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Nanotechnological applications and
pharmacogenetic research
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“The past is of no importance. The present is of no importance. It is with the future that we have to deal…”

Oscar Wilde (1854-1900)

“If it were not for the great variability among individuals medicine might as well be a science and not an art.”

Sir William Osler (1892)
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Abstract (Italian version)

Questo progetto di dottorato di ricerca è finalizzato allo studio dell'applicazione nella pratica clinica attuale della medicina di comunità, di tests farmacogenetici utili sia per la determinazione di polimorfismi noti per influenzare la dose iniziale di warfarin nella terapia anticoagulante orale, sia per l'analisi dei polimorfismi associati alla risposta di farmaci antidepressivi. Gli studi si sono perciò svolti in collaborazione con la medicina territoriale, al fuori dei centri di eccellenza e le rigorose condizioni in cui di solito vengono eseguiti i trials. In quest'ottica, i risultati finora ottenuti, in accordo con la letteratura corrente, supportano l'impiego dell'analisi dei polimorfismi per la determinazione della corretta dose iniziale di warfarin. Inoltre, il polimorfismo del trasportatore della serotonina (5-HTTLPR) è stato valutato in relazione alla risposta ai farmaci antidepressivi appartenenti alla classe degli inibitori selettivi della ricaptazione della serotonina (SSRI), e anche nella medicina di comunità, sia in pazienti oncologici che psichiatrici, l'analisi di 5-HTTLPR sembra essere uno strumento utile per predire l’esito della risposta al trattamento con SSRI.

Infine, con lo sviluppo di nuove tecnologie, i costi per l'analisi genetica sono diminuiti,e possono essere considerati limitati soprattutto in relazione ai vantaggi raggiunti con il loro impiego. In ogni caso, il rapporto costo-efficacia dei test farmacogenetici potrebbe essere migliorato con lo sviluppo di ulteriori devices che permettano un’analisi più veloce ed economica.
Abstract (English version)

This PhD project is aimed to the study of the application, in the current clinical practice of the community medicine, of pharmacogenetic tests known to be associated with the prediction of warfarin dose at the initiation of the oral anticoagulant therapy and moreover, the test for the analysis of the polymorphism related to the response to the antidepressant drugs. The study was carried out in the community care, out of the centers of excellence and the strict conditions in which usually the trials are performed. From this perspective, the results obtained so far, according to the current literature, support the use of the analysis of polymorphisms in determining the correct dose of warfarin.

Furthermore, polymorphism of the serotonin transporter (5-HTTLPR) has been assessed in relation to response to antidepressant drugs belonging to the class of Selective Serotonin Reuptake Inhibitors (SSRIs), and also in community medicine, both in oncological and psychiatric patients, the analysis of 5-HTTLPR seems to be a useful tool to predict the outcome of the response to treatment with SSRIs. Finally, Since the development of new technologies, the costs for genetic analysis have been decreased, and may be considered limited, particularly in relation to the benefits achieved with their use. In any case, the cost-effectiveness of pharmacogenetic tests could be improved by the development of further devices that allow a faster and cheaper analysis.
Chapter I

Background
1.1 Pharmacogenetics and its application in pharmacology

1.1.1 Introduction

Most patient populations show large inter-individual variability in drug response and toxicity. For all major classes of drugs given at standard doses, a substantial proportion of patients do not respond, respond only partially, or experience adverse drug reactions\(^1\) (ADRs) (see Fig 1).

**Figure 1.** The potential of pharmacogenetic is to identify patients within a population with the same diagnosis, who are genetically predisposed either not to respond to therapy or to develop unacceptable toxicity, and then to prospectively alter their therapy to avoid treatment that is not likely to be optimal. The remaining, now more homogeneous population, can then be treated with conventional therapy in which they are not genetically predisposed to fail.\(^1\)
Drug concentrations in plasma can vary more than 600-fold between two individuals of the same weight on the same drug dosage. This variation can be of genetic, physiological, pathophysiological, or environmental origin, but a drug’s absorption, distribution and metabolism, and interactions with its target can be determined by genetic differences. \(^1\) Genetic variation in humans was recognized as an important determinant of individual variability of drug response from clinical observations in late 1950s.\(^2\)\(^-\)\(^4\) In these cases, patients with very high or low plasma or urinary drug concentrations that correspond to a specific phenotype of a drug response were identified, and the biochemical traits leading to the variation of drug concentrations were found to be inherited. The observation that individual variation of a drug response is often larger among members in a population (population variability) than within the same person at different times (intrapatient variability) further supports inheritance as a major determinant of drug response.\(^5\)\(^-\)\(^6\) These clinical and population-based findings fostered the formation of pharmacogenetic to specifically address genetic contribution to individual variability in drug therapy.\(^7\) The human genome sequence provides a special record of human evolution that varies among populations and individuals. Sequence variations in drug target proteins, drug-metabolizing enzymes, and drug transporters can alter drug efficacy, drug side effects, or both to cause variable drug responses in individual patients.\(^8\)\(^-\)\(^14\) From this prospect, the availability of the complete human genome sequence has made it possible to analyze the impact of variations of the human genome sequence on the pathogenesis of important diseases and the response to drug therapy. Moreover, the rapid development of techniques in the area of genome analysis has facilitated the identification of pharmacogenetic
biomarkers that can provide predictive tools for improved drug response and fewer ADR (see Tab. 1).

<table>
<thead>
<tr>
<th>Protein or gene</th>
<th>Medications</th>
<th>Examples of altered drug effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C9</td>
<td>Non-steroidal anti-inflammatories, warfarin, tolbutamide, phenytoin</td>
<td>Increased anticoagulant effects of warfarin</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>Omeprazole, Mephenytoin</td>
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<tr>
<td>CYP2D6</td>
<td>Antidepressants, codeine, β-blockers</td>
<td>Increased antidepressant toxicity, decreased codeine analgesia</td>
</tr>
<tr>
<td>CYP3A4/3A5/3A7</td>
<td>Cyclosporin, tacrolimus, calcium channel blockers, midazolam, terfenadine, etoposide, lovastatin, lamotrigine, steroids</td>
<td>Decreased efficacy of tacrolimus in organ transplantation</td>
</tr>
<tr>
<td>Dihydropyrimidine dehydrogenase</td>
<td>Fluorouracil</td>
<td>Increased neurotoxicity</td>
</tr>
<tr>
<td>Glutathione transferases</td>
<td>Several anticancer agents</td>
<td>Increased response in breast cancer, more toxicity and poorer outcome in acute myeloid leukaemia</td>
</tr>
<tr>
<td>Thiopurine methyltransferase</td>
<td>Mercaptopurine, thioguanine, azathioprine</td>
<td>Increased haematopoietic toxicity, increased risk of secondary cancer</td>
</tr>
<tr>
<td>UGT1A4</td>
<td>Irinotecan</td>
<td>Increased gastrointestinal toxicity</td>
</tr>
<tr>
<td>Drug transporters and targets</td>
<td>Digoxin, HIV protease inhibitors, natural product anticancer drugs</td>
<td>Decreased CD4 response in HIV-infected patients, decreased digoxin bioavailability</td>
</tr>
<tr>
<td>ABCB1 (MDR-1)</td>
<td>β2-agonist (for example, albuterol, terbutaline)</td>
<td>Decreased bronchodilation</td>
</tr>
<tr>
<td>β1-adrenergic receptor</td>
<td>β2-agonist</td>
<td>Decreased cardiovascular response to β2-agonist</td>
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<td>G protein α</td>
<td>β-blockers</td>
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(Table adapted from Evans WE and Relling MV, Nature 2004)

Since their penetrance in the population, and the significant functional role, this thesis considered the variations in the genes associated with the oral anticoagulant agent warfarin, and those related to the pharmacogenetic of SSRIs antidepressant drugs.
1.1.2 Warfarin

Warfarin sodium is an anticoagulant drug which acts by inhibiting vitamin K-dependent coagulation factors. Chemically, it is a derivative of 4-hydroxycoumarin with a nonpolar carbon substituent at the 3 position (see Fig. 2) which is asymmetrical. The enantiomers differ in anticoagulant potency (the S form is more potent), metabolism, elimination, and interaction with other drugs. Commercial formulation is a racemic mixture of the R- and S-enantiomers.

![Warfarin structure](The red circle indicates the chiral centre)

Warfarin is indicated for the prophylaxis and/or treatment of venous thrombosis and its extension, and pulmonary embolism; for the prophylaxis and/or treatment of the thromboembolic complications associated with atrial fibrillation and/or cardiac valve replacement and to reduce the risk of death, recurrent myocardial infarction, and thromboembolic events such as stroke or systemic embolization after myocardial infarction.
Pharmacological properties

Mechanism of action

Warfarin acts by inhibiting the synthesis of vitamin K dependent clotting factors, which include Factors II, VII, IX and X, and the anticoagulant proteins C and S. Half-lives of these clotting factors are as follows: Factor II - 60 hours, VII - 4 to 6 hours, IX - 24 hours, and X - 48 to 72 hours. The half-lives of proteins C and S are approximately 8 hours and 30 hours, respectively. The resultant in vivo effect is a sequential depression of Factor VII, Protein C, Factor IX, Protein S, and Factor X and II activities. Vitamin K is an essential cofactor for the post ribosomal synthesis of the vitamin K dependent clotting factors. The vitamin promotes the biosynthesis of γ-carboxyglutamic acid residues in the proteins which are essential for biological activity. Warfarin is thought to interfere with clotting factor synthesis by inhibition of the C1 subunit of the vitamin K epoxide reductase (VKORC1) enzyme complex, thereby reducing the regeneration of vitamin K1 epoxide (see Fig. 3). Therapeutic doses of warfarin decrease the total amount of the active form of each vitamin K dependent clotting factor made by the liver by approximately 30% to 50%. An anticoagulation effect generally occurs within 24 hours after drug administration. However, peak anticoagulant effect may be delayed 72 to 96 hours. The duration of action of a single dose of racemic warfarin is 2 to 5 days. The effects of warfarin may become more pronounced as effects of daily maintenance doses overlap. Anticoagulants have no direct effect on an established thrombus, nor do they reverse ischemic tissue damage. However, once a thrombus has
occurred, the goal of anticoagulant treatment is to prevent further extension of the formed clot and prevent secondary thromboembolic complications which may result in serious and possibly fatal consequences.

