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Contents

General Introduction 1

Drug response is a complex trait that shows significant interpatient variability. It has been suggested that response rates to major therapeutic classes of drugs range from 25 to 60 percent [1]. To a certain extent this variability may be explained by genetic variation. The concept of interindividual differences in drug response was proposed as early as 1909 [2]. However, current clinical practice hardly considers genetic variation a relevant factor during the processes of drug prescribing and dispensing. Pharmacogenetics is the study of variations in DNA sequence as related to drug response [3]. The ultimate goal of pharmacogenetics is to predict and thereby improve drug response in the individual patient.

After the completion of the Human Genome Project in 2003, genomics has become a mainstay of biomedical research and pharmacogenetics has been forecasted to be one of the first clinical applications arising from the new knowledge [4]. Indeed, the research efforts in the field of pharmacogenetics expressed as the number of publications listed on PubMed have steadily increased until leveling out in 2009 at 1100-1200 publication per year (Figure 1.1) [5].

By contrast, the clinical use of pharmacogenetic testing did not meet the initial high expectations and has lagged considerably behind, despite the significant body of evidence supporting its usefulness. As a result of the unmet promises many clinicians have become somewhat disillusioned regarding pharmacogenetics in recent years. Indeed, expectations of the effect of a single polymorphism on drug response were unrealistically high [6]. Still, pharmacogenetics holds the promise of advancing drug therapy.

The aim of this thesis is to identify the reasons for the slow clinical translation of pharmacogenetics and to explore and expand possible solutions to address these obstacles.

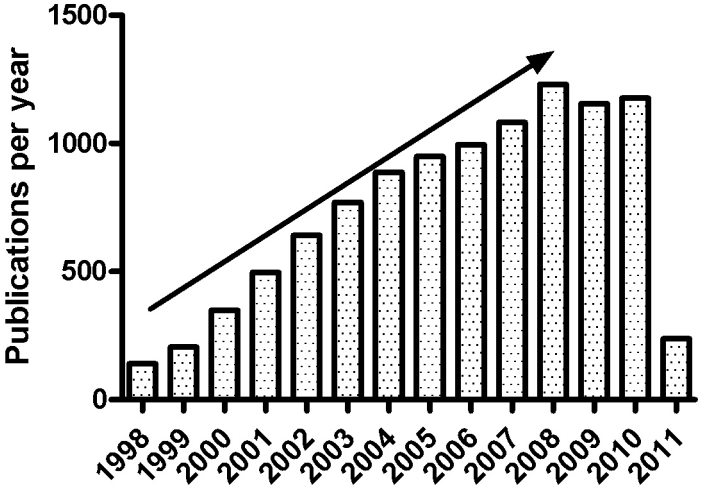

Figure 1.1 Hits on PubMed using the search string "pharmacogenetics OR pharmacogenomics".

Basic principles of pharmacogenetics

A gene is a part of the DNA that codes for a type of protein or for a RNA chain that has a specific function in the organism. There are two alleles per autosomal gene (one paternal and one maternal) with one allele on each of the two chromosomes of a chromosome pair [7]. Together the two alleles form the genotype. Heterozygotes have two different alleles, and homozygotes have two of the same alleles. Genetic variation can consist of deletions, insertions, inversions, and copy number variation [8]. Most sequence variations are single nucleotide polymorphisms (SNPs), a single DNA base pair substitution that may result in a different gene product. As a result of this genetic variation many genes have multiple variants. The most common allele in a population is referred to as the wild type. Some of the variant alleles code for non-functional or decreased functional proteins. Allele frequencies can vary greatly in different ethnic populations. Phenotype refers to the trait resulting from the protein product encoded by the gene.

Outline of the thesis

This thesis is divided into four parts. The first part aims at identifying obstacles and possible solutions for the clinical implementation of pharmacogenetics. In the second part, issues related to the quality control of pharmacogenetic testing are discussed. In the third part the influence of genetic variation on the response to sulfonylureas (SUs), a class of commonly used oral antidiabetic drugs used in the treatment of Type 2 diabetes mellitus (T2DM) patients, is used as a case model to investigate the possibilities for pharmacogenetics in primary care. The fourth part contains the general discussion and summary.

In **Chapter 2** possible obstacles for the clinical implementation of pharmacogenetics are investigated and solutions to overcome these obstacles are identified. In the next chapter one of the identified solutions, the development of clinical guidelines to aid the use of pharmacogenetic tests, is investigated in detail (**Chapter 3**). **Chapter 4** describes the results of a pilot experiment to investigate the technical feasibility of pharmacogenetic screening in primary care. In this chapter also the potential impact of the pharmacogenetic guidelines described in **Chapter 3** is investigated.

The application of pharmacogenetics in clinical practice may result in the adjustment of treatment of individual patients. Therefore, genotyping of patients in a routine clinical setting requires robust and reliable genotyping methods and good quality control is of great importance. **Chapter 5** discusses the use of plasmid-derived samples as quality controls. A second issue related to quality control of pharmacogenetic tests is the exclusion of SNPs because of poor genotyping. Several studies have reported difficulties in genotyping rs757210, a SNP in the gene coding for hepatocyte nuclear factor 1β. **Chapter 6** describes our experiments to find alternative methods to genotype this SNP.

The third part of this thesis is devoted to the investigation of the influence of genetic variation on the response to SUs. SUs are part of the mainstay of treatment with oral antidiabetic drugs. We selected SU treatment as a case model to investigate the potential role of pharmacogenetics in primary care for three reasons. First, most T2DM patients are treated in primary care. Secondly, there is significant interpatient variability in response to SUs, with approximately 10-20% of the patients experiencing primary failure. Thirdly, SUs are metabolized by the polymorphic enzyme CYP2C9. This enzyme also plays an important role in the metabolism of many other drugs frequently used in primary care. **Chapter 7** describes the application of the classic candidate gene approach to investigate the effect of SNPs in *CYP2C9* on the response to SUs. In **Chapter 8** a different approach is applied. In 2007, multiple T2DM risk alleles have been identified from genome-wide association studies. From the identified T2DM risk alleles a panel of 20 consistently replicated SNPs appears of which the majority has been associated with the process of insulin release from the pancreatic beta-cells. We hypothesized that this panel of 20 SNPs not only confers to an increased risk for T2DM but also influences response to SU treatment. Finally the results from the presented studies are put into perspective and a future outlook is described in **Chapter 9**.

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Clinical Implementation of Pharmacogenetics in Primary Care

2

Translating Pharmacogenomics: Challenges on the Road to the Clinic

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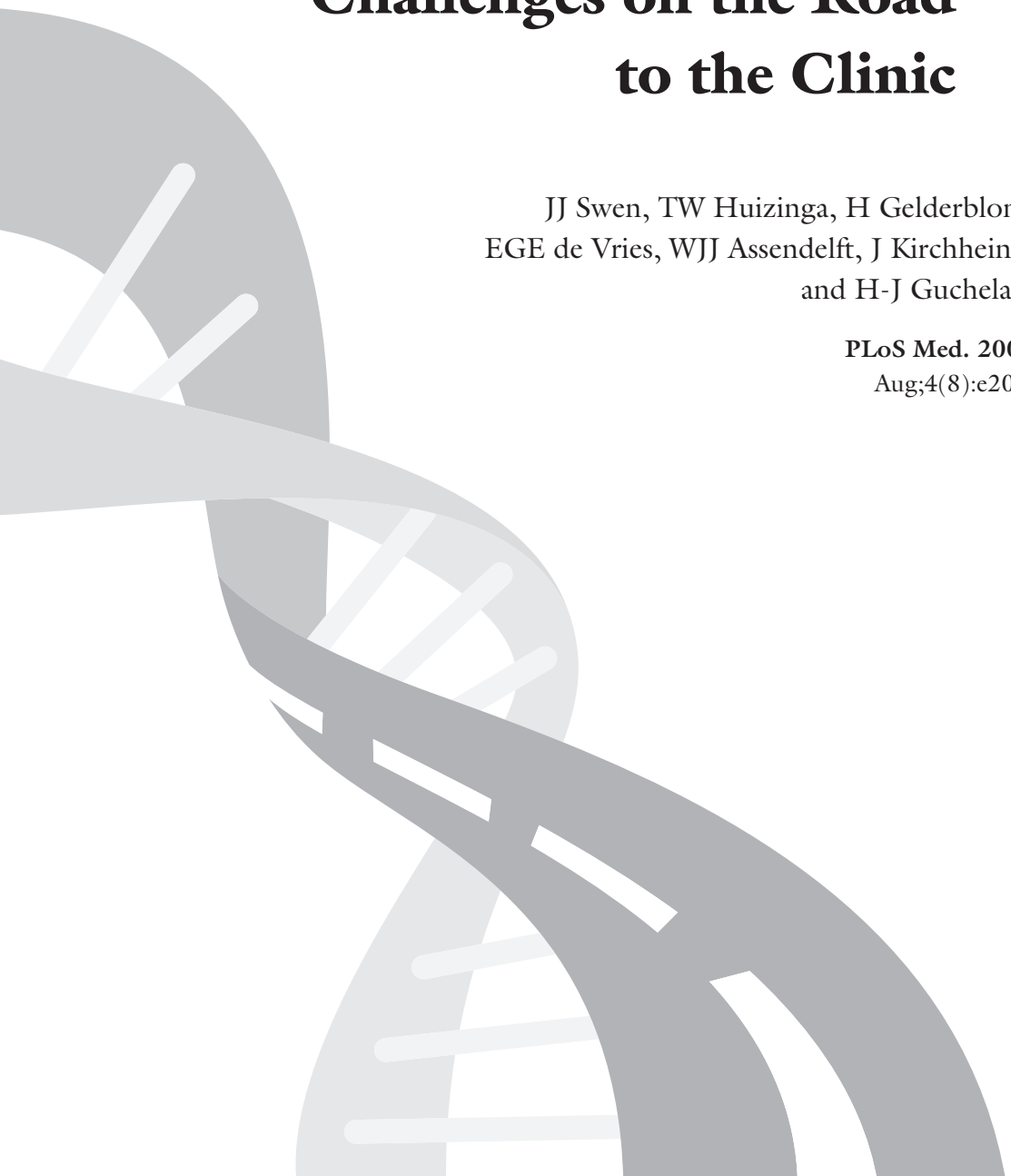

ABSTRACT

Pharmacogenomics is one of the first clinical applications of the postgenomic era. It promises personalized medicine rather than the established "one size fits all" approach to drugs and dosages. The expected reduction in trial and error should ultimately lead to more efficient and safer drug therapy. In recent years, commercially available pharmacogenomic tests have been approved by the Food and Drug Administration (FDA), but their application in patient care remains very limited. More generally, the implementation of pharmacogenomics in routine clinical practice presents significant challenges. This article presents specific clinical examples of such challenges and discusses how obstacles to implementation of pharmacogenomic testing can be addressed.

INTRODUCTION

In 2003 the International Human Genome Sequencing Consortium declared that the Human Genome Project had been completed, raising expectations of clinical application in the near future. Pharmacogenomics (PGx) (here used synonymously with pharmacogenetics [Box 2.1]), promising the end of "one size fits all" drugs and of trial and error in pharmacotherapy, is often predicted to be one of the first such applications [1].

The concept of interindividual differences in drug response was proposed as early as 1909 by Garrod in his book *The Inborn Errors of Metabolism* [2]. Today, the concept of PGx, namely that variation in drug response is related to genetic variation, is widely recognized. Two commercially available PGx tests that support the personalization of drug treatment have already received FDA approval. The tests detect variations in the genes coding for enzymes involved in drug metabolism: cytochrome P450 CYP2C19 and CYP2D6 (Roche AmpliChip, http://www.roche.com/), and UDP-glucuronosyltransferase (Invader UGT1AI Molecular Assay; Third Wave Technologies, http://www.twt.com/). Examples of these and other PGx tests actually being used in patient care are sparse, however. Recent surveys in Germany and Australia reported that only a small number of laboratories offer PGx testing for clinical use [3,4]. Current and potential future uses of PGx tests are summarized in Table 2.1.

This article focuses on challenges in the translation of PGx to clinical practice. Six challenges associated with consecutive phases in the translation process are discussed (Figure 2.1). Each of the identified challenges is exemplified by situations from clinical practice, and possible approaches to overcome them are discussed.

BOX 2.1

A matter of definitions

In many publications the terms pharmacogenetics (PGt) and pharmacogenomics (PGx) are used interchangeably while others distinguish between the two concepts [54–56]. We prefer to use the single term PGx with the following definition: "the individualization of drug therapy through medication selection or dose adjustment based upon direct (e.g., genotyping) or indirect (e.g., phenotyping) assessment of a person's genetic constitution for drug response." This definition includes tests operating at protein, metabolite, or other biomarker levels whenever these factors are affected by genetic variation (i.e., single nucleotide polymorphisms, insertions, deletions, microsatellites, variance in copy number, etc). Both germline (i.e., heritable mutations) as well as somatic mutations (i.e., nonheritable mutations in, for example, tumor specimens) are considered. Therefore, immunohistochemical tests such as that for HER2/neu are considered a PGx test in the context of this article.

Players in the field

In the challenges presented in Figure 2.1, several "players" can be identified [5], including the biotechnology and analytical industry, the pharmaceutical industry, research institutions, funding agencies, regulatory agencies, clinicians, and patients. These players each have substantial roles, both individually and in collaboration, in developing and implementing clinical applications of PGx.

Table 2.1 Use of PGx in clinical practice

Current	Future
Primarily diagnostic; retrospective	Prevention of toxicity and treatment optimization; prospective
Specific test in individual	Population-wide screening
Focus on adverse drug events	Focus on therapy selection

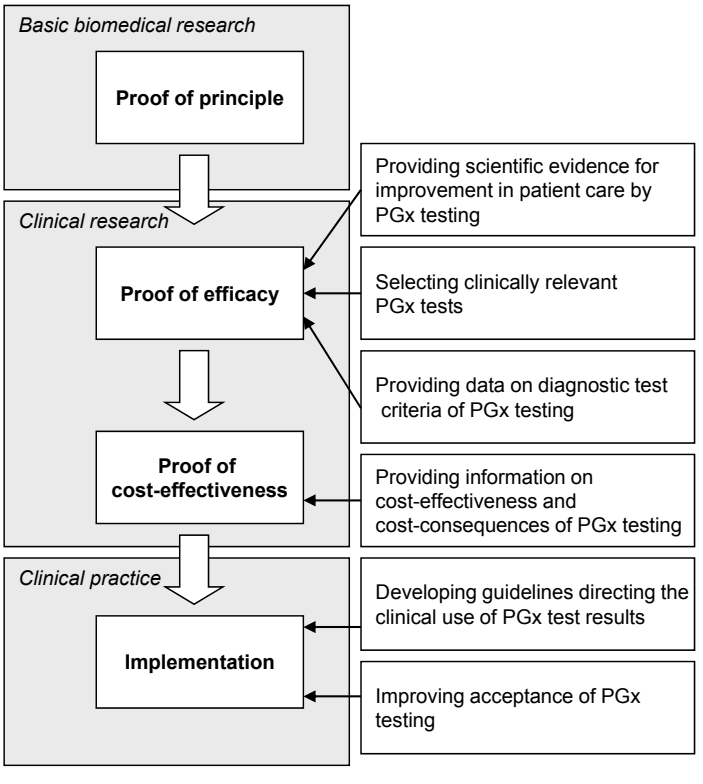

Figure 2.1 Consecutive phases and associated challenges on the road to clinical implementation of pharmacogenomics.

As an early step in this process, the biotechnology and analytical industry must develop fast, reliable, and affordable assays for routine PGx measurement. The reaction of the pharmaceutical industry to the concept of PGx has been reserved, possibly because of the potential for market segmentation and an end to the era of blockbuster drugs (Box 2.2) [5]. Nonetheless, a 2001 report stated that by applying genomics technologies, the investments to develop a drug could be reduced by as much as $300 million and two years [6]. Further, the influence of the pharmaceutical industry on the translation of PGx to the clinic, although considerable, should not be overestimated. Manufacturers can be expected to pursue development of PGx tests only for new compounds and not for drugs already marketed. The latter would most likely be of interest to research institutions, for example academic medical centers.

Indeed, most of our PGx knowledge comes from clinical studies initiated by research institutions. The importance of adequately designed original studies on associations between genetic variation and clinical drug response needs to be recognized by funding agencies, including health insurers and governmental agencies [7]. In recent years, many

BOX 2.2

PGx need not be financially unattractive from a drug manufacturer's point of view

The potentially smaller market for a drug could be compensated by (1) an increased rate of adoption of the drug; (2) the identification of patients who otherwise would not have been candidates for the drug; (3) increased compliance with improved efficacy; and (4) the possibility of premium pricing [57]. This process can be illustrated with preliminary calculations of the use of the tumor necrosis factor alpha-blocking drug adalimumab used in the treatment of rheumatoid arthritis.

The prevalence of rheumatoid arthritis in adults in The Netherlands is 1%, resulting in approximately 160,000 potential users of adalimumab. The estimated cost for the treatment of all these patients with adalimumab during one year is about €1,900,000,000. To limit the costs, the use of adalimumab has been restricted to treatment of patients with moderate to severe rheumatoid arthritis failing to respond on disease-modifying antirheumatic drugs or methotrexate. As a result, only 3,440 patients, or 2.15% of the potential 160,000, used the drug in 2005. When a certain PGx test enables predicting the response to adalimumab, there would be no legitimate reason to withhold the drug from the predicted responders; and if the prevalence of the responsive genotype were to exceed 2.15% in the rheumatoid arthritis patient population the revenues of the manufacturer would increase.

projects have been funded, and even prospective studies on dose recommendations are now being performed. In addition, these agencies will have to be convinced to reimburse routine PGx testing, which will require extensive information on cost-effectiveness and cost-consequences of PGx testing.

Regulatory agencies, such as the European Agency for the Evaluation of Medicinal Products and the FDA could play a role by recommending or requiring PGx testing for certain drugs, which would obviously provide a strong stimulus. In 2004 and 2005 the FDA approved label changes of 6-mercaptopurine and irinotecan to include PGx information; recommendations for other drugs, such as warfarin, may follow [8,9]. In the case of irinotecan, however, results not fully supporting the dose adjustment included in the label change have been reported [10]. To date, mandatory testing is mentioned only in the package insert of trastuzumab [11]. The FDA has issued a guidance for industry on the subject of PGx and is encouraging voluntary data submission [12]. More recently the FDA and the European Agency for the Evaluation of Medicinal Products have issued a joint procedure for the voluntary submission of PGx data [13].

Following the increase of evidence of clinical relevance and number of available tests, physicians and clinical pharmacists need to become informed about the usefulness and also the limitations of PGx tests in patient care. Patients and patient advocacy groups also can have significant influence on PGx implementation.

CHALLENGES FOR IMPLEMENTATION OF PGx

Providing scientific evidence for improvement in patient care by PGx testing

On 16 August 2006, a search we did of the medical literature with the MeSH term "pharmacogenetics" on PubMed resulted in 3,347 hits, of which 1,487 —almost 45%— were review articles. The relative paucity of original research articles is not the only problem. Many original articles involve a small, specific study population, administration of single doses, use of healthy volunteers instead of patients, or use of a different translation from genotype to phenotype. Moreover, most positive association studies lack validation of findings in an independent patient population.

A classic application of PGx, often used as an example of its potential clinical consequences, involves the variable effect of the antidepressant nortriptyline (NT) due to differences in the gene encoding cytochrome P450 family member CYP2D6. The plasma levels of NT may vary almost 10-fold depending on the number of functional *CYP2D6* alleles. However, the scientific literature reveals a lack of solid evidence that, in the case of NT, the *CYP2D6* polymorphisms actually lead to significant clinical consequences, such as increased toxicity or decreased drug efficacy.

The Pharmacogenetics Working Party of the Royal Dutch Society for the Advancement of Pharmacy is working to implement PGx into their automated medication control database, which is to be used in computerized physician and pharmacist order entry systems (http://farmacogenetica.knmp.nl/). Table 2.2 summarizes their recently conducted systematic literature search for evidence to define NT dose recommendations for different *CYP2D6* genotype-predicted phenotypes (search terms available upon request).

Only nine scientific articles concerning the interaction between *CYP2D6* and NT, encompassing a total study population of 193 participants, could be retrieved. Among these participants there were only 15 poor metabolizers and 12 ultrarapid metabolizers (UM). Furthermore, the studies frequently were single-dose experiments with healthy volunteers or were limited to specific populations, such as Korean inhabitants or geriatric patients. Most study end points were pharmacokinetic, confirming that *CYP2D6* genotype has an impact on NT pharmacokinetics. However, no drug efficacy or toxicity data were reported. Therefore, even for what is considered a classic example of PGx, solid scientific evidence for clinical relevance is still lacking. In a recent article Kirchheiner et al. [14] provide an overview of how better-designed studies are needed for the clinical breakthrough of PGx and how this breakthrough could be realized by a more systematic inclusion of PGx in drug development.

Selecting clinically relevant PGx tests

Research in the field of PGx should be focused on the development of diagnostic tests for clinically important problems. Not every association study leads to a potentially useful PGx test, and financial and technical resources may be wasted if the relevance of more readily measurable values is not excluded first [15]. For example, the 5-hydroxytryptamine 3 receptor antagonists used to prevent nausea and vomiting are known to be metabolized by CYP2D6. Kim et al. showed genotype-dependent pharmacokinetics in healthy volunteers for tropisetron [16], suggesting a hypothesis that cancer patients who are UM are undertreated by a standard dose of tropisetron. This hypothesis was studied by Kaiser and colleagues in 270 cancer patients. Patients with a high number of functional *CYP2D6* alleles experienced more nausea and episodes of vomiting [17]. A similar result was found in patients receiving 4 mg of ondansetron to prevent postoperative nausea and vomiting [18]. These findings clearly show the influence of UM phenotype on both pharmacokinetics and clinical effectiveness of 5-hydroxytryptamine 3 receptor antagonists. However, due to the low prevalence of UM genotype in people of northern European descent, the "number needed to genotype" (i.e., the number of patients needed to genotype in order to prevent one patient from unnecessary nausea and vomiting) appeared to be 50. This number is probably too high to implement this PGx test into routine clinical practice and, more importantly, easier methods such as dose titration or the use of an alternative antiemetic regimen are already available to prevent

Table 2.2 Evidence for Nortriptyline dose adjustments based on pharmacogenetics

Population	Dose (mg/d)	Single (S) or multiple dose (M)	End point	Outcome	Reference
Single patient	150	M	Clinical	Develops plasma concentration of 0.471 mg/ml, dry mouth, constipation, and dizziness	[61]
36 geriatric patients	Titrated to C_{ss} of 0.050–0.150 mg/ml	M	Kinetic	Dose corrected C_{ss} (IM + PM) was 2.2 times C_{ss} (EM) Average IM + PM dose was 30% lower than EM A correlation between the number of alleles encoding decreased metabolism, C_{ss}, dose, and dose corrected C_{ss}, Effect co medication not clear	[62]
Ten healthy native Korean volunteers	25	S	Kinetic	No significant difference in C_{max}, t_{max}, $t_{1/2}$, AUC for NT or 10-OH-NT between homo- and heterozygous	[63]
41 Japanese patients	15–120	M	Kinetic	Dose-corrected C_{ss} WT/mut was 1.4 times C_{ss} WT/WT Dose-corrected C_{ss} mut/mut was 2.1 times C_{ss} WT/WT Dose-corrected C_{ss} mut/mut E-10-OH-NT was 0.66 times C_{ss} WT/WT	[64]
15 healthy Chinese volunteers	25	S	Kinetic	No significant difference in $t_{1/2}$, AUC for NT between homo- and heterozygous EM IM: $t_{1/2}$ and AUC of NT were raised 1.8 and 2.2 times, respectively, compared to EM IM: $t_{1/2}$ of 10-OH-NT was 1.9 times $t_{1/2}$ EM	[65]
21 white patients	150	M	Kinetic	IM: Dose corrected C_{ss} of 10-OH-NT was 0.7 times C_{ss} EM PM: Dose corrected C_{ss} of NT was 2.5 times C_{ss} EM PM: Dose corrected C_{ss} of 10-OH-NT was 0.9 times C_{ss} EM	[66]
Eight patients with adverse drug reaction	10–100	M	Clinical	44% were carriers of ≥1 mutant allele compared to 21% in 56 control psychiatric patients Co-medication unknown	[67]

21 healthy white volunteers	25–50	S	Kinetic	IM: $t_{1/2}$ and AUC NT were raised 2.3 and 2.8 times respectively compared to EM IM: $t_{1/2}$ of 10-OH-NT was 1.9 times $t_{1/2}$ EM PM: $t_{1/2}$ and AUC NT were raised 2.6 and 3.3 times, respectively, compared to EM PM: $t_{1/2}$ of 10-OH-NT was 2.4 times $t_{1/2}$ EM UM (three alleles): $t_{1/2}$ and AUC NT were raised 0.9 and 0.76 times, respectively, compared to EM UM (three alleles): $t_{1/2}$ of 10-OH-NT was 0.82 times $t_{1/2}$ EM UM (13 alleles): $t_{1/2}$ and AUC NT were raised 0.9 and 0.20 times, respectively, compared to EM UM (three alleles): $t_{1/2}$ of 10-OH-NT was 0.4 times $t_{1/2}$ EM	[68]
20 healthy volunteers and 20 patients	25–150	Both	Kinetic	IM: Cl, $t_{1/2}$, F, NT were raised 0.8, 1.2, 1.2 times, respectively, compared to EM PM: Cl, $t_{1/2}$, F, NT were raised 0.6, 1.8, 1.4 times, respectively, compared to EM UM: Cl, $t_{1/2}$, F, NT were raised 1.3, 0.9, 0.8 times, respectively, compared to EM Co-medication unknown	[69]

10-OH-NT, 10-hydroxynortriptyline; AUC, area under the plasma drug concentration-time curve; Cl, clearance; C_{ss}, steady state plasma concentration; EM, extensive metabolizer; F, bioavailability; IM, intermediate metabolizer; mut, mutant allele; NT, nortriptyline; PM, poor metabolizer; $t_{1/2}$, elimination half-life; UM, ultrarapid metabolizer; WT, wild type.

nausea [19]. PGx studies should be encouraged in fields where the likelihood of a clinically relevant effect is high and its potential usefulness is evident in clinical practice (Table 2.3).

Providing data on diagnostic test criteria of PGx testing

To be clinically useful, a PGx test must predict the outcome of drug treatment. Complex pathways are involved in the action and metabolism of most drugs, and nongenetic influences also contribute to drug response [15]. Therefore, PGx testing for single polymorphisms may account for only part of the variability in drug response. The diagnostic test criteria sensitivity, specificity, and predictive value are applicable to tests for which response is determined as a dichotomous variable. However, drug response cannot always be considered an all-or-none phenomenon. In these situations the relative contribution of the genotype to the variability in response (the percentage explained variance, R^2) provides additional information. Diagnostic test criteria of PGx tests are not commonly reported, but are important for clinical implementation. Table 2.4 summarizes the characteristics of selected PGx tests.

It can be observed that the diagnostic test criteria for PGx tests are comparable to those of clinically available non-PGx tests (also shown in Table 2.4). Thus, while some consider current PGx tests as having inadequate value for clinical application, tests with comparable diagnostic test criteria are currently being used in patient care. The need for well-defined PGx test criteria has been previously discussed [20,21]. We maintain that demonstration of potential clinical usefulness requires the reporting of diagnostic test criteria in PGx association studies.

Providing information on cost-effectiveness and cost-consequences of PGx testing

Although funding agencies including health insurers have funded many PGx research projects in recent years, their willingness to reimburse routine PGx testing will require information on cost-effectiveness and cost-consequences. In 2004, Phillips performed a

Table 2.3 High likelihood of clinical relevance of PGx test

Drug characteristics
Narrow therapeutic index (i.e., high chance of toxicity)
Difficulty predicting response or adverse effect
Large interindividual variability in response
Consistent PK-PD relationship
Long-term treatment

Table 2.4 Comparison of diagnostic test criteria of a selection of PGx tests and non-PGx tests used in clinical practice

Test category	Biomarker	Form	Associated effect	*N*	Sensitivity	Specificity	PPV	NPV	R^2	Ref
PGx tests	*CYP2C9*3* polymorphism	SNPs	Risk of bleeding complication	185	0.17	0.94	0.40	0.82	NA	[39]
	Carrier of a *CYP2C9* and *VKORC1* polymorphism	SNPs	Acenocoumarol-induced overanticoagulation (INR>6)	226	0.48[a]	0.81[a]	0.20[a]	0.94[a]	39.1	[70]
	5-lipoxygenase (*Alox5*) genotype	Tandem repeat	Response to leukotriene antagonist ABT761	221	1[a]	0.17[a]	0.52[a]	1[a]	NA	[71]
	UGT1A1-3156AA genotype	SNP	Grade 4 neutropenia and irinotecan in whites	66	0.50	0.96	0.60	0.95	24	[72]
	β1 receptor Arg389Arg genotype	SNPs	Reduction in daytime diastolic blood pressure	40	0.78[a,b]	0.82[a,b]	0.78[a,b]	0.82[a,b]	15.8	[73]
	*HLA-B*5701* genotype	SNPs	Hypersensitivity to abacavir in whites	1,821	0.46–0.94	0.90–0.98	0.19–0.81	0.97–0.99	NA	[74]
Non-PGx tests used in clinical practice	Rheumatoid factor positivity		Radiologic progression	110	0.84	0.54	0.77[a]	0.75[a]	11	[75]
	Prostate specific antigen (> 4.0 ng/ml)		Prostate cancer	284	0.68–0.75	0.6–0.71	0.51–0.54	0.73–0.87	NA	[76]
	Troponin T (> 0.1 ng/ml)		Acute myocardial infarction	773	0.94[a]	0.89[a]	0.36[a]	1[a]	NA	[77]
	Borrelia burgdorferi antigen		Lyme disease	43	0.77	0.83-1	0.19-1[c]	0.99[c]	NA	[78]

[a]Calculated from reported results.
[b]Response defined as ≥10% reduction in daytime diastolic blood pressure from baseline.
[c]Calculated with a positive serum prevalence of 5%.
N, number of study participants; NA, not applicable; NAT, N-acetyltransferase 2; NPV, negative predictive value; PPV, positive predictive value; R^2, percentage explained variance; SNP, single nucleotide polymorphism; UGT1A1, UDP-glucuronosyltransferase.

systematic literature review on cost-effectiveness of PGx testing [22]. Only 11 published true cost-effectiveness analyses (CEAs) could be retrieved. Seven studies found a PGx-based strategy to be cost-effective, two showed equivocal results, and two concluded that a PGx-based strategy was not cost-effective. Despite the publication of additional CEAs of PGx, there is a need for more information [23–26]. The performance of such CEAs is problematic for two reasons. First, there are limited data on the rate at which PGx testing actually prevents adverse drug reactions. Second, PGx test prices are dropping continuously. Even without data from a comprehensive CEA, some simple calculations can be made and preliminary conclusions can be drawn on potential cost-effectiveness of PGx testing (Box 2.3).

The example in Box 2.3 indicates that screening for dihydropyrimidine dehydrogenase (DPD) deficiency in all 5-fluorouracil (5FU)-treated patients is not cost-effective, mainly due to the low incidence of DPD deficiency and the high cost of the phenotypic assay.

BOX 2.3

Estimated potential cost-effectiveness of DPD screening

The cytotoxic drug 5FU is widely used, for example in colorectal cancer. Severe neutropenia is associated with deficiency of the enzyme DPD, which metabolizes 5FU [58]. The deficiency of DPD is thought to be caused by germline mutations in the gene encoding DPD.

