (6.0%)	0.54 (0.20-1.42)	0.26
		*7/*7	0 (0%)	0 (0%)	-	-

OR, odds ratios; CI, confidence intervals

reference category

* Fisher's exact test

Between the two SNPs analysed for the CYP2C9 isoform (C430T and A1075C) there was no linkage disequilibrium (D'=0.37) as reported in Figure 26.

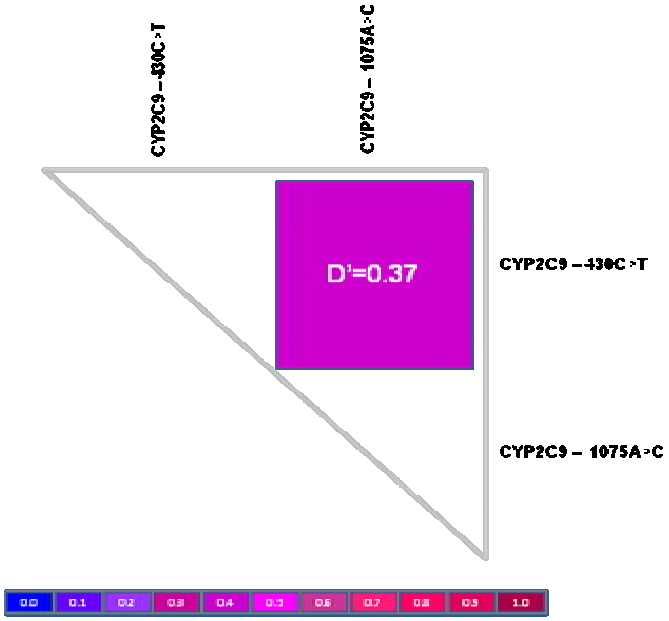

Fig. 26: Linkage Disequilibrium analysis for CYP2C9.

About the polymorphisms considered for CYP19, a complete LD (D'=1) was found among CYP19 – C47T and all the other variants analysed: CYP19 G410A, CYP19 C1558T and $(TTTA)_{>7}$ repeats (Fig. 27). However, a strong linkage disequilibrium was also found among the others genetic variants (D'=0.9).

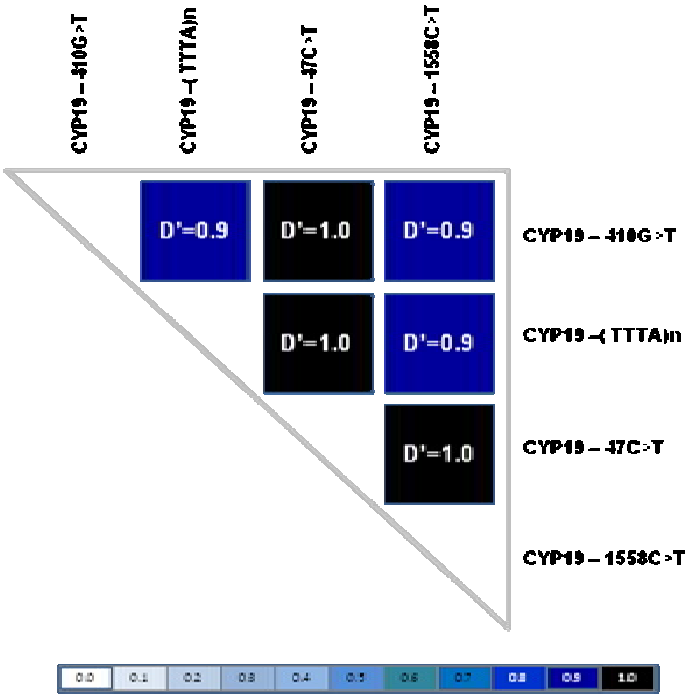

Fig.27: Linkage Disequilibrium analysis for CYP19.

The combination of these polymorphisms generates different haplotypes. The three most frequent haplotypes observed both in cases and in controls were reported in Table 12.
No significant association between frequencies and breast cancer risk was found compared to the three most frequent CYP19 haplotypes.

Table 12: Distribution of CYP19 haplotypes in controls and breast cancer patients.

HAPLOTYPE CYP19				Breast cancer N. (%)	Controls N. (%)	OR (95% CI)	p-value*
G410T	(TTTA)n	C47T	C1558T				
G	>7	C	T	89 (47.4%)	97 (46.6%)	1#	
C	≤7	C	C	38 (20.2%)	55 (26.5%)	0.75 (0.45-1.25)	0.31
A	≤7	C	C	61 (32.4%)	56 (26.9%)	1.19 (0.75-1.89)	0.48

OR, odds ratios; CI, confidence intervals

reference category

* Fisher's exact test

4.3 Treatment tolerance and response

In order to make toxicity evaluations homogeneous and more consistent and relevant from a clinical perspective, the tolerance to treatment was evaluated both during the 1st cycle of chemotherapy (acute toxicity) and over the entire course of chemotherapy (cumulative toxicity). Toxicity related to the 1st cycle and over the entire course of treatment was evaluated in all the 195 patients that entered the study.

Generally, patients well tolerated the treatment, with severe toxicity (G3-G4) only in 15 out of the 195 patients (7.7%) during the 1st cycle, and in 30 out of the 195 patients (15.4%) during the entire course of chemotherapy.

The most frequent severe hematological adverse effect was neutropenia, experienced by 8 patients (4.1%) during the 1st cycle (7 patients exhibited G3 and 1 patient had G4 neutropenia). During the entire course of the treatment, G3-G4 neutropenia was observed in 26 out of the 195 (13.3%) patients, 23 of which exhibited G3 and 3 others exhibited G4. Grade 3 leucopenia was observed in eight patients during the entire course of chemotherapy, whereas G3-G4 thrombocytopenia was never observed.

The severe (G3-G4) non-hematological toxicity were observed in 7 patients (3.6%) after the first cycle and in 4 patients (2.1%) after the entire course of chemotherapy. The predominant non-hematological adverse effects were hepatic toxicity. The G3-G4 hepatic side effects were only observed in 4 (2%) patients both after the first cycle and the end of treatment.

At the end of the study, all the 195 patients were evaluable for the Overall Survival (OS) analysis (median 101 months, range 4-160 months).

4.3.1 CYP2B6 polymorphisms and toxicity

The study evidenced no significant relationship between the three CYP2B6 polymorphisms analyzed and toxicity development during the 1st cycle and cumulatively over the entire course of treatment.

In particular, only one patient carrying the variant G516T had experienced severe hematological toxicity both after the 1st cycle and after the entire course of treatment. Differently, G3-G4 hematological toxicity at the end of treatment was observed in 3 patients homozygous for the variant CYP2B6 A785G. Not even the C1459T variant was associated with a development of severe toxicity: none of the patients carrying the genetic

variant exhibited hematological toxicity. Severe cumulative hepatic toxicity was observed only for one patient with 1459TT genotype. Not even considering haplotypes significative correlations have emerged (data not shown).

4.3.2 CYP2C9 polymorphisms and toxicity

Conversely to what observed for CYP2B6, acute toxicity and cumulative toxicity were significantly associated with both CYP2C9 polymorphisms analyzed. In particular, the variant C430T was found correlated with the severe acute hepatic toxicity as reported in Table 13. Moreover, patients carrying at least one mutated allele had a 3.62-fold increased risk (p=0.001) to develop hepatic toxicity (grade 1-3) compared to wild type patients.
The polymorphism CYP2C9 A1075C had also highlighted a significant correlation with the hepatic toxicity at the end of therapy as indicated in Table 13. Conversely, this significant association was not confirmed when comparing grade 1-3 versus grade 0.

4.3.3 CYP2C19 polymorphism and toxicity

The polymorphism was found significantly associated with the development of severe hematological toxicity (G3-G4) at the end of entire course of therapy. As reported in Table 13, patients carrying at least one mutated allele (681GA+681AA) had an increased risk of 3.05-fold for toxicity compared to wild type patients. In particular, severe hematological toxicity was observed in 1 mutated patient and in 11 heterozygous patients.
The analyses regarding the other types of toxicities were inconsistent to define a significant correlation with genotype.