Figure 3. Warfarin’s mechanism of action
Metabolism

The elimination is almost entirely by metabolism. Warfarin is stereoselectively metabolized by hepatic microsomal enzymes (cytochrome P-450) to inactive hydroxylated metabolites (predominant route) and by reductases to reduced metabolites (warfarin alcohols). The warfarin alcohols have minimal anticoagulant activity. The metabolites are principally excreted into the urine; and to a lesser extent into the bile. The metabolites of warfarin that have been identified include dehydrowarfarin, two diastereoisomer alcohols, 4′-, 6-, 7-, 8- and 10-hydroxywarfarin. The cytochrome P-450 isozymes involved in the metabolism of warfarin include 2C9, 2C19, 2C8, 2C18, 1A2, and 3A4. 2C9 is likely to be the principal form of human liver P-450 which modulates the in vivo anticoagulant activity of warfarin. The warfarin (S)-isoform is metabolised predominantly by the CYP2C9 enzyme, which converts the drug in the 7-hydroxy and 6-hydroxy inactive metabolites that are excreted in the urine.15
Pharmacogenetic of warfarin

CYP2C9

The human CYP2C9 gene is located on the chromosome 10 (10q24.2), is approximately 55 Kb long, contains 9 exons and encodes for a 60 kDa microsomal protein. More than 50 variants in CYP2C9 have been described and, among these, and the most common allele, designated as CYP2C9*1, is considered the wild-type genotype. Two single nucleotide polymorphisms (SNPs), CGT>TGT in the exon 3, inducing the Arg144Cys substitution, and ATT>CTT in the exon 7, encoding for the Ile359Leu variant, denoted as CYP2C9*2 and CYP2C9*3 allele, respectively, play an important role in warfarin metabolism and are relatively common among Caucasian populations. The allelic frequencies of CYP2C9*2 and CYP2C9*3 diverge considerably among different ethnic groups. In Caucasians, the allelic frequencies of CYP2C9*2 and CYP2C9*3 vary approximately from 8% to 20% and from 6% to 10%, respectively. They are less frequent in Asian and African-American populations. Indeed, CYP2C9*2 is not present in Asians and only 2-4% of African-Americans carry this allele. CYP2C9*3 is present in 1-4% of Chinese, Korean and Japanese populations, and in 1-2% of African-Americans. In vitro, CYP2C9*2 and CYP2C9*3 are functionally defective and, as compared with the wild-type enzyme, exhibit only 12% and 5% of metabolic efficiency, respectively. The decreasing in catalytic activity of the CYP2C9*2 allele can be attributed to its impaired ability to interact with the NADPH-CYP450 reductase in the oxidative metabolism cascade. The enzyme encoded by the CYP2C9*3 allele shows an altered affinity for the substrate. The clinical effects of these polymorphisms
are well documented in vivo. Furuya and co-workers were the first to report the influence of CYP2C9 polymorphisms on warfarin dose requirement in vivo. They reported that patients with the CYP2C9*1/*2 genotype required, on average, a 20% lower warfarin dose to maintain a target INR (international normalized ratio) between 2 and 4 compared to the anticoagulated patient population studied. Moreover, 90% of patients requiring the lowest warfarin dose were heterozygous for *2. Aithal et al. demonstrated that the odds ratio for an individual on low-dose warfarin having one or more of the variant CYP2C9 alleles as compared with the general patient population is 6.21 (95% CI 2.48–15.6). They also showed that individuals in the low-dose group were more prone to difficulties at the time of induction and were four times more likely to develop major bleeding complications than the general clinic group. In the 2002, Scordo et al. confirmed the association between CYP2C9 polymorphism and warfarin dose requirement that had, by then, been reported by several other studies. However, 30% of the patients with the wild-type CYP2C9 genotype were found to require low daily warfarin doses, indicating the possibility that other genetic, environmental, physiological, and pathological factors were contributing to warfarin dose requirement. A systematic review and meta-analysis of data relating to 2775 patients established that 20% of patients studied carried a CYP2C9 variant allele, with *2 at 12.2% (range: 9.7%–15.0%) and *3 at 7.9% (range: 6.5%–9.7%). Mean reduction in daily warfarin dose for the CYP2C9*2 genotype was 0.85 mg/day (0.60–1.11 mg), a 17% reduction, and for CYP2C9*3 it was 1.92 mg/day (1.37–2.47 mg), a 37% reduction. For the CYP2C9*2/*3 genotype the reduction in dose was 1.47 mg (1.24–1.71 mg), a 27% reduction. The relative bleeding risk for CYP2C9*2 was
1.91 (1.16–3.17), and for CYP2C9*3 it was 1.77 (1.07–2.91). For either variant, the relative risk was 2.26 (1.36-3.75).\textsuperscript{31}

**VKORC1**

The gene that encodes for the vitamin K epoxide reductase complex 1 (VKORC1), the target enzyme for warfarin, has been recently identified.\textsuperscript{32,33} VKORC1 gene maps to the short arm of chromosome 16 and contains three exons coding for an 18-kDa integral membrane protein. Several groups have demonstrated a dependence of warfarin dose on the polymorphic VKORC1 gene\textsuperscript{31,34-36} although other factors, including non-compliance, accelerated metabolism, and excessive dietary vitamin K, may contribute to a resistance phenotype independent of VKORC1 status. A number of different SNPs have been identified and Rieder and colleagues have created a widely used haplotype grouping system including five main haplotypes significantly associated with stable warfarin dose, which were segregated into two haplotype groups: a low-dose haplotype group (“A”) and a high-dose group (“B”). These haplotype groups explain approximately 20% to 25% of the variability in stable warfarin dose, and are strongly related to mRNA levels for VKORC1, with the high-dose group having higher mRNA levels.\textsuperscript{37} Two VKORC1 SNPs have been proven to be important genetic determinants of inter-individual warfarin dose variability and then are commonly considered in the study considering the genetic influence of VKORC1 on warfarin dose, the G>A substitution in position −1639 (rs9923231) and the C>T variation in 1173 (rs9934438).\textsuperscript{41} The A allele in position −1639 allele is associated with the need of lower doses than the G allele. The association is such that a homozygous carrier of the A allele requires a warfarin dose
approximately 50% of that of an individual that is homozygous for the G allele. Similar effects have been ascribed to the VKORC 1173C>T polymorphism.\textsuperscript{39} However, these two SNPs are in pronounced linkage disequilibrium and are interchangeably used as tag SNPs for differentiation between a low-dose haplotype A (−1639A and 1173T) and a high-dose haplotype B (−1639G and 1173C).\textsuperscript{36} Other SNPs have been independently associated with altered warfarin dose requirements, but their contribution to the inter-individual variability is small compared to the −1639G>A and 1173C>T polymorphisms.\textsuperscript{40,41} The warfarin maintenance dose differs significantly between the three combinations of haplotype groups, with a dose of 2.7±0.2 mg/day for group A/A, 4.9±0.2 mg/day for group A/B, and 6.2±0.3 mg/day for group B/B. Thus, VKORC1 haplotypes can be used to stratify patients into low-, intermediate-, and high-dose warfarin groups and may explain differences in dose requirements among patients. The VKORC1 haplotype group A is more frequent in Asians (89%), while the haplotype group B does in Caucasians (58%).\textsuperscript{37} All these data are concordant with the clinical observation that Asians require lower doses of warfarin than their Caucasian counterpart to achieve the same degree of anticoagulation and suggest that, in people from a different ethnic background, the VKORC1 plays a pivotal role in the modulation of inter-individual variability of warfarin response.\textsuperscript{42,43} In Caucasian and Asian populations, VKORC1 genotype predicts 25% of the variability in warfarin dose.\textsuperscript{44}
Other genes

In addition to CYP2C9 and VKORC1, there are several other genes that may influence variation in warfarin dose and response. The most widely replicated of these is a nonsynonymous SNP in CYP4F2 (rs2108622, V433M).\(^{45,46}\) The inclusion of CYP4F2 variant in the dosing models showed an improvement in the overall predictability of warfarin dose\(^{47-54}\), however some contradictory reports showing that the contribution of this variant to warfarin dose was negligible.\(^{55-58}\) Variants in CALU (gene encoding Calumenin) and GGCX (γ-glutamyl carboxylase) have been shown to affect warfarin dose in some but not all populations.\(^{59-60}\)
Influence of the genetic polymorphisms on warfarin dose

Warfarin metabolism is influenced by a number of pathological, physiological, and environmental factors. Although it had long been recognized that warfarin dose requirement falls with increasing age and liver size, and that body size is an indirect marker of this association, induction regimens have remained crude, and overanticoagulation during the initiation phase is common, especially in older patients. The clinical potential for recognition of genetic polymorphisms that account for extremes in phenotypic response in advance of therapy, and tailoring dosage appropriately, has been underpinned by studies showing a relationship between genotype and risk of adverse drug effects. The clinical importance of the association between CYP2C9 genotypes and bleeding was confirmed in a retrospective cohort study that established CYP2C9 genotype as an independent predictor of a first bleeding event during the initiation phase of warfarin therapy (HR 3.94; 95% CI 1.29–12.06). Patients carrying CYP2C9 variant alleles had a higher rate of above-range INR values, took longer to reach stable dosing, and had a higher risk of serious and life-threatening bleeding events than patients with the wild-type allele. Similar results were noted in a study of warfarin-treated patients where CYP2C9*2 or *3 compound heterozygous and homozygous had low warfarin requirements and increased rates of excessive (INR>6.0) anticoagulation and bleeding compared with wild-type patients. In a retrospective cohort analysis of 172 patients, patients with CYP2C9 variants, compared to those without, achieved stable dose 48% later (p < 0.01), spent a higher proportion of time above range in the first month of therapy (14% versus 25%, p = 0.07), and had an odds ratio of 4.15 for an INR>5. In contrast, although patients homozygous for the
VKORC1 low-dose haplotype (AA) had an odds ratio of 4.47 for an INR>5, no other influence was noted on outcomes measured. The recognition that genotype affects the likelihood of serious and life-threatening bleeding highlighted the inadequacy of current dosing regimes. The quantification of the impact of genetics on warfarin dose requirement led to the concept that information on genotype, or any other factor that influences the interindividual variability in dose requirement, could be used toward a more individualized approach to warfarin therapy. Several studies have quantified the contribution of various genetic, clinical, and environmental factors to warfarin dose requirement based on retrospective analysis of data obtained from patients on maintenance therapy. In a North American population, the extent of the influence of CYP2C9*2 and *3, age, and body surface area (BSA) on maintenance warfarin dose was quantified at 19% per *2 allele, 30% per *3 allele, 8% per decade of age, and 13% per standard deviation decrease in BSA, with dose being 29% lower in patients who took amiodarone (a potent inhibitor of warfarin metabolism commonly used in elderly patients with cardiac arrhythmia), 12% lower in patients who took simvastatin, 21% lower in patients whose target INR was 2.5 rather than 3.0, and 11% lower in whites compared to African American patients. Regression analysis of data that included these factors and sex (which was of borderline significance) explained 39% of the variance in warfarin maintenance dose. In 453 Caucasian patients the CYP2C9 genotype frequencies for *1/*1, *1/*2, *1/*3, *2/*2, *2/*3, and *3/*3 were 65.1%, 19.0%, 12.1%, 1.6%, 1.8%, and 0.4%, respectively. Mean maintenance doses for these genotypes were 36.5, 29.1, 23.5, 28.0, 18.1, and 5.5 mg/week, respectively. In univariate analysis, genotype alone accounted for 19.8% of the variability in maintenance dose. Age, BSA, and male sex
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accounted for 14.6%, 7.5%, and 4.7%, respectively, while cardiac valve replacement as the indication for warfarin accounted for 5.4% of the variability. Collectively, these factors accounted for 33.7% of the variability in dose requirement according to multiple regression analysis. The discovery of the contribution of the VKORC1 genotype to warfarin dose requirement allowed further assessment of the importance of genetic factors. Sconce et al. demonstrated that variables of age, height, and CYP2C9 and VKORC1 genotype altogether explained 55% of the inter-individual variability in dose requirement in a cohort of stable patients. A dosing equation based on regression analysis of data was subsequently developed. The validity of the dosing equation in predicting stable maintenance dose was confirmed in a second cohort of patients on warfarin therapy. In a prospective study of outpatients in the United States, genotype was the dominant predictor of warfarin dose, explaining 33% of dose variance compared to 12% for age, weight, and sex. Warfarin dose requirement was reduced by 18%–72% in patients with a single or double CYP2C9 variant allele and by 65% in those with a VKORC1 variant. Genetics-based modeling explained almost half of the variability in dose requirement. Caldwell et al. noted that CYP2C9 and VKORC1 along with clinical factors of age, sex, BSA, and the presence or absence of prosthetic heart valves or diabetes explained 50% of the variability of stable warfarin maintenance dose, whereas, in contrast, GGCX, factor VII, and APOE polymorphisms contributed little to the variability. In a Swedish study, during initiation of therapy, homozygosity for CYP2C9 and VKORC1 variant alleles increased the risk of overanticoagulation; hazard ratios were 21.84 (95% CI 9.46–50.42) and 4.56 (95% CI 2.85–7.30), respectively. One of eight patients with CYP2C9*3/*3 genotype (12.5%) experienced severe bleeding during the
first month compared with 0.27% of other genotypes. A multiple regression model using the predictors CYP2C9, VKORC1, age, sex, and drug interactions explained 59% of the variance in warfarin dose requirement. When this was applied to an independent sample of 181 Swedish individuals, it explained 53% of dose variance. In a cohort of 1015 patients, the independent predictors of maintenance dose were VKORC1 polymorphism −1639/3673G>A (−28% per allele), BSA (+11% per 0.25 m), CYP2C9*3 (−33% per allele), CYP2C9*2 (−19% per allele), age (−7% per decade), target INR (+11% per 0.5 unit increases), amiodarone use (−22%), smoker status (+10%), race (−9%), and current thrombosis (±7%). The above covariates explained 53%–54% of the variability in warfarin dose requirement. For comparison, a regression analysis including nongenetic factors explained only 17%–22% of the dose variability. In recognition of the interethnic difference in allele and haplotype frequencies of genes for CYP2C9 and VKORC1, Wu et al. developed algorithms that included Caucasian, African American, Asian, and Hispanic ethnicity, which all produced a similar degree of correlation; the exclusion of rare genotypes that are more associated with certain ethnicities improved the model to a minor extent. A genotyping approach based on SNPs in CYP2C9*9, and VKORC1 CC and TC genotypes at position 381, in combination with age and weight, accounted for 60.2% of the variability in warfarin dose requirement in an Asian population. A model based on these, when validated in a separate cohort, showed a mean underestimation in dose of 0.23 ± 1.21 mg/day.