A possible strategy would be to test all 5FU-treated patients, and we estimate the cost consequences for the Dutch situation as follows. About 7,000 patients per year are treated with 5FU. A phenotypic test measuring DPD activity in peripheral mononuclear cells is available, and normal values for enzyme activity in both wild-type and heterozygotes are known, but are relatively difficult to distinguish. The incidence of DPD deficiency is about 3% and, therefore, 210 patients of the 7,000 5FU-treated patients may be detected by this test [59].

In a meta-analysis on 5FU-related toxicity it was reported that the incidence of 5FU-related death is about 0.5%, and in 50% of the cases toxicity was explained by deficiency of the enzyme DPD [60]. The cost of the DPD assay is €850, which would result in an estimated cost of nearly €6 million to test all 7,000 patients for DPD status. This testing would save 17 patients per year, at a cost of €350,000 per saved life, which may be unrealistically high. Moreover, even then, 17 other patients will die from 5FU-related toxicity anyway, because their toxicity is not related to DPD deficiency. Although this example is evaluated in a Dutch setting the data and conclusion can be applied to other settings.

It might become cost-effective if the cost of the assay decreases. Circumstances that favor the cost-effectiveness of PGx testing include high prevalence of the relevant allelic variant in the target population, good correlation between genotype and phenotype, satisfactory diagnostic test criteria, phenotype associated with significant morbidity or mortality if left untreated, and significant reduction in adverse drug reactions reduction by PGx testing [27].

Although the necessity of CEAs for every new clinical technique is debatable, and several innovations have found their way to application without proof of their cost-effectiveness [28,29], more research on the cost-effectiveness and cost-consequences of PGx testing will nonetheless stimulate its further implementation into clinical practice.

Developing guidelines directing the clinical use of PGx test results

PGx studies published to date usually report that carriers of a specified genotype in a particular patient population have an increased likelihood of a desired (or undesired) outcome of drug treatment. Such studies have not, however, resulted in the distillation of practical prescribing recommendations based on genotype. In particular, very little data are available on effective and safe dose adjustment for the different metabolizer phenotypes, although a 2001 consensus paper on deriving CYP2D6 phenotype-related dose recommendations for antidepressants from pharmacokinetic study data represents an early step [30]. Coumarins used in the treatment and prevention of venous and thromboembolic disorders constitute one case in which the application of dose recommendations is relatively far advanced. Coumarins (e.g., warfarin, phenprocoumon, acenocoumarol) are primarily metabolized by CYP2C9, and treatment outcome is known to be associated with *CYP2C9* genotype [31–39]. More recently, the gene coding for the vitamin K epoxide reductase subunit 1 (*VKORC1*) was found to contribute to the variability in response observed in warfarin users [40].

The effect of *CYP2C9* and *VKORC1* genotype combined with patient height explained up to 55% of variance in warfarin dose [41]. Two prospective (pilot) studies concluded that the use of an algorithm including *CYP2C9* genotype for warfarin dosing is feasible [42,43], and prospective research is ongoing in the UK. Therefore, prospectively validated coumarin dosing algorithms that include PGx information might become available in the near future. In more recent developments, Wessels et al. have developed a clinical scoring system based on seven factors, including four genetic polymorphisms, to predict efficacy of methotrexate monotherapy in rheumatoid arthritis patients. They provide a tool that translates the outcome of the model into individual treatment recommendations [44]. De Leon et al. have published clinical guidelines for using *CYP2D6* and *CYP2C19* genotypes in the prescription of antidepressants or antipsychotics [45]. Further translational research aimed specifically at the practical application of PGx in clinical situations is warranted.

Improving acceptance of PGx testing

A newly introduced drug or technology is normally first applied by a small group of clinicians. In time it may become standard treatment incorporated into guidelines and consequently into wider clinical use. The time from introduction to acceptance of new methods may vary widely, as illustrated by a comparison of the implementation of Calvert's formula with that of HER2/neu testing. Carboplatin is currently dosed using the formula of Calvert, published in 1989, for area-under-the-curve targeted dosing [46]. Attention was called to Calvert's formula several times but it was not until 1996 that it was reported by the American Hospital Formulary Service, a widely used source of drug information [47,48]. Assuming that uptake into guidelines to some extent represents clinical acceptance, this time course shows that it took no less than seven years for Calvert's formula to be accepted. This relatively slow acceptance is further exemplified by the limited use of the formula in clinical trials with carboplatin during the early 1990s (Figure 2.2).

A contrasting example is the implementation of testing of breast cancers for HER2/neu overexpression with immunohistochemistry or fluorescence in situ hybridization to select

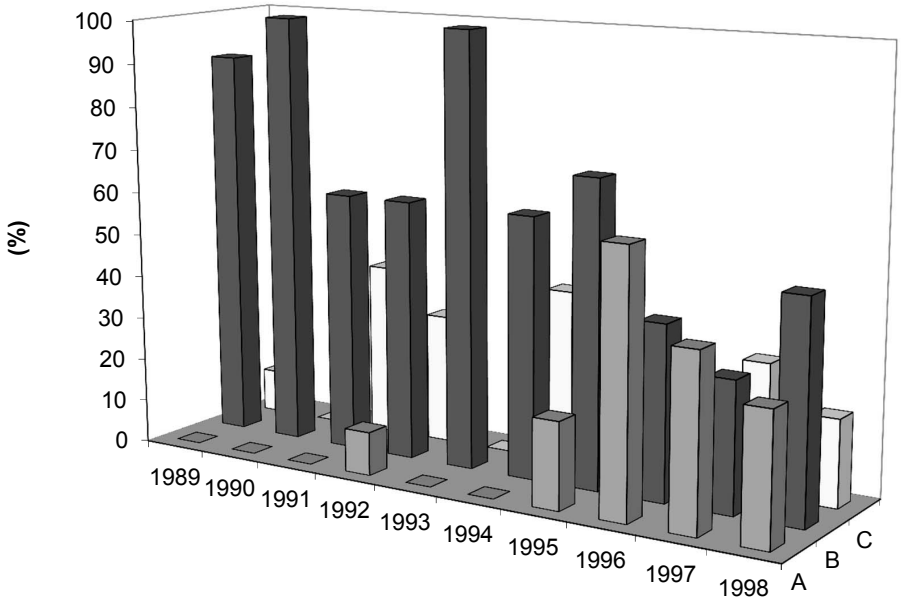

Figure 2.2 The use of the Calvert formula in clinical trials from 1989 to 1998. A PubMed search for the dosing of carboplatin in clinical trials was performed for the period 1989–1998. For each year the first ten results of PubMed were screened for the use of the Calvert formula. Bars represent the percentage of results in which the Calvert formula was used to dose carboplatin (A), the Calvert formula was not used (B), or no dosing information could be retrieved electronically (C).

patients with metastasized breast cancer eligible for treatment with trastuzumab. In the late 1980s and early 1990s, several studies demonstrated that breast cancers with HER2/neu overexpression showed poor prognosis [49–53]. In 1998 trastuzumab, a monoclonal drug directed against the HER2 protein, was launched on the US market. One year later, testing for HER2/neu overexpression was included in the American Hospital Formulary Service trastuzumab monograph. Testing for HER2/neu overexpression has become standard practice for guiding drug therapy for metastatic breast cancer. In contrast to the lengthy time line for acceptance of Calvert's formula, the short time line of acceptance of testing for HER2/neu overexpression indicates that fast uptake is possible. The two examples differ in many respects (e.g., one results in a dose adjustment while the other results in the decision whether or not to prescribe the drug). Nonetheless, two differences might be observed to present potential opportunities for improved clinical uptake of PGx. First, the use of testing for HER2/neu overexpression was required by the regulatory agencies upon market introduction of trastuzumab. With regard to PGx testing, this requirement suggests that obligatory testing prior to drug prescribing might give a strong stimulus to the clinical uptake of PGx. Second, HER2/neu testing was actively advocated by the pharmaceutical company manufacturing the drug and by patient advocacy organizations. Similarly active support for the use of clinically established PGx tests by pharmaceutical companies or patient advocacy organizations might be expected to improve clinical uptake of PGx testing.

CONCLUSIONS

Because variation in drug responses is, at least to some extent, related to genetic variation, PGx testing has the potential to result in safer and more effective use of drugs by permitting individualized therapy. In recent years FDA-approved PGx tests have become available, but the use of PGx testing has remained limited, largely by a lack of scientific evidence for improved patient care by PGx testing. Providing this scientific evidence presents a significant challenge. The development of novel tests should be aimed at solving important clinical problems. To demonstrate potential for clinical use, PGx studies should report diagnostic test criteria. For PGx tests shown to improve patient care, guidelines directing the clinical use of PGx test results should be developed. Information on cost-effectiveness and cost-consequences of PGx testing should be provided to facilitate reimbursement by insurance companies. Finally, uptake in clinical practice will be given a stimulus if regulatory agencies recommend testing prior to prescribing the drug, and if pharmaceutical companies or patient groups advocate for use of the test. If the outlined challenges can be met, the incorporation of PGx in routine clinical practice may prove an achievable goal in the near future.

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Pharmacogenetics: From Bench to Byte

3

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Despite initial enthusiasm [1–3], the use of pharmacogenetics has remained limited to investigation in only a few clinical fields such as oncology and psychiatry [4–8]. The main reason is the paucity of scientific evidence to show that pharmacogenetic testing leads to improved clinical outcomes [9,10]. Moreover, for most pharmacogenetic tests (such as tests for genetic variants of cytochrome P450 enzymes) a detailed knowledge of pharmacology is a prerequisite for application in clinical practice, and both physicians and pharmacists might find it difficult to interpret the clinical value of pharmacogenetic test results. Guidelines that link the result of a pharmacogenetic test to therapeutic recommendations might help to overcome these problems, but such guidelines are only sparsely available. In 2001, an early step was taken to develop such guidelines for the therapeutic use of antidepressants, and these included *CYP2D6*-related dose recommendations drawn from pharmacokinetic study data [11]. However, the use of such recommendations in routine clinical practice remains difficult, because they are currently outside the ambit of the clinical environment and are not accessible during the decision-making process by physicians and pharmacists, namely the prescription and dispensing of drugs.

It was for these reasons that the Royal Dutch Association for the Advancement of Pharmacy established the Pharmacogenetics Working Group (PWG) in 2005. In this 15-member multidisciplinary working group, clinical pharmacists, physicians, clinical pharmacologists, clinical chemists, epidemiologists, and toxicologists are represented. The objective of the PWG is to develop pharmacogenetics-based therapeutic (dose) recommendations on the basis of a systematic review of literature, and to assist the drug prescribers as well as the pharmacists by integrating the recommendations into computerized systems for drug prescription and automated medication surveillance. The recommendations do not indicate patients who are eligible for genotyping, but merely aim to optimize drug use in the small but ever-increasing group of patients whose genotypes are known.

In the Netherlands, computerized drug prescription and automated medication surveillance are well organized, and the majority of general practitioners as well as nearly all the community and hospital pharmacists use such a system [12]. Most of these automated medication systems use the G-standard, an extensive electronic drug database [13]. The therapeutic (dose) recommendations composed by the PWG are incorporated into the G-standard, thereby directly linking the pharmacogenetics-based therapeutic (dose) recommendations to the decision-making process. The first recommendations were released with the October 2006 edition of the G-standard. To our knowledge, the PWG initiative is the first to integrate pharmacogenetic test results and therapeutic (dose) recommendations into automated medication surveillance systems to be applied nationwide. In this article, we describe the procedures followed by the PWG for structured pharmacogenetic data collection, assessment, and subsequent synthesis of therapeutic (dose) recommendations. Furthermore, we report the first 26 defined recommendations included in the G-standard.

STRUCTURED ASSESSMENT OF GENE-DRUG INTERACTIONS

Scope

The scope of the PWG comprises the compilation of therapeutic (dose) recommendations on the basis of gene–drug interactions. It was decided to commence with the polymorphisms that affect pharmacokinetics. A list of polymorphic enzymes involved in phases I and II of the metabolic process, including an overview of drug substrates, was compiled. The criteria for inclusion were: (i) that the enzyme is known to play an important role in the metabolic process in vivo, and (ii) that data relating to the gene–drug interaction are available in the published literature. The following sources were used for assessing whether these criteria were fulfilled:

- PubMed (http://www.ncbi.nlm.nih.gov)
- Website (http://medicine.iupui.edu/flockhart/table.htm, http://www.genemedrx.com, http://www.druginteractioninfo.org, http://www.themedicalletter.com)
- Drug interaction textbook [14]
- Pharmacogenetics textbook [15]

Data collection

For each drug, a systematic search of PubMed and Frisbee (a bibliography of Dutch medical literature) [16] was carried out. The articles included in the reference lists were individually screened for additional material or papers. Wherever information relating to gene–drug interaction was present in the European Public Assessment Report, the manufacturer was asked to provide further details. Review articles, studies involving non-human subjects and in vitro experiments were excluded.

Data assessment

For data assessment, a method earlier described was adapted [13]. Two core parameters were defined:

- Level of evidence of the gene–drug interaction. This indicates the quality of the evidence found in literature for the gene–drug interaction, and was scored on a five-point scale with a range from 0 (lowest evidence) to 4 (highest evidence) (Table 3.1) [17].
- Clinical relevance of the potential adverse drug event, decreased therapeutic response, or other clinical effect resulting from the gene–drug interaction.

The clinical relevance was scored on a seven-point scale derived from the National Cancer Institute's Common Toxicity Criteria [18]. A clinical or pharmacokinetic effect

Table 3.1 Assigned levels of evidence

	Criteria for assigning levels of evidence
4	Published controlled studies of good quality[a] relating to phenotyped and/or genotyped patients or healthy volunteers, and having relevant pharmacokinetic or clinical endpoints
3	Published controlled studies of moderate quality[b] relating to phenotyped and/or genotyped patients or healthy volunteers, and having relevant pharmacokinetic or clinical endpoints
2	Published case reports, well documented, and having relevant pharmacokinetic or clinical endpoints. Well documented case series
1	Published incomplete case reports
	Product information
0	Data on file
-	No evidence

[a]The study is deemed to be of "good quality" if:
(i) the use of concomitant medication with a possible influence on the phenotype is reported in the manuscript.
(ii) other confounders are reported (e.g., smoking status).
(iii) the reported data are based on steady-state kinetics.
(iv) the results are corrected for dose variability.
[b]Wherever one or more of these "good quality" criteria were missing, the quality of the study was considered to be "moderate."

that was not statistically significant was classified as AA (lowest impact), whereas death, for example, was classified as F (highest impact) (Table 3.2). At every level of this point scale, new events are added after assessment by the PWG.

Status report and therapeutic (dose) recommendation

For each of the assessed gene–drug interactions, a status report was prepared that presented an overview of key findings from selected articles from the published literature, along with the scores representing level of evidence and clinical relevance. Based on these scores, each gene–drug interaction was coded with the highest scored level of evidence and clinical relevance. After a final assessment of the information presented in the status report, a decision was made whether or not a therapeutic (dose) recommendation was required. These recommendations could include (i) a dose adjustment, (ii) advice on therapeutic strategy (e.g., the advice for therapeutic drug monitoring or a warning for increased risk of adverse drug event or diminished therapeutic efficacy), or (iii) the recommendation to select an alternative drug. In order to clarify how the PWG had arrived at the final therapeutic (dose) recommendation, a concise rationale was provided.

A specific procedure was followed in the preparation of the status report. After data collection, the level of evidence and clinical relevance of each article were independently

Table 3.2 Classification of clinical relevance

Classification of clinical relevance	
AA	Clinical effect (NS) Kinetic effect (NS)
A	Minor clinical effect (S): QTc prolongation (<450 ms ♀, <470 ms ♂), INR increase <4.5 Kinetic effect (S)
B	Clinical effect (S): short-lived discomfort (<48 h) without permanent injury, for example, reduced decrease in resting heart rate, reduction in exercise tachycardia, diminished pain relief from oxycodone and ADE resulting from increased bioavailability of atomoxetine (decreased appetite, insomnia, sleep disturbance, etc.)
C	Clinical effect (S): long-standing discomfort (48–168 h) without permanent injury, for example, increase risk of failure of therapy with tricyclic antidepressants or atypical antipsychotic drugs: extrapyramidal side effects, parkinsonism: ADE resulting from increased bioavailability of tricyclic antidepressants, metoprolol, propafenone (central effects, e.g., dizziness).
D	Clinical effect (S): long-standing effect (>168 h), permanent symptom or invalidating injury, for example, failure of prophylaxis of atrial fibrillation; deep vein thrombosis
E	Clinical effect (S): Increased risk of failure of lifesaving therapy; expected bone marrow depression
F	Clinical effect (S): death; arrhythmia; unexpected bone marrow depression

ADE, adverse drug event; INR, international normalized ratio; NS, not statistically significant difference; S, statistically significant difference.

scored by two PWG members. In order to prevent interobserver variation, a seven-member subgroup of the PWG discussed the scores of each selected paper and composed a preliminary status report. This preliminary report was then evaluated by the complete PWG during one of its three-monthly meetings, resulting in the final consensus-based status report and inclusion into the G-standard.

Calculation of dose adjustments

The calculation of dose adjustments was based on four rules:

- Pharmacokinetic data only from papers with a level of evidence of 3 or 4 were used.
- Data from selected papers reporting both statistically significant and not statistically significant differences were used. Results showing differences that were not statistically significant were considered as having been caused by limited sample size per genotype. Dose recommendations were calculated only if statistically significant data were available, so as to rule out the possibility of making dosage calculations from data generated purely by chance.

- Dose calculations were based on the sum of parent drug and active metabolites for atomoxetine (4-hydroxyatomoxetine), clomipramine (desmethylclomipramine), imipramine (desipramine), nortriptyline (10-hydroxynortriptyline), propafenone (5-hydroxypropafenone), risperidone (9-hydroxy-risperidone), and venlafaxine (O-desmethylvenlafaxine).
- For prodrugs, pharmacokinetics of the active metabolite were used (e.g., morphine when codeine is used for analgesia).

We assumed that currently used standard doses are representative for extensive metabolizers. For calculating dose adjustments for the CYP2D6 PM phenotype (D_{PM}), we started by making a dose adjustment calculation from each selected paper from the published literature, using the formula below:

$$D_{PM}\,(\%) = (AUC_{EM} / AUC_{PM}) \times 100\%$$

After calculating dose adjustments from the data in each individual paper, a final dose recommendation was calculated as the population size-weighted mean of the individual dose adjustments:

$$D_{PM}\,(\%) = \frac{(N_{(a)} \times D_{PM(a)}) + (N_{(b)} \times D_{PM(b)}) + (N_{(c)} \times D_{PM(c)}) + \ldots + (N_{(x)} \times D_{PM(x)})}{N_{(a)} + N_{(b)} + N_{(c)} + \ldots + N_{(x)}}$$

N = number of subjects with corresponding phenotype in article a, b, c, … x

Dose recommendations of drugs for other genotypes and phenotypes were calculated using analogous equations, except in the case of prodrugs (e.g., codeine for analgesia) and drugs with metabolites whose contribution to the clinical effect is unknown (e.g., tamoxifen).

Consequences for automated medication systems

On the basis of the information collated in the status report, the PWG classified the gene–drug combination according to whether or not there was interaction between gene and drug (interaction: yes/no) and whether or not any alerts that were generated had to be tagged for action (action: yes/no). Wherever action is required, the alert with the therapeutic (dose) recommendation appears on the screen during prescription and dispensing (Figure 3.1). Where no action is required, the alert is only logged in the system.

Alerts will be generated only if a certain gene–drug combination occurs. Therefore, the recording of a patient's genotype in the computerized drug prescription or automated medication surveillance system is a prerequisite for the generation of an alert. The classifications and their consequences for the computerized drug prescription and automated medication surveillance system have been described earlier [13]. Four different types of alerts, each with its own text, are provided by the PWG; a prescriber

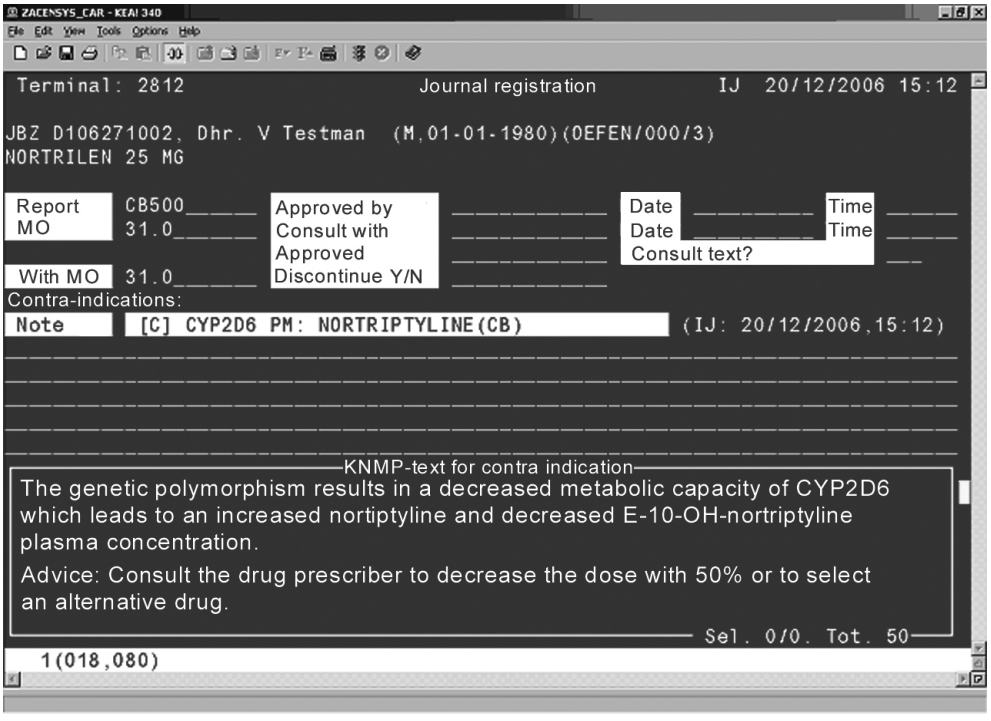

Figure 3.1 Typical alert generated by automated medication surveillance after prescription of nortriptyline to a patient known to be a poor metabolizer of CYP2D6 (translated from Dutch).

text, a pharmacy counter text, a hospital text, and a background text. Each of these is specifically designed to meet the requirements of its user. After a prescription has been issued by a physician (prescriber text), the prescription is transferred to the pharmacy either electronically or physically (by the patient). In the Netherlands, the prescription is then processed electronically by a pharmacy assistant (pharmacy counter text in a pharmacy, hospital text in a hospital setting), and the prescribed drug is dispensed. Prescriptions are checked for medication errors by the pharmacist (background text in community pharmacy, hospital text in hospital).

Composed therapeutic (dose) recommendations

To date, we have used this method of assessment for 85 genotype/phenotype–drug combinations comprising 26 drugs (Table 3.3, please note that the table in this thesis contains the information for 53 drugs from the updated 2011 Cinical Pharmacology & Therapeutics paper). The assessed drugs were substrates for CYP2D6 (n = 21), CYP2C19 (n = 1), CYP2C9 (n = 3), and UGT1A1 (n = 1). After assessment of the literature,

Table 3.3 Updated results for *CYP2D6*, *CYP2C9*, *CYP2C19*, *UGT1A1*, *TPMT*, *HLA-B44*, *HLA-B*5701*, *CYP3A5*, *VKORC1*, *Factor V Leiden*, *DPYD*

Drug	Subjects (n)	Genotype or phenotype	Level of evidence	Clinical relevance	Gene-drug interaction	Therapeutic (dose) recommendation	References
CYP2D6							
Amitriptyline	459	PM	3	A	Yes	Insufficient data to allow calculation of dose adjustment. Select alternative drug (e.g. citalopram, sertraline) or monitor amitriptyline and nortriptyline plasma concentration	[26-28]
		IM	3	C	Yes	Reduce dose by 25% and monitor plasma concentration or select alternative drug (e.g. citalopram, sertraline)	[26-31]
		UM	3	C	Yes	Insufficient data to allow calculation of dose adjustment. Select alternative drug (e.g. citalopram, sertraline) or monitor (E-10-hydroxy)amitriptyline plasma concentration	[28,32,33]
Aripiprazole	124	PM	4	C	Yes	Reduce maximum dose to 10 mg/day (67% of the maximum recommended daily dose)	[34-37]
		IM	4	A	Yes	No	[35,38-40]
		UM	—	—	Yes	No	—
Atomoxetine	10,081	PM	3	B	Yes	Standard dose. Dose increase probably not necessary, be alert to ADE	[41-46]
		IM	4	A	Yes	No	[47]
		UM	—	—	Yes	Insufficient data to allow calculation of dose adjustment. Be alert to reduced efficacy or select alternative drug (e.g. methylphenidate, clonidine)	—
Carvedilol	135	PM	4	B	Yes	No	[48,49]
		IM	4	A	Yes	No	[50-54]
		UM	—	—	Yes	No	—
Clomipramine	272	PM	4	C	Yes	Reduce dose by 50% and monitor (desmethyl) clomipramine plasma concentration	[55-60]
		IM	4	C	Yes	Insufficient data to allow calculation of dose adjustment. Monitor (desmethyl)clomipramine plasma concentration	[57,61,62]

		UM	2	C	Yes	Select alternative drug (e.g. citalopram, sertralin) or monitor (desmethyl)clomipramine plasma concentration	[63,64]
Clozapine	297	PM	4	AA	No	No	[65-69]
		IM	4	AA	No	No	[66,69]
		UM	4	AA	No	No	[68,69]
Codeine	453	PM	4	B	Yes	Analgesia: Select alternative drug (e.g. acetaminophen, NSAID, morphine not tramadol or oxycodone) or be alert to symptoms of insufficient pain relief Cough: No	[70-80]
		IM	3	A	Yes	Analgesia: Select alternative drug (e.g. acetaminophen, NSAID, morphine not tramadol or oxycodone) or be alert to symptoms of insufficient pain relief Cough: No	[71,81]
		UM	3	F	Yes	Analgesia: Select alternative drug (e.g. acetaminophen, NSAID, morphine not tramadol or oxycodone) or be alert to ADE Cough: Be extra alert to ADE due to increased morphine plasma concentration	[70,82-85]
Doxepin	76	PM	3	F	Yes	Reduce dose by 60%. Adjust maintenance dose in response to (nor)doxepin plasma concentration	[32,86-89]
		IM	3	A	Yes	Reduce dose by 20%. Adjust maintenance dose in response to (nor)doxepin plasma concentration	[88]
		UM	3	A	Yes	Select alternative drug (citalopram, sertraline), or increase dose by 100%. Adjust maintenance dose in response to (nor)doxepin plasma concentration	[87]
Duloxetine	0[b]	PM	0	AA	Yes	No	[90]
		IM	—	—	Yes	No	—
		UM	—	—	Yes	No	—

Table 3.3 continues on next page

Table 3.3 – Continued

Drug	Subjects (n)	Genotype or phenotype	Level of evidence	Clinical relevance	Gene-drug interaction	Therapeutic (dose) recommendation	References
Flecainide	145	PM	4	A	Yes	Reduce dose by 50%, record ECG, monitor plasma concentration	[91-95]
		IM	3	A	Yes	Reduce dose by 25%, record ECG, monitor plasma concentration	[96,97]
		UM	—	—	Yes	Record ECG and monitor plasma concentration or select alternative drug (e.g. sotalol, disopyramide, quinidine, amiodarone)	—
Flupenthixol	0	PM	—	—	No	No	—
		IM	—	—	No	No	—
		UM	—	—	No	No	—
Haloperidol	1,411	PM	4	C	Yes	Reduce dose by 50% or select alternative drug (e.g. pimozide, flupenthixol, fluphenazine, quetiapine, olanzapine, clozapine)	[98-105]
		IM	4	A	Yes	No	[98-102,106-114]
		UM	4	C	Yes	Insufficient data to allow calculation of dose adjustment. Be alert to decreased haloperidol plasma concentration and adjust maintenance dose in response to haloperidol plasma concentration or select alternative drug (e.g. pimozide, flupenthixol, fluphenazine, quetiapine, olanzapine, clozapine)	[98,99]
Imipramine	268	PM	4	C	Yes	Reduce dose by 70% and monitor imipramine and desipramine plasma concentration	[57,115-119]
		IM	4	A	Yes	Reduce dose by 30% and monitor imipramine and desipramine plasma concentration	[115,117, 119]
		UM	4	A	Yes	Select alternative drug (e.g. citalopram, sertraline) or increase dose by 70% and monitor imipramine and desipramine plasma concentration	[117,119]

Metoprolol	1,966	PM	4	C	Yes	*Heart failure*: Select alternative drug (e.g. bisoprolol, carvedilol) or reduce dose by 75% *Other indications:* Be alert to ADE (e.g. bradycardia, cold extremities) or select alternative drug (e.g. atenolol, bisoprolol)	[120-135]
		IM	4	B	Yes	*Heart failure:* Select alternative drug (e.g. bisoprolol, carvedilol) or reduce dose by 50% *Other indications:* Be alert to ADE (e.g. bradycardia, cold extremities) or select alternative drug (e.g. atenolol, bisoprolol)	[121-125,127, 132,133, 135-140]
		UM	4	D	Yes	*Heart failure*: Select alternative drug (e.g. bisoprolol, carvedilol) or titrate dose to max. 250% of the normal dose in response to efficacy and ADE *Other indications:* Select alternative drug (e.g. atenolol, bisoprolol) or titrate dose to max. 250% of the normal dose in response to efficacy and ADE	[123,125-128]
Mirtazapine	333	PM	3	B	Yes	No	[32,55,141-145]
		IM	3	A	Yes	No	[144,146]
		UM	3	A	Yes	No	[32,141,143]
Nortriptyline	270	PM	3	C	Yes	Reduce dose by 60% and monitor nortriptyline + 10-hydroxynortriptyline plasma concentrations	[147-152]
		IM	4	C	Yes	Reduce dose by 40% and monitor nortriptyline + 10-hydroxynortriptyline plasma concentrations	[147-149, 151,153-157]
		UM	3	C	Yes	Select alternative drug (e.g. citalopram, sertraline) or increase dose by 60% and monitor nortriptyline + 10-hydroxynortriptyline plasma concentrations	[64,148, 149,153]
Olanzapine	201	PM	3	AA	No	No	[158-160]
		IM	3	AA	No	No	[159,161, 162]
		UM	—	—	No	No	—

Table 3.3 continues on next page

Table 3.3 – Continued

Drug	Subjects (n)	Genotype or phenotype	Level of evidence	Clinical relevance	Gene-drug interaction	Therapeutic (dose) recommendation	References
Oxycodone	78	PM	3	B	Yes	Insufficient data to allow calculation of dose adjustment. Select alternative drug (not tramadol or codeine) or be alert to symptoms of insufficient pain relief	[163-167]
		IM	3	AA	Yes	Insufficient data to allow calculation of dose adjustment. Select alternative drug (not tramadol or codeine) or be alert to symptoms of insufficient pain relief	[165]
		UM	1	A	Yes	Insufficient data to allow calculation of dose adjustment. Select alternative drug (not tramadol or codeine) or be alert to ADE (e.g. nausea, vomiting, constipation, respiratory depression, confusion, urinary retention)	[168]
Paroxetine	633	PM	4	A	Yes	No	[144,169-176]
		IM	4	A	Yes	No	[144,170, 176-179]
		UM	4	C	Yes	Insufficient data to allow calculation of dose adjustment. Select alternative drug (e.g. citalopram, sertraline)	[169,173, 175, 176, 180]
Propafenone	257	PM	4	C	Yes	Reduce dose by 70%, record ECG, monitor plasma concentration	[181-190]
		IM	3	A	Yes	Insufficient data to allow calculation of dose adjustment. Adjust dose in response to plasma concentration and record ECG or select alternative drug (e.g. sotalol, disopyramide, quinidine, amiodarone)	[190-193]
		UM	3	D	Yes	Insufficient data to allow calculation of dose adjustment. Adjust dose in response to plasma concentration and record ECG or select alternative drug (e.g. sotalol, disopyramide, quinidine, amiodarone)	[184,190]