4.3.4 CYP3A polymorphisms and toxicity

For what concerns the polymorphisms of CYP3A isoform, only the CYP3A5 G6986A was correlated with the development of non-hematologic toxicity at the end of the 1st cycle. Conversely, this significant association was not confirmed when comparing grade 1-3 of hepatic toxicity versus grade 0 (Table 13).
For CYP3A4 polymorphism no significant association was observed among toxicities and genotypes: none of heterozygous patients experienced severe toxicity (G3-G4).

Table 13: Association between CYP polymorphisms and toxicity (hematological, hepatic and non-hematological) according to NCI-CTC classification.

SNP			GRADE					p-value *	OR (95% CI)	p-value	TYPE OF TOXICITY
			0	1	2	3	4				
CYP2C9	C430T	CC	103 (79.3%)	18 (13.8%)	7 (5.4%)	2 (1.5%)	0 (0.0%)	0.016	1#	0.001**	HEPATIC TOXICITY 1ST CYCLE
		CT	20 (52.6%)	13 (34.2%)	3 (7.9%)	2 (5.3%)	0 (0.0%)		3.624 (1.70-7.73)§		
		TT	0 (0.0%)	1 (100%)	0 (0.0%)	0 (0.0%)	0 (0.0%)				
	A1075C	AA	132 (83.5%)	14 (8.9%)	9 (5.7%)	3 (1.9%)	0 (0.0%)	0.031	1#	0.19**	HEPATIC TOXICITY END OF THERAPY
		AC	20 (74.1%)	6 (22.2%)	0 (0.0%)	1 (3.7%)	0 (0.0%)		1.934 (0.77-4.87)§		
		CC	1 (50%)	0 (0.0%)	1 (50%)	0 (0.0%)	0 (0.0%)				
CYP2C19	G681A	GG	13 (9.1%)	47 (33.1%)	68 (47.9%)	12 (8.5%)	2 (1.4%)	0.22	1#	0.014***	HEMATOLOGICAL TOXICITY END OF THERAPY
		GA	5 (11.4%)	7 (15.9%)	21 (47.7%)	10 (22.7%)	1 (2.3%)		3.05 (1.30-7.17)§§		
		AA	0 (0.0%)	3 (42.9%)	3 (42.9%)	1 (14.2%)	0 (0.0%)				
CYP3A5	G6986A	GG	71 (44.4%)	62 (38.7%)	20 (12.5%)	7 (4.4%)	0 (0.0%)	0.023	1#	0.89**	NON - HEMATOLOGICAL TOXICITY 1ST CYCLE
		GA	9 (40.9%)	5 (22.7%)	8 (36.4%)	0 (0.0%)	0 (0.0%)		1.152 (0.47-2.85)§		

reference category

* p-value calculated with Chi-squared Test (wild type vs heterozygous vs homozygous mutated, all grade of toxicity)

** p-value calculated with Fisher's Exact Test (heterozygous + homozygous mutated vs wild type, grade 0 vs grade 1-3)

*** p-value calculated with Fisher's Exact Test (heterozygous + homozygous mutated vs wild type, grade 0-2 vs grade 3-4)

§ Odds Ratio (OR) and Confidence Intervals (CI) calculated comparing heterozygous + homozygous mutated vs wild type, grade 0 vs grade 1-3

§§ Odds Ratio (OR) and Confidence Intervals (CI) calculated comparing heterozygous + homozygous mutated vs wild type, grade 0-2 vs grade 3-4

4.3.5 Overall survival and genotypes

A significant association between Overall Survival and genotypes was found significant only for the isoform CYP2C9 A1075C. Adverse event (death for disease) occurred in 27 patients (13.8%): 6 of these carried at least one mutated allele (Fig. 28). For these patients the Hazard Ratio for death was 1.5-fold increased compared to wild type (Table 14).
On the contrary, no significant associations between the other polymorphisms of CYP2B6, CYP2C9 and CYP2C19 and Overall Survival were observed.

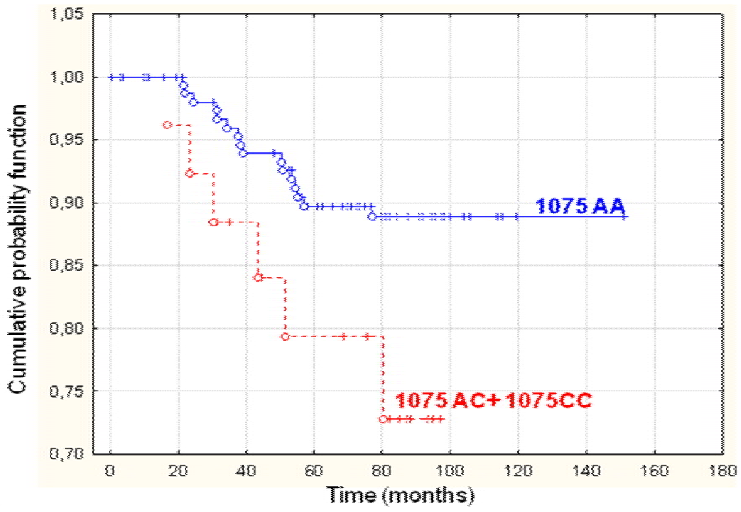

Fig. 28: Overall Survival according with the genotype of CYP2C9 A1075C.

Table 14: Hazard Ratio (HR) and corresponding 95% confidence intervals (95% CI) for overall survival according to CYP2C9 A1075C.

SNP			Alive/Dead	Median (range)	HR (95% CI)	p-value *
CYP2C9	A1075C	AA	144/21	105 (4-160)	1#	
		AC	22/6	93 (17-120)	1.52 (0.86-1.97)	0.04
		CC	2/0			

HR, Hazard Ratios; CI, confidence intervals

reference category * Fisher's exact test

5. DISCUSSION

Pharmacogenetics could represent a useful and innovative tool for optimization of schedule and dosage of anticancer chemotherapy. The individualization of chemotherapy based on pharmacogenetic informations could discern among subgroups of patients in which the treatment may provide a real benefit limiting side effects. Indeed, it appears to be an easy method of analysis and could prove to be an alternative to the traditional modalities of dosing, such as those based on body surface area and other methods studied in phase I-II trials, which have not always proven satisfactory.

There are several candidate genes with the potential to affect response and toxicity to cyclophosphamide. The metabolism of this drug involves several enzyme systems: phase I metabolic enzymes (CYP) for bioactivation, and phase II metabolic enzymes (GST, ALDH) for inactivating reactions of metabolites (Fig. 2, page 15). At the present, there are differents levels of evidence for utility of pharmacogenetic markers to predict response and toxicity from breast cancer treatment. The potential for pharmacogenetics to influence drug prescription has become a reality with recent technological advances. For breast cancer, several SNP genotyping platforms are now available (such as onco*type* DX™ or Mammaprint®). This combined with knowledge from the Human Genome Project and linkage disequilibrium maps offer increasing possibilities for clinical pharmacogenetics. The most compelling evidence for the use of pharmacogenetics is with CYP2D6 genotype and tamoxifen therapy[58]. Several studies performed in this field have stimulated the FDA to update the label for the breast cancer drug tamoxifen to include information about CYP2D6 genotype testing. These latter results lead to identify new predictive and prognostic molecular markers of breast clinical outcome, especially for the enzymes that are involved in bioactivation of drugs (CYP) mostly used in breast cancer treatment.

In this work of thesis, a gene pathway approach is used to define the genetic variants that could have a significant role in response or in development of toxicity. This study is focused on the bioactivation pathway mediated by several isoforms of CYP. In particular, the bioactivation to 4-hydroxycyclophosphamide is catalyzed primarily by isoforms CYP2B6, CYP2C9 and CYP3A4, whereas CYP2C19 and CYP3A5 make a minor contribution. Several polymorphisms in all these isoforms of CYP have been described.

Moreover, preliminary results for the group of patients treated with exemestane concerning the relative risk to develop breast cancer have been reported.