The future clinical utility of pharmacogenetics will require the development of a dosing equation that incorporates information on genetic as well as clinical and patient factors. Although a number of such equations have been proposed, most are highly geographically confined,
and none was developed from robust data in a population containing patients of Asian, European, and African descent. A dosing equation derived from a large, geographically and ethnically diverse population would be a step toward global clinical utility of a pharmacogenetics-guided dosing regimen. Recently, the International Warfarin Pharmacogenetics Consortium (IWPC), made up of researchers from 21 centers in nine countries on four continents (Asia, Europe, North America, and South America), developed an algorithm for estimating warfarin dose based on both clinical and genetic data from a broad patient population base. Data belonging to 4043 patients, with a target INR of 2–3, were used to create a dosing algorithm that was based on clinical variables only and an algorithm in which genetic information was added to the clinical variables. The accuracy of dose prediction by the algorithms was assessed using a validation cohort of 1009 patients. In the validation cohort, the pharmacogenetic algorithm accurately identified larger proportions of patients who required low or high doses of warfarin (weekly totals of 21 mg or less, or 49 mg or more) to achieve the target INR than did the clinical algorithm (49.4% versus 33.3%, p < 0.001, among patients requiring 21 mg per week or less; and 24.8% versus 7.2%, p<0.001, among those requiring at least 49 mg per week). The greatest benefits were observed in these low- and high-dose patients, who totaled 46.2% of the population. These are the patients in whom standard dosing regimens are most likely to produce underdosing or overdosing of warfarin with adverse clinical consequences.
1.1.3 Antidepressant Selective Serotonin Reuptake Inhibitors (SSRIs)

Depressive disorders constitute a major public health issue and the World Health Organization (WHO) has estimated that it is the fourth major cause of disability worldwide, and may become second only to cardiovascular diseases in the next two decades, thus contributing heavily to the global burden of diseases in man, according to Murray and Lopez\textsuperscript{77}, who conducted a study for the WHO. Even though antidepressant drugs (AD) have successfully been used to treat depressive disorders, there is still substantial need for improvement. Response to antidepressant therapy is often incomplete with approximately 30–40% not responding at all to the first AD given and about 60–70% not achieving remission.\textsuperscript{78} Moreover, AD pharmacotherapy is characterized by a delayed time of onset of the clinical improvement and by a variety of adverse effects. Such shortcomings of AD medication not only lead to personal suffering in both individuals and their families, but also impose considerable costs on society.

The first class of antidepressant drugs developed was the tricyclic antidepressants (TCAs). The TCAs were the result of an unsuccessful attempt to improve on the antipsychotic effectiveness of phenothiazines (medication used in the treatment of schizophrenia). Molecular modifications of phenothiazines led to synthesis of imipramine, the first clinically useful tricyclic antidepressant. The Selective Serotonin Reuptake Inhibitors were developed in response to the need for better tolerated, safer antidepressants than the TCAs, but no less effective for the symptoms of depression. The first SSRI, fluoxetine, was released in
1987. Each of the SSRIs was the product of a development strategy in which the goal was to produce a drug capable of inhibiting the reuptake of serotonin, but without affecting the various other neuroreceptors (i.e., histamine, acetylcholine, and alpha1-adrenergic receptors), affected by the TCAs.

The development of the SSRIs, with their selective mode of action, has resulted in a class of antidepressant drugs possessing an improved side-effect profile, while retaining good clinical efficacy. The fact that SSRIs were designed to avoid affecting other neuroreceptors explains many of the pharmacological differences between the SSRIs and the TCAs and explains the similarities among the SSRIs. The benefits of SSRIs include therapeutic effectiveness, a wide therapeutic index, good tolerability, and less toxicity in overdose situations. Moreover, SSRIs induce significantly less anticholinergic, antihistaminergic and cardiotoxic side-effects than TCAs.\textsuperscript{79} Despite their low adverse effects, SSRIs have been characterized by some side actions including nausea, weight gain and sexual dysfunction, such as decreased sexual desire, erectile difficulties and delayed ejaculation, which is one of the most frequent and persistent SSRI adverse effect.\textsuperscript{80,81} Furthermore, SSRIs have been associated to the serotonin syndrome, characterized by changes in autonomic, neuromotor, and cognitive-behavioral function triggered by increased serotonergic stimulation.

In addition, SSRI increased the risk compared to placebo of suicidal thinking and behavior (suicidality) in children, adolescents, and young adults in short-term studies of major depressive disorder (MDD) and other psychiatric disorders. Anyone considering the use of SSRI antidepressant in a child, adolescent, or young adult must balance this risk with the clinical need. Short-term studies did not show an increase in
the risk of suicidality with antidepressants compared to placebo in adults beyond age 24; there was a reduction in risk with antidepressants compared to placebo in adults aged 65 and older. Depression and certain other psychiatric disorders are themselves associated with increases in the risk of suicide. Patients of all ages who are started on antidepressant therapy should be monitored appropriately and observed closely for clinical worsening, suicidality, or unusual changes in behavior. Families and caregivers should be advised of the need for close observation and communication with the prescriber.  

Anyway, nowadays the SSRIs are considered the mainstay treatment for depressive and anxiety disorders due to the therapeutic effectiveness, a wide therapeutic index, good tolerability, and less toxicity in overdose situations. Six medications (fluoxetine, paroxetine, sertraline, fluvoxamine, citalopram, and escitalopram) are currently available in the SSRI and five of them (excluding fluvoxamine) are used as first-line drug therapy for major depression. They share the same mechanisms of action but differ in severity of adverse effects and drug interactions.
Figure 4. Chemical Structure of the most common SSRIs.
A. Sertraline; B. Fluoxerine; C. Paroxetine; D. Fluvoxamine;
E. Citalopram; F. Escitalopram
Pharmacological properties

Mechanism of action

As their name implies, the SSRIs selectively block serotonin (5HT) reuptake. This occurs through inhibitory actions on the Na⁺/K⁺ adenosine triphosphatase-dependent carrier on presynaptic neurons (see Fig. 5). Among the six available SSRIs, citalopram, escitalopram and paroxetine are the most potent blockers of 5HT reuptake. Some SSRIs have additional antagonist effects on neurotransmitter receptors. For example, paroxetine and citalopram have moderate anticholinergic effects and sertraline blocks presynaptic dopamine receptors. Escitalopram is a stereoisomer of citalopram and has been shown to exert actions at both the primary binding sites for the serotonin transporter, and also on secondary allosteric binding sites, a property not shared by other SSRIs.84 There is also evidence from Position Emission Tomography (PET) using a ligand for the serotonin transporter, that 80% or greater occupancy of the transporter occurs with citalopram, paroxetine, and sertraline at standard doses, and no additional binding occurs at higher doses.85 These findings are based on a small sample of depressed patients, and the SSRIs were not examined across a wide range of doses. The SSRIs, like their predecessors the TCAs, inhibit neurotransmitter reuptake almost immediately, but often take 2–3 weeks to exert clinically meaningful benefit. This has been linked to down regulation of the 5HT1A terminal autoreceptors. In addition, SSRIs, like other antidepressants, stimulate neurogenesis, particularly in the CA3 layer of the hippocampus after 2–3
weeks of exposure. Animal models of depression using stress paradigms show suppression of neurogenesis which is reversed by antidepressants.86

Figure 5. SSRIs antidepressant drugs’ mechanism of action
Metabolism

The SSRIs are generally well absorbed and not affected by food administration, with the exception of sertraline, where food can increase the plasmatic levels of the drug. They are metabolized by hepatic microsomal enzyme that are part of the cytochrome P450 system, particularly the CYP2D6 isoenzyme, although the 2C9, 2C19 and 3A4 isoenzymes are also substrates for several SSRIs (see Tab. 2).