Risperidone	1,721	PM	4	D	Yes	Insufficient data to allow calculation of dose adjustment. Select alternative drug (e.g quetiapine, olanzapine, clozapine) or be extra alert to ADE and adjust dose to clinical response	[194-200]
		IM	4	C	Yes	Insufficient data to allow calculation of dose adjustment. Select alternative drug (e.g quetiapine, olanzapine, clozapine) or be extra alert to ADE and adjust dose to clinical response	[198,199, 201-209]
		UM	4	C	Yes	Insufficient data to allow calculation of dose adjustment. Select alternative drug (e.g quetiapine, olanzapine, clozapine) or be extra alert to decreased response and titrate dose in response to clinical effect and ADE	[198-200, 210]
Tamoxifen	5,020	PM	4	E	Yes	Increased risk for relapse of breast cancer. Consider aromatase inhibitor for postmenopausal women	[211-221]
		IM	4	E	Yes	Increased risk for relapse of breast cancer. Avoid concomitant use of CYP2D6 inhibitors. Consider aromatase inhibitor for postmenopausal women	[212,214-222]
		UM	4	A	Yes	No	[217,222]
Tramadol	968	PM	4	B	Yes	Select alternative drug (not oxycodone or codeine) or be alert to symptoms of insufficient pain relief	[223-236]
		IM	4	B	Yes	Be alert to decreased efficacy. Consider dose increase. If response is still inadequate select alternative drug (not oxycodone or codeine) or be alert to symptoms of insufficient pain relief	[223-225, 233,236-238]
		UM	3	C	Yes	Reduce dose by 30% and be alert to ADE (e.g. nausea, vomiting, constipation, respiratory depression, confusion, urinary retention) or select alternative drug (e.g. acetaminophen, NSAID, morphine not oxycodone or codeine)	[224,231, 236,239, 240]

Table 3.3 continues on next page

Table 3.3 – Continued

Drug	Subjects (n)	Genotype or phenotype	Level of evidence	Clinical relevance	Gene-drug interaction	Therapeutic (dose) recommendation	References
Venlafaxine	251	PM	4	C	Yes	Insufficient data to allow calculation of dose adjustment. Select alternative drug (e.g. citalopram, sertraline) or adjust dose to clinical response and monitor (O-desmethyl)venlafaxine plasma concentration	[241-247]
		IM	4	C	Yes	Insufficient data to allow calculation of dose adjustment. Select alternative drug (e.g. citalopram, sertraline) or adjust dose to clinical response and monitor (O-desmethyl)venlafaxine plasma concentration	[243-246, 248-250]
		UM	4	A	Yes	Be alert to decreased venlafaxine and increased O-desmethylvenlafaxine plasma concentration. Titrate dose to max 150% of the normal dose or select alternative drug (e.g. citalopram, sertraline)	[243,245]
Zuclopenthixol	231	PM	4	A	Yes	Reduce dose by 50% or select alternative drug (e.g. flupenthixol, quetiapine, olanzapine, clozapine)	[251-255]
		IM	4	A	Yes	Reduce dose by 25% or select alternative drug (flupenthixol, quetiapine, olanzapine, clozapine)	[252-254]
		UM	—	—	Yes	Insufficient data to allow calculation of dose adjustment. Be alert to low zuclopenthixol plasma concentrations or select alternative drug (flupenthixol, quetiapine, olanzapine, clozapine)	—
CYP2C9							
Acenocoumarol[a]	6,811	*1/*2	4	F	Yes	Check INR more frequently after initiating or discontinuing NSAIDs	[256-274]
		*2/*2	4	F	Yes	Check INR more frequently after initiating or discontinuing NSAIDs	[256-261, 263-274]
		*1/*3	4	F	Yes	Check INR more frequently after initiating or discontinuing NSAIDs	[256-275]

		*2/*3	4	F	Yes	Check INR more frequently after initiating or discontinuing NSAIDs	[257-275]
		*3/*3	4	F	Yes	Check INR more frequently during dose titration and after initiating or discontinuing NSAIDs	[256-259, 263, 267-270, 272,275, 276]
Glibenclamide	86	*1/*2	3	AA	Yes	No	[277-281]
		*2/*2	3	AA	Yes	No	[277,279]
		*1/*3	3	B	Yes	No	[277-280]
		*2/*3	3	AA	Yes	No	[277,279, 281]
		*3/*3	3	A	Yes	No	[279,281]
Gliclazide	912	*1/*2	3	AA#	Yes	No	[282-284]
		*2/*2	3	AA#	Yes	No	[282,284]
		*1/*3	3	AA#	Yes	No	[282-285]
		*2/*3	3	AA#	Yes	No	[282]
		*3/*3	3	AA#	Yes	No	[282]
Glimepiride	442	*1/*2	3	AA	Yes	No	[277,278, 281,283]
		*2/*2	4	AA	Yes	No	[277]
		*1/*3	4	AA#	Yes	No	[277,278, 281,283, 286,287]
		*2/*3	3	D	Yes	No	[277,278, 281]
		*3/*3	3	D	Yes	No	[281,287]
Phenprocoumon[a]	1,802	*1/*2	4	F	Yes	No	[264-267, 288-296]
		*2/*2	4	F	Yes	Check INR more frequently	[264-267, 289-294, 296]

Table 3.3 continues on next page

Table 3.3 – Continued

Drug	Subjects (n)	Genotype or phenotype	Level of evidence	Clinical relevance	Gene-drug interaction	Therapeutic (dose) recommendation	References
		*1/*3	4	F	Yes	No	[265-267, 288-294, 296]
		*2/*3	4	F	Yes	Check INR more frequently	[265-267, 288-292, 294, 296]
		*3/*3	4	D	Yes	Check INR more frequently	[289-292, 294]
Phenytoin	1,354	*1/*2	4	A	Yes	Standard loading dose. Reduce maintenance dose by 25%. Evaluate response and serum concentration after 7-10 days. Be alert to ADE (e.g. ataxia, nystagmus, dysarthria, sedation)	[297-303]
		*2/*2	4	A	Yes	Standard loading dose. Reduce maintenance dose by 50%. Evaluate response and serum concentration after 7-10 days. Be alert to ADE (e.g. ataxia, nystagmus, dysarthria, sedation)	[297-299, 301-303]
		*1/*3	4	D	Yes	Standard loading dose. Reduce maintenance dose by 25%. Evaluate response and serum concentration after 7-10 days. Be alert to ADE (e.g. ataxia, nystagmus, dysarthria, sedation)	[297-300, 303-311]
		*2/*3	4	A	Yes	Standard loading dose. Reduce maintenance dose by 50%. Evaluate response and serum concentration after 7-10 days. Be alert to ADE (e.g. ataxia, nystagmus, dysarthria, sedation)	[298,302]
		*3/*3	4	D	Yes	Standard loading dose. Reduce maintenance dose by 50%. Evaluate response and serum concentration after 7-10 days. Be alert to ADE (e.g. ataxia, nystagmus, dysarthria, sedation)	[297,299-301,311-315]

Tolbutamide	544	*1/*2	3	A	Yes	No	[277,316-320]
		*2/*2	3	A	Yes	No	[277,316, 318,319]
		*1/*3	3	B	Yes	No	[277,316-322]
		*2/*3	3	A	Yes	No	[277,319, 320]
		*3/*3	3	A	Yes	No	[319-321]
CYP2C19							
Citalopram / Escitalopram	2,396	PM	4	A	Yes	No	[323-330]
		IM	4	A	Yes	No	[323-325, 327,330, 331]
		UM	4	A	Yes	Monitor plasma concentration and titrate dose to max. 150% in response to efficacy and ADE or select alternative drug (e.g. fluoxetine, paroxetine)	[324,332]
Clopidogrel	11,785	PM	4	F	Yes	Increased risk for reduced response to clopidogrel. Consider alternative drug. Prasugrel is not or to a much smaller extent metabolized by CYP2C19 but is associated with an increased bleeding risk compared to clopidogrel	[333-351
		IM	4	F	Yes	Increased risk for reduced response to clopidogrel. Consider alternative drug. Prasugrel is not or to a much smaller extent metabolized by CYP2C19 but is associated with an increased bleeding risk compared to clopidogrel	[323-353]
		UM	3	A	Yes	No	[333,340-342,354]

Table 3.3 continues on next page

Table 3.3 – Continued

Drug	Subjects (n)	Genotype or phenotype	Level of evidence	Clinical relevance	Gene-drug interaction	Therapeutic (dose) recommendation	References
Esomeprazole	975	PM	4	AA#	Yes	No	[355-364]
		IM	4	AA#	Yes	No	[355-363, 365]
		UM	—	V	Yes	*Helicobacter pylori eradication:* increase dose by 50-100%. Be extra alert to insufficient response *Other:* Be extra alert to insufficient response. Consider dose increase by 50-100%	—
Imipramine	541	PM	3	A	Yes	Reduce dose by 30% and monitor plasma concentration of imipramine and desipramine or select alternative drug (e.g. fluvoxamine, mirtazapine)	[118,366-371]
		IM	3	A	Yes	Insufficient data to allow calculation of dose adjustment. Select alternative drug (e.g. fluvoxamine, mirtazapine)	[118,367-370]
		UM	—	—	Yes	No	—
Lansoprazole	2,304	PM	4	AA#	Yes	No	[372-394]
		IM	4	AA#	Yes	No	[372-393, 395,396]
		UM	—	—	Yes	*Helicobacter pylori eradication:* increase dose by 200%. Be extra alert to insufficient response. *Other:* Be extra alert to insufficient response. Consider dose increase by 200%	—
Moclobemide	31	PM	3	A	Yes	No	[397-399]
		IM	—	—	Yes	No	—
		UM	—	—	Yes	No	—
Omeprazole	2,522	PM	4	AA#	Yes	No	[356,378, 380,383, 384,386, 389,400-414]

		IM	4	AA#	Yes	No	[356,378, 380,383, 384,386, 389,396, 400-404, 406-410, 412-415]
		UM	3	A	Yes	*Helicobacter pylori eradication:* increase dose by 100-200%. Be extra alert to insufficient response *Other:* Be extra alert to insufficient response. Consider dose increase by 100-200%	[416-418]
Pantoprazole	829	PM	3	AA#	Yes	No	[361,419-423]
		IM	3	AA#	Yes	No	[361,365, 415,420-423]
		UM	3	AA	Yes	*Helicobacter pylori eradication:* increase dose by 400%. Be extra alert to insufficient response *Other:* Be extra alert to insufficient response. Consider dose increase by 400%	[423]
Rabeprazole	2,239	PM	4	AA#	Yes	No	[359,377, 382,384, 386,389, 401,405, 406,410, 413,419, 424-435]
		IM	4	AA	Yes	No	[359,377, 382,384, 386,389, 401,406, 410,413, 424-428, 430-434]
		UM	—	—	Yes	No	—

Table 3.3 continues on next page

Table 3.3 – Continued

Drug	Subjects (n)	Genotype or phenotype	Level of evidence	Clinical relevance	Gene-drug interaction	Therapeutic (dose) recommendation	References
Sertraline	26	PM	3	C	Yes	Reduce dose by 50%	[32,436]
		IM	3	A	Yes	Insufficient data to allow calculation of dose adjustment. Be extra alert to ADE (e.g. nausea, vomiting, diarrhea)	[436]
		UM	—	—	Yes	No	—
Voriconazole	314	PM	3	A	Yes	Monitor serum concentration	[437-446]
		IM	3	A	Yes	Monitor serum concentration	[437,438, 441, 444-446]
		UM	3	A	Yes	No	[443,445]
UGT1A1							
Irinotecan	3,883	*1/*28	3	F	Yes	No	[447-473]
		*28/*28	3	E	Yes	*Dose > 250 mg/m²:* Reduce initial dose by 30%. Increase dose in response to neutrophil count *Dose ≤ 250 mg/m²:* No dose adjustment	[447,448, 450-460, 462, 464-470,472-479]
TPMT							
Azathioprine / Mercaptopurine	2,853	PM	4	F	Yes	Select alternative drug or reduce dose by 90%. Increase dose in response of hematologic monitoring and efficacy	[480-492]
		IM	4	E	Yes	Select alternative drug or reduce dose by 50%. Increase dose in response of hematologic monitoring and efficacy	[480,481, 483,484, 486,487, 489-491, 493-502]
Thioguanine	792	PM	2	F	Yes	Select alternative drug. Insufficient data to allow calculation of dose adjustment	[503,504]
		IM	3	D	Yes	Select alternative drug. Insufficient data to allow calculation of dose adjustment	[505-508]

HLA-B44							
Ribavirine	130	HLA-B44 negative	4	C	Yes	No	[509]
*HLA-B*5701*							
Abacavir	3,791	HLA-B*5701 positive	4	E	Yes	Select alternative drug	[510-523]
CYP3A5							
Tacrolimus	1,302	*1/*1	4	B	Yes	No	[524-536]
		*1/*3	4	D	Yes	No	[524-537]
VKORC1							
Acenocoumarol[a]	776	CT	4	A	Yes	No	[258,275, 538-540]
		TT	4	A	Yes	Check INR more frequently	[258,275, 538-540]
Phenprocoumon[a]	391	CT	4	D	Yes	No	[294,539]
		TT	4	D	Yes	Check INR more frequently	[294,539]
Factor V Leiden							
Estrogen containing oral contraceptive	7,441	FVL homozygous	3	D	Yes	*Positive (family)history of thrombotic events:* Avoid estrogen containing OC and select alternative (e.g. copper intrauterine device or progestin-only contraceptive) *Negative (family)history of thrombotic events:* Avoid additional risk factors (e.g. obesity, smoking etc.)	[541-548]
		FVL heterozygous	4	D	Yes	*Positive (family)history of thrombotic events:* Avoid estrogen containing OC and select alternative (e.g. copper intrauterine device or progestin-only contraceptive) *Negative (family)history of thrombotic events:* Avoid additional risk factors (e.g. obesity, smoking etc.)	[541-545, 547-560]

Table 3.3 continues on next page

Table 3.3 – Continued

Drug	Subjects (n)	Genotype or phenotype	Level of evidence	Clinical relevance	Gene-drug interaction	Therapeutic (dose) recommendation	References
DPYD							
Fluorouracil / Capecitabine	3,733	PM	3	F	Yes	Select alternative drug. Tegafur is not a suitable alternative because this drug is also metabolized by DPD	[561-569]
		IM	3	F	Yes	Reduce dose by 50% or select alternative drug. Tegafur is not a suitable alternative because this drug is also a substrate for DPD. Increase dose in response to toxicity and efficacy	[561-567, 569-580]
Tegafur/uracil combination	0[b]	PM	3	AA	Yes	Select alternative drug. Fluorouracil or capecitabine are not suitable alternatives because both are also metabolized by DPD	[581]
		IM	3	AA	Yes	No	[581]

Level of evidence: assigned level of evidence (0–4) for the gene–drug interaction. If scored "—" no data was retrieved with the literature search.
Clinical relevance: assigned level of clinical relevance (AA–F) for the gene–drug interaction. If scored "—" no data were retrieved with the literature search. Positive clinical effects were scored as AA#.
ADE, adverse drug event; ECG, electrocardiogram; FVL, factor V Leiden; IM, intermediate metabolizer; INR, international normalized ratio; NSAID, nonsteroidal anti-inflammatory drug; OC, oral contraceptive; PM, poor metabolizer; UM, ultrarapid metabolizer.
CYP2C19 IM, *1/*2, *1/*3, *17/*2, *17/*3; CYP2C19 PM, *2/*2, *2/*3, *3/*3; CYP2C19 UM, *17/*17; CYP2D6 IM, patients carrying two decreased-activity (*9, *10, *17, *29, *36, *41) alleles or carrying one active (*1, *2, *33, *35) and one inactive (*3-*8, *11-*16, *19-*21, *38, *40, *42) allele, or carrying one decreased-activity (*9, *10, *17, *29, *36, *41) allele and one inactive (*3-*8, *11-*16, *19-*21, *38, *40, *42) allele; CYP2D6 PM, patients carrying two inactive (*3-*8, *11-*16, *19-*21, *38, *40, *42) alleles; CYP2D6 UM, patients carrying a gene duplication in absence of inactive (*3-*8, *11-*16, *19-*21, *38, *40, *42) or decreased-activity (*9, *10, *17, *29, *36, *41) alleles; DPD PM, patients carrying two inactive (*2A, *3, *7, *8, *10, *11, *12, *13, 496A>G, IVS10-15T>C, 1156G>T, 1845G>T) alleles, two decreased-activity (*9B, *10) alleles, or one inactive (*2A, *3, *7, *8, *10, *11, *12, *13, 496A>G, IVS10-15T>C, 1156G>T, 1845G>T) and one decreased-activity (*9B, *10) allele; DPD IM, patients carrying one active (*1, *4, *5, *6, *9A) allele and one inactive (*2A, *3, *7, *8, *10, *11, *12, *13, 496A>G, IVS10-15T>C, 1156G>T, 1845G>T) or decreased-activity (*9B, *10) allele. For the inactive *DPYD* alleles *3, *7, *8, *11, *12, *13, 1156G>T, 1845G>T and decreased-activity *DPYD* alleles *9B, *10, toxicity has been described in case reports but has not been confirmed in independent studies or pharmacokinetic analyses. TPMT IM, patients carrying one active (*1, *1S, *1A) and one inactive (*2, *3A-*3D, *4-*18) allele; TPMT PM, patients carrying two inactive (*2, *3A-*3D, *4-*18) alleles.
[a]Therapeutic (dose) recommendations for acenocoumarol and phenprocoumon solely based on *CYP2C9* genotype without knowledge of *VKORC1* status. Advice based on situation in the Netherlands. [b]Therapeutic (dose) recommendation based on information from the Summary of Product Characteristics.

therapeutic (dose) recommendations were composed for 17 of the 26 drugs. It was decided that for four of the drugs (clozapine, duloxetine, flupenthixol, and olanzapine) no gene–drug interaction was present and therefore no therapeutic (dose) recommendation was required. For aripiprazole, tamoxifen, acenocoumarol, phenprocoumon, and voriconazole, although a gene–drug interaction was present, no therapeutic (dose) recommendation was made.

Overview and caveats

We have developed a method to interpret the results of structured assessment of gene–drug interactions, and translate them into therapeutic recommendations. These recommendations have been included in the G-standard since October 2006, and are applied in clinical practice for patients whose genotype is known. The availability of these guidelines as part of most computerized drug prescription and automated medication surveillance systems in the Netherlands will facilitate the use of pharmacogenetic information in therapeutic decision-making. Recommendations relating to other drugs such as sulfonylureas, angiotensin II receptor blockers, and proton pump inhibitors, are currently under evaluation and will be released along with future three-monthly updates.

Many of the studies that were assessed did not have pharmacogenetics as their primary objective, and this resulted in underpowered studies. Even where pharmacogenetics was the primary study objective, the assessed endpoints were mostly pharmacokinetic; also, the results related to single-dose experiments in healthy volunteers and was therefore not representative of daily clinical practice. A third limitation was the frequent use of specific study populations such as Asians, involving the investigation of genotypes which occur only rarely in Caucasian populations. In particular, there is a dearth of data relating to intermediate and ultrarapid metabolizers. Because we did not allow extrapolation of dose recommendations if a phenotype was not present in the studied population, only a few dose recommendations could be calculated for ultrarapid and intermediate metabolizers. The number of research papers per gene–drug combination retrieved during our searches and eligible for assessment was lower than we had expected, varying from 0 to 21. For nortriptyline, a widely used example for demonstrating the possible impact of pharmacogenetics, only 10 original papers were found eligible for assessment. These findings demonstrate that there remains a need for more studies to provide data on the clinical consequences of pharmacogenetics. These studies should be adequately designed with regard to sample size and clinically relevant endpoints [19]. Also, initiatives such as the cataloging of pharmacogenetic information, introduced by the Pharmacogenomics and Pharmacogenetics Knowledge Base (http://www.pharmgkb.org/), are a valuable approach to providing research studies with adequate power to demonstrate the clinical relevance of pharmacogenetics.

Currently there is only limited evidence to justify prospective pharmacogenetic testing or population-wide screening. The justification for such testing and screening will depend upon the availability of sufficient data demonstrating that pharmacogenetic testing actually improves clinical outcome and is cost-effective [20]. Producing such evidence presents a significant challenge. Long-term monitoring of the clinical outcome of the PWG dose recommendations might provide such data. However, there are indications that patients with non-wild-type genotypes are more often prone to an aberrant drug response. Therefore, we chose to formulate therapeutic recommendations for the situation where the patient's genotype is known. Currently, the infrastructure for genotyping is available only in a limited number of centers and needs to be expanded or made accessible for other centers [4,21].

Obviously, tests for single polymorphisms that affect pharmacokinetics may account for only part of the variability in drug response, and the pharmacogenetic tests that are currently available cannot replace other methods for dose individualization such as therapeutic drug monitoring [22,23]. We have described only genetic polymorphisms that affect the pharmacokinetics of a drug. The available literature on polymorphisms that affect pharmacodynamics, and the implications of these effects, is limited and sometimes contradictory [24,25].

In summary, our initiative to develop pharmacogenetics-based therapeutic (dose) recommendations and to make them accessible during electronic drug prescription and automated medication surveillance represents an important step forward toward the application of pharmacogenetic information in daily patient care.

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4

Feasibility of Pharmacy Initiated Pharmacogenetic Screening for *CYP2D6* and *CYP2C19*

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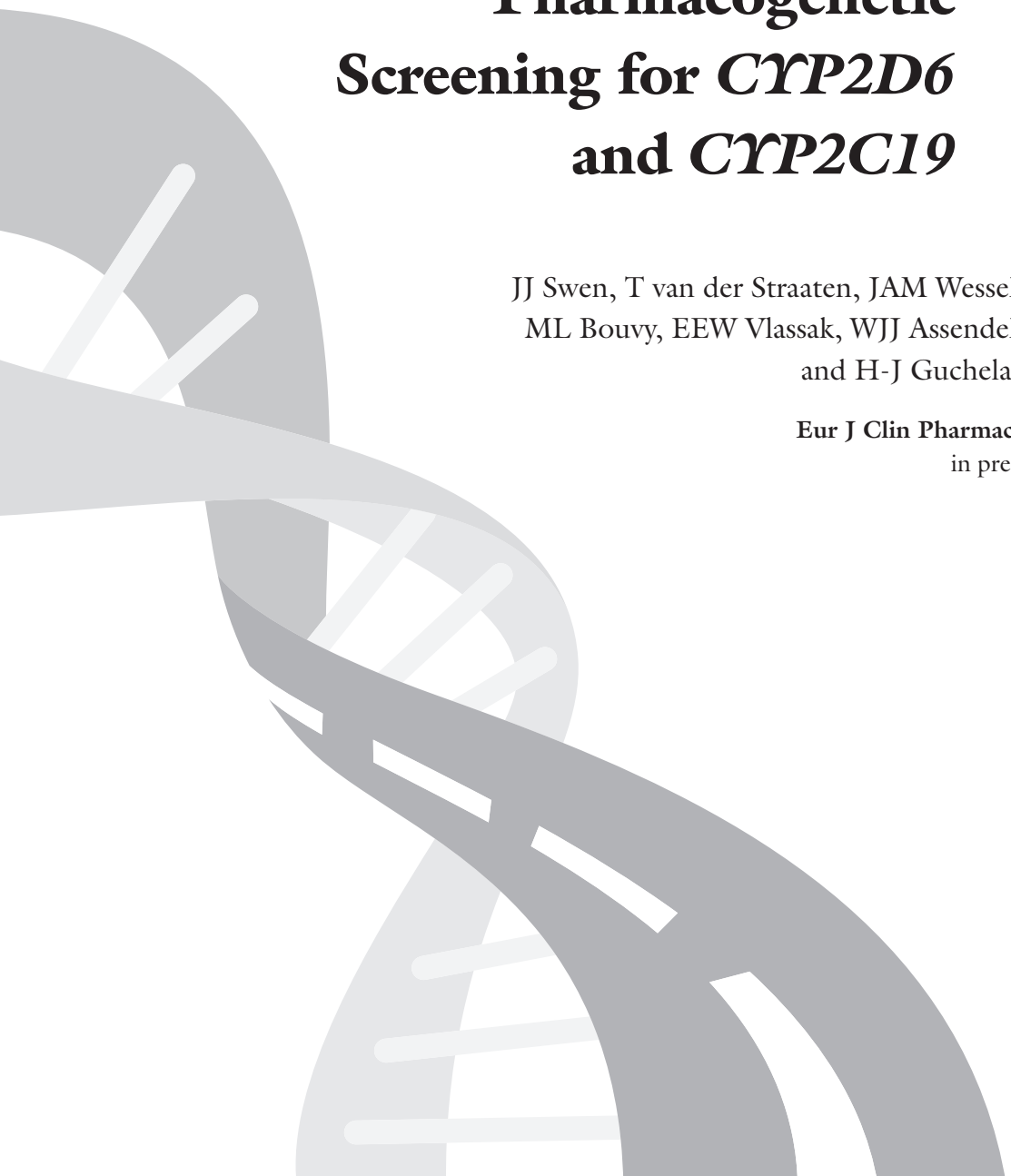

ABSTRACT

Purpose: To investigate the feasibility of pharmacy initiated pharmacogenetic screening in primary care with respect to patient willingness to participate, quality of DNA collection with saliva kits, genotyping, and dispensing data retrieved from the pharmacy.

Methods: Polypharmacy patients, age > 60 years that used at least 1 drug with ATC code N06AA01-N06AX19 (anti-depressants), A02BC01–A02BC05 (proton pump inhibitors), N05AA01-N05AH04 (anti-psychotics) or C07AB02 (metoprolol) in the preceding 2 years were randomly selected. DNA was collected with saliva kits and genotyped for *CYP2D6* and *CYP2C19* with the AmpliChip. Pharmacy dispensing records were retrieved and screened for drugs interacting with the patient's *CYP2D6* and *CYP2C19* genotype by using the evidence-based pharmacogenetic guidelines from the Dutch Pharmacogenetics Working Group.

Results: Out of the 93 invited patients, 54 patients (58.1%) provided informed consent. 9 saliva samples (16.7%) contained too little DNA. Call rates for *CYP2D6* and *CYP2C19* were 93.3% and 100% respectively. The frequencies of genotype-predicted-phenotype were 2.4%, 38.1%, 54.8%, and 4.8% for CYP2D6 poor-metabolizers (PM), intermediate-metabolizers (IM), extensive-metabolizers (EM), and ultrarapid-metabolizers respectively. For *CYP2C19* genotype-predicted-phenotype frequencies were 2.2%, 15.6%, and 82.2% for PM, IM, and EM, respectively.

Conclusions: This study shows that pharmacy initiated pharmacogenetic screening is feasible for a primary care setting.

INTRODUCTION

Pharmacogenetics promises an exciting approach to a more individualized drug therapy ultimately leading to a more efficient and safer application of drugs. To date, many efforts within the field of pharmacogenetics have been aimed at the improvement of drug therapy with "high risk" medications such as within the field of oncology. By contrast, multiple pharmacogenetic interventions for drug therapies with less high risk might also prove beneficial [1]. Yet the clinical use of genotyping prior to drug prescription and dispensing is not widely practiced [2].

Currently, pharmacogenetic information is accumulating rapidly and it was reported that based on the available pharmacogenetic information it is possible to generate advice for nearly 100 drugs for a patient with a completely sequenced genome [3]. In addition, Philips et al. reported that 16 out of 27 (59%) of the drugs most commonly associated with adverse drug events (ADEs) were metabolized by a polymorphic gene [4]. A subsequent study published by Grice et al. investigated the frequency of use of these 16 pharmacogenetic ADE-associated drugs in a primary care setting [5]. It was reported that 28.6% of the patients took more than one of the pharmacogenetic ADE-associated drugs indicating a high potential for pharmacogenetics to make drug therapy safer. A disadvantage of this study was that it did not include drugs for which pharmacogenetic testing is recommended to enhance efficacy instead of to avoid ADEs. In a study aimed at gaining insight into the feasibility of informing physicians reporting ADEs to the Netherlands Pharmacovigilance Centre about possible pharmacogenetic involvement and genotyping their patient Van Puijenbroek et al. reported that 39.5% of the reporting health care professionals actually initiated genotyping [6]. This illustrates that incorporating pharmacogenetic information in drug prescribing could increase safety of drug therapy.

More recently, two large (inter)national initiatives aimed at providing pharmacogenetics based guidelines and recommendations concerning drug prescribing have been published [7,8]. The Clinical Pharmacogenetics Implementation Consortium (CPIC) recently published its first guideline [9] and has the goal to provide peer-reviewed, evidence-based, freely accessible guidelines for gene/drug pairs. CPIC published a list of 29 gene/drug pairs that were ranked highest in a survey of American Society for Clinical Pharmacology and Therapeutics members in 2010 [7]. The Dutch Pharmacogenetics Working group recently published updated evidence-based guidelines with pharmacogenetics recommendations concerning 53 drugs [8]. From the list of 53 drugs of the Dutch Pharmacogenetics Working group, the list of highest-ranked priority gene/drug pairs from CPIC and the article by Grice et al., it can be observed that many drugs used in primary care are to some extend influenced by pharmacogenetics, again illustrating a potential role for pharmacogenetics in primary care.

The aim of the current study is to test the feasibility of pharmacy initiated pharmacogenetic screening in primary care with respect to patient willingness to participate, quality of DNA collection with saliva kits, genotyping, and quality of dispensing data retrieved from the pharmacy records.

METHODS

Study setting

In the Netherlands the vast majority of the population obtains their medication from only one community pharmacy, enabling collection of complete medication histories [10]. The pharmacy keeps an electronic patient record that covers all dispensing data. Polypharmacy patients were recruited from a community pharmacy located in the city of Leiden, the Netherlands. Patients were selected from the pharmacy records if they used at least one drug that is metabolized by CYP2D6 or CYP2C19 and at least four additional drugs in the preceding 2 years. Drugs were selected on their Anatomical Therapeutic Chemical (ATC) classification code [http://www.whocc.no/atc_ddd_index/]. To identify eligible ATC codes textbooks, an academic website [http://medicine.iupui.edu/flockhart/table.htm] and a review article by Kirchheiner et al. were used [11-13]. Codes eligible for inclusion comprised N06AA01 - N06AX19 (anti-depressants), A02BC01 – A02BC05 (proton pump inhibitors), N05AA01 - N05AH04 (anti-psychotics) and ATC-code C07AB02 (metoprolol). Patients had to be 60 year or older at the start of the study. This age was chosen for the practical reason that elderly patients are more frequently at home simplifying saliva collection.

Sample collection

Samples of 2 ml saliva were collected during a 30 min house visit. Samples were collected in the Oragene DNA self-Collection kit (DNA Genotek Inc Ottawa, Ontario, Canada). A sample of 125 patients was drawn from the selected patients by randomization with the aim to invite 100 patients by mail and finally obtain DNA samples from 50 patients. General practitioners were informed of our study prior to contacting the patient, and asked to exclude patients with terminal disease status. Patients selected for inclusion received a letter from the pharmacy explaining the background of the study and the study objectives. Approximately 1-2 weeks after the letter was sent, patients were contacted by phone by one of the pharmacists participating in the study (EV). During this phone call patients were asked if they agreed to participate. If patients agreed, a 30 minute house visit was planned to collect informed consent and a saliva specimen. If the first attempt to contact a patient by phone was unsuccessful a maximum of three consecutive attempts were made. After that the patient was considered not willing to participate. The study was approved by the ethics committee of the Leiden University Medical Center. Informed consent was obtained from all participants.