5.1 CYP polymorphisms and relative risk to develop breast cancer

The polymorphisms analyzed for all the CYP isoforms involved in cyclophosphamide bioactivation or as exemestane target, could be associated with the risk to develop breast cancer. These enzymes are active both in the metabolism of precarcinogens and drug and thus could be related to the development of disease. The comparaison among the frequencies of genotypes in cases and in controls (healthy subjects matched for age and sex) could provide useful information in this field. However, the analyses performed in this study have not underlined a significant association among CYP genotypes and breast cancer risk.

5.2 CYP polymorphisms and toxicity

As mentioned above, new trend in the pharmacogenetic research indicates the importance of considering more than a single genetic variant to exhaustively describe the complex pharmacodynamic effect of a drug.

The bioactivation of cyclophosphamide has a pivotal role in the bioavailability of active metabolite of the drug. This important reaction is mediated by several isoforms of CYP with a different contribute. All the isoforms CYP2B6, CYP2C9, CYP2C19, CYP3A4 and CYP3A5 are active in the hepatic tissue. An alteration in the bioactivation of prodrug could lead to an accumulation of cyclophosphamide in the liver, with a possible increase of toxicity. Several polymorphisms in CYP enzymes have been described in literature and their phenotypic profile in terms of alteration of enzyme expression or activity are clearly defined.

In particular, the isoform CYP2C9 is characterized by two polymorphisms that are quite rare in Caucasian population (1-3%). These SNPs (C430T and A1075C) have demonstrated an enzyme activity reduction both *in vitro* and *in vivo*. Few studies have evaluated the role of these polymorphisms in cyclophosphamide pharmacokinetcs with controversial results[8,20]. However, in this work both the variants showed a significant association with the development of hepatic toxicity after adjuvant treatment with cyclophosphamide. In our group of 195 breast cancer patients, subjects carrying at least one variant allele had a 3.62-fold increased risk to develop acute hepatic toxicity (grade 1-3). Moreover, there was a significant correlation among CYP2C9 A1075C genotypes and hepatic toxicity at the end of the therapy. This association was not confirmed when comparing grade 1-3 of toxicity versus grade 0 in subjects with at least one variant allele. These results could be related to the decreased activity of CYP enzymes consequently to the genetic alteration. The reduction of bioactivation of cyclophosphamide could induce an accumulation of prodrug in the liver causing hepatic toxicity. At present, in literature there are no data to confirm our results.

Another isoform involved in cyclophosphamide bioactivation with a minor impact was correlated with the development of severe hematological toxicity (neutropenia, grade 3-4): CYP2C19 G681A. Patients with at least one variant allele had a 3.05-fold increased risk to develop severe neutropenia at the end of therapy compared to wild type subjects. However, these data must be evaluated considering that among the seven patients

homozigous for the variant, only one patient exhibited severe hematological toxicity. Thus, these data have been confirmed in a larger study.

Other polymorphisms of CYP2B6 isoform have been considered in this work. At present, only few studies have been conduced to verify their functional relevance. Among these polymorphisms, three are considered interesting because of their decrease of the enzyme expression: G516T, A785G and C1459T. The combination of these SNPs generates four different alleles: CYP2B6*4 (A785G), CYP2B6*5 (C1459T), CYP2B6*6 (G516T and A785G) and CYP2B6*7 (G516T, A785G and C1459T). The presence of both G516T and A785G was indicated as the best marker for a reduction of hepatic expression of CYP[8]; however a more recent study reveals that only the C1459T variant was significantly correlated with a lesser expression compared to the other variants[59]. None of the variant considered in this study had underlined an association with the development of toxicity neither after the 1st cycle of therapy nor at the end of treatment.

5.3 CYP polymorphisms and survival

Because of the involvement of CYPs in bioavailability of the active metabolite of cyclophosphamide, their polymorphic variants could have a role in survival after chemotherapy. Overall Survival data had a predictive value because it could be unreleated to the treatment; data of Time To Progression (TTP) should be more informative. At the moment, we have not yet collected these data.

However, a strong impact of CYP2C9 A1075C was observed in the overall survival of breast cancer patients considered in this study. Subjects carrying the 1075AC or 1075CC genotypes showed a 1.5-fold increased risk of death after adjuvant tretament with cyclophosphamide compared to wild type subjects (median survival 93 months vs 105 months, respectively). As above mentioned, the polymorphism defines a phenotype with a reduced enzyme acitvity in bioactivation of prodrug, leading to a lesser bioavailability of active metabolite of cyclophosphamide. This effect could explain the negative impact of this polymorphism in survival after treatment with the alkylating agent.

5.4 Conclusions

In conclusion, data from this study indicate that some polymorphisms affecting CYP isoforms are involved in the development of cyclophosphamide toxicity, both acute and cumulative. It was also found that CYP2C9 polymorphisms have an impact in survival after adjuvant chemotherapy with cyclophosphamide. Several isoforms are involved in the bioactivation pathway of the prodrug, the essential step for the production of active cytotoxic metabolite.

What could emerge from this study is that molecular markers of toxicity and response to chemotherapy exist and could be taken into consideration to design a chemotherapy tailored to the patients, by predicting the individual attitude to respond and to develop toxic effects to treatment. The single-polymorphism approach is no longer sufficient to describe the complex action of the drug and a comprehensive assessment of all genetic variants implicated in the drug pathway is needed. Therefore powerful mathematic and statistical tools should be employed, in the near future, to put together all of these information and to possibly elaborate an algorithm able to predict pharmacokinetics and pharmacodynamics of the drug. Prospective trials calculating the proper cyclophosphamide dosage *a priori*, based on the genetic and molecular characteristics of each patient, will be designed to test the validity of this approach, in comparison to the use of classically employed parameters.