It is important to note that certain SSRIs inhibit their own clearance through the inhibition of their metabolizing enzyme, resulting in elevated plasma levels and increased side effects. Both fluoxetine and paroxetine are strong inhibitors of the CYP2D6 enzyme, and fluvoxamine is a potent inhibitor of CYP1A2, CYP3A4, and CYP2C19. Therefore, caution should be exercised when combining these drugs with other medications that are metabolized through any of these enzymes. The half-life of SSRIs ranges from about 15h (fluvoxamine) to over 60h (fluoxetine), with other agents in the 30'-6 h range. This means that in all cases, SSRIs can be prescribed at least once daily and in the case of fluoxetine, the drug remains capable of causing drug-drug interactions 2-3 weeks after the last dose.
<table>
<thead>
<tr>
<th>Drug</th>
<th>% Bioavailability</th>
<th>Metabolism</th>
<th>Active Metabolites</th>
<th>Half life (in Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citalopram</td>
<td>80</td>
<td>CYP3A4, CYP2C19, CYP2D6</td>
<td>Desmethylicitalopram</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weakly inhibits CYP2D5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escitalopram</td>
<td>80</td>
<td>CYP3A4, CYP2C19, CYP2D5</td>
<td>Demethylascitalopram</td>
<td>27-32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Weakly inhibits CYP2D5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>72</td>
<td>CYP2D5, CYP2C19, Inhibits CYP2D6, 2C9, 2C19</td>
<td>Norfluoxetine</td>
<td>27-72</td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>53</td>
<td>CYP2D6, CYP2C9, Inhibits CYP2D6, 2C9, 1A2, 3A4</td>
<td>None</td>
<td>15</td>
</tr>
<tr>
<td>Paroxetine</td>
<td>50</td>
<td>CYP2D6</td>
<td>None</td>
<td>21-24</td>
</tr>
<tr>
<td>Sertraline</td>
<td>44</td>
<td>CYP3A4, Weakly inhibits CYP2D6, 2C9</td>
<td>Norsertraline</td>
<td>25</td>
</tr>
</tbody>
</table>
Pharmacogenetic of SSRIs antidepressant drugs

Serotonin transporter linked polymorphic region (5-HTTLPR)

The human serotonin transporter (5-HTT), regulates serotonin (5-HT) neurotransmission in the brain by removing it from the intercellular cleft. It is a target of primary interest in the pharmacogenetics of antidepressants because it is the principal site of action of many antidepressant drugs (e.g. SSRIs). Ramamoorthy and co-workers identified and cloned a single gene encoding the human SERT, named SLC6A4, localized to chromosome 17q11.1–q12.88 The gene spans 31 kb and consists of 14 exons (see Fig. 6, panel A).89

[Diagram of the 5-HTTLPR gene and its variants]
In the 1996, Heils reported a polymorphism in the transcriptional control region upstream of the 5-HTT coding sequence. The polymorphism is located approximately 1 kb upstream of the transcription initiation site and consist in a 44bp insertion/deletion involving 2 units in a sequence of 16 repeated elements which lead to a significantly less production of 5-HTT mRNA and consequently less protein (see Fig. 6, panel B). The presence of different alleles could affect 5-HTT expression: the long (“l”) 5-HTTLPR allele has twice the 5-HTT expression in the basal state than the short (“s”) allele. It has been consistently reported that this functional variation influences the antidepressant effect of different classes of antidepressant drugs. Moreover, a growing body of evidence links 5-HTTLPR genotypes to a variety of psychiatric disorders with affective symptomatology (e.g., depression, bipolar disorder, anxiety disorders, eating disorders, substance abuse) and to pathological behaviors and personality traits related to anxiety, impulsivity and stress. A recent meta-analysis confirmed the role of 5-HTTLPR in antidepressant response, showing that patients homozygous for the short allele have a selective and slower improvement of depressive “core” and somatic anxiety symptoms. The short/long genotype was recently found to be associated with an odds ratio (OR) of 2.37 concerning adverse effects during treatment with SSRIs (dermatologic reactions, weight change and fatigue above all), and the homozygous short genotype showed an OR of 1.77. These findings are generally well replicated in studies involving white populations, although opposite or inconsistent findings have also been reported. On the other hand, studies involving Asian populations usually report conflicting results: some studies reported that the short 5-HTTLPR allele was associated with better outcomes, some found no effect of 5-
HTTLPR genotype on treatment efficacy\textsuperscript{117-119} and some reported that the long 5-HTTLPR allele was associated with better outcomes.\textsuperscript{120-125} Interestingly, Lotrich and colleagues\textsuperscript{126} recently reported that paroxetine blood concentration was positively associated with Hamilton Rating Scale for depression (HAM-D) response in a sample of elderly patients, but this association was found to be significant only in carriers of the short allele. In contrast, when augmentation strategies have been investigated, the short allele has been associated with a better response in patients prescribed pindolol or lithium.\textsuperscript{127,128} This finding may be difficult to explain, and to make this challenge even more complex it has been reported that women are more sensitive to mood imbalances after tryptophan depletion if they are homozygous for the short or long allele, with heterozygotes having the most protective genotype.\textsuperscript{129} Finally, the adverse events occurring in newborns of mothers treated with SSRIs had a mixed association with genotype, with those with the heterozygous long genotype being at higher risk of respiratory distress and those with the homozygous short genotype being at higher risk for neuromotor symptoms.\textsuperscript{130} Giraldi and colleagues analyzed the pharmacotherapy of SSRIs in relation to the 5-HTTLPR polymorphism in groups of cancer patients in their terminal phase of illness, and in particular the different response to sertraline, and citalopram.\textsuperscript{131,132} After two weeks of treatment with sertraline, the scores of anxiety of the Hospital Anxiety and Depression Scale (HADS), hopelessness-helplessness and anxious preoccupation of the Mini-Mental Adjustment to Cancer (Mini-MAC) were significantly reduced only in patients homozygous for the “l” allele for which there has been also a significant increase in fighting spirit scores (Mini-MAC).\textsuperscript{131} In the cohort of patients treated with citalopram, there was a statistically significant reduction of depression scores (HADS)
only in patients homozygous for “l” allele, and an increase of fatalism in patients with at least one “s” allele.\(^{132}\)

Regarding the inconsistent findings between white and nonwhite populations, it must be remembered that, compared with Western populations, carrying the long allele is much less frequent in Asian populations: inconsistent results found in Asian populations could be influenced by this event, and further studies with larger samples are needed. Moreover, relevant stratification factors may be strictly genetic: in 2005 Hu and colleagues\(^ {133}\) reported that only carriers of the A allele at the A>G SNP within the long allele of the 5-HTTLPR insertion polymorphism yielded high mRNA levels, whereas carriers of the G allele were similar to carriers of the low-expressing short allele. This finding could partially explain the inconsistent evidence throughout the studies, and it mandates a reconsideration of all the investigations published before the identification of this mutation. A study by Hu and colleagues\(^ {109}\) reported that the low expression alleles (short allele and G variant within the long allele) were one of the strongest risk factors associated with adverse effects from antidepressants. Finally, a genetic oriented pretreatment test based on 5-HTTLPR has been found to be associated with a better clinical outcome.\(^ {134}\)
1.2 Application of pharmacogenetic in medical practice

Due to technological advances and large-scale DNA sequencing projects, pharmacogenetics research has made tremendous progress in recent years, with the identification of numerous inherited variants that influence drug response. As a result, many drug labels have been updated with information about the relevance of pharmacogenetic biomarkers (see Tab. 3).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Marker</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abacavir</td>
<td>HLA-B*5701</td>
<td>Required</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>HLA-B*1502</td>
<td>Required (HAN Chinese)</td>
</tr>
<tr>
<td>Maraviroc</td>
<td>CCR5</td>
<td>Required</td>
</tr>
<tr>
<td>Niotinib</td>
<td>UGT1A1</td>
<td>Required</td>
</tr>
<tr>
<td>Rasburicase</td>
<td>G6PD</td>
<td>Required</td>
</tr>
<tr>
<td>Azathioprine</td>
<td>TPMT</td>
<td>Recommended</td>
</tr>
<tr>
<td>Irinotecan</td>
<td>UGT1A1</td>
<td>Recommended</td>
</tr>
<tr>
<td>Warfarin</td>
<td>CYP2C9/VKORC1</td>
<td>Recommended</td>
</tr>
<tr>
<td>Atomoxetine</td>
<td>CYP2D6</td>
<td>Information only</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>LDL receptor</td>
<td>Information only</td>
</tr>
<tr>
<td>Caecitabine</td>
<td>DPD</td>
<td>Information only</td>
</tr>
<tr>
<td>Capecitabine</td>
<td>DPD</td>
<td>Information only</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>CYP2C9</td>
<td>Information only</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>CYP2C19</td>
<td>Information only</td>
</tr>
<tr>
<td>Codeine</td>
<td>CYP2D6</td>
<td>Information only</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>CYP2D6</td>
<td>Information only</td>
</tr>
<tr>
<td>Isoniazide</td>
<td>NAT</td>
<td>Information only</td>
</tr>
<tr>
<td>Primaquine</td>
<td>G6PD</td>
<td>Information only</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>CYP2D6/CYP2C19</td>
<td>Information only</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>CYP2C19</td>
<td>Information only</td>
</tr>
</tbody>
</table>

Table 3. FDA valid genomic biomarkers. **CYP2D6-C9-C19**, Cytochrome P-450 (isoenzyme D6, C9, C19); **CCR5**, Chemokine C-C motif receptor; **DPD**, dihydropyrimidine dehydrogenase; **G6PD**, glucose-6-phosphate dehydrogenase; **NAT**, N-acetyltransferase; **TPMT**, thiopurine methyltransferase; **UGT1A1**, glucuronosyl transferase 1 family polypeptide 1; **VKORC1**, vitamin K epoxide reductase complex 1.
Moreover, in the field of oncology, genetic alterations are often characteristic for a specific tumor type and allow a molecular characterization of tumors that can provide information regarding disease prognosis, treatment response or new targets for drug development\(^{135}\) (see Tab. 4).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Marker</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anastrozole</td>
<td>ER</td>
<td>Required</td>
</tr>
<tr>
<td>Busulfan</td>
<td>Ph1 chromosome</td>
<td>Required</td>
</tr>
<tr>
<td>Cetuximab</td>
<td>EGFR/KRAS</td>
<td>Required</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>Ph1 chromosome</td>
<td>Required</td>
</tr>
<tr>
<td>Exemestane</td>
<td>ER</td>
<td>Required</td>
</tr>
<tr>
<td>Lapatinib</td>
<td>HER2</td>
<td>Required</td>
</tr>
<tr>
<td>Letrozole</td>
<td>ER</td>
<td>Required</td>
</tr>
<tr>
<td>Panitumumab</td>
<td>EGFR/KRAS</td>
<td>Required</td>
</tr>
<tr>
<td>Tamoxifene</td>
<td>ER</td>
<td>Required</td>
</tr>
<tr>
<td>Transtuzumab</td>
<td>HER2</td>
<td>Required</td>
</tr>
<tr>
<td>Imatinib</td>
<td>c-Kit expression</td>
<td>Required</td>
</tr>
<tr>
<td>Tretinoin</td>
<td>PML/RAR</td>
<td>Recommended</td>
</tr>
</tbody>
</table>

**Table 4. FDA valid genomic biomarkers.** *EGFR,* epidermal growth factor receptor; *ER,* estrogen receptor; *HER-2,* human epidermal growth factor receptor 2; *PhI,* Philadelphia chromosome; *PML/RAR,* promyelocytic leukaemia-retinoic acid receptor.