Genotyping

DNA was extracted from saliva using the Oragene DNA self-Collection kit according to the manufacturer's instruction at the Leiden University Medical Center (Leiden, The Netherlands). DNA concentrations were measured with nanodrop (Isogen, Maarssen, The Netherlands) and diluted with water to a concentration of 10 ng/μl. The DNA was tested for 29 known polymorphisms in the *CYP2D6* gene, including gene duplication and gene deletion, as well as two major polymorphisms in the *CYP2C19* gene. Genotyping was performed at the department of clinical chemistry of the Erasmus MC (Rotterdam, The Netherlands) by use of the AmpliChip CYP450 test (Roche Molecular Systems, Alameda, California) according to the manufacturer's instruction. A genotype-predicted-phenotype (phenotype) was assigned to each patient [8]. For *CYP2D6* intermediate metabolizers (IMs) were defined as patients carrying two decreased-activity (*9, *10, *17, *29, *36, *41) alleles or carrying one active (*1, *2, *33, *35) and one inactive (*3-*8, *11-*16, *19-*21, *38, *40, *42) allele, or carrying one decreased-activity (*9, *10, *17, *29, *36, *41) allele and one inactive (*3-*8, *11-*16, *19-*21, *38, *40, *42) allele. Poor metabolizers (PMs) were defined as patients carrying two inactive (*3-*8, *11-*16, *19-*21, *38, *40, *42) alleles. Ultra rapid metabolizers (UMs) were defined as patients carrying a gene duplication in absence of inactive (*3-*8, *11-*16, *19-*21, *38, *40, *42) or decreased-activity (*9, *10, *17, *29, *36, *41) alleles. All other patients were considered extensive metabolizers (EMs). For *CYP2C19* IMs were defined as patients with one active (*1) and one inactive (*2, *3) allele. PMs were defined as patients carrying two inactive alleles.

Medication analyses

Data were extracted from the pharmacy dispensing records. Drugs for topical application were excluded from the analysis. The number of unique prescribed drugs per patient was calculated as the number of unique ATC codes prescribed to each patient in the studied period of 2 years. For each unique prescribed drug it was checked if a recommendation was available in the guidelines of the Dutch Pharmacogenetics Working Group of the Royal Dutch Association for the Advancement of Pharmacy [8]. These evidence-based guidelines contain a comprehensive evaluation of pharmacogenetic gene-drug interactions involving 53 drugs and 11 genes. To illustrate the potential impact of pharmacogenetics on primary care, the percentage of prescribed drugs with any pharmacogenetic recommendation was calculated for each patient and in detail for pharmacogenetic recommendations regarding *CYP2D6* and *CYP2C19*. To evaluate if patients with a non-EM CYP2D6 or CYP2C19 status had been empirically switched to non CYP2D6 / CYP2C19 substrates, the percentage of CYP2D6/CYP2C19 substrates with a pharmacogenetic recommendation of the total number of prescribed drugs was compared between EMs and non-EMs.

Statistical analysis

The Student's t-test was used to evaluate differences in the percentage of prescribed CYP2D6 and CYP2C19 substrates, and the number of drugs with a recommendation in the guideline of the Dutch Pharmacogenetics Working Group between EMs and non-EMs. A p-value < 0.05 was regarded as significant. Statistical analysis was conducted with the SPSS statistical package (version 17.0, SPSS, Chicago, IL, USA).

RESULTS

Patient response

Five hundred and seven patients representing approximately 5% of the total registered patient population, were prescribed at least one drug from the selected ATC-codes and at least four additional drugs (Figure 4.1). Out of these patients a random sample of 125 patients was selected. Twenty two patients were excluded because they visited a general practitioner that did not participate in the study. A further ten patients were excluded because of terminal disease status as judged by their general practitioner. Out of the 93

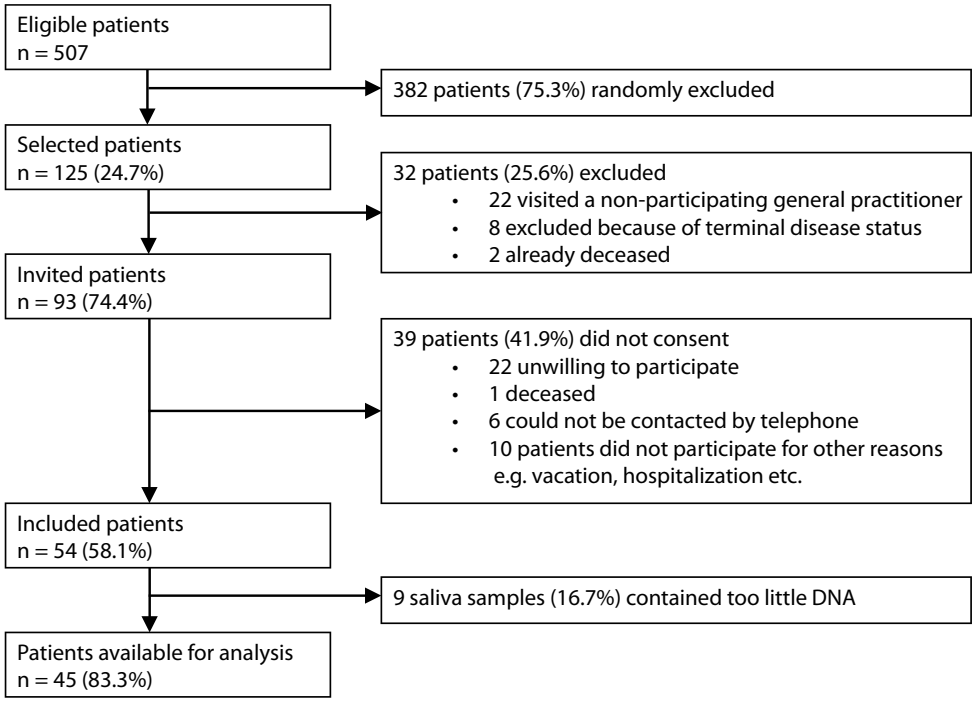

Figure 4.1 Study population.

invited patients, 54 patients (58.1%) provided informed consent. Twenty two patients (23.7%) of the 93 invited patients refused to participate, and 17 patients (18.3%) could not be included for other reasons (Figure 4.1). The mean age of the included patients was 71 years (range 60-91 years). Ethnicity was not routinely recorded but all patients were of European ancestry as observed during sample collection (by EV). There were more females (61.1%) than males (38.9%) in the cohort. The percentage of invited males and females that agreed to participate was 54.1% and 65.6% respectively (p = 0.29).

Genotyping

Nine saliva samples contained too little DNA for genotyping with the AmpliChip (< 50 ng). The call rate after a single run was 93.3% for *CYP2D6* (three no-calls) and 100% for *CYP2C19*. As expected the most frequent alleles were the *CYP2D6*1* and *CYP2D6*2* with a frequency of 0.35 and 0.21 respectively. The other functional allele, *CYP2D6*35* was found with a prevalence of 0.10. The *CYP2D6*4* allele was the most frequent zero activity allele followed by the *CYP2D6*5* and *CYP2D6*3* allele (0.17, 0.02, 0.01 respectively). The inactive alleles *CYP2D6*41*, *CYP2D6*9*, and *CYP2D6*10* had a prevalence of 0.06, 0.04 and 0.02 respectively. Most of the patients (54.8%) were predicted to have the EM phenotype, followed by IM (38.1%), UM (4.8%) and PM (2.4%). Prevalence of the *CYP2C19*1* and *CYP2C19*2* allele was 0.90 and 0.10 respectively. No carriers of *CYP2C19*3* allele were found. One patient was homozygous for the *CYP2C19*2* allele and therefore categorized as PM. Seven patients were heterozygous carriers of the *CYP2C19*2* allele and therefore categorized as IM. All other patients were considered EMs.

Medication history

The mean number of unique prescribed drugs per patient was 15.2 (95% CI 13.4-17.1) in the studied two year period with an average of 4.6 (95% CI 4.0-5.2) prescriptions per unique prescribed drug. The percentage of CYP2D6 substrates with a pharmacogenetic recommendation of the total number of prescribed drugs was not different between CYP2D6 non-EMs, and patients with a predicted CYP2D6 EM-phenotype with 4.70% and 4.86% respectively (p = 0.95). For CYP2C19 similar results were found with 8.53% and 9.26% of the prescribed drugs having a pharmacogenetic recommendation for non-EMs and EMs respectively (p = 0.74). For patients with a predicted CYP2D6 or CYP2C19 non-EM phenotype detailed information about prescriptions for drugs for which a CYP2D6 or CYP2C19 pharmacogenetic recommendation was available are provided in Table 4.1. The CYP2D6 PM did not use any drugs with a CYP2D6 pharmacogenetic recommendation. Of the CYP2D6 UMs one patient did not use any of the drugs with a CYP2D6 pharmacogenetic recommendation and one patient used codeine 10 mg three times daily and paroxetine 20 mg once daily. The codeine was limited to a single prescription

Table 4.1 Patients with non-extensive metabolism for CYP2D6 or CYP2C19 and prescribed drugs with a CYP2D6 or CYP2C19 pharmacogenetic recommendation in the Dutch Pharmacogenetics Working Group guidelines [8]

Subject	CYP2D6 phenotype	No of CYP2D6 drugs with recommendation (% total drugs)	Prescribed CYP2D6 drugs with recommendation	CYP2C19 phenotype	No of CYP2C19 drugs with recommendation (% total drugs)	Prescribed CYP2C19 drugs with recommendation	Total No of prescribed different drugs
601	**IM**	3 (15.0)	Metoprolol 200g retard qd Tramadol 100mg/ml drops	EM	2 (10.0)		20
2798	**IM**	1 (5.6)	Tramadol 50 mg qd	EM	2 (11.1)		18
11375	**IM**	0 (0.0)		EM	1 (12.5)		8
11793	**IM**	1 (7.1)	Metoprolol 100 mg retard qd	EM	1 (7.1)		14
15750	**IM**	1 (4.8)	Codeine 20 mg tid	EM	1 (4.8)		21
17777	**IM**	0 (0.0)		EM	1 (12.5)		8
27160	**IM**	0 (0.0)		EM	1 (6.7)		15
30182	**IM**	1 (11.1)	Metoprolol 100 mg retard qd	EM	1 (11.1)		9
30310	**IM**	0 (0.0)		EM	2 (13.3)		15
30430	**IM**	0 (0.0)		**PM**	1 (4.3)	Pantoprazole 40 mg bid	23
123890	**IM**	0 (0.0)		EM	1 (5.9)		17
125010	**IM**	2 (6.7)	Tramadol 50 mg bid Amitriptyline 10 mg bid	EM	0 (0.0)		30

125073	**IM**	0 (0.0)		EM	1 (4.30		23
138981	**IM**	0 (0.0)		**IM**	2 (7.7)	Pantoprazole 40 mg qd Omeprazole 40 mg qd	26
143823	**IM**	2 (6.7)	Paroxetine 20 mg qd Tramadol 50 mg tid	EM	2 (6.7)		30
294439	**IM**	2 (18.2)	Codeine 10 mg qid Metoprolol 50 mg retard qd	EM	1 (9.1)		11
158869	**PM**	0 (0.0)		EM	1 (5.3)		19
272	**UM**	0 (0.0)		EM	1 (4.8)		21
10639	**UM**	2 (14.3)	Codeine 10 mg tid Paroxetine 20 mg qd	**IM**	2 (14.3)	Clopidogrel 75 mg qd Omeprazole 40 mg bid	14
600	EM	0 (0.0)		**IM**	1 (7.7)	Omeprazole 20 mg qd	13
2872	EM	0 (0.0)		**IM**	1 (4.8)	Pantoprazole 20 mg qd	21
4634	EM	0 (0.0)		**IM**	1 (10.0)	Omeprazole 20 mg qd	10
122600	EM	0 (0.0)		**IM**	1 (11.1)	Esomeprazole 40 mg qd	9
214384	EM	1 (8.3)		**IM**	1 (8.3)	Omeprazole 20 mg qd	12

Non extensive phenotypes are indicated in bold. Empty cells indicate that the subject had no prescription for any drug substrate with a recommendation in the Dutch Pharmacogenetics Working Group guidelines. PM, poor metabolizer; IM, intermediate metabolizer; EM, extensive metabolizer; UM, ultra-rapid metabolizer; QID, four times a day; TID, three times a day; BID, two times a day; QD, once daily.

only. The latter patient was also predicted to be a CYP2C19 IM and used clopidogrel 75 mg once daily and omeprazole 40 mg twice daily. The reduced metabolic capacity for CYP2C19 and the drug-drug interaction between clopidogrel and omeprazole both lead to reduced formation of the active metabolite of clopidogrel and subsequent increased risk for therapeutic failure [14,15]. The CYP2D6 IMs were often prescribed tramadol or codeine, drugs that both are expected to have a reduced analgesic effect in CYP2D6 IMs. Metoprolol was also often prescribed but according to the guidelines of the Dutch Pharmacogenetics Working Group only requires a dose adjustment when used for heart failure. The CYP2C19 IMs and PM were mostly prescribed proton pump inhibitors that do not require a dose adjustment since decreased metabolism results in increased therapeutic efficacy.

Since we were interested in the potential impact of pharmacogenetics on primary care the medication history of all patients was further evaluated for drugs metabolized by other enzymes than CYP2D6 and CYP2C19. On average patients used 2.3 (15.9%, 95% CI 1.9-2.7) drugs for which a recommendation of the Dutch Pharmacogenetics Working Group was available. The most frequently prescribed drugs with a pharmacogenetic recommendation were the proton pump inhibitors followed by phenprocoumon and metoprolol (Table 4.2). The number of drugs with a therapeutic recommendation did not differ between EMs and non-EMs with 2.2 versus 2.5 drugs for CYP2D6 EMs and

Table 4.2 Frequency of patients with a prescription for a drug with a recommendation in the Dutch Pharmacogenetics Working Group guidelines [8]

ATC	Drug	Patients (n)
A02BC01	Omeprazole	37
A02BC02	Pantoprazole	19
B01AA04	Phenprocoumon	14
C07AB02	Metoprolol	12
N02AX02	Tramadol	7
N06AB05	Paroxetine	5
N06AA09	Amitriptyline	5
R05DA04	Codeine	5
B01AC04	Clopidogrel	4
G03CA03	Estrogen	4
B01AA07	Acenocoumarol	2
N06AB06	Sertraline	2
N02AA05	Oxycodone	1
N06AA10	Nortriptyline	1
N06AA02	Imipramine	1
N06AB04	Citalopram	1
A02BC05	Esomeprazole	1
L04AX01	Azathioprine	1

non-EMs respectively ($p = 0.48$) and 2.5 versus 1.8 drugs for CYP2C19 EMs and non-EMs respectively ($p = 0.24$).

DISCUSSION

The available evidence of genetic variants with clinical relevance regarding both efficacy and toxicity of drug therapy is accumulating rapidly. Elaborating on this information, multiple initiatives to develop clear cut therapeutic guidelines translating available evidence to therapeutic recommendations have been initiated [7,8,16]. However, there is little information regarding the potential impact of these recommendations in primary care. This study indicates that a majority of patients is willing to participate in a pharmacogenetic screening study and that pharmacy initiated pharmacogenetic screening is feasible in a primary care setting.

Of the invited patients 58.1% was willing to participate in our study. This is a relatively large percentage given the fact that screening was not directly related to a clinical problem. For patients presenting with an adverse drug event or lack of therapeutic effect, willingness to participate in pharmacogenetic screening may be even higher. Age has also been reported to be of influence on the attitude towards pharmacogenetic testing, with younger patients being more likely to be optimistic about the usefulness of pharmacogenetic testing [17]. Therefore, willingness to participate may be higher in a population younger than 60 years. On the other hand, we collected DNA samples during a visit at the patients' home. Collection by mail may result in lower response rates.

There were more females than males included in our study although there was no statistically significant difference in the willingness to participate between the sexes. Sex differences concerning pharmacogenetic testing have been reported, with female patients being more likely to have concerns regarding the possible negative consequences of pharmacogenetic testing and being less willing to participate than males [17,18]. However, the finding that more females were included in this study might simply be explained by the fact that from 65 years onward the female to male ratio is starting to increase due to higher life expectancy of females.

The reported allele, genotype and phenotype frequencies are comparable to previously reported results obtained with the AmpliChip [19] or other methods such as PCR- RFLP in comparable populations of mainly white subjects [20,21]. These results indicate that no selection bias has occurred and that our patient cohort is representative.

Our study is limited in that we used a dichotomous outcome parameter to categorize drugs as either "with" or "without" a pharmacogenetic recommendation in the Dutch Pharmacogenetics Working Group guidelines. All drugs for which according to the guidelines a gene-drug interaction is present and an action is required were put in

a single category. This is an oversimplification because the guidelines provide many different types of recommendations, e.g. advice to adjust the dose, be extra alert to diminished therapeutic efficacy or increased risk for an ADE. Also, the recommendation depends on the metabolism of the drug, e.g. does the drug have active metabolites or is it a prodrug? For example, codeine is used for both pain and cough. The analgesic effect requires the formation of morphine by CYP2D6. Therefore, CYP2D6 UMs will require a dose reduction for an equal pain reduction compared to EMs whereas no dose reduction is required for the effect on cough. Dose adjustments were not investigated in this feasibility study. In addition, patients that used at least one drug that is metabolized by CYP2D6 or CYP2C19 were selected for inclusion. As a result, this study does not provide quantitative estimates of the incidence of the use of drugs with a pharmacogenetic recommendation for CYP2D6 or CYP2C19 in the Dutch pharmacogenetic guidelines. This study was not designed to and therefore does not provide direct evidence that the use of pharmacogenetic recommendations results in improved efficacy or decreased toxicity. That requires further study.

In our study we identified some potential pitfalls for pharmacogenetic screening in primary care as performed in this study. First, for a number of patients the production of the required 2 ml saliva was difficult. Since the included patients used at least five different medications this might be explained by the use of anticholinergic medication or other drugs that cause a dry mouth. Indeed five of the nine patients that failed to provide sufficient DNA used this type of medication.

Secondly, the no-call rate for the AmpliChip was 6.7% for *CYP2D6*. This is relatively high when compared to results reported by other groups. In a study with 158 breast cancer patients treated with tamoxifen Borges et al. report a no-call rate of 0.7%. Serrano et al. report a no-call rate of 1.6% in a of 182 Italian breast cancer patients treated with tamoxifen. Both studies used DNA extracted from whole blood for genotyping. However, in a large study of 4,532 psychiatric patients the no-call rate for *CYP2D6* with the AmpliChip was 6.0% after three rounds of testing [22]. In that study it was first attempted to genotype patients with DNA extracted from buccal swabs or saliva specimens. When saliva/buccal DNA failed to provide a genotype, blood DNA was tested. The no-call rate after primary genotyping was as high as 13%. According to the authors, this was mainly the result of DNA collection with buccal swabs since this DNA tends to be contaminated by bacterial DNA [22]. DNA extracted from saliva has been reported to be of high quality and a suitable alternative to blood DNA [23]. In our laboratory we compared genotyping results of an additional set of 24 DNA samples for which both blood and saliva were available. The no-call rate for saliva was 4% higher compared to the no-call rate for blood samples with 3/24 and 2/24 for saliva vs. blood respectively. Although these results are inconclusive and further investigation is required, the results may indicate that DNA extracted from saliva results in slightly less successful genotyping with the AmpliChip.

According to the guidelines of the Dutch Pharmacogenetics Working Group approximately 5-10% of the drugs prescribed to patients with aberrant CYP2D6 or CYP2C19 metabolism require an action such as a dose adjustment or extra awareness for an ADE [8]. In our study there was no difference in the percentage of prescribed CYP2D6 and CYP2C19 substrates between EMs and non-EMs of CYP2D6 or CYP2C19. This suggests that physicians have not empirically identified patients with aberrant metabolism e.g. by switching patient with ADE to medications that were not substrate for CYP2D6 or CYP2C19. From Table 4.1 it can also be observed that patients with a non-EM phenotype were prescribed regular drug dosages. The current data do not allow an in depth analysis of switching behavior since this requires a complete medication history, including the first prescription, and not just a cross-sectional period of two years.

Of the total number of prescribed medications 15.9% had a pharmacogenetic therapeutic recommendation in the Dutch guidelines. This number is comparable to a previously published estimate that 15-20% of prescribed drugs are metabolized by genetically polymorphic enzymes [24]. However, our results should be interpreted with extreme caution since one of the inclusion criteria was the use of at least one drug metabolized by CYP2D6 or CYP2C19. Therefore, the number of drugs with a pharmacogenetic recommendation may be overestimated in our data.

In conclusion, this study shows that pharmacy initiated pharmacogenetic screening is feasible for a primary care setting.

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Quality Control of Pharmacogenetic Testing

Use of Plasmid-derived External Quality Control Samples in Pharmacogenetic Testing

5

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ABSTRACT

Objectives: Genetic variation in genes encoding for drug-metabolizing enzymes, drug targets and signaling pathways have proven to contribute significantly to differences in drug response. Pharmacogenetics is now expanding from clinical pharmacological research to its application in clinical practice. Genotyping of patients in a routine clinical setting requires robust and reliable genotyping methods.

Materials & methods: A survey of pharmacogenetic association studies for quality control samples published from 2005 to 2007 in the two most prominent pharmacogenetic journals, and development of plasmid-derived external controls.

Results: Surveying journals revealed that only a minority of papers report the use of quality controls, and no standard procedures are applied. We established 12 plasmid-derived external controls and applied these in pharmacogenetic testing.

Conclusion: There still is a need for quality control materials, especially for application in pharmacogenetic testing. We hope that our initiative to create plasmid-derived controls will help to facilitate quality in the pharmacogenetic genotyping tests applied in research, as well as in routine patient care.

INTRODUCTION

Pharmacogenetic testing prior to drug treatment is not a routine practice, and is largely confined to academic hospitals and specialized laboratories [1]. Several reasons for the relatively slow translation of pharmacogenetics to the clinic may be considered [2,3]. The most important reason is the lack of scientific evidence of improvement in patient care by pharmacogenetic testing. Also, the limited insight into cost consequences and cost–effectiveness of a pharmacogenetic strategy presents an obstacle for implementation. Other reasons include the limited number of studies reporting diagnostic test criteria such as sensitivity, specificity and predictive value of pharmacogenetic tests, the lack of guidelines that help clinicians to link the sometimes complex results of a pharmacogenetic test to therapeutic recommendations, and finally the limited acceptance of pharmacogenetic testing by clinicians. However, three US FDA approved pharmacogenomic tests have recently become available (Amplichip®, Roche [Basel, Switzerland] [101], Invader® UGT1A1 molecular assay, Third Wave Technologies [WI, USA] [102] and Verigene® system, Nanosphere [IL, USA] [103]). Moreover, the FDA has included pharmacogenetic test information to the labels of several older and new drugs such as 6-mercaptopurine, azathioprine, atomoxetine and irinotecan [4], and recently the first large, randomized, double-blind prospective study providing evidence that pharmacogenetics can improve patient care has been published [5]. These points illustrate that, though not as fast as once anticipated, pharmacogenetics has reached the clinic and implicates that more pharmacogenetic tests will be introduced in the near future. Clinical application of pharmacogenetics will result in adjustment of treatment for individual patients. For example, patients at a high risk for having an undesired drug reaction or therapeutic failure owing to polymorphisms in *CYP2D6* might receive an adjusted dose or change of drug therapy. Therefore, pharmacogenetic tests for clinical use need to be even more robust and reliable than testing methods for investigational use. Since 1999, the Center for Disease Control and Prevention (CDC) has initiated studies to assess the status of quality assurance practices of laboratories performing genetic testing [6]. One of the core recommendations from these studies was to improve the availability of quality control materials with utmost urgency [6,7]. Several organizations, such as The National Academy of Clinical Biochemistry [104] and the FDA [105], provide guidelines or survey programs [106] for pharmacogenetic testing. One of the main aims of these organizations is to provide quality control material for genetic testing [7]. Indeed, Jarvis et al. inserted targeted sequences of the cystic fibrosis gene into a plasmid to use that as quality control in clinical molecular testing [8]. They propose to use this approach as quality control for genetic testing.

However, despite the existence of commercially available reference material (i.e., from Coriell [NJ, USA] [107]; Gentris [NC, USA] [108]; or GeT-RM cell lines collected by the CDC [109]) this does not seem to be widely used for pharmacogenetic testing.

In order to evaluate the use of qualified controls for genotyping studies, we surveyed all association studies that were published in the two most prominent pharmacogenomics journals from 2005 to 2007, to assess how often and which controls are used as quality controls. We found that only a minority of studies reported the use of quality controls, and overall no standard procedures are used. We established plasmid controls for frequently determined alleles: *TPMT*2*,**3B/C*; *CYP2D6*3*, **4*, **6*, **9*, **41*; *CYP2C9*2*, **3*; *CYP2C19**2, and **3* and argue for the use of plasmids as standard genotyping controls.

METHODS

Literature study

In March 2008, we manually checked papers that have been published in the journals Pharmacogenomics and Pharmacogenetics and Genomics in 2005, 2006 and 2007. The materials and methods sections were searched for the use of genotyping quality controls. The retrieved papers in which samples were genotyped, were marked as using 'no controls', 'intern controls' (own material) or 'external controls' (well-characterized material).

Materials

Plasmid pGEM-Teasy was obtained from Promega (Leiden, the Netherlands). Primers and pyrosequence materials were obtained from Isogen (IJsselstein, the Netherlands). Sepharosebeads were bought from Amersham (Buckinghamshire, UK), Taqman® kits were bought from Applied Biosystems (Nieuwerkerk aan de IJssel, the Netherlands). PCR reagents and plasmid isolation kit was obtained from Qiagen (Breda, the Netherlands). Materials used to create the plasmid were obtained from previously collected and genotyped controls. Informed consent was obtained from all participants.

Generation & genotyping of plasmids

In order to establish plasmid controls that can be used for several techniques, we choose primers approximately 500 nucleotides up and downstream of the SNP. See Table 5.1 for sizes of the different amplicons and the position of the SNP herein. All obtained plasmids were numbered and sequenced. All plasmids are created by ligation of a specific PCR product into pGEM-Teasy and transformation to competent cells. Primers are chosen approximately 500 nucleotides up- and down-stream of the specific SNP. Primer sequences are listed in Table 5.1. PCR conditions were as follows: activation of polymerase at 95°C for 15 min, 35 cycles of 95–55–72°C, each for 1 min, followed by a final extension of 10 min at 72°C. Each PCR reaction consisted of 5 pmol of each primer, 6 μl hotstart mastermix, 10 ng of chromosomal DNA in a total volume of 12 μl. All products were

Table 5.1 Primer names and sequences

Nr	Name	SNP	Sequence 5′-3′	Size	Position SNP
0363	*TPMT*2* (G>C)	forward	TTCACTTTAGTACAGTAGCTAC	1150	525
0364	*TPMT*2*	reverse	TCACCATGCTTCAGGAAGC		
0365	*TPMT*3B* (G>A)	forward	ATTACACACTCGTCTGCACAC	1150	554
0366	*TPMT*3B*	reverse	GGTCTCAAACTCCTGGG		
0367	*TPMT*3C* (A>G)	forward	ACAATTCAGAGTTCAGGAAATT	1150	570
0368	*TPMT*3C*	reverse	ATCACCTGAACCTGGGAGGC		
0369	*CYP2C19*2* (A>G)	forward	AAAAGCTTTGAAATCCCCAACTA	1090	552
0370	*CYP2C19*2*	reverse	ATTCCTAACCAGCTGTCTCATC		
0371	*CYP2C19*3* (A>G)	forward	ACAGAAGTCATTTAACTGCTCTG	1092	558
0372	*CYP2C19*3*	reverse	TTTGCATTTCTCCAATGACTTC		
0373	*CYP2C9*2* (C>T)	forward	GCCATCTGAGTGGCAAGTAT	1150	610
0374	*CYP2C9*2*	reverse	AGAAACCCCAGAGAAGTCAG		
0375	*CYP2C9*3* (A>C)	forward	TCCATCCAGGTCAGTAACAG	1150	521
0376	*CYP2C9*3*	reverse	AAGTTGACAGATTAACATCATC		
0377	*CYP2D6* (**6*4*3*9*41*)	forward	CACCTGCACTAGGGAGGT	2330	*6: 519
0378	*CYP2D6*	reverse	CCCTGCCTATACTCTGGAC		*4: 658
					*3: 1370
					*9: 1434
					*41: 1809

checked by agarose gel electrophoreses. Ligation reaction consisted of 4 μl PCR product, 0.5 μl pGEM-Teasy, 0.5 μl ligase and 5 μl water. Incubation for at least 1 h at room temperature, prior to transformation to DH5α cells and plating at ampicillin, X-gal and isopropylthiogalactoside (IPTG) containing Luria Broth (LB) agar plates. After overnight incubation at 37°C, white colonies were picked, grown in LB broth and checked for insert. Next, plasmids were sequenced using conventional methods and genotype was confirmed by pyrosequencing (*TPMT* and *CYP2D6*), restriction analysis (*CYP2C9*) or Taqman analysis (*CYP2C19*) according to manufacturers' protocol. Of note, heterozygous controls were obtained by mixing two plasmid controls.

RESULTS

Literature study

In volumes 6 (1–8; 2005), volumes 7 (1–6; 2006) and volumes 8 (1–12; 2007) of Pharmacogenomics, and in volumes 15 (1–12; 2005), volumes 16 (1–12; 2006) and

volumes 17 (1–12; 2007) of Pharmacogenetics and Genomics, a total of 547 papers were published and 135 of these involved studies in which samples were genotyped. From these, 116 (86%) did not use or mention genotyping quality control, 12 (9%) did use quality controls but did not define them, in three studies (2%) a representative sample was sequenced as an additional control, and four studies (3%) used a defined control which was a previously sequenced sample (n = 2) or a reference panel from The Centre d'Etude du Polymorphism Humain (CEPH) (n = 2).

Sequencing plasmids

The generated plasmids are shown in Table 5.2. The sequences of all plasmids are available on request.

Table 5.2 Plasmids

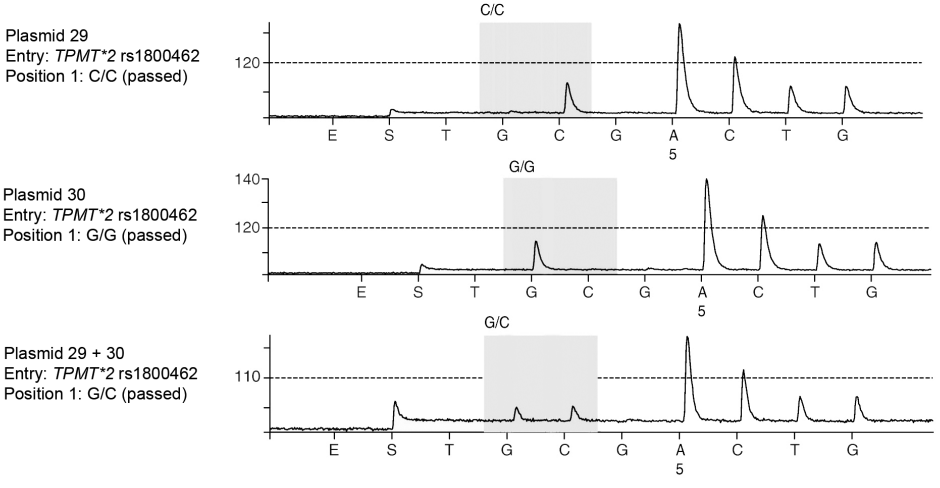
Gene	SNP	Synonym	rs nr	Plasmid nr	Genotype
CYP2D6[a]	1707Tdel	*6	rs5030655	40,41,42	1707Tdel
	G1846A	*4	rs3892097	1	1846A
	2549Adel	*3	rs4986774	2	2549Adel
	2613-2615AGAdel	*9	rs5030656	3,4	2613-2615AGAdel
	G2988A	*41	rs28371725	5	2988A
CYP2C9	C3608T	*2	rs1799853	6	3608C
				7	3608T
	A42614C	*3	rs1057910	9,10,11	42614A
				12,13	42614C
CYP2C19	G19154A	*2	rs4244285	18	19154G
				20	19154A
	G17948A	*3	rs4986893	22,23,24	17948G
				25,26,27,28	17948A
TPMT	G238C	*2	rs1800462	29	238G
				30,31,32	238C
TPMT	G460A	*3B	rs1800460	33,34,35,36	460G
				43	460A
	A719G	*3C	rs1142345	39	719A
				37,38	719G

[a]As a reference for *CYP2D6* SNPs a plasmid control was used with the wild-type nucleotides at designated positions (available on request).