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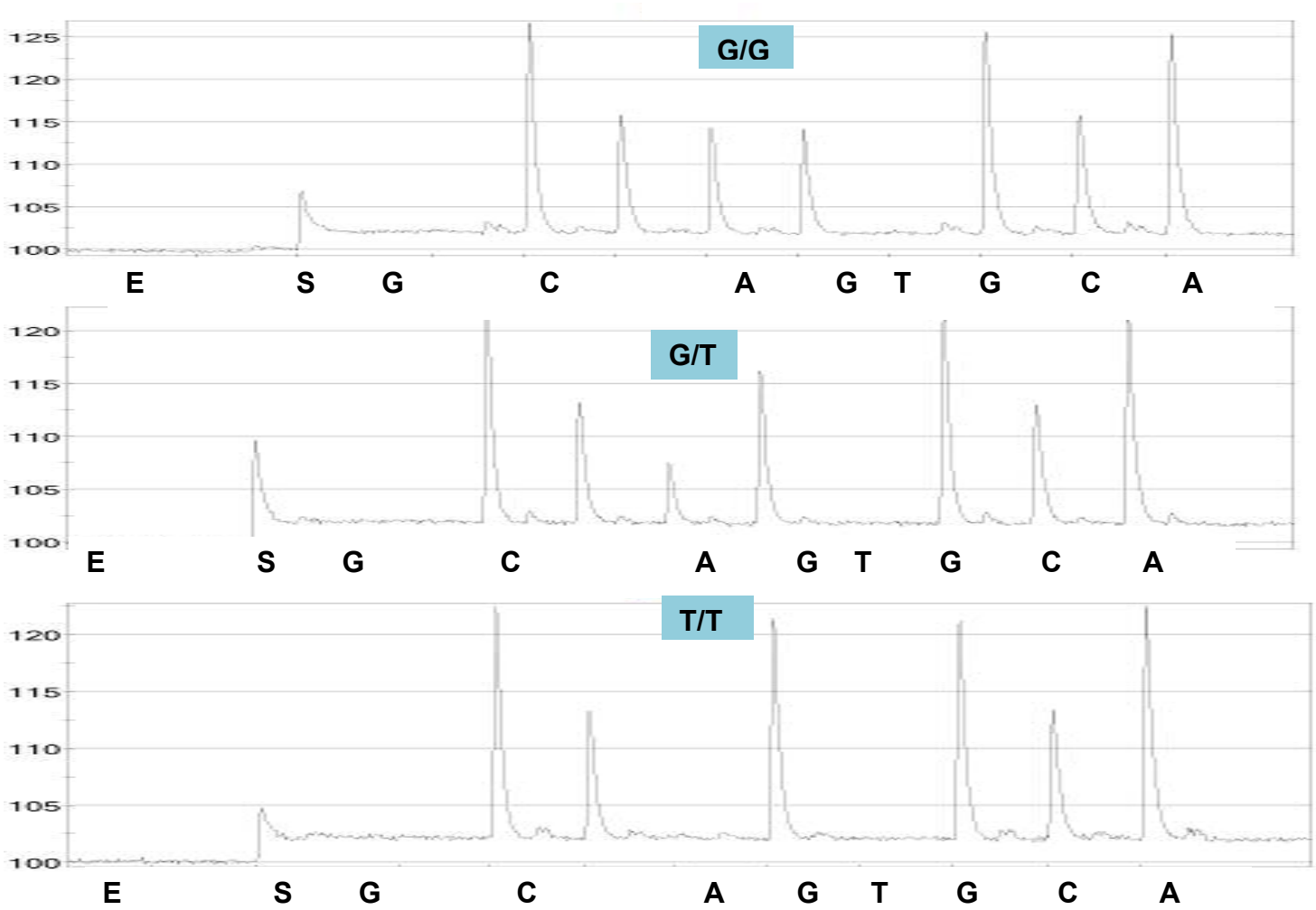
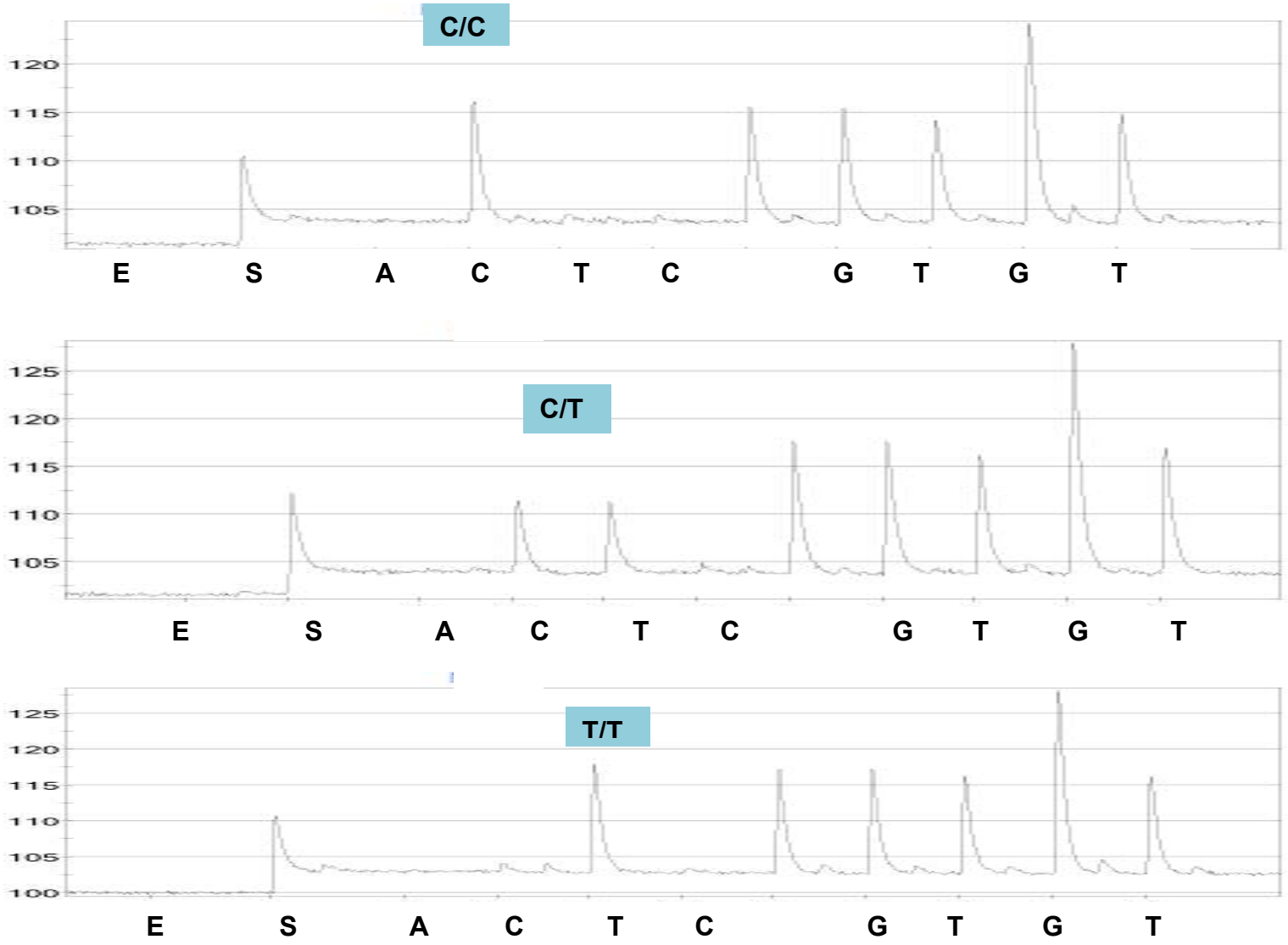
ATTACHMENTS