However, the introduction of pharmacogenetic testing into clinical practice has been relatively slow and there are few pharmacogenetic tests being carried out in the clinic.\(^{136-138}\) Many reasons have been cited for the lack of translation of pharmacogenetic into clinical practice, including the lack of awareness about the utility, the scarcity of trials proving the utility and cost-effectiveness, the lack of clinical testing services, the lack of incentives for diagnostic companies to invest in the development and
licensing of tests, the unclear regulatory framework and concerns regarding costs and the reimbursement system.\textsuperscript{137-142}

**Pharmacoeconomic issue**

As previously mentioned, the existence of finite healthcare budgets drives the need to consider the opportunity cost of decisions about which healthcare interventions to use. Then, there is a need to demonstrate that pharmacogenomic interventions offer added value, in terms of the relative costs and benefits, compared with current practice. Cost effectiveness analysis is the most commonly used method of economic evaluation.\textsuperscript{143} Another factor to consider with respect to the clinical utility and cost-effectiveness of pharmacogenetic testing is the actual cost of obtaining genetic information. These costs have been decreasing at a stunning rate. Only 10 years ago, sequencing of the first complete human genome cost over $3 billion and took years to accomplish. Today, the same can be obtained in less than a week for less than $20000. A few years from now, sequencing a human genome is estimated to cost only $1000. Already, a complete human genome can be sequenced for a few thousand dollars, a cost that is likely to drop further.\textsuperscript{144} Cost-effectiveness studies may thus lose some of their relevance in the near future with the decreasing costs of genetic testing. Furthermore, the increasing availability of direct-to-consumer genetic tests enables patients to obtain information about their genetic background without consulting their physician. This information, once obtained, could be used to optimize therapeutic decisions, irrespective of the evidence regarding cost-effectiveness of pharmacogenetic testing. However, to put this information to optimal use, physicians should be able to rely on
rigorously created evidence-based CPGs that inform health professionals about the relevance of a given genotype in a specific clinical context. Without such guidelines, physicians are left with the information provided by genotyping companies, in which the evidence on which a specific recommendation is based may not always be clear.\textsuperscript{144}

**Educational aspects**

Clinicians tend to ignore the large amount of new pharmacogenetic information and view it as an additional burden and complication of the complex process of therapeutic decision-making. This appears to be largely due to the lack of education on the science and potential of genomics by all parties involved in the medical application of this technology.\textsuperscript{144} This is a major obstacle that has hampered the widespread clinical application of PGx. Education in genetics at the undergraduate, postgraduate, and continuing medical education levels has trailed behind the enormous scientific and technical advances in the field.\textsuperscript{145} In addition to clinicians, this lack of education involves all stakeholders, including: a) other healthcare professionals (including researchers); b) patients and concerned individuals; c) media journalists, who often transmit incorrect information due to their lack of knowledge; d) government-employed regulators and politicians; e) hospital administrators; and f) health insurance executives and decision-makers. The latter are a very important group, since they are the ones who will decide to include pharmacogenetic tests in their coverage. A report issued in 2002 by the Consortium on Pharmacogenetics in the UK stated that: “Perhaps the greatest single factor affecting the penetration of pharmacogenomics into clinical practice and the pace at which it will occur will be the knowledge
and acceptance of physicians. Studies indicate that many physicians lack basic knowledge of genetics and also frequently fail to take into account available information about drugs”\(^\text{146}\). This urgent need was pinpointed by the participants of a recent Pharmacogenomics Education Forum of the International Society of Pharmacogenomics (ISP), who issued a set of recommendations and a Call for Action addressed to Medical, Pharmaceutical and Health Schools Deans of Education\(^\text{147}\). This document urges Deans of Education to incorporate PGx in the core teaching curricula of pharmacology without further delay. This step is vital for ensuring rapid and successful implementation of personalized medicine into medical practice, in pace with the emergence of the latest genomic diagnostics tools, and for the benefit of society at large\(^\text{144}\).

**Need for prospective clinical studies**

Numerous investigators and other specialists in the field have called for prospective clinical studies\(^\text{148-152}\). This is connected to the need for incentives for the pharmaceutical and the diagnostics industry to develop genotyping tools and validate them in the clinical setting via clinical studies. However, it has been pointed out that – unless co-developing a diagnostic to accompany a pharmacogenetic-based drug – pharmaceutical companies have few incentives to sponsor randomized controlled clinical trials of pharmacogenetic-based diagnostics\(^\text{149}\). On the other hand, the diagnostics industry has great interest in developing new pharmacogenetic-based diagnostic tests, but often has insufficient resources to sponsor major clinical trials and is not accustomed to testing the value of its products using randomized clinical research. Overall, it appears that governments should act soon. A thorough analysis of the
types of studies required and related problems has recently been published, to which the interested reader is referred.\textsuperscript{149} As stated by Gardiner and Begg: “In addition to clinical studies, formal pharmacoeconomic studies need to be performed whenever a strong evidence-based case is made for pharmacogenetic testing. This is valuable from a population perspective when there are limited funds available for health care expenditure. However, it is also recognized that the business model may intervene, with aggressive marketing, e.g., of genetic tests encouraging clinical uptake before the evidence supports use of the test. From a “best evidence” perspective, it would be useful for pharmaco economists to define standards for conducting such studies that are both feasible and readily comprehensible”.\textsuperscript{150} Furthermore, the advancement of clinical pharmacogenetic creates an urgent need for the establishment of a solid and clearly defined regulatory framework.
1.3 Application of nanotechnology in medical practice

Nanotechnology is the creation and utilization of materials, devices, and systems through the control of matter on the nanometer scale, i.e., at the level of atoms, molecules, and supramolecular structures. Applications of nanotechnology in the screening, diagnosis, and treatment of disease are collectively referred as ‘nanomedicine’. Key issues of nanomedicine include miniaturization of devices, novel nanosized materials, chip-based technologies, imaging techniques, drug delivery, new analytical tools that could quickly lead to a better understanding of initiation and progression of disease as well as to the personalization of drug’s therapy. A handful of nanomaterials are being studied in clinical trials or have already been approved by the FDA for use in humans (see Fig. 7), and many proof-of-concept studies of nanomaterials in cell-culture and small-animal models for medical applications are under way.
Many of these nanoproducts are designed to target tumors in vivo and are intended for use either as drug carriers for therapeutic applications or as contrast agents for diagnostic imaging. Furthermore, application of nanomaterials is as a label for measuring molecules of interest in biologic samples. Nanomaterials are used to either simplify the readout or amplify the detection threshold of the diagnostic device. Important nanotechnological applications for personalized medicine are now considered for the development of targeted drug formulations that achieve maximum efficacy and optimal safety profiles. For example, through conjugation of nanovehicles with an antibody, the guided accumulation of the carrying drug in its target antigen located in diseased
specific tissue (e.g., cancer) can be directed efficiently.\textsuperscript{156,157} Furthermore, such therapeutic advantages of nanovehicles are also used for imaging applications and theranostic approaches, i.e., for systems and strategies in which both disease diagnosis and therapy are combined to benefit personalized medicine.\textsuperscript{158} At the same time, specific nanomaterials, including nanotubes, dendrimers, liposomes, and quantum dots, are being developed as molecular diagnostic probes to target in vivo specific tissues or cells, thus implementing imaging techniques and improving clinical outcomes.\textsuperscript{159}

\textbf{Nanoengineered devices}

Particle-based systems for drug delivery are limited in a large part by the amount of drug that can be loaded on a single particle and, although there are several so-called multifunctional particles, their ability to control drug release is based on a single actuation such as dissolution of the particle. There is also a growing need for more intelligent systems that can carry a large payload of active molecules and that can release specific amounts in response to pulsatile stimuli over a longer period of time. Although such systems are larger in overall size than nanoparticles, the fact that nanoscale architecture is used to achieve some of the desired properties makes such systems fall into the general class of nanotechnology.\textsuperscript{160} Also in this class of nanotechnology are devices that use nanoscale architecture to confer certain desirable surface characteristics. Such nanofabricated devices may be prepared either by a bottom-up approach, that is, by assembling single molecules into a nanostructure, or by a top-down approach, that is, etching away parts of a macromaterial to yield the desired nanoarchitecture. Such surface
characteristics can influence the adhesion of bacteria and subsequent biofilm formation\textsuperscript{161} or may influence the adhesion of surrounding cells in the case of tissue implants or tissue engineering scaffolds.\textsuperscript{162,163}

**Biosensors**

There is currently much interest in miniaturizing diagnostic devices to improve the portability of such systems and also to reduce the cost of such devices. Portable systems capable of providing accurate estimates of traditional blood parameters and antigen levels with a single drop of blood represent just one potential arena in which a small-scale sensor may find application.\textsuperscript{160} Such ‘biosensor’-based devices would be a major advance in medical technology for use in remote locations with limited access to medical laboratories in both civilian and military situations. Toward this end, nanofabrication methods are currently being employed to design devices that are capable of serving as highly sensitive biosensors both in vitro and in vivo.\textsuperscript{160} Although this technology is still not as advanced in its clinical application as the particulate systems, it nevertheless holds much promise for the future. The current challenges in the development of such systems are associated with detection sensitivity. Several different approaches are being explored that include novel detection modalities or innovative use of biomolecules. In general, biosensors consist of a substrate material to which capture molecules are attached. The capture molecules are usually proteins or DNA molecules. Once a biomarker is captured, it must then produce a directly detectable change in the physical properties of the device or might have to be subject to some development process to visualize the capture. Electrochemical detection appears to be the most common means of detecting such
changes. The simplest approach to such a device is the immobilization of a capture molecule such as a protein on the surface of an electrode. Biomarker binding to the capture protein produces a detectable change in the electrochemical signal measured at the electrode. This approach is, however, subject to signal loss due to protein inactivation by the surface of the electrode.\textsuperscript{160} Nanotechnology-based approaches have been used to prevent surface–protein interaction-induced denaturing of the protein and improve sensor performance. For example, DNA dendrimers have been used to improve sensor performance in such a device.\textsuperscript{164} Limits of detection were improved by three orders of magnitude over a device without the DNA dendrimer interface in detecting salivary protein and mRNA markers.\textsuperscript{164} A similar approach has been applied to a glucose sensor using gold nanorods to immobilize glucose oxidase on cellulose acetate film\textsuperscript{165} and for DNA detection using polyaniline nanofibers and carbon nanotubes.\textsuperscript{166} As an alternative to electrochemical detection methods, surface plasmon resonance appears to be promising and has recently been reported to be sensitive enough for label-free single molecule detection.\textsuperscript{167,168} Also of note is the use of nanostructured devices to capture whole cells. A three-dimensional nanostructured support based on the use of silicon nanopillars generated by etching was modified with an epithelial cell adhesion molecule antibody.\textsuperscript{169} The resulting device was shown to be highly efficient at capturing circulating cancer cells from whole blood samples.\textsuperscript{169} Applied to the quantification of these cells in patient samples, such systems can offer an additional diagnostic parameter in cancer management.\textsuperscript{169} Continued improvements in the design and reliability are expected to make it possible to combine such sensors with drug reservoirs and microfluidic technology to create
systems that can be directly actuated in vivo by the binding of biomarkers.