Genotyping plasmids

The genotypes of all plasmids are listed in Table 5.2, and the result of a representative genotyping assay is shown in Figure 5.1. Results of the genotyping assays for the other plasmids are available on request.

A



B

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   1 TTCACTTTAG TACAGTAGCT ACTAGCCACA TGTGGCTATT TAAATGTAAA
  51 CTAAATAAAA TGTAAAATTC AGTTCCTCAA TCACGAGGAA CCAATGTGGC
 101 TAATGCCTAT TTTATTGGAC AGCGACAGAT ATAGACATTT TCATCAATGC
 151 AGAAAATTTT AATGGGCAGT GCTGATTTAG AGAAATTGAA AAGCATTTCC
 201 TCTAGTCAAA TCAATTTGTA TTAAATCAGT ATTTTGTTAT ATATCTATAA
 251 TTACATTCCA ACTGTTTCAT ACATAAAAAA AGATATATAT AATTTTCCAA
 301 ATTTTTATTG TTTCCTGAAT TCATATAAGT CAGTTTTTCA GAATTTTTAT
 351 AAGGTTTGAA AATAATATAG ATCTGCTTTC CTGCATGTTC TTTGAAACCC
 401 TATGAACCTG AATTCATATA AATTCCTCTA AATTAAAGAA AATATATGCT
 451 TACTCTAATA TAACCCTCTA TTTAGTCATT TGAAAACATA ATTTAAGTGT
 501 AAATGTATGA TTTTATGCAG GTTTGCAGAC CGGGGACACA GTGTAGTTGG
 551 TGTGGAAATC AGTGAACTTG GGATACAAGA ATTTTTTACA GAGCAGAATC
 601 TTTCTTACTC AGAAGAACCA ATCACCGAAA TTCCTGGAAC CAAAGTWTTT
 651 AAGGTTTGTT TTGATTTGGG TAAATAATTG TATCCATATC CCCACAAAAG
 701 TTTTTCTCAG CGTGAGTATT ATGAGGATAC CATTCATGTG TCCGATGGTT
 751 CCTATTTAGC ACGCAGATTC ACTGTAGATA CTATATAGTA TAAGAAGCAA
 801 GGGCTTAAAA ATATAGGTGA TAGCTACCTA AATAGGTATA GACATATGTA
 851 TATAAAAGCT GAGGTCAAAG CCTCTCTGTA CTCAAGCTTT TAGGCTTGTT
 901 TTATTTTTAT TAACACGCAT TTTCTGAGAA CCCATCATGT GCCAGACCCT
 951 GCCTAAGACA TTGAAGAGAT AAAGATAACA CAGCACACCC CCCTCACTCC
1001 CACCCCTAAA GAATCTCTTA GTTTAGGAGA GAAGAGAAAC AGGGACAAAG
1051 CTATTTGTAA TGCATGGAGC TAAGTGTAAA GACACAGGGT ATTAAGGGAA
1101 GAGATGGGAG TTACCTGCCC AACCTGGCGG TGCTTCCTGA AGCATGGTGA
```

Figure 5.1 Example of validation report of *TPMT*2*. Plasmids with insert (see sequence) are genotyped by pyrosequencing. Heterozygous genotype is the result of mixture of both homozygous plasmids 29 and 30. Pyrosequence primer was reverse orientated, therefore derived genotype is from complementary strain and should be reversed.

DISCUSSION

This study indicates that the use of external quality standards for genotyping assays is rare and not standardized. We found that in 2005, 2006 and 2007, 86% of papers in which samples were genotyped, and published in both the surveyed journals, did not use or define quality controls. We surveyed 20 other association studies, in randomly selected journals such as Rheumatism and Arthritis, Cancer and others, and found that one study mentioned the use of controls, but did not define them, and only one study was using CEPH DNA as controls [9]. Two studies declared that they sequenced a few samples to confirm the genotype. This indicates that the lack of using well-defined quality controls is a general observation in pharmacogenetic publications. In general, as a quality assurance, most studies duplicate 5–10% of the samples. This may serve as an internal control, for instance to exclude sample exchanging, but can not be regarded as an independent quality control sample to assure the validity of the pharmacogenetic test. We propose that independent external quality control samples are required to be included in (pharmaco) genetic testing, as was previously suggested by Jarvis et al. [8].

By cloning the SNP of interest in a plasmid we created a set of plasmids that can be used for different pharmacogenetic assays. We cloned the most important genotypes, which are used for diagnostic testing in a plasmid and sequenced the insert. In addition, we tested these plasmids by pyrosequencing and/or Taqman analysis and found 100% concordance. However, we are aware that this is only the proof of principle and external validation by an independent laboratory is required.

At present we have cloned *TPMT*2*,**3B/C*; *CYP2D6*3*, **4*, **6*, **9*, **41*; *CYP2C9*2*, **3*; and *CYP2C19*2*, **3*. In order to guarantee reliable genotyping results, independent of the assay or hospital, we argue for the use of these plasmids as external controls for genotyping testing. This selection of plasmids covers the most clinically relevant SNPs of *TPMT*, *CYP2C9*, *CYP2C19* and *CYP2D6* for pharmacogenetic testing in Caucasians at present. One should be aware that the exclusion of a SNP does not automatically identify a patient as wild-type for that gene, since not all mutations are covered in the assay.

The work described in this manuscript represents a new application for plasmid-derived control and expands their use to the field of pharmacogenetics. This should enable pharmacogeneticists to use standard genotype controls for diagnostic testing. Pharmacogenetic testing for diagnostic reasons demands good-quality controls. In the USA, the CDC initiated studies to assess the status of quality assurance practices of laboratories performing genetic testing, and to develop recommendations for improvement in genetic testing [6,7]. Quality-control materials are essential for validating new tests, monitoring test performance and for detecting errors in the testing process; therefore, one of the issues with utmost urgency is, as acknowledged by the CDC, to improve the availability of quality-control materials. In concordance with laboratories

in the Netherlands, quality control materials in the USA are obtained through a number of sources, such as commercially available cell lines or DNA (i.e., CEPH from Coriell [107]), previously tested patient materials and inter-laboratory exchanges. However, despite the availability of the desired appropriate quality control materials, these resources are still not adequate for all genetic testing, especially for pharmacogenetic testing. For example in Europe, Eurogentest (Leuven, Belgium) [110] has started its activities in 2005 (funded by the European Commission). The aims of Eurogentest are to harmonize and improve the overall quality for existing genetic services. The raising of Eurogentest was the direct result of the lack of structure and harmonization at the European level; diverse quality schemes and lack of reference systems. There are close collaborations with a European project to develop reference materials, the Certified Reference Materials for Molecular Genetic testing project (CRMGEN [111]). Despite the call of CRMGEN for samples with interesting mutations to be banked, their reference material database exists of the common disorders like cystic fibrosis, Factor V Leiden, Huntington disease and more (a reference material summary sheet can be downloaded at their website [111]) but did not yet contain pharmacogenetic-related genes. Thus, also in Europe there are initiatives to develop and make available reference materials for genetic tests, although they are restricted to disease-related mutations at present, and not for use in pharmacogenetic testing. From these initiatives we can conclude that there still is a need for quality-control materials, especially for application in pharmacogenetic testing. We hope that our initiative to create plasmid-derived controls will help to facilitate quality in the pharmacogenetic genotyping tests applied in research, as well as in routine patient care.

EXECUTIVE SUMMARY

- Pharmacogenetics is now expanding from clinical pharmacological research to its application in clinical practice.
- Clinical application of pharmacogenetics will result in adjustment of treatment of individual patients. Therefore, quality assurance for clinically applied test is even more important than for investigational tests. This can be achieved by the use of standardized quality controls.
- Screening of the materials and methods sections of papers published in 2005–2007 in Pharmacogenomics or Pharmacogenetics and Genomics revealed that only in a minority of papers the genotyping of quality controls is reported, and no standard procedures are applied.
- We developed plasmid-derived external controls for *TPMT*2*, **3B/C*; *CYP2D6*3*, **4*, **6*, **9*, **41*; *CYP2C9*2*, **3*; and *CYP2C19*2*, **3*, and applied these in clinical testing.

- The development of plasmid-derived external controls for pharmacogenetic testing represents a new application and expands the use of plasmid-derived controls to the field of pharmacogenetics.
- Plasmid-derived quality controls enable the general use of standardized external quality controls in pharmacogenetic testing.

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6

Alternative Methods to a TaqMan Assay to Detect a Tri-allelic SNP in the *HNF1ß* Gene

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Clin Chem Lab Med
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ABSTRACT

Background: Several studies report difficulties in genotyping *HNF1β* rs757210 using TaqMan probes. This is possibly due to the tri-allelic nature of this SNP. The aim of the present research was to develop alternative methods for genotyping rs757210.

Material & Methods: Pyrosequencing and High Resolution Melting analysis of small amplicons (HRM) were developed and tested in panels of type 2 diabetes mellitus patients (n = 258) and healthy blood donors (n = 183). Results were confirmed by Sanger sequencing.

Results: With pyrosequencing, allele frequencies for the A, G and C allele of 0.42, 0.56, 0.02, and 0.37, 0.62, 0.01 were established in the panel of type 2 diabetes mellitus patients and healthy blood donors, respectively. Similar results were found using the more routinely available HRM method. Results for pyrosequencing and HRM were in 99.6% concordance.

Conclusion: Pyrosequencing and HRM can be used to genotype the tri-allelic SNP rs757210 in the *HNF1β* gene and have the advantage over the commercially available TaqMan analysis that they can determine the rare C-allele variant.

INTRODUCTION

Rs757210 is located in the gene that codes for hepatocyte nuclear factor 1β (HNF1β). HNF1β is a transcription factor and alterations in this gene are associated with congenital anomalies of the kidney and urinary tract, as well as an increased risk for developing type 2 diabetes mellitus (T2DM) [1-12]. Rs757210 is an intronic A/G SNP with a reported minor allele frequency of 0.43 (HapMap-CEU, accessed 03-12-2010). Genotyping of rs757210 with commercially available TaqMan probes was found problematic in multiple studies. Stancakova et al. reported genotyping results of 20 risk alleles for T2DM using TaqMan probes in a large cohort of non-diabetic men. *HNF1β* rs757210 had to be excluded from the analyses because of failure to achieve Hardy-Weinberg equilibrium (HWE) [13]. In an investigation of the influence of T2DM risk alleles on the risk for the development of diabetes after renal transplantation, Ghisdal et al. reported a low call rate for rs757210 compared to a call rate of > 99% for the 10 other genotyped SNPs [14]. Again, rs757210 was the only SNP that deviated from HWE. Holmkvist et al. report that genotyping rs757210 with matrix-assisted laser desorption/ionization-time-of-flight mass spectroscopy (MALDI-TOF) was successful in subjects from the Malmö Preventive Project whereas genotyping rs757210 with a TaqMan assay failed in subjects from the Botnia study. These authors noted that the SNP is tri-allelic what is considered the reason for problematic genotyping [15]. In our own experience, repeated genotyping of rs757210 with a TaqMan assay also failed to meet the pre-defined quality criteria of a call rate ≥ 95% in a recent study on the effect of T2DM associated risk alleles on the response to sulfonylureas in T2DM patients [16].

The finding that rs757210 is tri-allelic has been reported previously [1,15] but has received limited attention in the literature. Furthermore, the NCBI SNP database [http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=757210, accessed 27-06-2011] also does not mention that rs757210 is tri-allelic.

The aim of the present research was to develop alternative methods for genotyping rs757210. First we developed a pyrosequencing method, and since this methodology is not always available, also the feasibility of a high resolution melting method of small amplicons (HRM) to genotype rs757210 was investigated.

RESULTS

Pyrosequencing

Pyrosequencing confirmed rs757210 as being tri-allelic. Using the given nucleotide dispensation order we identified the rare variant C allele, with an allele frequency of 0.02 (95% confidence interval (CI) 0.01-0.03) in a cohort of 258 T2DM patients (Figure 6.1).

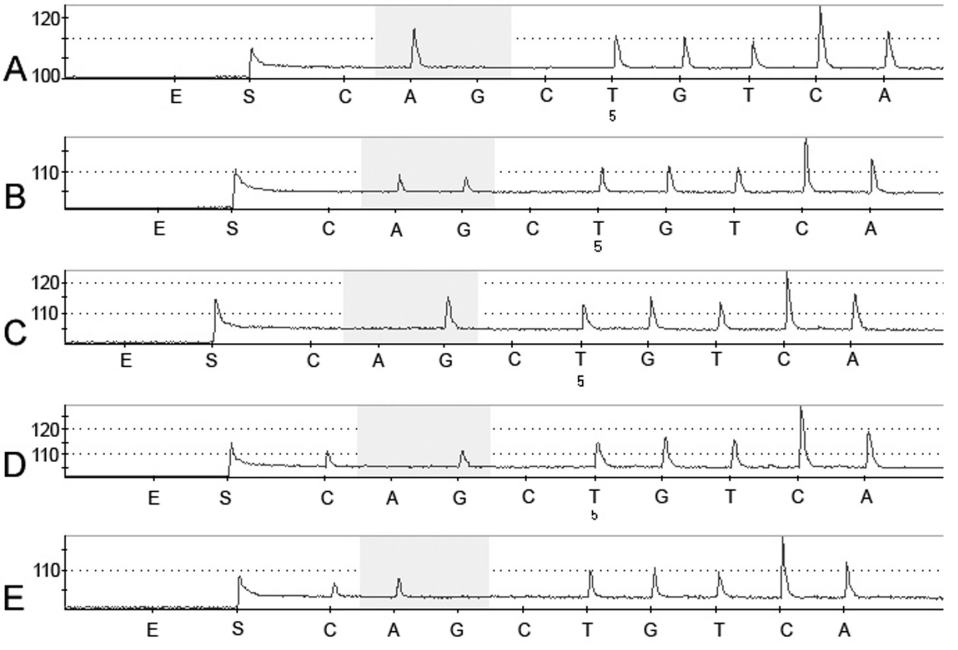


Figure 6.1 Pyrograms for *HNF1β* rs757210. Panels depict typical pyrograms obtained for HNF1β rs757210 for patients with AA genotype (panel A); AG genotype (panel B); GG genotype (panel C); CG genotype (panel D); AC genotype (panel E).

Allele frequencies for the A and G allele were 0.42 (95% CI 0.37-0.46) and 0.56 (95% CI 0.52-0.61), respectively. No homozygous carriers of the allele C were found. Results were in Hardy-Weinberg equilibrium ($p = 0.30$). To ascertain that the pyrosequencing results were valid, genotypes of the 8 patients carrying a copy of the variant C-allele and 13 (5%) other randomly chosen samples were confirmed by conventional Sanger sequencing. Results were in 100% concordance (data not shown). Subsequently, genotype frequencies of 0.37 (95% CI 0.32-0.42), 0.62 (95% CI 0.56-0.67), and 0.01 (0.002-0.03) for the A, G, C allele respectively, were established in a cohort of 183 healthy blood donors. No statistically significant differences were observed between the TDM population and healthy blood donors for any of the alleles (Table 6.1).

High Resolution Melting

Figure 6.2 depicts the typical melting curves of the 6 different *HNF1β* genotypes. Since no patient with the CC genotype was included, the CC melting curve was obtained from genomic plasmid control. HRM results were obtained for all of the 258 TDM patients. Results were in 99.6% concordance with the pyrosequencing results confirming the feasibility of this method.

Table 6.1 Allele frequencies of *HNF1β* rs757210 alleles in 258 type 2 diabetes mellitus patients and 183 healthy blood donors determined with pyrosequencing

	Type 2 diabetes mellitus patients (n = 258)		Healthy blood donors (n = 183)		
Allele	**Mean**	**95% CI**	**Mean**	**95% CI**	**p-value**
A	0.42	0.37-0.46	0.37	0.32-0.42	0.19
G	0.56	0.52-0.61	0.62	0.56-0.67	0.17
C	0.02	0.01-0.03	0.01	0.002-0.03	0.82

95% CI, 95% confidence interval.

MATERIALS AND METHODS

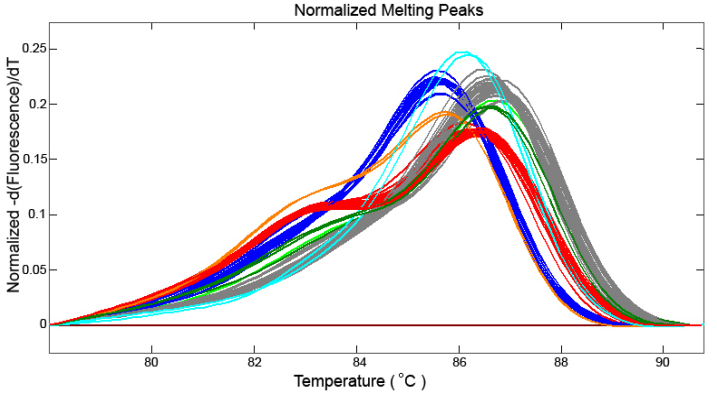
DNA samples and isolation

A total of 258 DNA samples was obtained from a panel of T2DM patients. Panel ascertainment and sample collection have been described in detail previously [17]. Briefly, patients that had at least one prescription of the oral antidiabetic drugs tolbutamide, glibenclamide, glimepiride or gliclazide between January 1992 and June 2008, were at least 18 years of age and without insulin use at the time of first sulfonylurea prescription were included. Patients were recruited from four university affiliated primary care centers located in the vicinity of Leiden, The Netherlands. Patients received a written invitation by mail from their general practitioner. All patients provided informed consent and the study was approved by the institutional ethics committee. DNA was extracted from saliva specimens using Oragene kits (Westburg, Veenendaal, The Netherlands). To compare allele frequencies between T2DM patients and healthy volunteers an additional set of 183 anonymized DNA samples was obtained from a panel of residual blood samples obtained from healthy blood donors (Sanquin, Leiden, The Netherlands). DNA was extracted with the Magnapure Compact instrument (Roche, Almere, The Netherlands) according to the manufacturers' protocol.

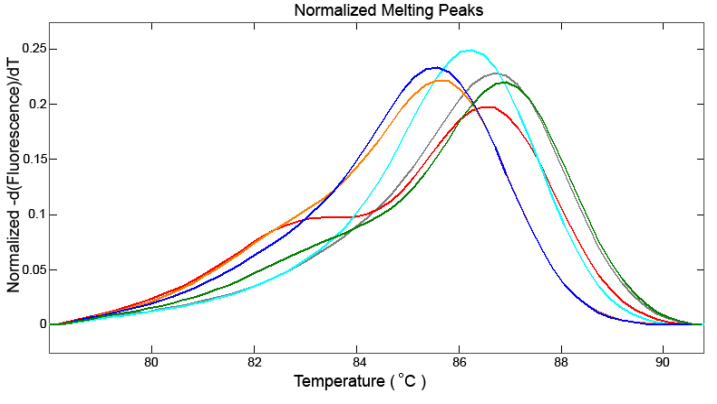
Pyrosequencing

First a pyrosequencing method was developed. Oligonucleotides were obtained from Eurogentec (Maastricht, The Netherlands). Primer sequences for polymerase chain reaction (PCR) and pyrosequence reactions are listed in Table 6.2. HotStar PCR mastermix and pyrosequencing reagents were obtained from Qiagen (Hilden, Germany) and PCR reactions were performed on the MyCycler (Biorad, Veenendaal, The Netherlands). The PCR reactions consisted of 10 ng DNA and 5 pmol of each primer in a total volume of 12μl. Cycle conditions were, initial enzyme activation for 15 minutes at 95°C, followed by

Panel A Mixture of samples from type 2 diabetes mellitus patients and genomic plasmid controls.



Panel B Typical melting peaks obtained from type 2 diabetes mellitus patients.



Panel C Typical melting peaks obtained from genomic plasmid controls.

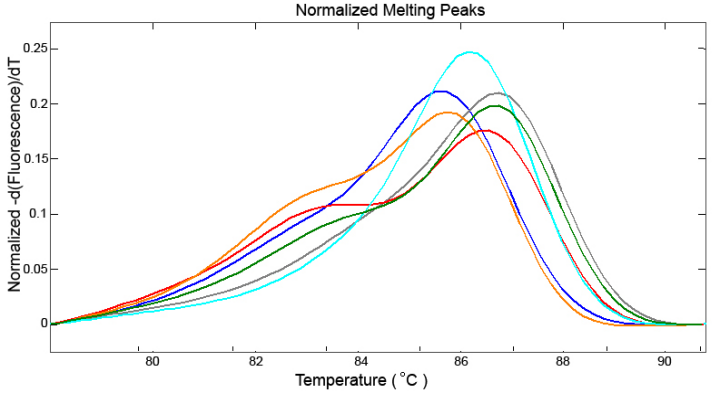


Figure 6.2 Typical melting peaks for the 6 different *HNF1β* rs757210 genotypes obtained with High Resolution Melting of the small amplicons. Lines represent results for AA (•);GG (•);AG (•); CG (•); AC (•), genotypes respectively, obtained from type 2 diabetes mellitus patients or plasmid controls, and CC (•) genotype obtained from genomic plasmid control.

Table 6.2 Primers for PCR and pyrosequencing of *HNF1β* rs757210

Name	Purpose	Modification	Orientation	Sequence 5′-3′
783	PCR		forward	CAGCTGCGAGAGTGTCTC
784	PCR	5′-Biotine	reverse	CTCCATGTGAGAAGTATAGC
785	Pyrosequencing		forward	AGCCTGCAGGCTGGGCTC
804	Plasmid		forward	TGAACACTGTTCAGCCTGC
805	Plasmid		reverse	TCTAAATGCAGTTACTTGAGG
806	HRM		forward	AAGCCTGACAGGCTGGGCTC
807	HRM		reverse	TTGGGGCAGGAGCCTGGAC

35 cycles of 95°C-55°C-72°C each for 30 seconds, and a final extension of 10 minutes at 72°C. Samples were genotyped on the Pyrosequencer 96 MA (Biotage, Uppsala, Sweden). The pyrosequence reactions were performed according to the manufacturers' protocol. Sequence to analyze was C/A/GTGTCCAGGCT and nucleotide dispensation order was TCAGCTGTCA. As a quality control 5% of the samples were genotyped in duplicate and no inconsistencies were observed. To ascertain that pyrosequencing results were valid, genotypes from more than 5% of patients were confirmed with Sanger sequencing, including all patients with the rare third allele.

High Resolution Melting of small amplicons

Secondly, the DNA samples from 258 T2DM patients were genotyped based on melting plots obtained by HRM of small amplicons. Results were compared to the results of pyrosequencing. Oligonucleotides used for small amplicon (+ 40 bp) genotyping were chosen adjacent to the SNP. Internal oligonucleotide calibrators were used to improve small amplicon genotyping. Oligonucleotide sequences are listed in Table 6.2. Amplification was performed in a 10 μl reaction volume in a HRM suitable 96-well plate (Thermofast ® 96 Skirted, ABgene, Westburg, Leusden, The Netherlands). The reactions included HotStar PCR mastermix (Qiagen, Hilden, Germany), 5 pmol primers, 1x LC-Green® Plus (Bioke, Leiden, The Netherlands), 0.05 mM internal oligonucleotide Calibrator (IDT, Coralville, USA) and 20 ng DNA. Reactions were overlaid with mineral oil (Sigma-Aldrich, Zwijndrecht, The Netherlands). PCR conditions was as follows: 15 minutes at 95°C, followed by 40 cycles of 95°C (10 sec)-55°C (20 sec)-72°C (30 sec) and a final extension of 5 minutes at 72°C followed by 1 minute at 95°C. Finally, the samples were cooled down to room temperature. High resolution melting was performed on the Lightscanner® (HR-96, Idaho Technology, Salt Lake City, USA). Melting was

done from 50°C to 98°C with ramp rate of 0.1°C/sec. Melting curves were analyzed with Lightscanner® Software using Call-IT 2.0. Again, 5% of the samples were genotyped in duplicate and no inconsistencies were observed.

Genotype controls

HRM requires the use of well characterised controls. Therefore plasmid controls with a 639 bp insert of the *HNF1β* gene, with the three variants of the SNP at position 240 were created. Preparation has been described in detail previously [18]. In short, plasmid controls were made by ligation of *HNF1β* PCR product into pGEM-T easy vector (Promega, Leiden, The Netherlands) and plasmid DNA was isolated by standard procedures. Genotypes of the plasmid controls were determined by conventional sequencing on the ABI 3130 analyser using BigDye® Terminator v3.1 Cycle Sequencing Kit From Applied Biosystems (Applied Biosystem, Nieuwerkerk aan de IJssel, The Netherlands).

Statistical analysis

The data were analyzed using the SPSS statistical package (version 17.0, SPSS, Chicago, IL, USA). Possible deviation from Hardy–Weinberg equilibrium was tested by the χ^2 test. A p-value < 0.05 was considered a deviation. Difference in allele frequencies between patients with T2DM and healthy blood donors were tested with an unpaired Student's t-test. A p-value < 0.05 was regarded as statistically significant.

DISCUSSION

In this study, we showed the feasibility and accuracy of pyrosequencing and HRM to genotype the tri-allelic rs757210 in the *HNF1β* gene. With the pyrosequencing method the presence of the rare variant C allele was confirmed and the allele frequencies were established in T2DM patients and healthy blood donors. To the best of our knowledge, this is the first study to report pyrosequencing and HRM as useful alternatives methods to TaqMan to genotype rs757210 with the latter method having the disadvantage to fail to determine the rare C-allele variant.

The presence of the tri-allelic nature of rs757210 was noted by Winckler et al. recently [1]. Holmkvist et al. established genotype frequencies in a Swedish population of 2293 healthy individuals of whom 132 developed type 2 diabetes [15]. Reported allele frequencies were 0.36, 0.61, and 0.03 for the A, G, and C allele respectively. These results are comparable to the pyrosequencing results reported in this article. However, genotyping by Holmkvist et al. was performed by MALDI-TOF. These techniques are not standard for most laboratories. For this reason we extended our investigations to

test the feasibility of HRM of small amplicons to detect these variants. HRM of small amplicons is an attractive genotyping method because it is fast, relatively cheap (no labelled primers are required), no sample processing is required after amplification and most new generation real-time machines, present in many laboratories performing genotyping studies, are equipped with this possibility making it more accessible for other investigators [19,20].

The problems of genotyping rs757210 with the commercially available TaqMan assays probably originate from the fact that the assay was designed without knowledge of the tri-allelic nature of the SNP. Both pyrosequencing and HRM are more flexible concerning probe design and therefore better suited to genotype this SNP.

A possible limitation of our study is that no homozygous carriers of the C allele were included. Therefore we were unable to test the suitability of the pyrosequencing and HRM method to genotype subjects with this genotype. Based on the frequency of the C allele, 0.04% of the population is expected to be homozygous carrier of the C allele and as a result very large cohorts are required to include them. Also, both methods were perfectly capable of identifying heterozygous carriers.

Carriers of the A allele of rs757210 have an increased risk to develop T2DM (OR 1.12 $p = 5*10\text{-}6$) [1,13]. Models that use *HNF1β* genotype and other genetic markers can explain approximately 5-10% of T2DM heritability [21]. In our cohort no statistically significant difference in allele frequencies was observed between patients with T2DM and healthy blood donors. This is most likely due to the limited sample size of our study. Post-hoc power analysis revealed that the power tot detect a statistically significant difference of 0.05 in allele frequency with an α of 0.05, was 48.8%.

HNF1β is an important gene. Besides its contribution to T2DM risk prediction it might also provide opportunities for new therapeutic interventions and management i.e. pharmacogenetics. Currently there are insufficient data to support the latter, but the knowledge of the pharmacogenetics of oral anti-diabetic drugs has increased significantly in recent years [22-24]. Combined with the ever increasing incidence of T2DM worldwide, these developments might anticipate a greater clinical role for genotyping *HNF1β* and other genetic variants, and endorse the relevance of easily available genotyping methods such as our pyrosequencing or HRM method.

Conclusion

In conclusion, we have shown that pyrosequencing and HRM can both be successfully applied to genotype the tri-allelic SNP rs757210 in the *HNF1β* gene.

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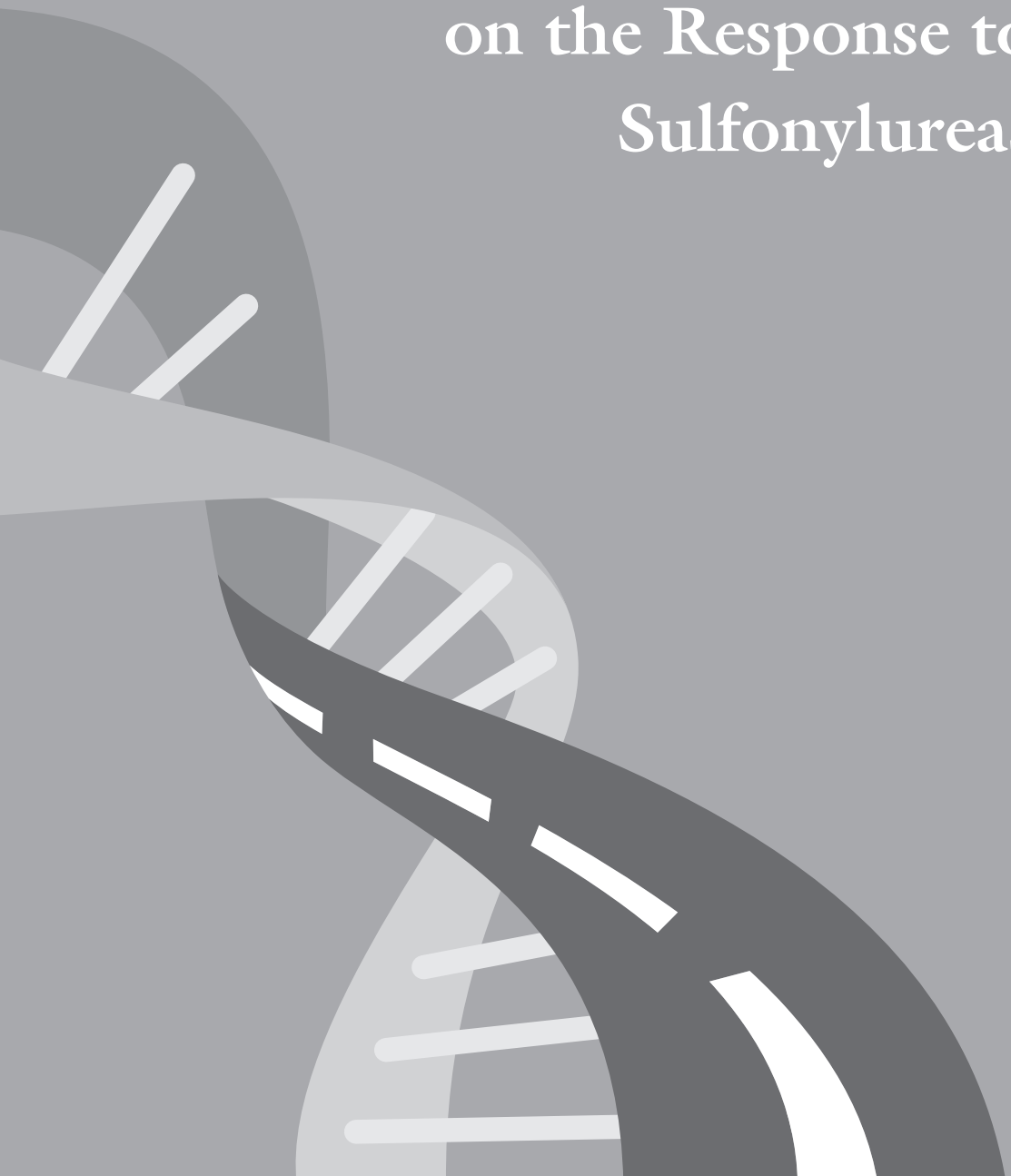
The Influence of Genetic Variation on the Response to Sulfonylureas



7

Effect of *CYP2C9* Polymorphisms on Prescribed Dose and Time-to-stable Dose of Sulfonylureas in Primary Care Patients with Type 2 Diabetes Mellitus

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ABSTRACT

Aims: Sulfonylureas are mainly metabolized by the enzyme CYP2C9. Two allelic variants, *CYP2C9*2* and *CYP2C9*3*, result in decreased metabolic capacity and have been associated with elevated sulfonylurea serum levels. However, most of the available data originates from pharmacokinetic analyses performed in healthy individuals. In this study, the effect of *CYP2C9*2* and *CYP2C9*3* alleles on prescribed dose and time-to-stable dose of sulfonylureas was investigated.

Materials & methods: A group of 207 incident sulfonylurea users treated in four university affiliated primary care centers were identified. The effect of the *CYP2C9*2* and *CYP2C9*3* alleles on prescribed dose and time-to-stable dose was then assessed.

Results: No significant effects of the *CYP2C9*2* and *CYP2C9*3* alleles were found. However, a trend towards a lower stable glimepiride dose for carriers of the CYP2C9*3 allele was observed.

Conclusion: Genotyping for the *CYP2C9*2* and *CYP2C9*3* alleles currently appears to have no clinical implications for dosing of sulfonylureas in primary care patients with type 2 diabetes mellitus.

INTRODUCTION

Sulfonylureas (SUs) are part of the mainstay of treatment of type 2 diabetes mellitus (T2DM) with oral antidiabetic drugs. They act by closing the pancreatic β-cell potassium channels, stimulating insulin secretion [1]. SUs are initiated at a low dose and titrated up to the optimal dose with intervals of 2–4 weeks until the glycemic target is achieved. Undertreatment will increase the risk of long-term microvascular and macrovascular complications, whereas overtreatment will lead to hypoglycemia, a well recognized adverse event that limits rapid dose escalation and is reported to be fatal in 1.4–10% of cases [2].