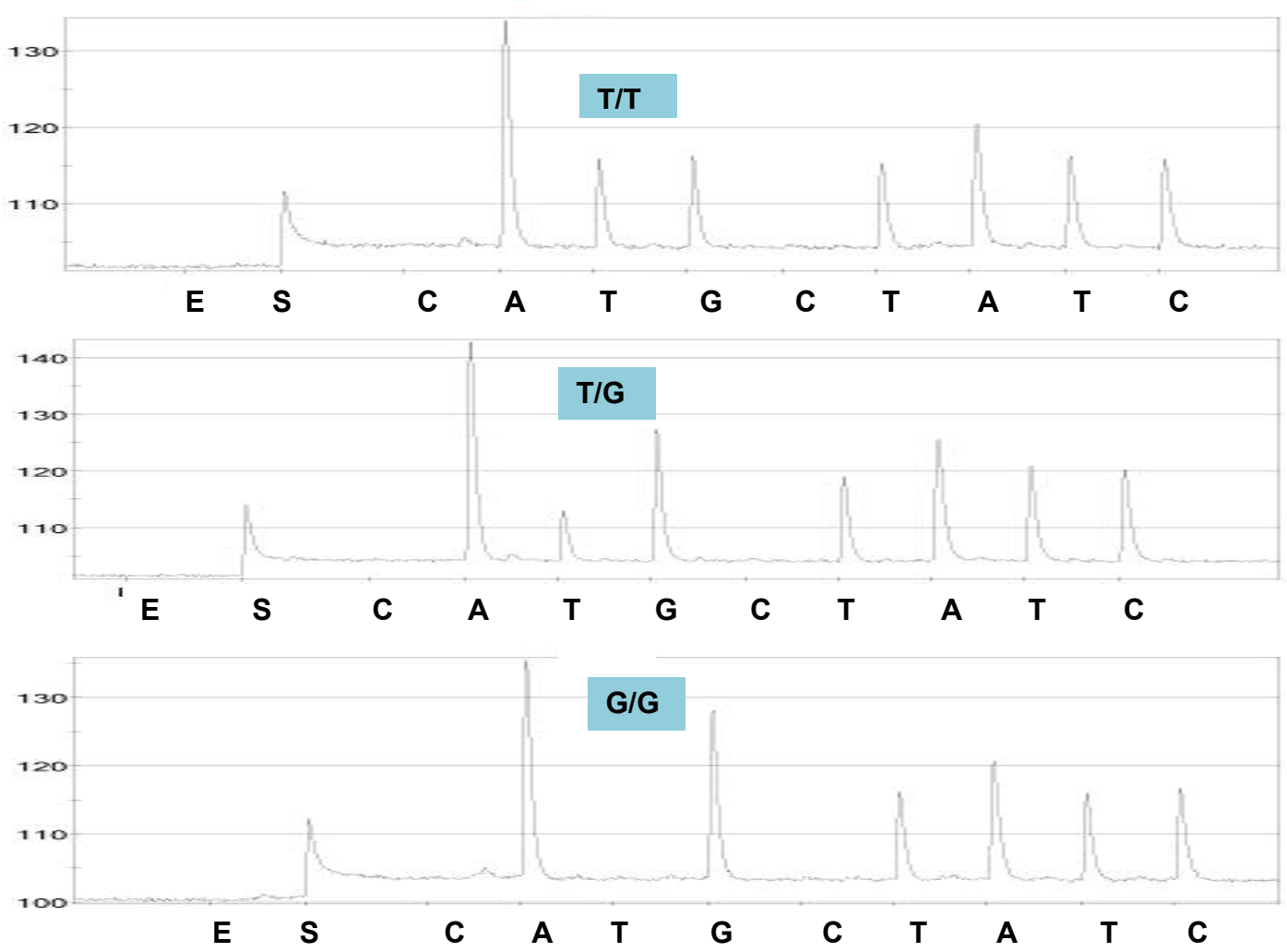
Attachment 1: CYP2B6 – G516T genotypes



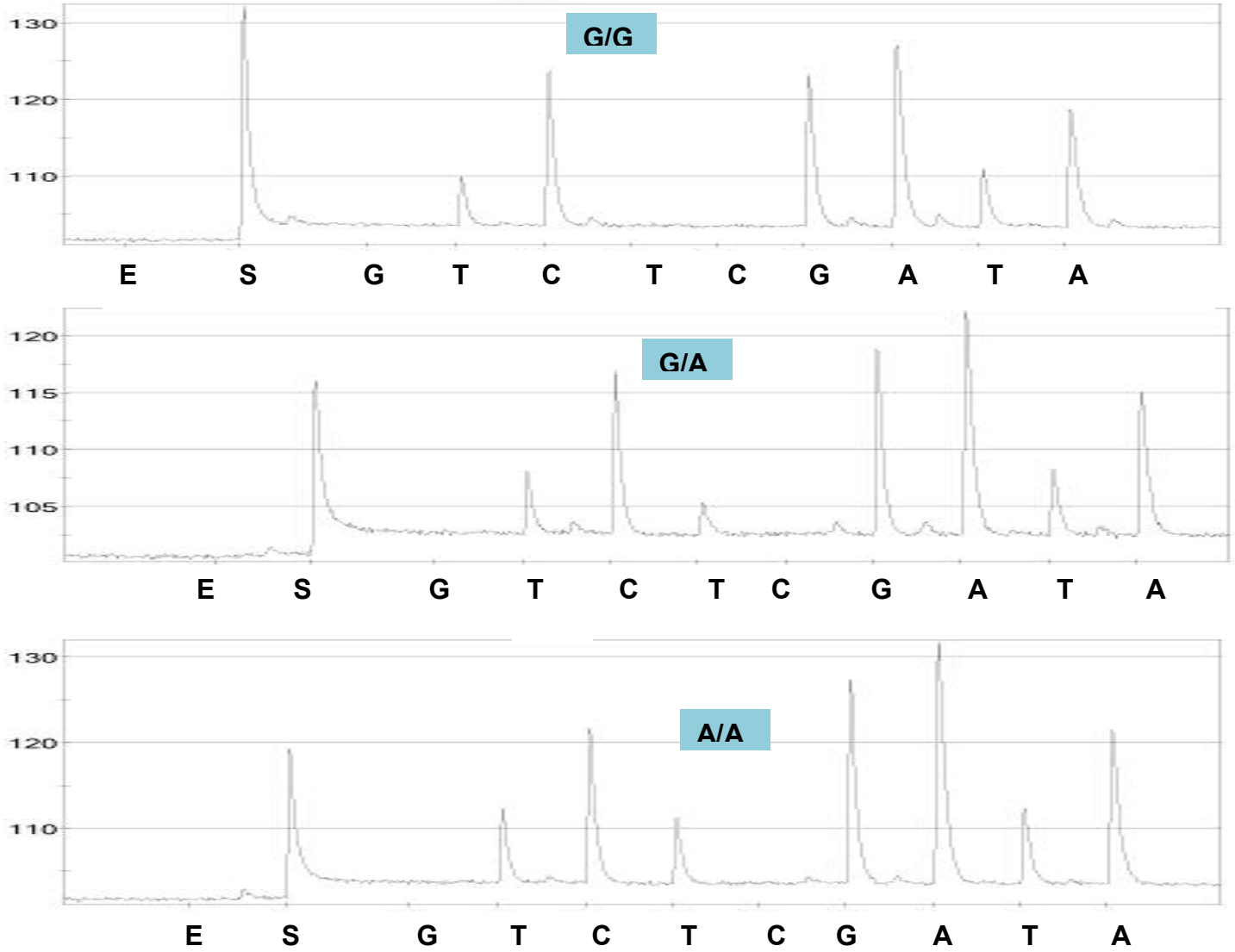
Attachment 2: CYP2C9 - C430T genotypes



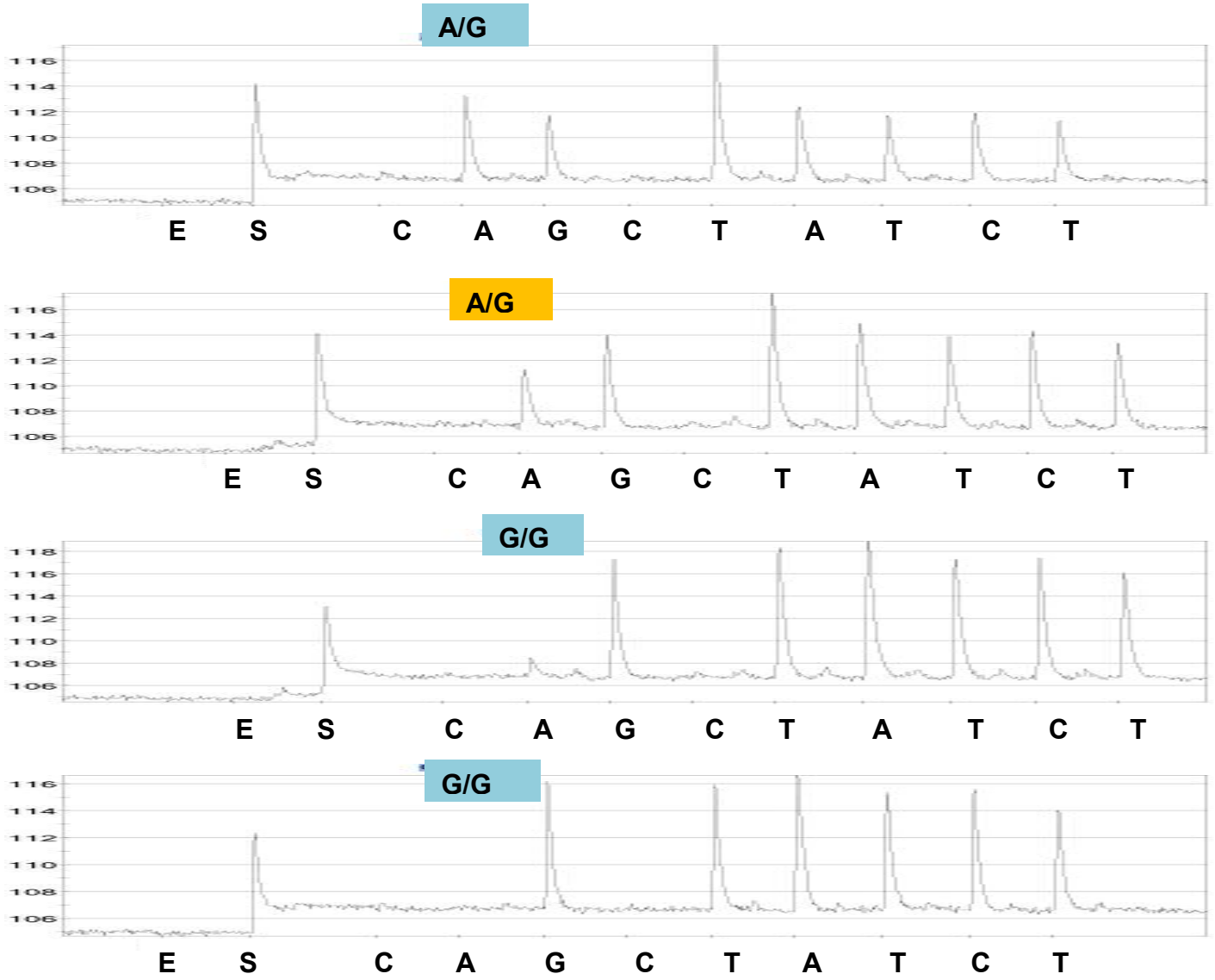
Attachment 3: CYP2C9 – A1075C genotypes