**Role of Nanobiotechnology in Personalized Medicine**

Personalized medicine simply means the prescription of specific therapeutics best suited for an individual. It is usually based on pharmacogenetics, pharmacogenomics and pharmacoproteomics, but other individual variations in patients are also taken into consideration. Apart from refinements in molecular diagnostics, an important basis of personalized medicine, nanobiotechnology also helps in the discovery of biomarkers that are crucial for the development of personalized medicine. A good example of the application of nanobiotechnology for personalized medicine is that of cancer: variation in the behavior of cancer of the same histological type from one patient to another is also taken into consideration. Personalization of cancer therapies is based on a better understanding of the disease at the molecular level and nanotechnology will play an important role in this area.

Applications of nanobiotechnology are beginning to show an impact on the practice of conventional medicine. Promoted by the National Institutes of Health of the United States, nanomedical research is providing easy access to innovative nanodevices and nanosystems based on the rational design and precise integration of functional nanomaterials for the further development of clinical nanomedicine. Nanotechnology will enable design and delivery of more effective drugs with targeted delivery increasing efficacy and reducing toxicity. Although considerable progress has been made in identifying the molecular components of the mitochondrial machinery, no effective treatment for diseases caused by
mitochondrial dysfunction have been developed. An impediment to manipulating mitochondria within living cells is their limited accessibility to direct physical, biochemical and pharmacological approaches. Advances in nanotechnology are providing new tools that have the potential for the diagnosis and therapy of mitochondrial disorders.\textsuperscript{172} Nanotechnology provides the basis of computer-controlled molecular tools that are much smaller than a human cell and built with the accuracy and precision of drug molecules. Such tools will be used for interventions in a refined and controlled manner at the cellular and molecular levels.\textsuperscript{153} They could remove obstructions in the circulatory system, kill cancer cells, or take over the function of subcellular organelles. Instead of transplanting artificial hearts, a surgeon of the future would be transplanting artificial mitochondria. Refinements in nanodiagnostics will enable routine detection of single particles of viruses or bacteria in minuscule samples. Nanotechnology will also provide devices to examine tissue in minute detail. Biosensors that are smaller than a cell would give us an inside look at cellular function. Tissues could be analyzed down to the molecular level, giving a completely detailed ‘snapshot’ of cellular, subcellular and molecular activities. Such a detailed diagnosis would guide the appropriate treatment. Although several nanomedicine-related applications of nanobiotechnology are in development or nearing commercialization, they face the usual regulatory approval hurdles encountered in the introduction of other innovative technologies and products. Judging from the progress and the increasing interest in this area during the past decade, further positive developments are predicted in nanomedicine in the next decade.\textsuperscript{153} For example, gold nanoparticles are used in high throughput genomic detection devices without the need for polymerase-chain-reaction (PCR) amplification but with a sensitivity
similar to that of PCR-based assays (see Fig. 8). This technology has been approved by the FDA for genetic screening to determine drug sensitivity and to detect genetic mutations but it is not still approved for employment in European countries.

**Figure 8 The Verigene System®** Nanomaterials such as gold nanoparticles can be coated with biorecognition molecules to target either a patient’s DNA or a protein sample. Here, gold nanoparticles are coated with a complementary oligonucleotide (single-stranded DNA) that recognizes the variant gene sequence captured on a surface. Once nanoparticles are bound to the surface, the signal is amplified by means of a silver nitrate reduction reaction. This technique has been reported to have sensitivity equivalent to that of the polymerase-chain-reaction assay for genetic analysis.
Chapter II

Aim of the research
Warfarin is the most widely prescribed oral anticoagulant in North America and Europe.\textsuperscript{174} Despite the availability of the international normalized ratio (INR), a laboratory test that is universally used to measure the anticoagulant effect of warfarin, serious adverse responses, including hemorrhagic and thromboembolic events, continue to complicate therapy, making warfarin one of the drugs most often responsible for emergency room visits.\textsuperscript{174} The relationship between the dose of warfarin prescribed and the individual response is regulated by genetic and environmental factors that can influence the absorption of warfarin, its pharmacokinetics, and pharmacodynamics. Since the Food and Drug Administration revised the label for warfarin to note the importance of VKORC1 and CYP2C9 polymorphisms,\textsuperscript{175} several groups have proposed genotype-guided maintenance dose algorithms that incorporate both genetics and demographic parameters, such as age, weight, and body surface area.\textsuperscript{73,176}

Selective Serotonin Reuptake Inhibitors (SSRIs) antidepressant drugs are the mainstay treatment for the depressive and anxiety disorders. A large number of studies report the association between the 5-HTTLPR genetic polymorphism of the serotonin transporter and the different response to treatment with the antidepressant drugs belonging to the class of the SSRI. It is known that the antidepressant effect exert by the SSRIs is more effective and occurs more rapidly\textsuperscript{99,177,84} in individuals homozygous for the L allele, which confers a high functional activity of the serotonin transporter.\textsuperscript{96,97,121} Furthermore, subjects who carrying at least one copy of the S allele, which confers a reduced functional activity of the serotonin transporter, are at increased risk of no remission of depressive symptoms when treated with SSRIs\textsuperscript{178,83} and they have a higher incidence of adverse effects following the treatment.\textsuperscript{179}
Evidence-based medicine applies guidelines developed on the basis of consensus from randomized clinical trials (RCTs). These RCTs are usually performed in carefully selected patient-population studied under strongly regulated conditions. It often is a challenge to translate the results of the RCTs to a generic cohort of patients in the real world.\textsuperscript{180} Since that considerations, the aims of this thesis were:

- To provide evidence in a translational perspective of the feasibility of the application in the community care of pharmacogenetic tests for the routine use of oral anticoagulant therapy with warfarin and Selective Serotonin Reuptake Inhibitors (SSRI) antidepressant drugs in oncology and psychiatry;
- To evaluate the genetic polymorphisms of CYP2C9 and VKORC1 and to develop and evaluate algorithms based on these genetic information;
- To evaluate the genetic polymorphism of the Serotonin Transporter (SERT) for the assessment of the SSRIs antidepressant response in oncology and psychiatry;
- To assess the applicability in community care of the pharmacogenetic of oral anticoagulant and antidepressant drugs in the perspective of the development of nanotechnological devices.
Chapter III
Materials and methods
Chapter III - Materials and methods

3.1 Protocols of the studies and patients recruitment

The studies described were conducted in accordance with the principles laid down in the Helsinki Declaration and the International Guidance for Good Clinical Practice. Written informed consent was obtained from all subjects prior to study enrollment. The research protocols for the studies were approved by the Ethical Committee of the Azienda per i Servizi Sanitari n.1 Triestina (ASS1).

- **Warfarin**

The study was a retrospective and observational investigation including patients treated with warfarin who referred to the Centre for the Cardiovascular disease (CCV) and to the Distretto n.1 of the ASS1 of the National Health System.

Inclusion criteria:

- Subjects either male or female between the age of xx and yy;
- INR stability, defined as 6 consecutive INR determination between the therapeutic range (+/- 0.2 INR units);
- Subjects provide written informed consent.

Exclusion criteria:

- Subjects with an inadequate compliance to the oral anticoagulant therapy;
- Period of treatment less than 1 month;
Subjects do not provide written informed consent.

Subjects who met the inclusion criteria provide a biological sample obtained from oral mucosa by means of standard brush. Finally, the study included 101 patients.

SSRIs antidepressant drugs

Oncological patients

The subjects initially included in this study were 46 consecutive patients, 25 men (54%) and 21 women (46%), who were admitted to the hospice of the Azienda per i Servizi Sanitari 6, S. Vito al Tagliamento (Pordenone, Italy). At admission and recruitment, all patients had a diagnosis of cancer and their tumors were located in the lung (9 patients), breast (6 patients), prostate (5 patients), colon (5 patients), ovary (4 patients), pancreas (3 patients), brain (2 patients), kidney (2 patients), stomach (2 patients), biliary tract (1 patient), tongue (1 patient), 4 patients were leukemic and one tumor had an unreported origin. Eleven patients (61%) were treated with chemotherapy, 5 (27%) with radiotherapy, and 9 (50%) with surgery; 15 patients (83%) had metastases. Each patient was carefully evaluated at admission by the palliative care team by means of the Hospital Anxiety and Depression Scale (HADS) and the Mini Mental Adjustment to Cancer (Mini-MAC), and those who met the criteria for a depressive disorder according to DSM-IV and who were clinically judged to potentially benefit from antidepressant treatment were treated with escitalopram. Eighteen patients (10 men and 8 women) completed a period of 2 weeks’ treatment.
Psychiatric patients

The study was a retrospective, observational investigation including a sample of subjects who have been diagnosed a depressive mood disorder according to the criteria of the ICD-10 (International Classification of Diseases 10 - chapter V, F32 depressive episode or F33 recurrent depressive disorder). Subjects were identified through the database of Siasi Web-DSM in collaboration with the Department of Mental Health (DSM) of the Azienda per i Servizi Sanitari No.1 "Triestina" (ASS1) belonging to the National Health System. The database including socio-demographic and clinical characteristics of patients taken into care at the four Mental Health Centres (CSM) in Trieste.

Inclusion criteria:

- Subjects either male or female between the age of 20 and 85;
- Subjects not previously taken into care by the DSM of ASS1;
- Subjects who have received the first contact with the CSM of Maddalena or Via Gambini with an initial diagnosis of F32 and F33;
- Subjects who received a prescription for an antidepressant drugs.

Subjects were psychometrically evaluated by means of the Montgomery-Asberg Depression Rating Scale and a biological sample was obtained from oral mucosa by rubbing the buccal wall with a standard brush. Finally, 132 subjects have been identified, however the number was significantly reduced due to several factors such as death, change of residence address or the not expression of the consent for the
participation in the study. At the end, this study considered 43 patients recruited from October, 2010 to May, 2011.
3.2 Methods

3.2.1 DNA extraction

Biological samples were obtained from oral mucosa cells by means of standard brushes (MasterAmp™ buccal swab brushes, Epicentre Biotechnologies). Genomic DNA was isolated from the biological samples using a commercial kit (GenElute™ Blood genomic DNA kit; Sigma-Aldrich Co). Cells are lysed with a chaotropic salt-containing buffer to ensure denaturation of macromolecules. DNA is bound to the spin column membrane and the remaining lysate is removed by centrifugation. A filtration column is used to remove cell debris. After washing to remove contaminants, the DNA is eluted with buffer into a collection tube. The purified DNA was frozen at -20°C until the genotyping.