The enzyme CYP2C9 plays an important role in the pharmacokinetics of SUs. Two allelic variants, *CYP2C9*2* and *CYP2C9*3*, result in decreased metabolic capacity. Both alleles are relatively common in Caucasians [101]. Most of the available data regarding the effect of *CYP2C9* polymorphisms on SU treatment originate from pharmacokinetic analyses performed in healthy individuals [3–9]. Only four studies have assessed the effect of *CYP2C9* polymorphisms in T2DM patients. Presence of the *CYP2C9*3* allele was associated with hypoglycemia [10,11]. Furthermore, the *CYP2C9*3* allele is associated with the absence of tolbutamide dose escalation and carriers of the *CYP2C9*2* or *CYP2C9*3* allele are less likely to fail on SU monotherapy [12,13]. However, none of these studies have assessed the effect of the CYP2C9 genotype on the time required for SU dose titration. Therefore, the aim of this study was to investigate the effect of *CYP2C9*2* and *CYP2C9*3* alleles on prescribed dose and time-to-stable dose of SUs in T2DM patients in a primary care setting.

MATERIALS AND METHODS

Study setting

In The Netherlands patients are listed with one family physician (FP) who is consulted for all healthcare problems and indicates whether referral to secondary care is appropriate. The FP keeps an electronic patient record (EPR) that covers all medical information, including prescription data concerning the patient. T2DM patients are treated according to the T2DM guideline of the Dutch College of General Practitioners [14].

Study population

Patients were recruited from four university affiliated primary care centers located in the vicinity of Leiden, The Netherlands. Approximately 37,000 patients were enlisted. Retrospective clinical and prescription data were retrieved from the EPR. Patients were eligible for the study if they had received at least one prescription of tolbutamide, glibenclamide, glimepiride or gliclazide after 1992, were at least 18 years of age and

were without insulin use at the time of first SU prescription and had at least 270 days of follow-up registered in the EPR. To ascertain that a SU prescription was the first, a period of at least 6 months without SU prescriptions prior to that prescription recorded in the EPR was required.

Sample collection

Eligible patients received a written invitation by mail from their FP. After consent, a saliva collection kit (DNA Genotek, ON, Canada) was mailed. The ethics committee of the Leiden University Medical Center approved the study and informed consent was obtained from all participants.

Genotyping

Genotyping of *CYP2C9*2* (rs1799853) and *CYP2C9*3* (rs1057910) was performed by a TaqMan® allelic discrimination assay (Applied Biosystems, CA, USA), independently and without knowledge of the patient data. Assays were used according to the manufacturer's instructions and performed on 10 ng genomic DNA. Fluorescence detection and genotype calling were performed using an ABI Prism® 7750 Sequence Detection System (Applied Biosystems).

Definition of effect of *CYP2C9*2* and *CYP2C9*3* alleles on SU dose and time-to-stable dose

The primary end point of our study was the effect of the *CYP2C9*2* and *CYP2C9*3* alleles on the stable SU dose. This was defined as the first period of 270 consecutive days or more without SU dose adjustment, or initiation or adjustment of therapy with another SUs, insulin or metformin. Dose was normalized to allow for the pooling of different SUs by dividing the prescribed daily dose with the standard daily dose used by the Dutch Healthcare Insurance Board (10 mg glibenclamide; 1000 mg tolbutamide; 160 mg gliclazide; 2 mg glimepiride) [15]. The period of 270 days or more was chosen because prescriptions in The Netherlands are limited to a maximum of 90 days, and 270 days or more equals three consecutive prescriptions.

The secondary end points of our study were the effect of *CYP2C9*2* and *CYP2C9*3* alleles on the time to the first stable SU dose, and the effect of the *CYP2C9*2* and *CYP2C9*3* alleles on the number of dose adjustments during the first year of SU treatment. Finally, the effect of the *CYP2C9*2* and *CYP2C9*3* alleles on the change in fasting glucose levels was analyzed in a subset of the cohort with measurements available 90 days before and during stable dose.

Statistical analysis

A difference in stable dose of 0.33 was considered to be clinically relevant. According to the T2DM guidelines of the Dutch College of General Practitioners this equals a difference in titration time of approximately 4 weeks. In the power analysis, we calculated that 120 patients were to be included in order to test for statistical difference at a two-sided 5% significance level with at least 80% power.

The data were analyzed using the SPSS statistical package (version 16.0, SPSS, IL, USA). Possible deviation from Hardy–Weinberg equilibrium was tested by the χ^2 test. Differences in stable SU dose, number of dose adjustments, and fasting glucose levels were analyzed with Kruskal–Wallis nonparametric tests and multivariate linear regression analysis. All demographic and clinical variables were tested univariately against stable SU dose. The variables with a p-value of less than 0.1 were selected for multivariate analysis. In addition, age, gender and genotype were included in the multivariate analysis regardless of their univariate p-value.

Associations between the *CYP2C9*2* and *CYP2C9*3* alleles and time-to-stable dose were evaluated using Kaplan–Meier survival analysis techniques.

RESULTS

Figure 7.1 depicts the study population. In total, 207 T2DM patients were available for data analysis. Table 7.1 presents the patient characteristics. The mean age at the time of first SU prescription was 61.5 years and 52.2% of the study population were men. Mean follow-up was 6.0 years, reflecting that most patients (74.4%) were included after 1st January 2000. The majority of patients started with tolbutamide (42.5%) or glimepiride (39.6%). Patients received an average of 26 SU prescriptions with a median duration of 90 days per prescription. In total, 30.4% of the patients used metformin when they started SU treatment. The population was in Hardy–Weinberg equilibrium ($\chi^2 = 5.50$; $p = 0.14$), indicating a low likelihood of selection bias or errors in genotyping.

Table 7.2 summarizes the effect of the *CYP2C9*2* and *CYP2C9*3* alleles on the SU dose. The mean starting dose was 0.62. As expected, no differences in mean starting dose were observed between the genotype groups. Of the 207 T2DM patients, 152 (73.4%) achieved stable dose. There were no statistically significant differences in the percentage of patients that achieved stable dose between carriers of the *CYP2C9*2* (70.7%) and/or *CYP2C9*3* (77.5%) allele compared with homozygous carriers of the *CYP2C9*1* (79.4%) allele ($p = 0.48$). For mean stable glimepiride dose, a trend towards a lower dose for carriers of a *CYP2C9*3* allele in comparison to homozygous carriers of the *CYP2C9*1* allele was observed (1.01 vs. 0.61; $p = 0.07$).

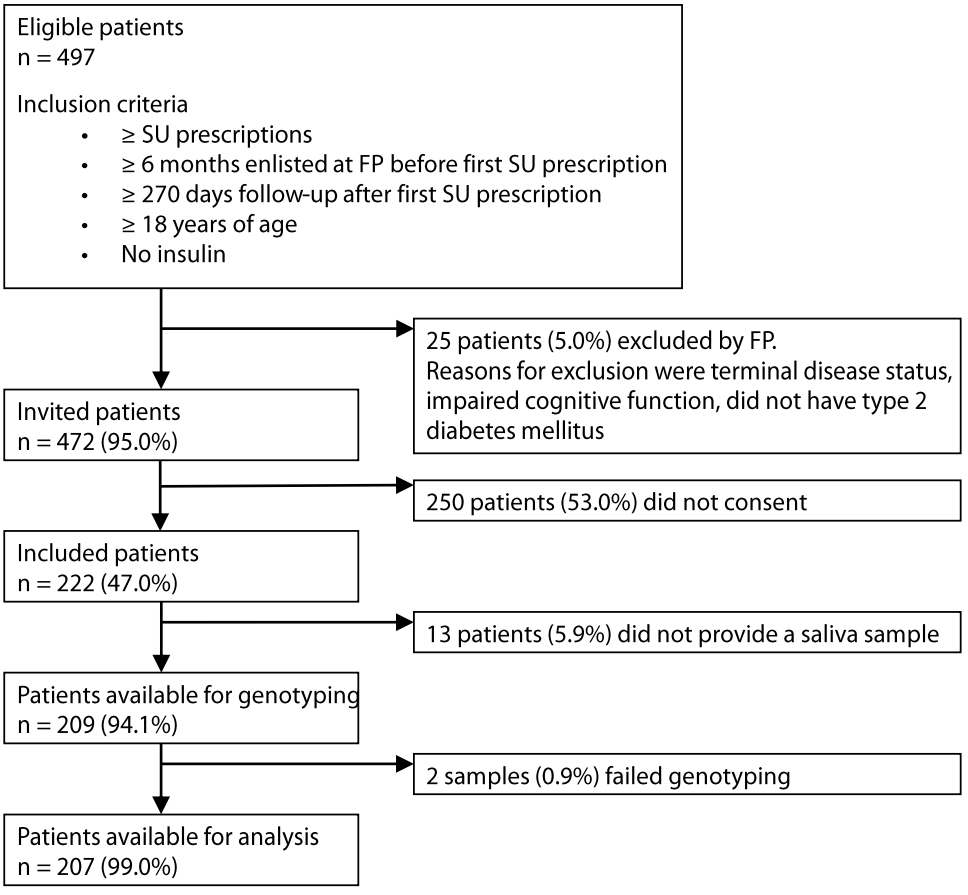

Figure 7.1 Flowchart of the study population. FP, family physician; SU, sulfonylurea.

To identify possible associations between demographic and clinical variables and stable SU dose, univariate regression analysis was performed. The SU starting dose and the use of metformin were associated with the mean stable SU dose (Table 7.3). To adjust for the effects of metformin and initial SU dose, a multivariate regression analysis with *CYP2C9* genotype, age and sex was performed. In summary, the results remained similar to the data as presented in Table 7.2.

Since SUs are titrated to the optimal dose, an analysis of the time-to-stable SU dose and *CYP2C9* genotype was performed. Median time-to-stable dose was 56, 50 and 48 days for homozygous carriers of the *CYP2C9*1* allele, carriers of the *CYP2C9*2* allele or the *CYP2C9*3* allele, respectively. The Kaplan–Meier curves for time-to-stable dose

Table 7.1 Characteristics of the type 2 diabetes mellitus patient population in primary care

Variable, n (%)[a]	All patients	*CYP2C9*1/*1*	*CYP2C9*1/*2* or *CYP2C9*2/*2*[b]	*CYP2C9*1/*3* or *CYP2C9*2/*3*[c]	p-value
Subjects	207	133 (64.3)	40 (19.3)	34 (16.4)	NA
Men	108 (52.2)	73 (54.9)	18 (45.0)	17 (50.0)	0.53
Women	99 (47.8)	60 (45.1)	22 (55.0)	17 (50.0)	
Age, mean (SD), years	61.5 (10.7)	61.2 (11.1)	61.8 (10.7)	62.3 (8.8)	0.85
Follow-up, mean (SD), years	6.0 (3.0)	5.8 (3.0)	6.3 (3.1)	6.1 (2.9)	0.68
Visits year 1 (SD)	9.6 (4.67)	9.9 (5.03)	9.5 (3.73)	8.8 (4.23)	0.20
Metformin	63 (30.4)	45 (33.8)	7 (17.5)	11 (32.4)	0.14
Primary sulfonylureas					
Glibenclamide	12 (5.8)	8 (6.0)	3 (7.5)	1 (2.9)	0.03[d]
Tolbutamide	88 (42.5)	45 (33.8)	24 (60.0)	19 (55.9)	
Gliclazide	25 (12.1)	29 (15.8)	2 (5.0)	2 (5.9)	
Glimepiride	82 (39.6)	59 (44.4)	11 (27.5)	12 (35.3)	

[a]Unless stated otherwise; [b]Four patients were carriers of the CYP2C9*2/*2 genotype; [c]One patient was a carrier of the CYP2C9*2/*3 genotype; [d]χ^2 for primary sulfonylureas vs. genotype group. NA, not applicable; SD, standard deviation.

Table 7.2 Prescribed dose of sulfonylurea at first and stable prescription stratified by CYP2C9 genotype group in type 2 diabetes mellitus patients in primary care[a]

Dose, mean (SD)		*CYP2C9*1/*1*	n	*CYP2C9*1/*2* or *CYP2C9*2/*2*	n	p-value	*CYP2C9*1/*3* or *CYP2C9*2/*3*	n	p-value
All patients									
	First	0.61 (0.25)	133	0.66 (0.31)	40	0.55	0.61 (0.26)	34	0.91
	Stable	0.92 (0.59)	94	0.94 (0.45)	31	0.49	0.80 (0.37)	27	0.48
Glibenclamide									
	First	0.63 (0.23)	8	0.58 (0.38)	3	0.63	0.25 (NA)	1	0.06
	Stable	0.75 (0.42)	6	1.00 (0.41)	4	0.30	1.50 (NA)	1	0.16
Tolbutamide									
	First	0.58 (0.20)	45	0.65 (0.23)	24	0.23	0.63 (0.28)	19	0.59
	Stable	0.90 (0.47)	35	0.78 (0.31)	15	0.56	0.86 (0.41)	14	0.86
Gliclazide									
	First	0.64 (0.35)	21	0.88 (0.88)	2	0.95	0.75 (0.35)	2	0.48
	Stable	0.78 (0.45)	13	0.88 (0.88)	2	0.86	0.83 (0.29)	3	0.78
Glimepiride									
	First	0.63 (0.26)	59	0.66 (0.36)	11	0.96	0.58 (0.19)	12	0.65
	Stable	1.01 (0.74)	40	1.15 (0.53)	10	0.21	0.61 (0.22)	9	0.07

Patients were allowed to switch from SU in the period between the first prescribed SU and stable dose. As a consequence the number of patients may differ within SU.
[a]To allow pooling of different SUs, dose was normalized by dividing the prescribed daily dose with the standard daily dose used by the Dutch Health Care Insurance Board (10 mg glibenclamide; 1,000 mg tolbutamide; 160 mg gliclazide; 2 mg glimepiride).
NA, not applicable; SD, Standard deviation; SU, Sulfonylurea.

Table 7.3 Analysis of multiple factors relevant for stable sulfonylurea dose in type 2 diabetes mellitus patients in primary care

Factor	Multivariate			Univariate		
	Difference in change[a]	95% CI	p-value	Difference in change	95% CI	p-value
Constant	0.912	0.307–1.516	0.003			
Male vs. female gender	-0.190	-0.355– -0.025	0.024	-0.15	-0.32–0.02	0.083
The effect of age (per year increase)	-0.005	-0.013–0.004	0.280	-0.005	-0.014–0.003	0.218
Metformin use vs. no metformin use at stable sulfonylurea dose	0.142	-0.038–0.321	0.120	0.177	-0.01–0.361	0.060
Carrier of *CYP2C9*2* allele vs. wild-type	0.001	-0.209–0.211	0.992	0.041	-0.171–0.254	0.701
Carrier of *CYP2C9*3* allele vs. wild-type	-0.088	-0.307–0.130	0.425	-0.129	-0.352–0.094	0.254
First sulfonylurea dose	0.583	0.266–0.899	0.000	0.569	0.253–0.885	0.000

[a]This considers the change in stable sulfonylurea dose (expressed as the prescribed daily dose divided by the standard daily dose used by the Dutch Healthcare Insurance Board).

demonstrated no significant differences between carriers of the *CYP2C9*1*, *CYP2C9*2* or *CYP2C9*3* alleles (p = 0.58) (Figure 7.2). For all *CYP2C9* genotypes, approximately 35% of the patients achieved stability without any dose adjustment from the first prescription. The mean number of dose adjustments in the first year of SU therapy was 1.02 (range: 0–6) for all patients, and did not differ between the genotype groups with 0.95 dose adjustments for homozygous carriers of the *CYP2C9*1* allele, 1.22 dose adjustments for carriers of the *CYP2C9*2* allele and 1.06 dose adjustments for carriers of the *CYP2C9*3* allele (p = 0.24).

In addition, differences in fasting glucose levels were assessed for the *CYP2C9* genotypes. For 75 patients (49.3%), fasting glucose level measurements were available in the period 90 days prior to the first SU prescription and during stable SU dose. Fasting glucose levels decreased with 2.8, 2.6 and 2.4 mmol/l for homozygous carriers of the *CYP2C9*1* allele, carriers of the *CYP2C9*2* allele, and carriers of the *CYP2C9*3* allele, respectively (p = 0.89).

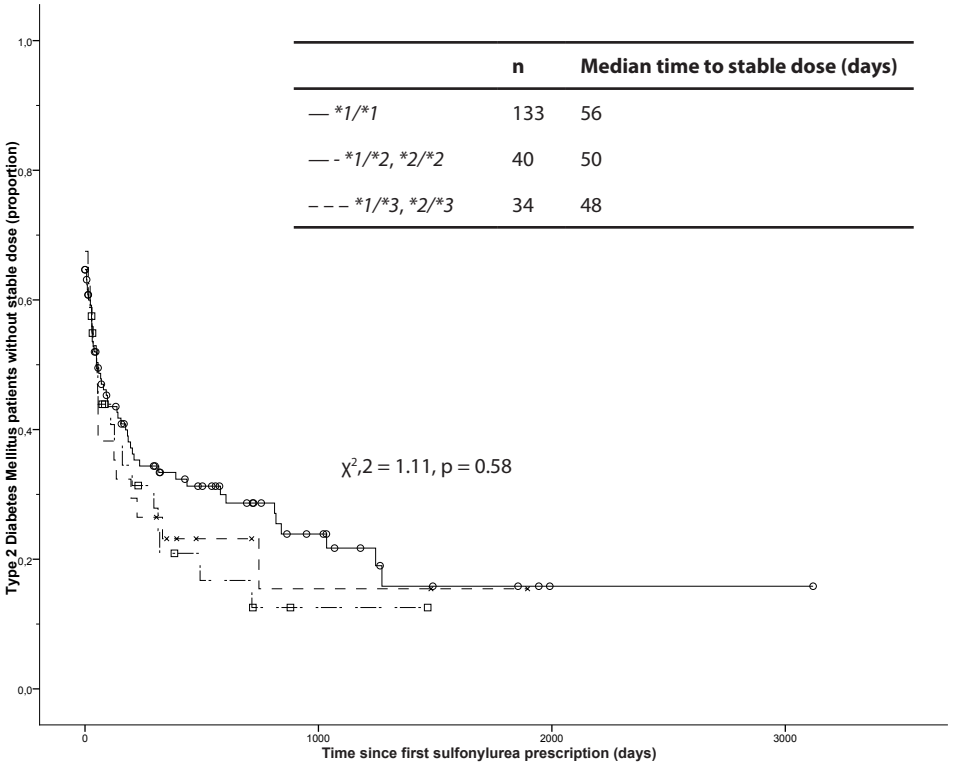

Figure 7.2 Kaplan-Meier survival plots of time to the first stable dose of sulfonylurea in type 2 diabetes mellitus patients in primary care stratified by *CYP2C9* genotype.

DISCUSSION

In this retrospective study of 207 primary care patients with T2DM, no statistically significant effect of the *CYP2C9*2* and *CYP2C9*3* alleles on the prescribed stable dose or time-to-stable dose of SUs was found. However, a trend towards a lower stable dose for carriers of the *CYP2C9*3* allele was observed, in the subgroup of patients treated with glimepiride. Since no difference in stable dose or time-to-stable dose between the different genotypes was observed, we hypothesized that carriers of the *CYP2C9*2* and *CYP2C9*3* alleles might have a larger decrease in fasting glucose levels. There was however, no significant difference in decrease in fasting glucose levels during stable dose between carriers of the *CYP2C9*2* or *CYP2C9*3* alleles and homozygous carriers of the *CYP2C9*1* allele in the relatively small subgroup of patients with fasting glucose level measurements available.

Our study has some limitations. In general, observational studies may potentially be affected by bias. The FPs were unaware of the genotype, thereby excluding this information bias. In our study, no data was available for patients who had switched to another family practice or who died in the period after 1992. As a consequence, we cannot completely rule out the possibility of selection bias. However, our population was in Hardy–Weinberg equilibrium, suggesting that no selection bias on genotype occurred. Moreover, a nonresponse analysis on age, gender, type of first prescribed SU, metformin use and FP revealed no differences between participants and patients who did not consent to our study, indicating that no selection bias on any of these parameters occurred.

The analysis of time-to-stable dose assumes that FPs adhere to the T2DM guideline of the Dutch College of General Practitioners. In general, adherence to guidelines by Dutch FPs is good [16]. If FPs do not adhere to the guideline, they can initiate treatment with a different dose or follow different titration intervals. In both situations this could introduce an error to our analyses of time-to-stable dose. However, there is no reason to assume that this error is not divided randomly over the different genotype groups. Therefore it does not affect the comparison of time-to-stable dose between the genotype groups but can only affect the absolute results of the time-to-stable dose analyses.

The *CYP2C9* genotype is known to have a significant effect on the pharmacokinetics of SUs in healthy volunteers [3–7,9]. Less information is available regarding the effect of the *CYP2C9* genotype in T2DM patients. Two retrospective studies assessed the effect of the *CYP2C9*2* and *CYP2C9*3* alleles on treatment outcomes with SUs. Becker et al. found that carriers of the *CYP2C9*3* allele treated with tolbutamide received significantly lower doses on the arbitrarily chosen 10th prescription compared with patients with the wild-type genotype, No such effect was found for any of the other assessed SUs [12]. In addition, Zhou et al. observed a trend towards a 5% dose increase for patients with none or one copy versus no dose increase in carriers of two copies of the *CYP2C9*2* or *CYP2C9*3* alleles in patients who were mainly treated with gliclazide monotherapy [13]. We report similar findings towards a lower stable SU dose for patients with a *CYP2C9*3* allele for a population of whom approximately 40% of the patients is treated with glimepiride. These findings indicate that the *CYP2C9*3* allele influences the treatment of T2DM patients with SUs. However, although inconclusive, our study suggests that the effect is probably small and we therefore feel that there is currently insufficient evidence to support the genotyping of *CYP2C9* prior to prescribing SUs to an individual.

The pathogenesis of T2DM is not yet fully understood. Current theories include defects in insulin-mediated glucose uptake in muscle, dysregulation of the adipocyte as a secretory organ, dysfunction of the pancreatic β-cell and impaired insulin action in the liver [17]. Several studies including linkage analysis, candidate gene approaches and genome-wide association studies have identified 20 common genetic variants associated with T2DM

reflecting the disease's multifaceted genetic background [18]. Probably as a consequence of this heterogeneity, interpatient variability of drug response remains largely unexplained. It is possible that the multifaceted genetic background of T2DM surpasses the effect of the *CYP2C9* genotype on SU response. Therefore, there may be subpopulations of T2DM patients in which the effect of the *CYP2C9*2* and *CYP2C9*3* alleles may be of clinical relevance.

Conclusion

In conclusion, no association between the *CYP2C9*2* or *CYP2C9*3* alleles and time-to-stable dose was found in T2DM patients in primary care, whereas carriers of a *CYP2C9*3* allele showed a trend towards a lower stable glimepiride dose. However, there are many other factors influencing SU treatment outcome. Therefore, the effect of the *CYP2C9*2* and *CYP2C9*3* alleles currently has no clinical implications to dosing of SUs in T2DM patients.

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Genetic Risk Factors for Type 2 Diabetes Mellitus and Response to Sulfonylurea Treatment

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ABSTRACT

Objective: After the identification of type 2 diabetes mellitus (T2DM) risk alleles from genome-wide association studies, models have been developed to identify subjects at high risk to develop T2DM. We hypothesize that a panel of 20 repeatedly associated T2DM risk alleles influences response to sulfonylureas (SUs).

Methods: Two hundred and seven incident SU (tolbutamide, glibenclamide, glimepiride, gliclazide) users with T2DM were recruited from four primary care centers. A genetic risk score per patient was calculated based on the number of risk-alleles. With this score, patients were categorized into three predefined genetic risk groups. The effect of the genetic risk group on the achievement of stable SU dose, prescribed stable SU dose, and time to stable SU dose was analyzed.

Results: Carriers of more than 17 T2DM risk alleles had a 1.7-fold reduced likelihood to achieve stable SU dose ($p = 0.044$). No significant effect of the number of T2DM risk alleles on prescribed dose was found. Carriers of more than 17 T2DM risk alleles showed a marginally significant increased time to stable dose (hazard ratio: 0.81; 95% confidence interval 0.75–1.01, $p = 0.058$).

Conclusion: T2DM risk alleles are associated with response to SUs in primary care T2DM patients. This suggests that individualization of T2DM treatment according to genetic profile may be an opportunity to improve clinical outcome.

INTRODUCTION

The incidence of type 2 diabetes mellitus (T2DM) is increasing at an alarming rate. Worldwide, the number of patients is expected to increase from 171 million in 2000 to 366 million in 2030 [1]. The therapeutic goal of treating T2DM patients is to prevent or delay long-term microvascular and macrovascular complications by achieving the best possible glycemic control.

Sulfonylureas (SUs) are part of the mainstay of treatment with oral antidiabetic drugs. Tolbutamide, glibenclamide (glyburide), gliclazide, and glimepiride are the most commonly used representatives of this group. These drugs act by closing the pancreatic β-cell potassium channels, stimulating insulin secretion [2]. SUs are initiated at a low dose and escalated to the optimal dose with intervals of 2–4 weeks until the glycemic target (HbA1c < 7%) is achieved. However, there is significant interpatient variability in response to SUs, with approximately 10–20% of the patients experiencing primary failure (decrease in fasting glucose level < 1.1 mmol/l) and a similar percentage having an above average response (mean reduction HbA1c 1.5–2%) [3–5].

With the completion of multiple genome-wide association studies (GWAS) the knowledge of the complex genetic background of T2DM has increased. These studies report associations between genetic variants and the risk for the development of T2DM. A panel of 20 T2DM associated single nucleotide polymorphisms (SNPs) comprising 19 genes out of the GWAS data appears, that has been replicated in several studies [6–17]. These SNPs are used in models with the ultimate goal to identify subjects at high risk to develop T2DM. Although marginally, the addition of genetic information to clinical T2DM risk factors increased the ability to predict future diabetes [18–24].

From the panel of 20 T2DM risk-associated SNPs, the majority is involved in the process of insulin release from the pancreatic β-cells (Table 8.1). As SUs act by stimulating insulin secretion, response to SU treatment may also be influenced by these genetic variants. Indeed, two of the 19 T2DM risk-associated genes, encoding *KCNJ11* and *TCF7L2*, have been previously correlated with variation in SU response [4]. Furthermore, in subjects analyzed for genetic variation in the genes *TCF7L2*, *PPARG*, *FTO*, *KCNJ11*, *NOTCH2*, *WFS1*, *CDKAL1*, *IGF2BP2*, *SLC30A8*, *JAZF1*, *HHEX*, it was reported that subjects with 12 or more T2DM risk alleles did not increase their insulin secretion to compensate for the increased insulin resistance as efficiently as those with 8 or less risk alleles [19]. Therefore, patients with a greater number of risk alleles may show less response to SU treatment and individualization of T2DM treatment according to genetic profile may be an opportunity to improve clinical outcome.

We hypothesize that the genetic variants associated with the development of T2DM are also associated with response to SU treatment. Therefore, we investigated the effect of T2DM risk alleles on the response to SU treatment in T2DM patients in a primary care setting.

Table 8.1 Selected single nucleotide polymorphisms associated with type 2 diabetes mellitus

Gene	rs number	Chromosome	Risk allele	Year	Mechanism	References
NOTCH2	rs10923931	1	T	2008	Unknown	[9,19-24]
THADA	rs7578597	2	T	2008	Unknown	[9,19-24]
IGF2BP2	rs4402960	3	T	2007	β-cell dysfunction	[8,9,11-13,18-24]
PPARG	rs1801282	3	C	2000	Insulin sensitivity	[8,9,11-13,19-24]
ADAMTS9	rs4607103	3	C	2008	Unknown	[9,19-24]
WFS1	rs10010131	4	G	2007	Unknown	[9,13,18-20,22,24]
CDKAL1	rs7754840	6	C	2007	β-cell dysfunction	[7-9,11-13,18-24]
JAZF1	rs864745	7	A	2008	β-cell dysfunction	[9,19-24]
SLC30A8	rs13266634	8	C	2007	β-cell dysfunction	[7-9,11-14,18-24]
CDKN2A/CDKN2B	rs10811661	9	T	2007	β-cell dysfunction	[8,9,11-13,18-24]
	rs564398	9	A			[8,18,20,24]
TCF7L2	rs7903146	10	T	2006	β-cell dysfunction	[7-9,11-14,18-24]
HHEX/IDE	rs1111875	10	G	2007	β-cell dysfunction	[7-9,11-14,18-24]
CDC123/CAMK1D	rs12779790	10	G	2008	Unknown	[9,19-24]
KCNJ11	rs5219	11	T	2003	β-cell dysfunction	[8,9,11-13,19-24]
KCNQ1	rs2237892	11	C	2008	β-cell dysfunction	[10,17,24]
MTNR1B	rs10830963	11	G	2009	Disturbance of circadian rhythm	[6,16,24]
TSPAN8/LGR5	rs7961581	12	C	2008	Unknown	[9,19-24]
FTO	rs8050136	16	A	2007	Obesity	[8,9,11,13,19-22,24]
HNF-1β (TCF2)	rs757210	17	A	2007	β-cell dysfunction	[15,20,21,24]

MATERIALS AND METHODS

Study setting

In the Netherlands the general practitioner (GP) plays a central role in the provision of health care. Patients are listed with one GP who is consulted for all healthcare problems and indicates whether a referral to secondary care is appropriate. Typically, the GP keeps an electronic patient record (EPR) that covers all medical information concerning the patient including prescription information and reports from laboratories and specialists. GP's have adopted the practice guideline T2DM of the Dutch College of General Practitioners [25]. Tailoring the treatment to the individual patient is an important part of the therapy.

Cohort ascertainment

A total of 207 T2DM patients from four university-affiliated primary care centers (17 GPs) located in the vicinity of Leiden, the Netherlands were recruited. The ascertainment of the cohort has been described in detail previously [26]. In brief, patients that had at least one prescription of tolbutamide, glibenclamide, glimepiride, or gliclazide between January 1992 and June 2008, were at least 18 years of age and without insulin use at the time of first SU prescription, and had at least 270 days of follow-up registered in the EPR, were included. Ethnicity was not routinely recorded in the EPR but most patients in the Netherlands are from European ancestry. Patients received a written invitation by mail from their GP. Of the 472 invited patients, 222 (47%) agreed to participate (see Figure 8.1). After consent, a saliva collection kit (DNA Genotek Inc., Ottawa, Ontario, Canada) was mailed. The study was approved by the ethics committee of the Leiden University Medical Center.

Genotyping

We selected a panel of 20 SNPs in 19 genes that have been associated with the development of T2DM in at least three GWAS and were consistently replicated in later studies aimed at estimating the predictive value of these SNPs on the development of T2DM [6–24]. The selected SNPs are listed in Table 8.1. DNA was isolated from the saliva according to the protocol provided by the manufacturer (DNA Genotek Inc.). Taqman genotyping assays for 19 SNPs were designed by and obtained from Applied Biosystems (Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands). SNP rs757210 could not be designed as a Taqman genotyping assay and therefore was genotyped by pyrosequencing (Isogen Life Science, Maarssen, the Netherlands). Taqman genotyping assays were performed on the LightCycler 480 II Real-Time PCR System (Roche Diagnostics, Almere, the Netherlands) according to standard procedures. Genotyping was performed without knowledge of the clinical data. We obtained an average genotyping success rate

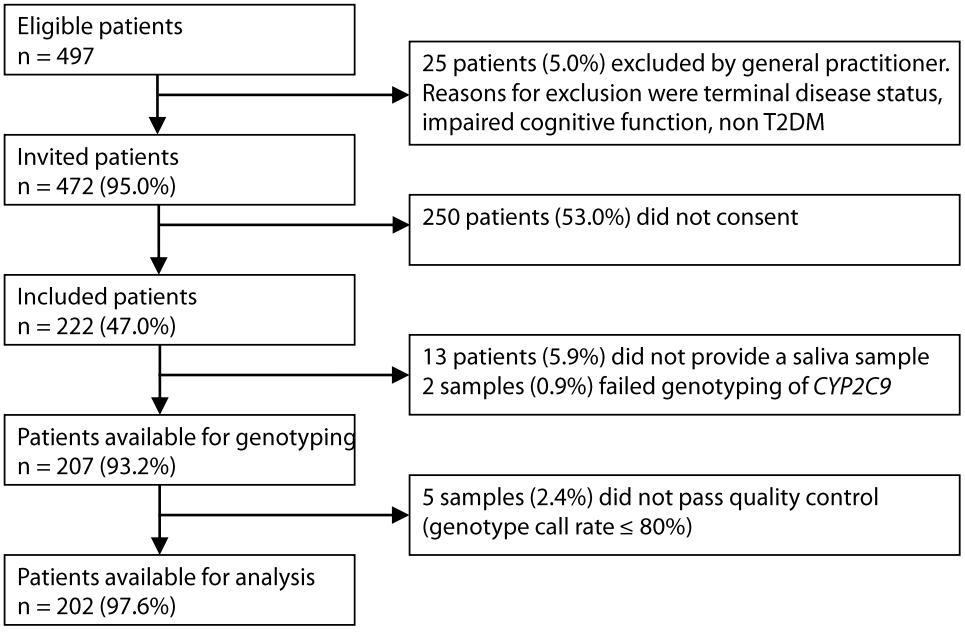

Figure 8.1 Flowchart of the study population.