Attachment 4: CYP2C19 – G681A genotypes

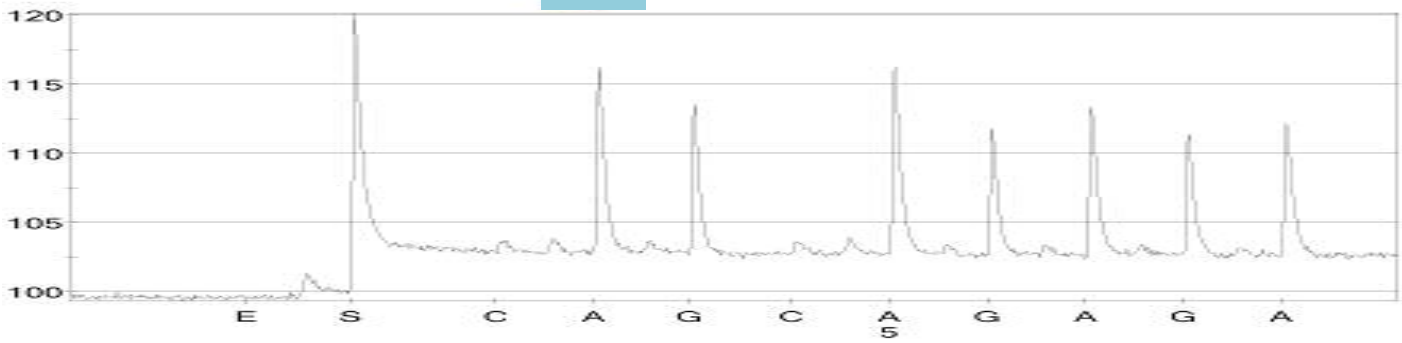


Attachment 5: CYP3A5 – G6986A Annealing temperature gradient experiment

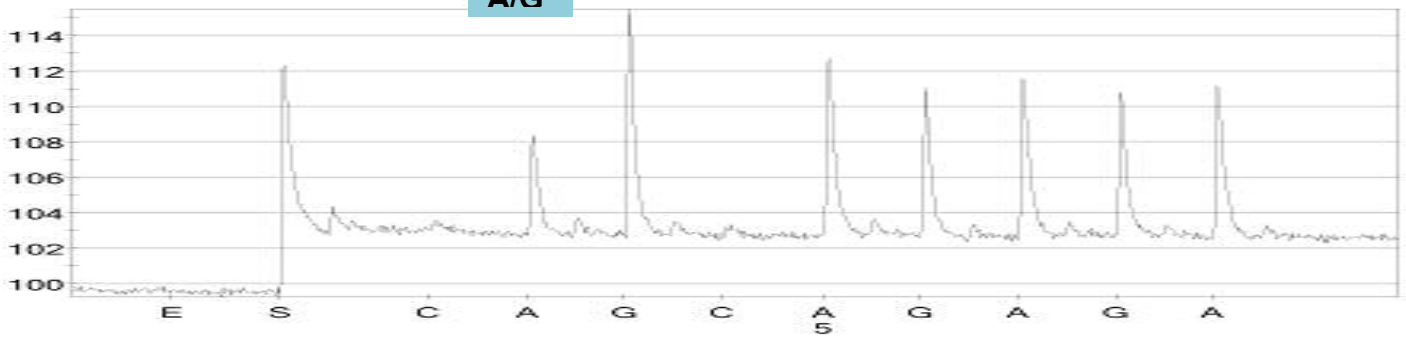


Attachment 6: CYP3A4 – A392G genotypes

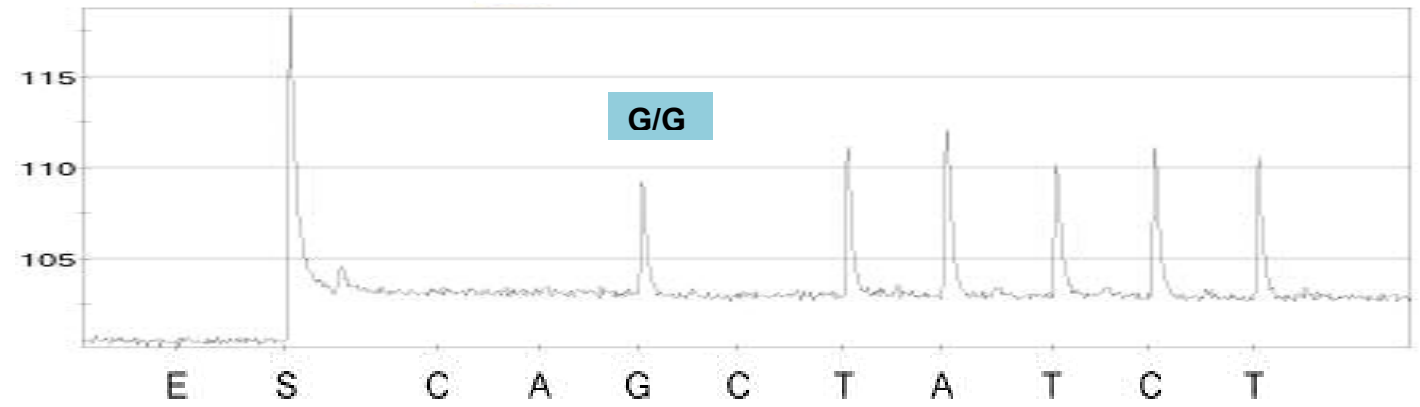