3.2.2 Single Nucleotide Polymorphisms detection

- Polymerase Chain Reaction (PCR)

Genotyping of the allelic variations in the 5-HTTLPR has been carried out by standard polymerase chain reaction (PCR) amplification (see Fig. 9) using the primers described by Gelernter\textsuperscript{181}, and with the GC-rich PCR System (Roche Molecular Biomedicals) in a 50-µL reaction containing 20-100 ng of DNA, 100 µm deoxyribonucleoside triphosphate (dNTPs), 20 pmol for each primer, and 1.5 mM MgCl\textsubscript{2}. DNA was denatured at 95°C for 10 minutes and subjected to 40 cycles of 40 seconds of denaturation at 94°C, 45 seconds of annealing at 56°C, 40 seconds of extension at 72°C, and 10 minutes of final extension at 72°C. The PCR
amplification products were separated on a 2% agarose gel and visualized in ultraviolet light after Gel Red™ staining.

Figure 9. Schematic overview of PCR cycles
**Real-Time PCR-TaqMan® assay**

The discrimination of the allelic variants within the VKOC1 gene have been performed on an Applied Biosystems HT7900 real-time PCR (Applied Biosystems, Foster City, CA) with commercially available and validated Applied Biosystems TaqMan® SNP Genotyping Assays for VKORC1 −1639 G>A (rs9923231). TaqMan® probes consist of a fluorophore attached to the 5’-end of the oligonucleotide probe and a quencher at the 3’-end (see Fig. 10).

![Schematic overview of TaqMan® probe](image)

*Figure 10. Schematic overview of TaqMan® probe*
The quencher molecule quenches the fluorescence emitted by the fluorophore when excited by the cycler’s light source. As long as the fluorophore and the quencher are in proximity, quenching inhibits any fluorescence signals (see Fig. 10). TaqMan® probes are designed such that they anneal within a DNA region amplified by a specific set of primers. As the Taq polymerase extends the primer and synthesizes the nascent strand, the 5’ to 3’ exonuclease activity of the polymerase degrades the probe that has annealed to the template. Degradation of the probe releases the fluorophore from it and breaks the close proximity to the quencher, thus relieving the quenching effect and allowing fluorescence of the fluorophore. Hence, fluorescence detected in the real-time PCR thermal cycler is directly proportional to the fluorophore released and the amount of DNA template present in the PCR.

- **Pyrosequencing**

Genotyping of CYP2C9 *2 and *3 alleles was performed by PCR followed by a pyrosequencing analysis of the amplified DNA. Pyrosequencing technique is usually suited for sequencing of shorter fragments as compared to the Sanger sequencing method. The principle (see Fig. 11) is based on the extension of single nucleotides using the polymerase reaction and the release of pyrophosphate (PPI).
In brief, a sequencing primer is annealed to a single-strand PCR fragment and deoxynucleotide triphosphates (dNTPs) are added in a user predefined order. If the nucleotide is complementary to the base on the DNA strand, the released PPI is converted to ATP by ATP-sulfurylase in the presence of adenosine 5′ phosphosulfate (APS). The ATP generated will be used by luciferase to convert luciferin to oxyluciferin during which light is produced. The light created in the reaction is proportional to the amount of incorporated nucleotide and detected by a charge coupled device (CCD) camera. Unincorporated dNTPs and ATP are degraded between each cycle by apyrase. The result is displayed in a
pyrogram and the height of each peak is used for the determination of the sequence (see Fig. 11).

### 3.3 Statystical analysis

The analysis of the clinical, socio-demographic and psychometric characteristics was conducted by means of the use of SPSS software version 15.0 (SPSS Inc., Chicago, IL, USA). Results are presented as mean±SEM unless stated otherwise. A p value of less than 0.05 was taken as statistically significant.
Chapter IV

Results and discussion
4.1 Pharmacogenetic of warfarin

4.1.1 Clinical, demographic and genetics characteristics of the patients

The first part of this study was focused on the enrolment of the patients from the Center for cardiovascolar disease (CCV) and the distretto 1 both of the Azienda per i Servizi Sanitari n.1 Triestina of the National Health System. 101 patients who met the criteria have been identified for the inclusion in the study. Their demographic and clinical characteristics are summarized in Table 5. Out of the 101 subjects included, 56 (55.4%) were male and 45 (44.6%) female with a mean age of 69.55±0.98. The main indications for anticoagulation with warfarin were atrial fibrillation or flutter (78.4%), cardiac valve replacement (16.7%), pulmonary embolism (2.9%) and deep vein thrombosis (2.0%). The mean dose administered was 28.77±1.47 mg/wk. Genotyping has been conducted in order to identify the allelic variants of the gene coding CYP2C9 and VKORC1. The allelic frequencies of CYP2C9 and VKORC1 are reported in the table 5. The observed genotype frequencies showed no deviation from Hardy-Weinberg equilibrium.
4.1.2 Association between CYP2C9 and VKORC1 allelic variants and warfarin dose

In order to identify the role of genetic in the variability of warfarin, the dose required was analyzed in relation to the allelic variants of each gene considered. The mean warfarin weakly dose requirement was 36.86±3.46 mg in patients with the VKORC1 (-1639) GG genotype, which was significantly higher (p=.000) than that in the GA (26.96±1.44 mg) and AA (19.58±2.21 mg) genotype patients. The results extend the known association between CYP2C9 genotype and warfarin dose. There were significant differences in mean dose requirements between each of the variant alleles compared with the wild type. The mean warfarin weakly dose requirement was 32.82±2.20 mg/wk in CYP2C9 homozygous wild-
type patients, which was significantly higher (p=.005) than that in *1/*2 (28.69±2.22 mg), *1/*3 (25.16±3.52 mg), *2/*2 (43.75±27.5 mg), and *2/*3 patients (13.40±2.42 mg). Distributions of dose within the different genotypes are illustrated in Table 6 and 7. The data were analyzed by means of the parametric test Analysis of the Variace (ANOVA).

Table 6. Effect of VKORC1 G-1639A variants on warfarin dose

<table>
<thead>
<tr>
<th>VKORC1</th>
<th>N</th>
<th>Weekly warfarin dose, mg (mean ± SEM)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>G/G</td>
<td>36</td>
<td>36.86±3.46</td>
<td></td>
</tr>
<tr>
<td>G/A</td>
<td>54</td>
<td>26.96±1.44</td>
<td>.000</td>
</tr>
<tr>
<td>A/A</td>
<td>13</td>
<td>19.58±2.21</td>
<td></td>
</tr>
</tbody>
</table>

Table 7. Effect of CYP2C9 variants on warfarin dose

<table>
<thead>
<tr>
<th>CYP2C9 Haplotype</th>
<th>N</th>
<th>Weekly warfarin dose, mg (mean ± SEM)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>*1/*1</td>
<td>50</td>
<td>32.82±2.20</td>
<td></td>
</tr>
<tr>
<td>*1/*2</td>
<td>25</td>
<td>28.69±2.22</td>
<td></td>
</tr>
<tr>
<td>*1/*3</td>
<td>19</td>
<td>25.16±3.52</td>
<td>.005</td>
</tr>
<tr>
<td>*2/*2</td>
<td>3</td>
<td>43.75±27.5</td>
<td></td>
</tr>
<tr>
<td>*2/*3</td>
<td>6</td>
<td>13.40±2.42</td>
<td></td>
</tr>
</tbody>
</table>
Moreover, combined effect of both VKORC1 and CYP2C9 variants on the dose has been examined. Table 8 and Figure 12 reported the distribution of warfarin dose among the possible combination of genotypes. Among variants, the highest dose is required by patients carrying the wild-type alleles both for VKORC1 and CYP2C9.

### Table 8. Combined effect of VKORC1 and CYP2C9 variants and warfarin dose [mean±SEM (mg/wk)]

<table>
<thead>
<tr>
<th>VKORC1</th>
<th>CYP2C9</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>*1/*1</td>
<td>*1/*2</td>
<td>*1/*3</td>
<td>*2/*2</td>
<td>*2/*3</td>
</tr>
<tr>
<td>G/G</td>
<td>42.52±5.92</td>
<td>36.92±4.12</td>
<td>28.51±5.96</td>
<td>/</td>
<td>16.25±8.84</td>
</tr>
<tr>
<td></td>
<td>(N=13)</td>
<td>(N=9)</td>
<td>(N=11)</td>
<td></td>
<td>(N=2)</td>
</tr>
<tr>
<td>G/A</td>
<td>31.11±1.73</td>
<td>25.38±2.19</td>
<td>22.67±4.56</td>
<td>16.25±0.00</td>
<td>13.06±5.44</td>
</tr>
<tr>
<td></td>
<td>(N=30)</td>
<td>(N=13)</td>
<td>(N=7)</td>
<td>(N=1)</td>
<td>(N=3)</td>
</tr>
<tr>
<td>A/A</td>
<td>20.96±3.22</td>
<td>21.60±3.85</td>
<td>17.50±0.00</td>
<td>/</td>
<td>8.75±0.00</td>
</tr>
<tr>
<td></td>
<td>(N=7)</td>
<td>(N=3)</td>
<td>(N=1)</td>
<td></td>
<td>(N=1)</td>
</tr>
</tbody>
</table>

Figure 12. Distribution of the warfarin dose in relation to VKORC1 and CYP2C9
4.1.3 Association between clinical and genetic data with warfarin dose

An ordinary stepwise linear regression method incorporates both genetic and clinical data was used to develop a pharmacogenetic algorithm for the prediction of warfarin dose. The best regression dose model is reported in the Table 9. As reported in the table, the multivariate regression model including the variables weight, smoking habits, VKORC1 and CYP2C9 genotype produced the best model for estimating warfarin dose, having the largest $R^2$ value (0.50). Among clinical characteristics, smoking habits appears to be the major determinants of the dose required by patients.

<table>
<thead>
<tr>
<th>Table 9. Regression equation for modeling warfarin dose requirements based on clinical and genetic characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variables in the equation</td>
</tr>
<tr>
<td>CYP2C9 *2/*3</td>
</tr>
<tr>
<td>smoking habits</td>
</tr>
<tr>
<td>weight</td>
</tr>
<tr>
<td>VKORC1 G/A</td>
</tr>
<tr>
<td>VKORC1 A/A</td>
</tr>
<tr>
<td>CYP2C9 *1/*3</td>
</tr>
</tbody>
</table>

Weekly Warfarin Dose [mg/wk]; $I$ (statement)= 1 if statement is true, =0 if statement is false.
4.2 Pharmacogenetic of SSRIs

Oncological patients.