of more than 95%. As a quality control 5% of the samples were genotyped in duplicate for all assays and no inconsistencies were observed. Five patients were excluded for quality reasons (genotype call rate ≥ 80%). All SNPs were in Hardy–Weinberg equilibrium ($p > 0.05$), with the exception of rs2237892 ($p = 0.011$). This is most probably ascribed to the very low minor allele frequency of rs2237892, which was 0.025 in our study and comparable with previously reported minor allele frequencies of 0.056–0.075 (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=2237892) accessed 5 October 2010.

Definition of effect

For each patient a cumulative genetic risk score was calculated based on the number of present risk alleles. Each person could have 0, 1, or 2 of them for each SNP, resulting in a theoretical individual cumulative risk score between 0 and 40. This approach assumes an equal and additive effect of each allele on the risk of T2DM. To allow categorization of patients, we predefined three genetic risk groups on the basis of the frequency distribution of risk alleles. We defined a low genetic risk group and a high genetic risk group as the quintiles with the lowest and highest number of T2DM risk alleles, respectively. All other patients (three quintiles) were categorized in the intermediate risk group.

The primary endpoint of our study is the effect of the genetic risk group on achieving stable SU dose. Stable SU dose was defined as the first period of more than or equal to 270 consecutive days without SU dose adjustment, or initiation or adjustment of therapy with other SUs, insulin or metformin. If therapy with insulin was initiated patients were censored. The period of more than or equal to 270 days was chosen because prescriptions in the Netherlands are limited to a maximum of 90 days and more than or equal to 270 days equals three consecutive prescriptions. Stable SU dose was calculated as normalized dose by dividing the prescribed daily dose with the standard daily dose used by the Pharmaceutical Aid Committee of the Dutch Health Care Insurance Board (10 mg glibenclamide; 1,000 mg tolbutamide; 160 mg gliclazide; 2 mg glimepiride). Secondary endpoints of our study are the stable SU dose and the time required for dose escalation (time to stable SU dose).

Statistical analysis

The data were analyzed using the SPSS statistical package (version 16.0, SPSS, Chicago, Illinois, USA). Deviation from Hardy–Weinberg equilibrium was tested by the χ^2 test. Achievement of stable SU dose was analyzed with the χ^2 test and multivariate logistic regression analysis. Differences in mean stable SU dose between genetic risk groups were analyzed using the Kruskal–Wallis test and multivariate linear regression analysis. Associations between the genetic risk groups and time to stable SU dose were evaluated using the Cox survival regression analysis. Before multivariate analysis, all demographic and clinical variables were tested univariately against the selected outcome. Variables with a p-value of 0.1 or less, age, and sex were selected for multivariate analysis. All multivariate analyses were corrected for age and sex.

RESULTS

Data from 202 T2DM patients were available. The range of the calculated genetic risk score was 10–26. The quintiles with the lowest ($\leq$ 17) and highest ($\geq$ 21) number of T2DM risk alleles consisted of 59 patients and 62 patients, respectively (Figure 8.1). Table 8.2 presents the characteristics of the 202 patients. There were no differences between the different genetic risk groups observed in any of the patient characteristics except for age. Patients in the high-risk group were younger at the time of first SU prescription compared with patients in the low-risk and intermediate-risk group, respectively ($p = 0.001$). Mean follow-up was 5.9 years, reflecting that most patients (75.2%) were included after 2000. Our patients received an average of 26 SU prescriptions during the follow-up period with a median duration of 90 days per prescription.

The results of achieving stable SU dose and the T2DM genetic risk groups are presented

Table 8.2 Characteristics of the 202 patients with type 2 diabetes mellitus in primary care

		Genetic risk group			
Variable no (%)[a]	**All patients**	**Low-risk**	**Intermediate-risk**	**High-risk**	**p-value**
Subjects	202	59 (29.2)	81 (40.1)	62 (30.7)	NA
Men	106 (52.5)	30 (50.8)	45 (55.6)	31 (50.0)	0.77
Women	96 (47.5)	29 (49.2)	36 (44.4)	31 (50.0)	
Age in years, mean (SD)	61.4 (10.7)	64.0 (9.5)	62.6 (10.4)	57.3 (11.1)	**0.001**
Follow-up in years, mean (SD)	5.9 (3.0)	6.0 (3.0)	5.7 (3.0)	6.2 (3.0)	0.52
Visits in year one (SD)	9.6 (4.7)	8.6 (3.7)	10.0 (5.2)	10.0 (4.6)	0.35
Metformin use	62 (30.7)	18 (30.5)	27 (33.3)	17 (27.4)	0.75
Primary sulfonylurea					0.098[b]
Glibenclamide	12 (5.9)	7 (11.9)	1 (1.2)	4 (6.5)	
Tolbutamide	85 (42.1)	18 (30.5)	41 (50.6)	26 (41.9)	
Gliclazide	24 (11.9)	7 (11.9)	10 (12.3)	7 (11.3)	
Glimepiride	81 (40.1)	27 (45.8)	29 (35.8)	25 (40.3)	

Low-risk group: patients with 17 or less risk alleles. Intermediate-risk group: patients with 18-20 risk alleles. High-risk group: patients with at least 21 risk alleles. NA not applicable; SD, standard deviation, [a]Unless stated otherwise; [b]χ^2 for primary sulfonylurea vs genetic risk group. P-value < 0.05 is regarded as significant and indicated with bold font;

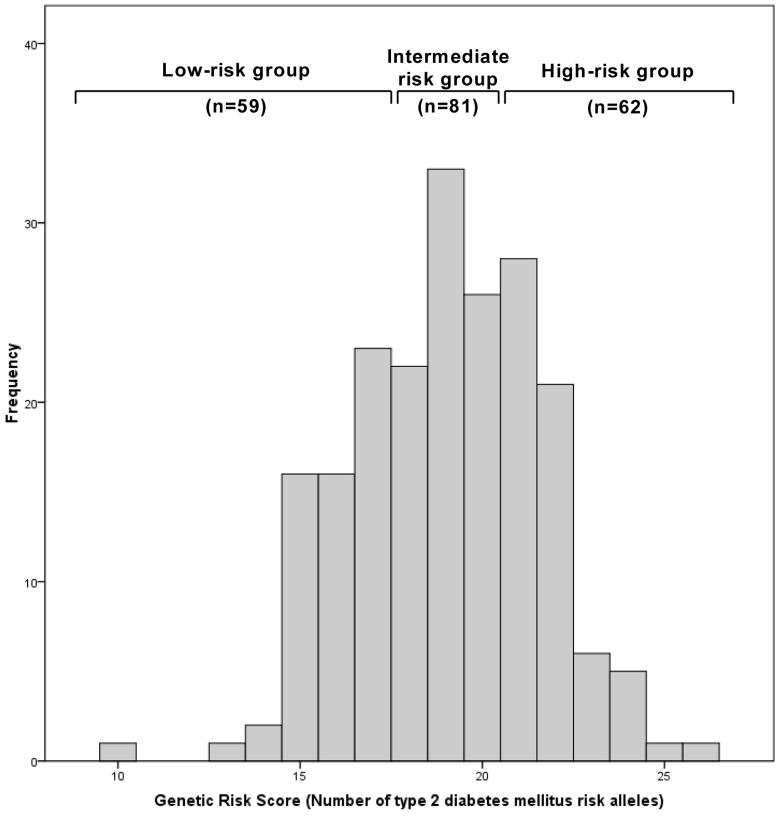

Figure 8.2 Distribution of type 2 diabetes mellitus risk alleles and subsequent classification in risk groups. Patients were categorized in three genetic risk groups. Low-risk group; quintile with the lowest (≤ 17) number of type 2 diabetes mellitus risk alleles. High-risk group; quintile with the highest (≥ 21) number of type 2 diabetes mellitus risk alleles. Remaining patients were categorized in the intermediate-risk group (18-20 type 2 diabetes mellitus risk alleles).

in Figure 8.3. Of the patients, 148 (73.3%) achieved stable SU dose. The percentage of patients achieving stable SU dose was lower in the high-risk group compared with the intermediate-risk and low-risk groups (61.3 vs. 74.1 vs. 84.7%, respectively, p = 0.004). In the multivariate logistic regression analysis age at first SU prescription, the concomitant use of metformin, and the T2DM genetic risk group were independently significantly associated with achieving stable SU dose (Table 8.3). The regression model explained 28.7% of the variation in achievement of stable dose. Data show that patients with a higher T2DM risk had a 1.7-fold reduced likelihood to achieve stable SU dose (p = 0.044).

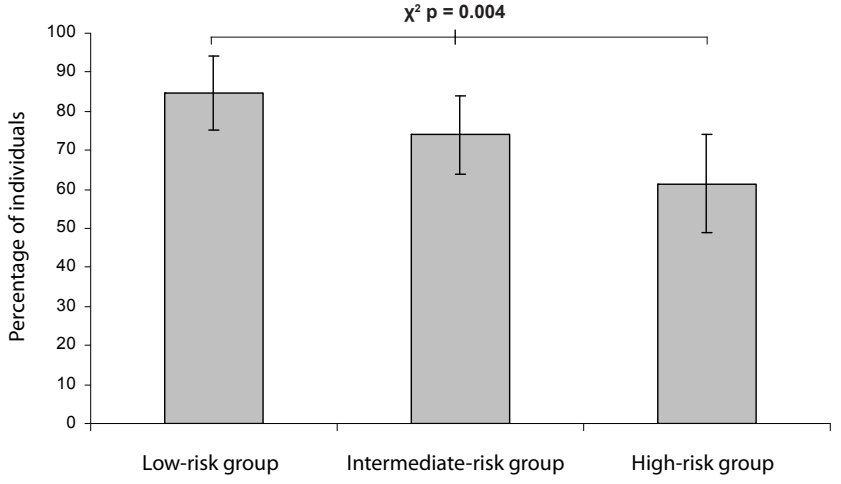

Figure 8.3 Percentage of type 2 diabetes mellitus patients that reached stable sulfonylurea dose. Low-risk group; patients with 17 or less risk alleles. Intermediate-risk group: patients with 18-20 risk alleles. High-risk group; patients with at least 21 risk alleles.

Next, the mean SU starting dose was analyzed. The mean SU starting dose for all patients was 0.61 (95% CI 0.58–0.65). As expected, no differences in SU starting dose were found between the different genetic risk groups. No differences in mean stable SU dose were found between the different T2DM genetic risk groups [low-risk group 0.90, 95% CI 0.75–1.05 vs. intermediate-risk group 0.84, 95% CI 0.74–0.94 vs. high-risk group 0.95, 95% CI 0.72–1.17, p = 0.97]. In multivariate linear regression, only the effect of the SU starting dose and sex were independently significant associated with stable SU dose, whereas the genetic risk group for T2DM was not associated with stable SU dose.

As SUs are escalated to the optimal dose, the effect of the genetic risk group on time to stable SU dose was evaluated. Carriers of the high-risk genetic profile (≥ 21 risk alleles) had a two-fold and five-fold longer time to stable dose compared with patients with the intermediate risk (18–20 risk alleles) and low-risk profile (≤ 17 risk alleles) (median time to stable SU dose 160 vs. 59 vs. 31 days, respectively, p = 0.007). In a multivariate Cox regression analysis including the factors such as age on first SU prescription, sex, and the concomitant use of metformin, patients with a higher number of risk alleles showed a marginally significant increased time to stable SU dose (hazard ratio: 0.81; 95% CI 0.75–1.01, p = 0.058) (Figure 8.4).

Table 8.3 Analysis of factors relevant for achieving stable sulfonylurea dose in patients with type 2 diabetes mellitus in primary care

Factor	Univariate				Multivariate[a]		
	OR	95% CI	R^2	p-value	OR	95% CI	p-value
Constant					1.81	NA	0.654
Male vs. female sex	1.55	0.83–2.91	0.009	0.17	1.54	0.72–3.29	0.262
Effect of age (per year increase)	1.06	1.03–1.09	0.069	**< 0.001**	1.04	1.00–1.08	**0.036**
Metformin use vs. no metformin use at stable sulfonylurea dose	0.07	0.03–0.16	0.238	**< 0.001**	0.07	0.03–0.17	**< 0.001**
Genetic risk group (low-risk → intermediate-risk → high-risk group)	0.54	0.35–0.82	0.042	**0.004**	0.59	0.35–0.99	**0.044**

→, stepwise increase from low-risk to intermediate-risk to high-risk group; CI, confidence interval; OR, odds ratio; R^2, variation in the achievement of stable dose. [a]R^2 for the complete model was 0.287.
P-value < 0.05 is regarded as significant and indicated with bold font.

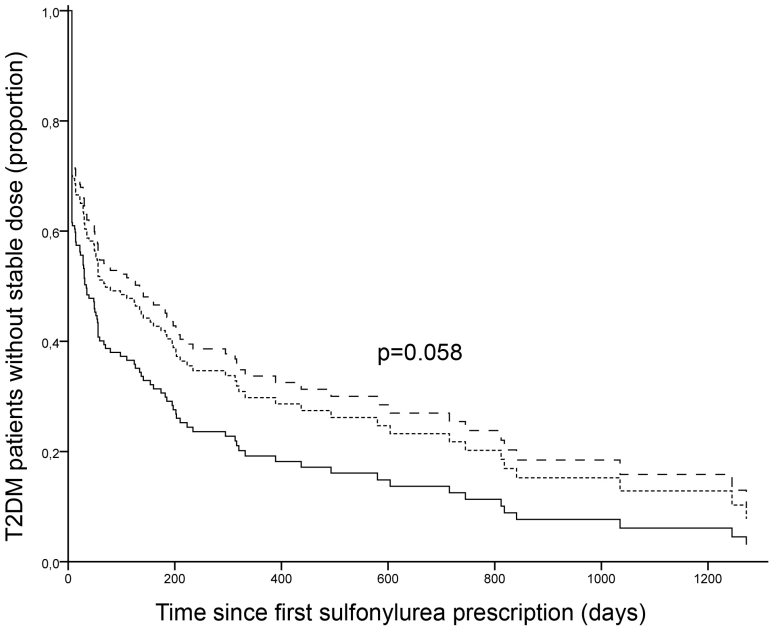

Figure 8.4 Multivariate Cox regression analysis plots of time to the first stable dose of sulfonylureas in type 2 diabetes mellitus patients in primary care.
Low-risk group (──); patients with 17 or less risk alleles. Intermediate-risk group (- - - -); patients with 18-20 risk alleles. High-risk group (– – –); patients with at least 21 risk alleles.

DISCUSSION

To the best of our knowledge, this is the first study exploring the relationship between response to treatment with SUs and T2DM risk alleles. In this retrospective cohort study of 202 T2DM patients, patients with more than 17 risk alleles have a 1.7-fold reduced likelihood to achieve a stable SU dose. These patients also show a marginally significant increased time to achieve stable SU dose compared with carriers of less than 17 risk alleles. However, the number of T2DM risk alleles does not seem to affect the average stable SU dose used. Therefore, our data suggest that patients with a higher number of T2DM risk alleles have a decreased and delayed response to SU treatment.

Drug response is determined by both pharmacokinetics and pharmacodynamics of a drug. Several groups have investigated genetic variation in genes affecting the pharmacokinetics of SU response. Two variants in *CYP2C9*, *CYP2C9*2* and *CYP2C9*3*, have been associated with a decreased SU metabolism in healthy volunteers [27]. Five studies assessed the effect of these polymorphisms in T2DM patients. Presence of the *CYP2C9*3* allele

was associated with an increased risk for hypoglycemia [28,29]. Tolbutamide users with a *CYP2C9*2* or *CYP2C9*3* allele have been shown to have a significantly lower dose escalation compared with homozygous carriers of the *CYP2C9*1* allele [30]. In a large cohort of 1,073 incident SU users with T2DM Zhou et al. [31] found that carriers of the *CYP2C9*2* or *CYP2C9*3* allele were less likely to fail on SU monotherapy. In a recent study we found no statistically significant effect of the *CYP2C9*2* or *CYP2C9*3* allele on the prescribed stable dose [26].

Variation in genes associated with the pharmacodynamics of SUs in T2DM patients has received considerably less attention. Genetic variants associated with SU response have been described for some monogenic forms of diabetes [32–34]. For polygenic T2DM, variants in the genes *KCNJ11*, *TCF7L2*, *ABCC8*, *IRS1*, and *NOS1AP* have been associated with SU response [35–38]. Of these, only the genes *KCNJ11* and *TCF7L2* were reported to contribute to an increased risk for T2DM in published GWAS. *KCNJ11* encodes the Kir6.2 subunit, one of the two subunits that form the ATP-sensitive potassium channel involved in insulin release. Carriership of the E23K variant of the *KCNJ11* gene has been associated with failure to SU therapy, but there are some conflicting results [39–41]. Variants in the TCF7L2 gene have also been associated with SU response. In a study with 901 incident SU users, patients with the TT genotype for rs7903146 were 1.73 times less likely to be treated to lower a target HbA1c of 7% in the first 3–12 months of treatment compared with patients with the CC genotype [42]. For a variant in linkage with rs7903146 an even larger effect (odds ratio = 1.95) was reported. In this study, none of the individual risk alleles were significantly associated with the achievement of stable dose (see Table 8.4), risk allele frequency and association with achievement of stable SU dose of the individual SNPs). This is most likely due to the limited sample size of our study and the probable small effect size of the individual risk alleles.

Our study has some limitations. No data were available for patients that switched to another GP or who died after 1992. Therefore, we cannot completely rule out the possibility of selection bias, although this is conceptually very unlikely. A nonresponse analysis with age, sex, type of first prescribed SU, metformin use, and GP showed no differences between participants and patients who did not consent to our study, suggesting that no selection bias has occurred on any of these parameters.

We selected stable SU dose as the primary endpoint of our analysis. Ideally macrovascular (e.g. diabetes-related death or myocardial infarction) or microvascular events (e.g. retinopathy or renal failure) would have been used. Alternatively, biomarkers related to these events, such as HbA1c or fasting plasma glucose (FPG) might have been used. However, as data concerning these parameters were not routinely recorded in the EPR, data were too sparse to be used in our analysis. Therefore, we selected stable SU dose as an alternative. Although, no SU pharmacogenetics studies have used stable SU dose as

Table 8.4 Risk allele frequency and association with achievement of stable SU dose of the individual SNPs

Gene	rs number	Risk allele	Risk allele frequency	Association with achievement of stable dose (p-value, χ^2)	Patients (n)
TCF7L2	rs7903146	T	0.359	0.188	199
KCNJ11	rs5219	T	0.418	0.338	202
HHEX/IDE	rs1111875	G	0.616	0.506	202
SLC30A8	rs13266634	C	0.730	0.523	201
CDKAL1	rs7754840	C	0.336	0.176	201
CDKN2A/CDKN2B	rs10811661	T	0.827	0.357	202
	rs564398	A	0.612	0.261	202
IGF2BP2	rs4402960	T	0.306	0.759	197
KCNQ1	rs2237892	C	0.027	0.665	199
PPARG	rs1801282	C	0.890	0.567	202
FTO	rs8050136	A	0.394	0.554	202
NOTCH2	rs10923931	T	0.108	0.755	202
WFS1	rs10010131	G	0.623	0.742	202
JAZF1	rs864745	A	0.538	0.813	202
THADA	rs7578597	T	0.903	0.538	202
CDC123/CAMK1D	rs12779790	G	0.185	0.242	195
TSPAN8/LGR5	rs7961581	C	0.279	0.244	202
ADAMTS9	rs4607103	C	0.752	0.319	199
HNF-1β (TCF2)	rs757210	A	0.428	0.918	190
MTNR1B	rs10830963	G	0.271	0.878	202

endpoint, this parameter closely reflects actual clinical practice. The time to stable SU dose analysis assumes that GPs adhere to the T2DM guideline of the Dutch College of General Practitioners and titrate SU dose in response to glucose and HbA1c levels. We have three arguments that support our assumption. Firstly, mean FPG was 7.77 mmol/l (95% CI 7.42–8.12, n = 95) for the subgroup of patients with a FPG measurement available during stable SU dose. Secondly, the adherence of GPs to guidelines is reported to be good in the Netherlands [43]. Finally, even if GPs do not adhere to the T2DM guideline, and bias would be introduced to our analysis, there is no reason to assume that the nonadherence of GPs is not divided randomly over the different genetic risk groups. Therefore, possible nonadherence does not affect the comparison of the time to stable dose between the different genetic risk groups but can only affect the absolute results of this analysis.

There are multiple known factors that predict a good response to SUs including baseline HbA1c, recently diagnosed diabetes, mild-to-moderate fasting hyperglycemia (< 12.2–13.3 mmol/l), good β-cell function (high fasting C-peptide level), no history of insulin therapy, and absence of islet cell or glutamic acid decarboxylase antibodies [3]. However, for none of these factors sufficient data were available in our retrospective

cohort study and we were unable to account for their effect. In addition, the available data on weight, a factor that is associated with the onset of T2DM, were too sparse to be included in the analysis as a covariate. As a consequence we cannot rule out that patients with a higher number of risk alleles also have a more severe form of T2DM that might confer to an a priori decreased probability to achieve stable SU dose. In our opinion, the only way to collect sufficient high quality data that cover all of these parameters would be to conduct a prospective observational study. Ideally such a study would include two treatment arms with pharmacological different drugs or placebo. Such a design would allow differentiating between the effect of T2DM risk alleles on disease progression and effect on treatment.

The results of different SUs were pooled in one analysis. Although SUs are generally reported to have equipotent glucose lowering effects when administered in maximally effective doses [3,5], it would be interesting to investigate if our hypothesis is valid for each of the individual SUs. However, due to the sample size of our study such a subgroup analysis was not possible.

We achieved a high success rate of genotyping with a call rate of more than 95% for all individual SNPs. After exclusion of five patients with a call rate of less than or equal to 80, 0.9% of the genotype data were missing. Missing genotype data were replaced with a risk score of 0. To test the sensitivity of our analysis for this replacement, we reanalyzed the data using two alternative approaches. First, as for some SNPs the wild-type allele is the risk allele, missing data were replaced with the score of the wild-type allele. As a result, two patients were reclassified from the low-risk to the intermediate-risk group, and one patient was reclassified from the intermediate-risk group to the high-risk group. Secondly, we excluded all patients with any missing data, resulting in the exclusion of an additional 31 (15.3%) patients. Similar results on all end points were obtained with all approaches, except for the effect of the genetic risk score that lost statistical significance in multivariate analysis after exclusion of all patients with missing data. These sensitivity analyses indicate that our results are valid.

The analysis of the effect of the genetic risk score on SU response assumes that each risk allele has an equal and additive effect, both within and between loci. This is clearly a simplification of the mechanism leading to variation of SU response. However, this approach is used in all GWAS studies concerning prediction of T2DM. Until it is clear what the true effect size of individual risk alleles is, the additive genetic model is probably the most appropriate and consistent method to analyze T2DM genetic data.

We chose to compare the quintile with the lowest (≤ 17, $n = 59$, low-risk group) and highest (≥ 21, $n = 62$, high-risk group) number of T2DM risk alleles, whereas patients with 18–20 risk alleles were pooled in one group ($n = 81$, intermediate-risk group) (Figure 8.1). The use of quintiles was based on a study by Lyssenko et al. [19] and allows

potentially easy translation to the clinic by clear classification of T2DM patients. The cutoffs for the quintiles with the highest and lowest number of T2DM risk alleles fell within the group of patients with 21 and 17 risk alleles respectively. We categorized patients with 17 risk alleles to the low-risk group and 21 alleles to the high-risk group, resulting in a slightly larger number of patients in both categories than anticipated. To ascertain that our results are not solely due to study design, we also analyzed the genetic risk score as a continuous variable instead of the analysis of risk groups. Next to this genetic risk score (range 10–26), sex, age on first SU prescription, and the use of metformin were included in the multivariate analysis. Data showed similar results for both the effect size and direction for the genetic risk score (odds ratio 0.88, 95% CI 0.76–1.02, p = 0.11). This suggests that with increasing number of risk alleles, the chance of achieving stable SU dose decreases.

The concept of disease-related genes influencing response to treatment is not new. For example, variation in the gene coding for the 5-hydroxytryptamine 2A receptor has been associated with variation of clozapine response and increased susceptibility to schizophrenia [44,45]. Variation in the gene coding for the β-2-adrenergic receptor has been associated with airway responsiveness to β-2-receptor agonists and susceptibility to lower airway reactivity in patients with asthma [46,47]. Our results show that patients with a higher number of risk alleles were younger at the date of their first SU prescription. This may be the result of a more 'aggressive' form of T2DM. For many complex diseases such as T2DM, there may be multiple genetic backgrounds resulting in similar phenotypic disease, each requiring a different drug treatment. Our results support this concept, and support the use of disease-related genes in pharmacogenetic studies. We should emphasize, however, the fact that we have only begun to unravel the genetic determinants of drug response in T2DM and that although many of the genes are associated with β-cell function, the exact mechanism behind our finding remains unclear. Our results do provide some 'proof of principle' that the complex background of T2DM may ultimately result in the identification of different genetic subgroups of T2DM patients that require different pharmacotherapy. However, replication in an independent cohort and further elucidation of the causal mechanisms underlying SU response are warranted.

In conclusion, T2DM-associated risk alleles are associated with response to SU treatment in primary care T2DM patients. This suggests that individualization of T2DM treatment according to genetic profile may be an opportunity to improve clinical outcome.

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General Discussion and Summary

9.1 Translating Pharmacogenetics from Concept to Clinic

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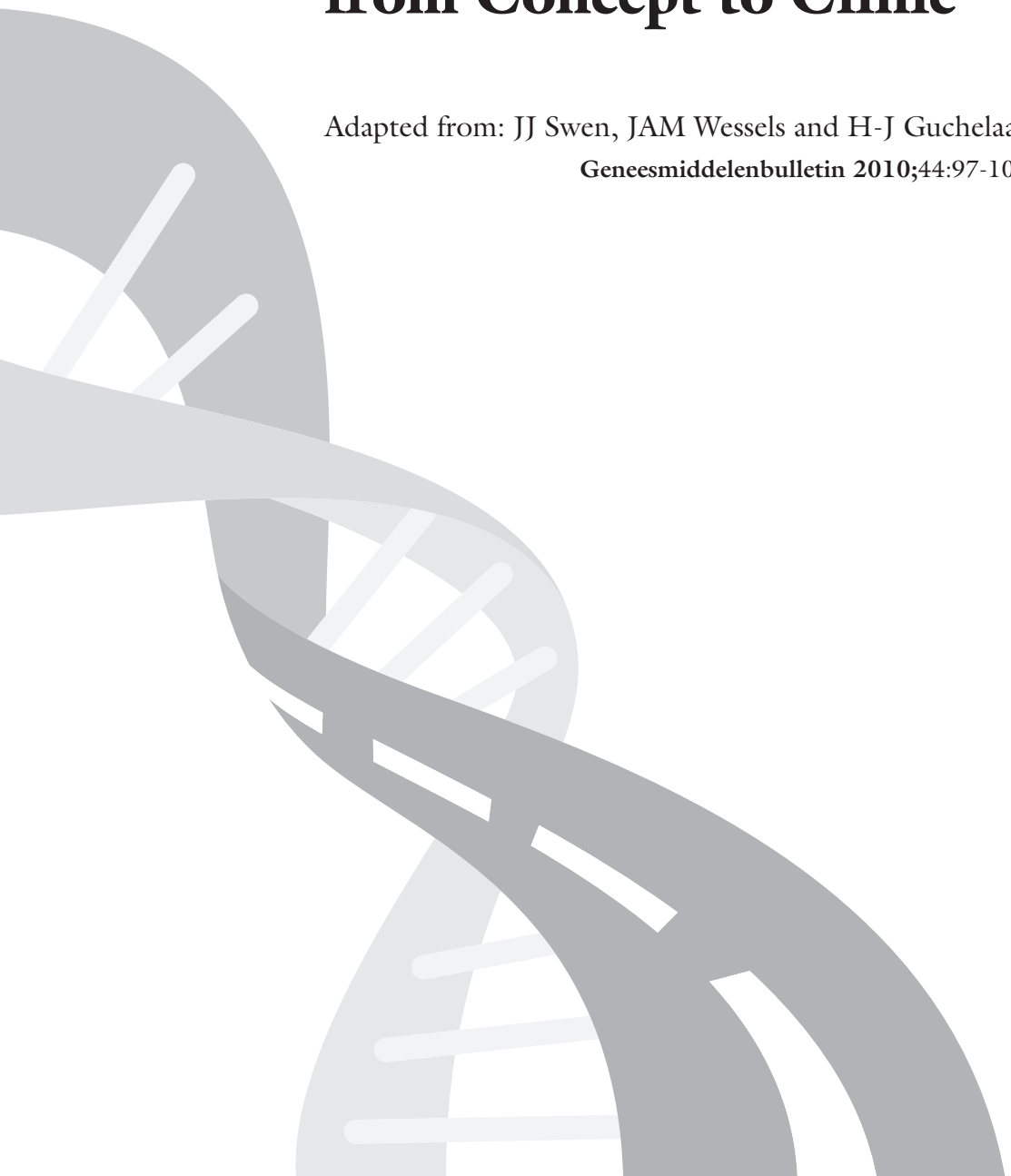

INTRODUCTION

Pharmacogenetics is the study of variations in DNA sequence as related to drug response [1]. The ultimate goal of pharmacogenetics is to predict and thereby improve the drug response in the individual patient. After the completion of the Human Genome Project pharmacogenetics was often thought to be one of the first clinical applications resulting from the new knowledge of the human genome [2]. This chapter will present an overview of the developments within the field of pharmacogenetics in recent years, discuss barriers for clinical uptake, and finally will provide a future perspective of the implementation of pharmacogenetics in clinical practice.

Gene-drug interactions

Gene-drug interactions can be divided into three different types; gene-drug interactions affecting the pharmacokinetics of a drug, gene-drug interactions affecting the pharmacodynamics of a drug, and idiosyncratic gene-drug interactions.

Pharmacokinetic gene-drug interactions

Initially the field of pharmacogenetics focused on drug metabolism [3]. Pharmacokinetics describes the processes to which a drug is exposed after administration. These processes are divided into absorption, distribution, metabolism (phase 1; biotransformation, phase 2; conjugation), and excretion. Drug plasma concentration is a result of these processes. Each of these processes can be affected by genetic variation resulting in a different plasma concentration, drug exposure and subsequently possibly a difference in drug response [4].

The cytochrome P450 enzyme system is involved in the phase I metabolism of numerous drugs [5]. Genetic variation in genes coding for cytochrome P450 enzymes can affect the metabolic capacity of these enzymes. In general four different genotype inferred phenotypes are distinguished; ultrarapid metabolizers (UMs) with increased metabolic capacity, extensive metabolizers (EMs) with a normal metabolic capacity, intermediate metabolizers (IMs) with a decreased metabolic capacity, and poor metabolizers (PMs) with a strongly decreased metabolic capacity. Within each genotype inferred phenotype a significant variation in metabolic capacity remains [6]. UM status results in faster and increased formation of drug metabolites. As a result, efficacy can be decreased, or increased in the case of prodrugs. IM and PM status can result in adverse drug events due to increased plasma concentration or decreased efficacy in the case of prodrugs. The platelet aggregation inhibitor clopidogrel presents an example of a prodrug that requires metabolic activation. This reaction is catalyzed by the genetically polymorphic enzyme cytochrome P-450 CYP2C19. Several pharmacogenetic studies with clinically relevant endpoints have been reported (Box 9.1.1).

BOX 9.1.1

Clopidogrel

From an analysis of nearly 1,500 patients with acute coronary symptoms who were treated with clopidogrel it was concluded that carriers of a reduced function *CYP2C19* allele had an increase of 53% in the composite primary efficacy outcome of the risk of death from cardiovascular causes, myocardial infarction, or stroke, as compared with noncarriers (hazard ratio 1.53, 95% CI 1.07-2.19) [7]. In a study of 259 patients who survived a first myocardial infarction it was reported that the primary composite endpoint of death, non-fatal myocardial infarction, or urgent revascularization occurred more frequently in carriers of the *CYP2C19*2* allele (hazard ratio 5.38, 95% CI 2.32-12.47) [8]. In a cohort of approximately 2,200 clopidogrel treated patients presenting with an acute myocardial infarction it was observed that patients carrying any two *CYP2C19* loss-of-function alleles (*CYP2C19*2*, **3*, **4*, or **5*) had a higher occurrence of the composite endpoint of death from any cause, nonfatal myocardial infarction, or stroke compared to patients with no loss-of-function alleles (hazard ratio 1.98, 95% CI 1.10-3.58) [9]. The observed association between reduced function *CYP2C19* alleles and increased likelihood of a cardiovascular event has been confirmed in a genomewide association study [10]. By contrast, the *CYP2C19*17* allele results in an increased metabolic capacity of CYP2C19. In a cohort of 1,524 patients undergoing percutaneous coronary intervention after pretreatment with 600 mg clopidogrel it was observed that carriers of the *CYP2C19*17* allele had an increased risk for bleeding (odds ratio 1.80, 95% CI 1.03-3.14) [11]. A limitation of most clopidogrel data is that they are obtained from observational studies or subgroup analyses of studies not primarily designed to investigate the effect of genetic variation in *CYP2C19* rather than randomized controlled trials.