A/A

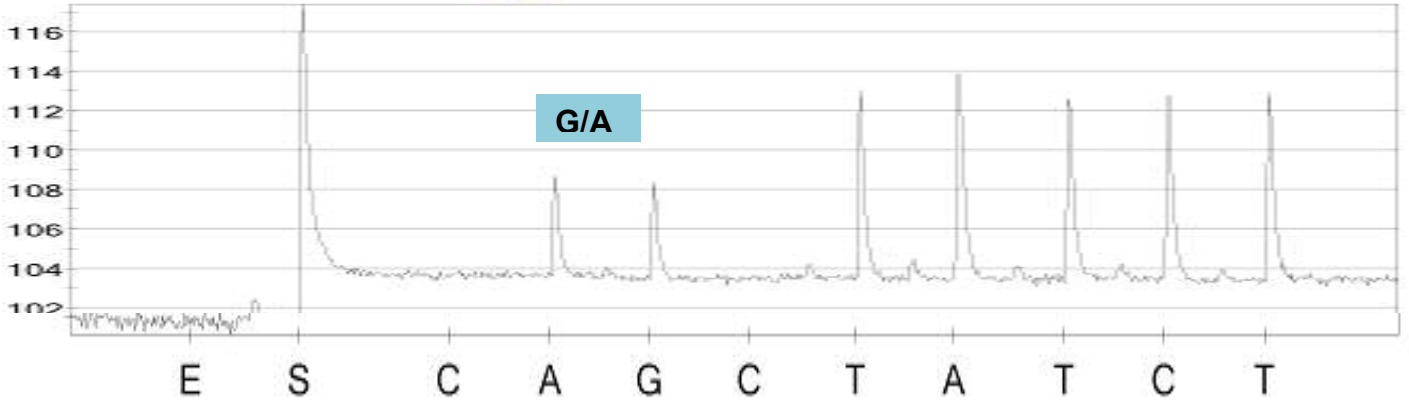


A/G

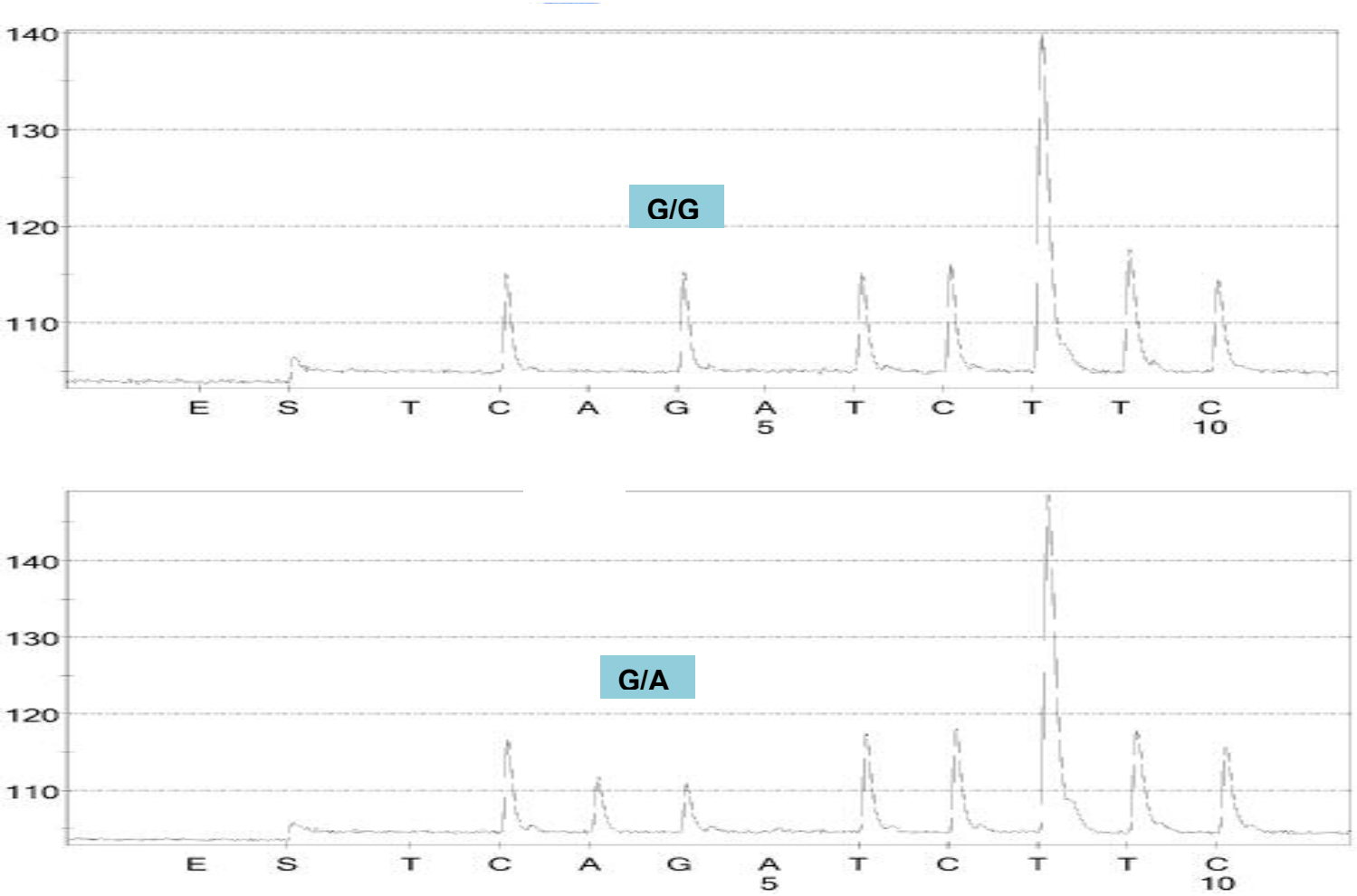


Attachment 7: CYP3A5 – G6986A genotypes

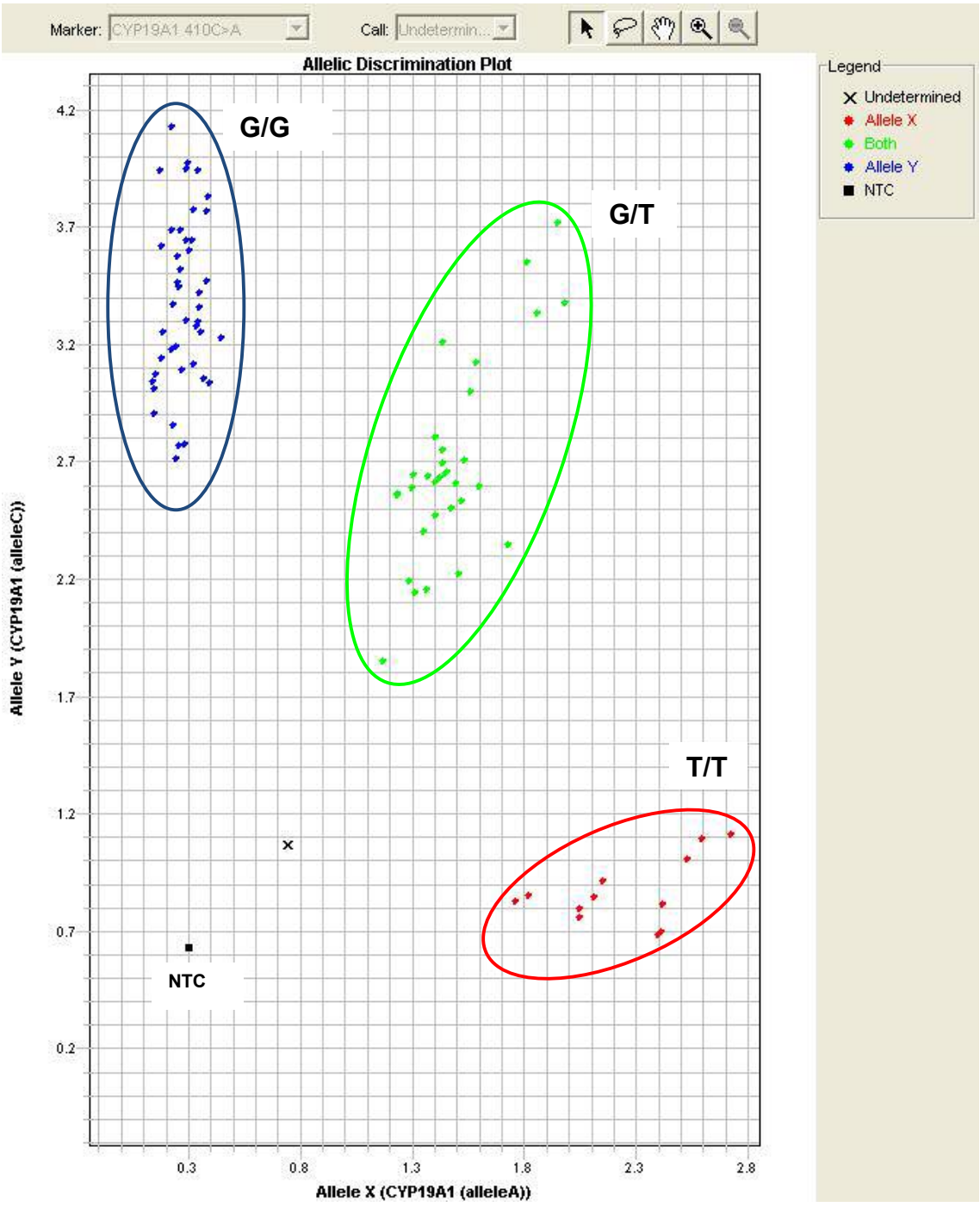




Attachment 8: CYP19 – C47T genotypes

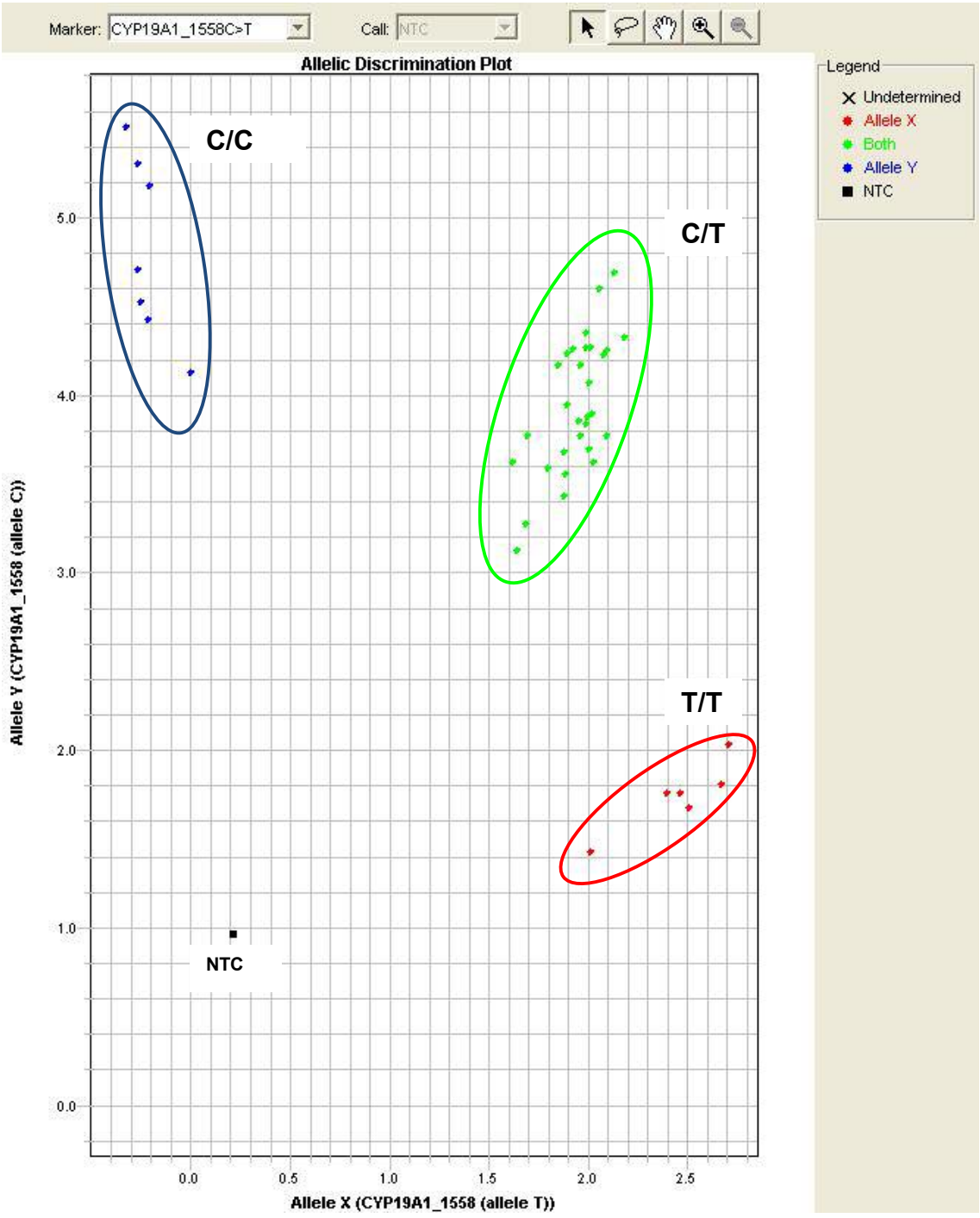