4.2.1 Clinical, demographic and genetics characteristics of the patients

Eighteen patients (10 men an were admitted to the hospice of the Azienda per i Servizi Sanitari 6, S. Vito al Tagliamento (Pordenone, Italy) and completed a period of 2 weeks’ treatment; their demographic, clinical and genetic characteristics are reported in the table 10. Out of the 18 patients, 10 were male (55.6%) and 8 female (44.4%) with a mean age of 71.6±2.01. At admission and recruitment, all patients had a diagnosis of cancer and their tumors were located in the lung (2 patients), breast (4 patients), prostate (2 patients), colon (2 patients), ovary (2 patients), brain (2 patients), kidney (2 patients), tongue (1 patient), and one tumor had an unreported origin. Eleven patients (61%) were treated with chemotherapy, 5 (27%) with radiotherapy, and 10 (55.6%) with surgery; 15 patients (83%) had metastases. Out of the 18 patients considered, 11 (61%) were found to carry at least one “s” allele, and 7 (39%) were homozygous for the “l/l” alleles.
4.2.2 Association between response to treatment with escitalopram and 5-HTTLPR allelic variants

After 2 weeks of treatment with escitalopram, a significant reduction was observed in anxiety scores of HADS and in anxious preoccupation and hopelessness-helplessness scores of the Mini-MAC (Table 11). The data were analyzed by means of the non-parametric test of Wilcoxon.
When the 5-HTTLPR polymorphism was considered, HADS anxiety scores were significantly decreased only in patients carrying the “s/s” and “s/l” variants, whereas those with “l/l” genotypes displayed a significant reduction of Mini-MAC anxious preoccupation (Table 12); no significant difference was found for the remaining subscales. The data were analyzed by means of the non-parametric test of Wilcoxon.

### Table 11. Effect of treatment with escitalopram

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Treatment (days)</th>
<th>Score (mean±SEM)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>HADS Anxiety</td>
<td>18</td>
<td>0</td>
<td>8.2±0.89</td>
<td>.006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>5.9±0.92</td>
<td></td>
</tr>
<tr>
<td>Mini-MAC Anxious preoccupation</td>
<td>18</td>
<td>0</td>
<td>19.3±0.92</td>
<td>.007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>16.3±0.94</td>
<td></td>
</tr>
<tr>
<td>Hopelessness-helplessness</td>
<td>18</td>
<td>0</td>
<td>22.8±0.92</td>
<td>.017</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>20.3±1.18</td>
<td></td>
</tr>
</tbody>
</table>

### Table 12. Response to treatment with escitalopram and SERT genotype

<table>
<thead>
<tr>
<th>Allelic variant</th>
<th>N</th>
<th>Treatment (days)</th>
<th>Score (mean±SEM)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>HADS Anxiety</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S/S, S/L</td>
<td>11</td>
<td>0</td>
<td>7.8±0.97</td>
<td>.024</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>5.7±0.89</td>
<td></td>
</tr>
<tr>
<td>L/L</td>
<td>7</td>
<td>0</td>
<td>8.8±0.64</td>
<td>.128</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>6.3±0.89</td>
<td></td>
</tr>
<tr>
<td>Mini-MAC Anxious preoccupation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S/S, S/L</td>
<td>11</td>
<td>0</td>
<td>18.3±0.99</td>
<td>.094</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>16.0±1.23</td>
<td></td>
</tr>
<tr>
<td>L/L</td>
<td>7</td>
<td>0</td>
<td>21.0±0.73</td>
<td>.018</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>16.7±0.38</td>
<td></td>
</tr>
</tbody>
</table>
Psychiatric patients

4.2.3 Clinical, demographic and genetics characteristics of the patients

Table 13 summarizes the demographic and clinical characteristics of the 43 subjects included in the study. The statistical analysis found no significant differences between subjects in relation to the two CSM (chi-square test). Subjects were aged between 20 and 85 years, with an mean age of 57.40±2.00. Out of the 34 subjects enrolled by the CSM of Via Gambini, 19 (55.9%) had received a diagnosis of depressive episode (F32) and 15 (44.1%) of recurrent depressive disorder (F33). Moreover, 15 (44.1%) were aged between 20 and 55 and 19 (55.9%) between 56 and 85 years. 26 patients (76.5%) were females and 8 (23.5%) were male. About the subjects identified by the CSM of Maddalena, 4 patients (44.4%) had received a diagnosis of F32 and 5 (55.6%) F33. Three subjects aged between 20 and 55 years (33.3%) and 6 (66.7%) aged between 56 and 85 years. Moreover, 8 (88.9%) were females, and only 1 (11.1%) was male.
The allelic frequencies of 5-HTTLPR are reported in the table 14. The observed genotype frequencies showed no deviation from Hardy-Weinberg equilibrium (Chi-square=0.22, df=1, p=0.636). 32 patients were carriers of at least one copy of the “s” allele, and 11 subjects were homozygous for the “l” allele.

Moreover, allelic variants were analyzed also in relation to initial diagnosis. No significant differences have been observed in the distribution of 5-HTTLPR genotypes among diagnosis (Chi-square=0.612, df=1, p=0.433).
df=1, p=434). The distribution of the allelic variants are reported in the table 15.

<table>
<thead>
<tr>
<th>Diagnosis (ICD-10 code)</th>
<th>Patients, n (%)</th>
<th>Allelic variant, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S/S; S/L</td>
</tr>
<tr>
<td>Depressive episode (F32)</td>
<td>23 (53.5)</td>
<td>16 (69.6)</td>
</tr>
<tr>
<td>Recurrent depressive disorder (F33)</td>
<td>20 (46.5)</td>
<td>16 (80.0)</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>32</td>
</tr>
</tbody>
</table>

Out of 23 patients who have received a diagnosis of depressive episode (F32), 16 (69.6%) carried at least one copy of the “s” allele, whereas 7 (30.4%) were homozygous for the “l” allele. Considering the subjects who were diagnosed with a recurrent depressive disorder (F33), out of the 20 considered, 16 (80.0%) were carriers of low functional activities variants of 5-HTTLPR (S/S or S/L) and 4 (20.0%) were homozygous for the “l” allele.
4.2.4 Association between 5-HTTLPR allelic variants and depression

To assess the influence of 5-HTTLPR on the severity of the depression, the scores of MADRS scale were analyzed in relation to allelic variants of the SERT. The data were analyzed by means of the non-parametric test of Mann-Whitney.

<table>
<thead>
<tr>
<th>Allelic variant</th>
<th>N (%)</th>
<th>Mean±SEM</th>
<th>Median (Min-Max)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>S/S; S/L</td>
<td>32 (77.1%)</td>
<td>18.08±2.12</td>
<td>17.00 (0-53)</td>
<td>.098</td>
</tr>
<tr>
<td>L/L</td>
<td>11 (22.9%)</td>
<td>12.00±2.54</td>
<td>13.00 (0-25)</td>
<td></td>
</tr>
</tbody>
</table>

The results (Table 16) show that subjects with genotype S/S and S/L have higher scores, with a slightly significant difference than those who are homozygous for the “l” allele (18.08±2.12 vs 12.00±2.54, p=0.098). Such difference indicates a greater severity of depressive disorder for patients who carrying at least one copy of the “s” allele.
Chapter V

Conclusions
5.1 Pharmacogenetic of warfarin

Warfarin therapy, particularly during the initiation period, is associated with a high incidence of overanticoagulation with resultant bleeding, as the inability to take into account interindividual and intraindividual variability in response to the drug makes accurate dose predication impossible. This is frequently a cause for concern. It has been estimated that almost half of patients with atrial fibrillation who are eligible for, and would benefit from, warfarin therapy are not receiving the drug because of the associated risks and monitoring costs. In this point of view, the contribution of CYP2C9, VKORC1 and other clinical and demographic factors has been investigated in patients in the contest of the community medicine. The analysis of the dose of warfarin in relation to each genetic factors confirmed that there were significant differences in mean dose requirements between each of the variant alleles compared with the wild type both for CYP2C9 and VKORC1 also in a cohort of patients from the community medicine. Moreover, this has been confirmed also when genetic, clinical and demographic factors have been analyzed in order to define the major determinant of warfarin dose and to identify an algorithm for the prediction of the dose. The results indicate the role in the determination of the warfarin dose of the allelic variants considered jointly with the weight of patients and their smoking status. These results appear to support the need of the pharmacogenetic characterization of the patients at the initiation of the warfarin therapy also in the general practice and in community medicine.
5.2 Pharmacogenetic of SSRIs

Oncological patients

Depressive mood disorders show an increasing incidence and prevalence in the general population and are extensively treated with antidepressants, in particular SSRIs. Difficulties in the mental adaptation to cancer are common in patients with advanced disease and may significantly and negatively affect quality of life; studies on the effects of treatment with SSRI antidepressants in these patients are scarce. In the present study, treatment with escitalopram significantly attenuated anxiety as identified using HADS and improved the mental adaptation to cancer by reducing anxious preoccupation and hopelessness-helplessness as determined with Mini-MAC. The results obtained also indicate that the effects of escitalopram on anxiety and anxious preoccupation depended on 5-HTTLPR genetic polymorphism. In fact, anxiety was significantly and markedly reduced only in carriers of at least one “s” allele, whereas a significant and marked reduction of anxious preoccupation occurred only in homozygotes for the “l” allele. The characterization of the patients’ genetic polymorphism of the serotonin transporter 5-HTTLPR thus appears to contribute significantly to the therapeutic response to SSRIs, indicating that pharmacogenetics can be usefully integrated in the palliative care for the treatment of mood disorders and difficulties in the mental adaptation to cancer in patients with advanced disease.
Psychiatric patients

Response to antidepressant therapy is often incomplete with approximately 30–40% not responding at all to the first antidepressant given and about 60–70% not achieving remission. Moreover, antidepressant treatment is characterized by a delayed time of onset of the clinical improvement and by a variety of adverse effects. Recent literature shown that in subjects with difficulties to adaptation to stressful life events, the development of depressive symptoms are related to the polymorphism in the gene coding for the serotonin transporter (SERT) with a specific Gene x Environment interaction.

In this study, the response to antidepressant treatment was analyzed in relation to the allelic variants of 5-HTTLPR genetic polymorphism of SERT. 43 subjects suffering from depressive disorders and previously treated with an antidepressant were genotyped for 5-HTTLPR and psychological evaluated by Montgomery-Asberg Depression Rating Scale (MADRS) to detect the severity of depression. The results indicate that the incidence of depressive mood disorder is greater in subjects carrying at least on copy of the “s” allele (S/L and S/S) and these patients have higher scores, with a marginally significant difference, compared to those who are homozygous for “l” allele.

The data obtained appear to be of interest because they refer to mental distress and its treatment in the field of community medicine, which is poorly represented in the literature. Moreover, these results seem to encourage future research aiming to consider a larger sample, in the perspective of a personalized intervention in the clinical practice of mood disorders.
General conclusions

- The results obtained for the warfarin pharmacogenetic in community care are in general agreement with those reported by the majority of the trials, which are performed in carefully selected patient population studied under strongly controlled conditions, and support the inclusion of genetic data in the algorithms used for choosing the initial dose of warfarin;

- The pharmacogenetic test of 5-HTTLPR has been evaluated in community care on oncological patients with advanced cancer and in psychiatric patients treated with antidepressants; 5-HTTLPR appears to be predictive of the response to treatment with SSRIs also in the community care;

- The pharmacoeconomic profile of these pharmacogenetic approaches appear to be favorable, for the limited cost of these tests in relation to the benefit reached with the optimization of the response and with the reduction of the adverse effects;

- These conclusions are in favor of the development of nanotechnological devices useful for the implementation of pharmacogenetic tests in general practice and community care.
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