Attachment 9: CYP19 - G410T genotypes



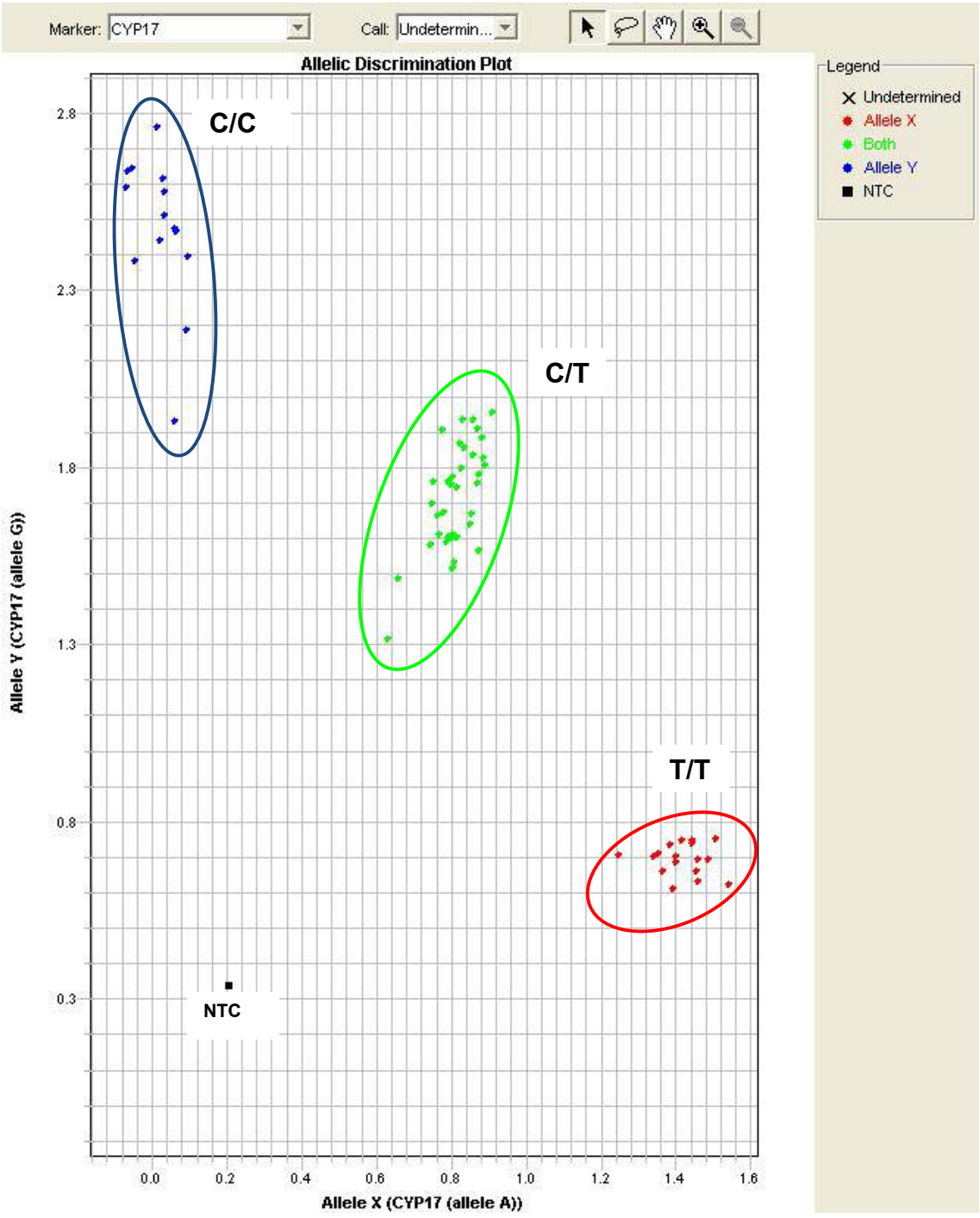
NTC= No Template Control

Attachment 10: CYP19 – C1558T genotypes



NTC= No Template Control

Attachment 11: CYP17 – T27C genotypes



NTC= No Template Control