During phase II metabolism an endogenous substrate (e.g. glucuronic acid, sulphate or glutathione) is incorporated in the drug molecule to facilitate excretion in the urine or bile. The N-acetylation of isoniazid by N-acetyltransferase is perhaps the most well-known example of genetic variability affecting phase II metabolism [3]. Other examples of genetic variation associated with phase II metabolism are thiopurine S-methyltransferase (TPMT), and UDP glucuronosyltransferase 1 family, polypeptide A1 (UGT1A1).

Transporter proteins play an important role in the absorption, distribution, and excretion phase of a wide variety of drugs. Drug plasma concentrations are particularly determined by drug transporters in the small intestine, liver, and kidney. For example, the P-glycoprotein encoded by the *ABCB1* gene transports many drugs out of the cell into the urine or bile.

Genetic variation in the *ABCB1* gene results in variable expression of P-glycoprotein and variation in drug plasma concentrations [4,12]. For many other transporter proteins pharmacogenetic effects have been described [13,14].

Pharmacodynamic gene-drug interactions

Pharmacodynamics describes the effects of the drug on the body i.e. its biological effect mediated through interaction with a receptor, enzyme or ionchannel. Pharmacodynamic gene-drug interactions occur at this level and can influence drug response i.e. by genetic variation resulting in increased expression of a receptor. This is exemplified by the screening of breast cancers for HER2/neu overexpression to select patients eligible for treatment with trastuzumab (although strictly this is not an example of pharmacogenetics according to the EMA definition since no variations in DNA sequence is involved). Trastuzumab is a monoclonal drug directed against the HER2 protein that is expressed on the surface of tumor cells and mediates uncontrolled growth [15]. Trastuzumab is approved for the treatment of HER-2-positive breast cancer. A second example of a pharmacodynamic gene-drug interaction is the variation in response to the treatment with the oral anticoagulant warfarin as a result of genetic variation in the gene coding for vitamin K epoxide reductase (VKORC1), a key enzyme in the vitamin K cycle [16].

Idiosyncratic gene-drug interactions

Finally, there are idiosyncratic adverse drug reactions. Idiosyncrasy is an abnormal response that is unpredictable and thought to have an underlying genetic etiology. pharmacogenetic research has increasingly focused on these types of adverse drug reactions but was hampered by a lack of sufficient numbers of patients with well characterized phenotypes [17,18]. A classic example of idiosyncratic gene-drug interactions is glucose 6-phosphate dehydrogenase and response to primaquine (Box 9.1.2).

The association of *HLA-B*5701* genotype with the hypersensitivity reaction to the antiretroviral drug abacavir presents a more recent example. For the latter gene-drug interaction the first randomized controlled pharmacogenetic trial was performed in 2008 [22]. 1,956 human immunodeficiency virus type 1 infected patients were randomly assigned to undergo prospective *HLA-B*5701* screening, with exclusion of *HLA-B*5701*–positive patients from abacavir treatment (prospective-screening group), or to undergo a standard-of-care approach. Both immunologically confirmed (2.7% vs. 0%) and clinically diagnosed (7.8% vs. 3.4%) hypersensitivity reactions were more frequent in the control group than in the prospective-screening group. In response of these results registration authorities have revised the package insert of abacavir to

BOX 9.1.2

Historic perspective

The concept of interindividual differences in drug response was proposed as early as 1909 by Garrod in his book The Inborn Errors of Metabolism [19]. But pharmacogenetics did not emerge as a distinct discipline until the late 1950s. For instance, it was then observed that a deficiency of glucose 6-phosphate dehydrogenase (G6PD) resulted in acute hemolytic crises in approximately 10% of the African-American soldiers but only a very small number of Caucasian soldiers who used primaquine as antimalarial drug [3]. G6PD deficiency was also suspected to account for favism - hemolysis after ingestion of Fava beans. Next, in 1956 Werner Kalow was the first to demonstrate interindividual variation in butyrylcholinesterase activity leading to clinical differences of patients in response to the muscle relaxant suxamethonium [20]. In 1977, Smith and his colleagues studied the antihypertensive effects of debrisoquine. Smith was the only among the study subjects to experience a very strong hypotensive reaction, and collapsed. When he recovered, he found out that the most important metabolite of debrisoquine was not detectable in his urine. In 1977 he published on the poor metabolizer and extensive metabolizer phenotypes of debrisoquine [21]. Since then many more SNPs in *CYP2D6* as well as other cytochrome P-450 polymorphisms have been identified.

include the advice to screen for *HLA-B*5701* prior to treatment initiation and to avoid prescription of abacavir to carriers of the *HLA-B*5701* polymorphism, unless there is no therapeutic alternative.

OBSTACLES FOR THE IMPLEMENTATION OF PHARMACOGENETICS IN ROUTINE CLINICAL PRACTICE

Since the completion of the human genome project in 2003, genomics has become a mainstay of biomedical research and pharmacogenetics has been predicted to be one of the first clinical applications arising from the new knowledge [2,23,24]. Indeed, the research efforts in the field of pharmacogenetics expressed as the number of publications listed on PubMed have steadily increased during the last decade until leveling out in 2009 at 1,100-1,200 publication per year (Figure 1.1, **Chapter 1**) [25]. By contrast, the clinical use of pharmacogenetic testing did not meet high expectations and has lagged considerably behind (**Chapter 2**).

To better understand the reasons for the limited clinical use of pharmacogenetics one should take into account the consecutive phases and associated challenges on the road to clinical implementation of pharmacogenetics presented in Figure 2.1. This figure represents the parts of the "life cycle" of a pharmacogenetic biomarker. After the proof of principle, a pharmacogenetic biomarker is applied in small scale clinical research allowing evaluation of efficacy and cost-effectiveness. Following positive results, clinical adoption on an increasing scale will initiate. The first, and major, obstacle precluding clinical application of pharmacogenetics is the relative lack of scientific evidence that pharmacogenetics results in improvement in patient care [26]. Although the number of pharmacogenetic publications is large, approximately 45% of these publications consist of review articles and only a limited proportion is original research. The relative paucity of original research articles is not the only problem. Many original articles involve small, specific study populations, administration of single doses, use of healthy volunteers instead of patients, use of a different translation from genotype to phenotype, or were not designed to investigate pharmacogenetics. Moreover, most positive association studies lack validation of findings in an independent patient population. However, in recent years the number of well-designed pharmacogenetic studies increased. Many of these studies are genome wide association studies (GWAS) designed to investigate the association between genetic variability and drug efficacy or adverse drug reactions. GWAS have revolutionized genetic research as they allow the discovery of multiple gene variants with individually small effects. The underlying rationale for GWAS is the hypothesis that common diseases are attributable in part to common genetic variants present in more than 1-5% of the population [27]. The advantage of GWAS is that it eliminates the need to choose, a priori, candidate genes or variants. GWAS are highly suitable to identify genetic variants contributing to complex phenotypes (Figure 9.1.1). At the end of 2010 1,212 published GWAS were listed at the National Human Genome Research Institute GWAS catalog [28]. The GWAS have identified hundreds of genetic variants associated with common diseases (type 2 diabetes mellitus (T2DM), inflammatory bowel disease, prostate cancer) as well as various individual traits (height, hair color, eye color) providing novel insights in the genetic architecture of complex traits in general. However, clinical application is limited since most SNPs only explain a small portion of genetic heritability [29]. For example despite extensive analyses by consortia that accrued cohorts of over 20,000 subjects only 5-10% of T2DM heritability can be explained by genetic variants [30]. Since 2007 GWAS have increasingly been applied to the field of pharmacogenetics (Table 9.1.1).

Drug response is a complex phenotype involving both known and unknown biological pathways. The GWAS approach enables novel and less obvious pharmacogenetic genes to be discovered, especially for genetic variation affecting drug pharmacodynamics which is more complex and often less well understood than drug pharmacokinetics. The GWAS

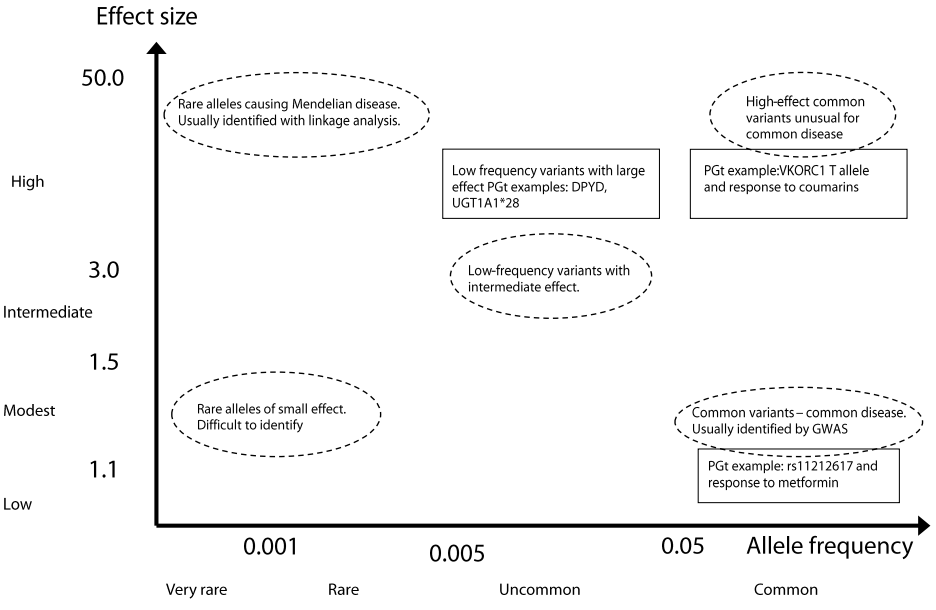

Figure 9.1.1 Feasibility of genome wide association studies to identify genetic variants by risk allele frequency and effect size (odds ratio). Genome wide association studies are especially suited to identify common variants of common disease. Figure adapted from [27,29]. Dashed circles indicate effect size vs. allele frequency for disease related genome wide association studies, boxes indicate pharmacogenetic genome wide association studies. PGt, pharmacogenetics.

approach also seems promising in the discovery of genetic variants predicting drug response since drug response is generally a complex trait that is influenced by numerous genetic and environmental factors. There are some important differences between pharmacogenetic GWAS and GWAS for complex diseases (Figure 9.1.1).

First, for a GWAS of complex diseases such as T2DM it is unlikely to detect a common allele with a large effect size since such an allele would have been eliminated from the population due to natural selection. Yet some of the first pharmacogenetic GWAS have reported relatively large effect sizes that involved fewer genes compared to those detected in GWAS for complex disease [31]. This could be explained by the fact that for drug response no "natural-selection" occurs unless involved genes are also part of normal physiological processes. For adverse drug events the process of natural selection is embodied by the drug registration authorities that do not approve drugs that cause frequent toxicity (as would be the case for common alleles resulting in adverse drug

Table 9.1.1 Published and replicated genome-wide association studies of drug response

Drug	Outcome	Patient population	Significant genes	Reference
Warfarin	Efficacy	All with drug	*VKORC1 CYP4F2 CYP2C9*	[76,77]
Acenocoumarol	Efficacy	All with drug	*VKORC1 CYP4F2 CYP2C9 CYP2C18*	[78]
Peginterferon α	Efficacy	Chronic hepatitis C infection	*IL28-β*	[79-81]
Clopidogrel	Efficacy	Percutaneous coronary intervention	*CYP2C19*	[10]
Methotrexate	Efficacy	Pediatric patients with acute lymphoblastic leukemia	*SLCO1B1*	[82]
Simvastatin	Toxicity	Patients with myopathy and drug	*SLCO1B1*	[83]
Flucloxacillin	Toxicity	Patients with liver injury and drug	*HLA-B*5701*	[43]
Carbamazepine	Toxicity	Patients with hypersensitivity syndrome and drug	*HLA-A*3101*	[54]
Metformin	Efficacy	All with drug	rs11212617	[32]

events). However observed difference in effect size between GWAS for complex disease and pharmacogenetics might be biased by the fact that most pharmacogenetic GWAS have been of limited sample size (often < 500) compared to GWAS for complex disease (typical sample size 4,000). For drug response similar results have been obtained with a recent GWAS investigating glycemic response (HbA1c ≤ 7%) to metformin (n = 3,920) [32]. A SNP (rs11212617) with an odds ratio of 1.35 for treatment success was reported. If more pharmacogenetic studies with larger sample sizes become available also more effect sizes similar to those in complex disease GWAS may be detected.

A second difference is related to the definition of the studied phenotype. Because drug response is influenced by numerous genetic and environmental factors it often shows a continuous phenotypic distribution. It is suggested that selectively genotyping patients at the extremes of the response distribution provides nearly equivalent power to complete genotyping [33]. Heterogeneity in phenotype can also be introduced because drug response can not always be measured in a quantitative way.

A concern for pharmacogenetic GWAS is that currently commercially available genotyping arrays do not sufficiently capture the genetic variation that is already known to be important to common pharmacokinetic and pharmacodynamic pathways. In a recent evaluation of seven commercially available genotyping arrays it was estimated

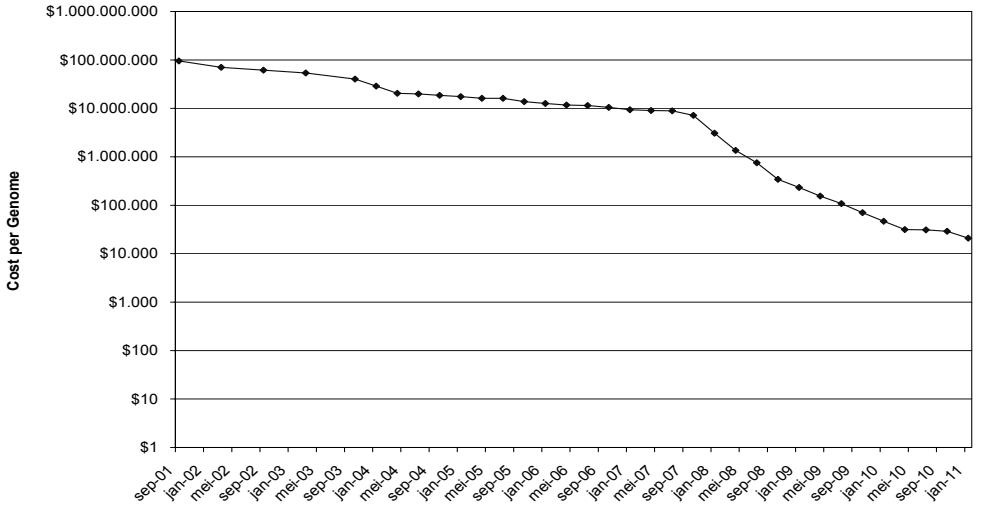

Figure 9.1.2 Cost of sequencing a human-sized genome. Reproduced from: Wetterstrand KA. DNA sequencing costs: data from the NHGRI large-scale genome sequencing program: <www.genome.gov/sequencingcosts>. Accessed 09-06-2011.

that coverage of 83 SNPs associated with 26 important pharmacogenes listed on PharmGKB coverage varied from 10-53% [34,35]. However, it is without doubt that GWAS offer tremendous possibilities for discovering new insights in the mechanisms of drug response.

A second obstacle for clinical implementation is the lack of information on cost-effectiveness and cost-consequences of pharmacogenetic testing. Cost-effectiveness has only been established for a very limited number of pharmacogenetic tests. The performance of cost-effectiveness analyses is problematic for two reasons. First, there are limited data on the rate at which pharmacogenetic testing actually prevents adverse drug reactions. Second, pharmacogenetic test prices are dropping continuously (Figure 9.1.2) [36]. A recent systematic review on cost-effectiveness of 20 pharmacogenetic studies found a lack of standardization regarding aspects such as the perspective of the analysis, factors included in the sensitivity analysis and the applied discount rates. In particular, an important limitation of several studies was related to the failure to provide a sufficient evidence-based rationale for an association between genotype and phenotype [37].

A third obstacle is that most pharmacogenetic tests (such as tests for genetic variants of cytochrome P450 enzymes) a detailed knowledge of pharmacology is a prerequisite for application in clinical practice, and both physicians and pharmacists might find it difficult

BOX 9.1.3

High likelihood of clinical relevance of pharmacogenetic testing

Pharmacogenetics can be useful in situations where the predictive ability of currently available biomarkers is low and the consequences, positive or negative, of a treatment are large. This can be exemplified by genotyping *DPYD* in patients treated with 5-fluorouracil or its oral prodrug capecitabine. *DPYD* codes for the enzyme DPD which metabolizes these drugs. Germline polymorphisms in *DPYD* are associated with deficiency of the enzyme DPD which results in severe neutropenia [40].

A second useful application of pharmacogenetics may be in situations where controlling or adapting a therapy on the basis of a phenotype is not possible. This is exemplified by genotyping *CYP3A5* in renal transplant patients prior to treatment with the calcineurin inhibitor tacrolimus. To prevent acute rejection, it is important to establish adequate tacrolimus plasma concentrations as early as possible. *CYP3A5* is polymorphic and over ten different alleles have been identified [41]. The *CYP3A5*3* allele, results in a non-functional CYP3A5 enzyme, and is the allele with highest gene frequency among Caucasians. In a randomized open label study with 280 renal transplant recipients treated with tacrolimus it was found that a larger proportion of patients receiving the genotype adapted dose had values within the targeted C0 at day 3 after treatment initiation (43.2% vs. 29.1%) [42].

A third example of a situation where pharmacogenetics can be useful is the lack of alternative drug treatments. Testing for *HLA-B*5701* in patients infected with HIV sensitive to abacavir and a viral load requiring treatment present an example.

Finally, pharmacogenetics can be useful in the elucidation and possibly prevention of idiosyncratic drug reactions such as the development of drug drug-induced liver injury due to flucloxacillin. Recently it was reported that approximately 85% of these cases can be explained by *HLA-B*5701* status [43].

to interpret the clinical value of pharmacogenetic test results. Guidelines that link the result of a pharmacogenetic test to therapeutic recommendations might help to overcome these problems, but such guidelines are only sparsely available (**Chapter 2 and 3**).

Other obstacles for clinical application of pharmacogenetics are unclear reimbursement status, and a lack of financial stimulus for the pharmaceutical industry to develop pharmacogenetic tests for drugs already marketed [26,38,39] (**Chapter 2**).

TRANSLATING PHARMACOGENETICS TO PRIMARY CARE

Quality control of pharmacogenetic testing

Pharmacogenetic testing prior to drug treatment is currently not a routine practice, and largely confined to academic hospitals and specialized laboratories. Clinical application of pharmacogenetics will result in adjustment of treatment of individual patients. Therefore quality control for clinically applied pharmacogenetic tests is even more important than for investigational pharmacogenetic tests. This can be achieved by the use of standardized quality controls. Yet after screening of the materials and methods sections of papers published in 2005-2007 in *Pharmacogenomics* or *Pharmacogenetics and Genomics* we found that only in a minority of papers the genotyping of quality controls is reported, and no standard procedures are applied (**Chapter 5**). Subsequently, we developed plasmid-derived external controls for *TPMT*2*, **3B/C*; *CYP2D6*3*, **4*, **6*, **9*, **41*; *CYP2C9*2*, **3*; *CYP2C19*2*, **3* to enable the general use of standardized external quality controls in pharmacogenetic testing. The availability of good quality control materials is of key importance for the clinical implementation of pharmacogenetics.

A second issue related to quality control is the exclusion of SNPs because of poor genotyping. In **Chapter 6** we report that the exclusion of rs757210, a SNP in *HNF1β*, because of difficulties in genotyping with TaqMan probes might be related to the tri-allelic nature of this SNP. Therefore we investigated the suitability of pyrosequencing and high-resolution melting as alternatives. These two chapters illustrate that ensuring the analytical validity of pharmacogenetic tests is essential. In addition they show that design of pharmacogenetic tests and operation of a pharmacogenetic laboratory are an evolving specialism in clinical laboratory medicine.

Pharmacogenetic dose recommendations in clinical practice

In 2005 the Royal Dutch Association for the Advancement of Pharmacy established the Pharmacogenetics Working Group (PWG) (**Chapter 3**). In this 15-member multidisciplinary working group, clinical pharmacists, physicians, clinical pharmacologists, clinical chemists, epidemiologists, and toxicologists are represented. The objective of the PWG is to develop pharmacogenetics-based therapeutic (dose) recommendations on the basis of a systematic review of literature, and to assist the drug prescribers as well as the pharmacists by integrating the recommendations into computerized systems for drug prescription and automated medication surveillance. The recommendations do not indicate patients who are eligible for genotyping, but merely aim to optimize drug use in the small but ever-increasing group of patients whose genotypes are known. Initially the project focused on genetic variations that affect pharmacokinetics, but this has been expanded to include genetic variations that affect pharmacodynamics for example the interaction

between genetic variation in the gene coding for factor V Leiden and estrogen-containing oral contraceptives. To date, the PWG has composed pharmacogenetic guidelines for 53 drugs associated with 11 genes [44]. The guidelines issued since October 2006 are available through most automated drug prescription, dispensing, and medication surveillance systems in The Netherlands making them accessible during the decision-making process by physicians and pharmacists, namely the prescription and dispensing of drugs. A detailed description of the background of the project, methods used for data collection, data assessment, and preparation of gene–drug monographs are provided in **Chapter 3**. Recently, another project aimed at the creation of pharmacogenetic dosing guidelines was launched: the Clinical Pharmacogenetics Implementation Consortium (CPIC) [45]. The consortium was created in 2009 with the aim of facilitating the clinical implementation of pharmacogenetic testing by providing guidance to test users and has recently published its first guideline [46]. Created guidelines will be publicly available in the Pharmacogenomics Knowledge Base [34].

Despite the availability of pharmacogenetic guidelines there is little information regarding the potential impact of these guidelines in primary care. To test the feasibility of pharmacogenetic screening in primary care we conducted a pilot study among 93 polypharmacy patients treated in primary care (**Chapter 4**). This study indicated that pharmacogenetic screening is feasible in a primary care setting with regard to patient willingness to participate, quality of DNA collection with saliva kits, genotyping, and dispensing data retrieved from pharmacy records. On average, included patients used 2.3 drugs with a recommendation in the PWG guidelines. Moreover, general practitioners had not empirically switched PMs to drugs that were not substrate of the aberrant enzyme. These findings indicate potential clinical relevance of pharmacogenetic for a primary care setting. However, this was only a small pilot study with a highly selected patient population of polypharmacy patients aged > 60 not representing the average primary care patient.

FUTURE PERSPECTIVE

Throughout this thesis it is noted that the initial enthusiasm with what pharmacogenetics was received, sometimes even described as "hype", has transformed into doubt and disbelief among some health care professionals in more recent years. In my opinion this is mainly due to the fact that pharmacogenetics has often been depicted as a "revolution" while in fact it is more an evolution, albeit a rapid evolution. Pharmacogenetic research is beginning to show consistent, reproducible results for an increasing number of genetic markers for drug response. In the upcoming years, we will see some important developments.

The first development concerns dosing algorithms that include pharmacogenetic as well as more traditional clinical variables such as age, renal function and bodyweight.

Approximately 10 years ago, the majority of pharmacogenetic research was aimed at investigating the effect of a single polymorphism on treatment outcome. Often, the investigated polymorphism had been previously associated with an enzyme or transporter involved in the metabolic pathway of the drug under investigation. Nowadays, pharmacogenetic research has progressed from searching for associations between individual polymorphisms and treatment outcome, through combinations of multiple polymorphisms affecting pharmacokinetics and pharmacodynamics, to the use of pharmacogenetic models including polymorphisms as well as more traditional clinical variables for therapy individualization such as age, renal function and bodyweight. The inclusion of these traditional clinical variables is essential since drug response is a complex trait and individuals belonging to the same "pharmacogenetic class" sometimes still vary about 10-fold in metabolic ratio. Therefore pharmacogenetics alone can not explain all interindividual differences in drug response. The progress from individual polymorphisms to pharmacogenetic models is well exemplified by the coumarin warfarin. This drug is metabolized by the polymorphic enzyme CYP2C9. The *CYP2C9* genotype by itself explained 5–18% of the variation in required warfarin dose [47]. It was already reported that genetic variability in *VKORC1*, a gene coding for vitamine K epoxide reductase, a key enzyme in the vitamine K cycle, could explain 15–37% of the variation in required warfarin dose [19,48-50]. Recently, dosing algorithms including genetic variants of *VKORC1*, *CYP2C9* and clinical factors such as age, sex, height and bodyweight have been reported to explain up to 50% of variation in required warfarin dose [51]. Currently, such dosing algorithms are under development for acenocoumarol and phenprocoumon, the coumarins used in The Netherlands [52].

A second development is the application of pharmacogenetic knowledge to idiosyncratic drug reactions [17]. Multiple relatively rare adverse drug events such as flucloxacilline induced liver injury [43], Stevens-Johnson syndrome induced by carbamazepine [53,54], and the hypersensitivity reaction to abacavir have all been associated with specific HLA-B genotypes. For the latter, a prospective study showed that the hypersensitivity reaction can be prevented by screening patients prior to treatment with abacavir [22].

A third development is the availability of guidelines that link the result of a pharmacogenetic test to specific dose recommendations. No matter what level of evidence is required for implementation of pharmacogenetics in clinical practice, it will be essential to develop algorithms that aid physicians in their interpretation and use of pharmacogenetic data. In 2001, an early step was taken to develop such guidelines for the therapeutic use of antidepressants, and these included *CYP2D6*-related dose recommendations drawn from pharmacokinetic study data [55]. In the Netherlands the PWG established in 2005 by the Royal Dutch Association for the Advancement of Pharmacy published guidelines concerning 53 drugs and 11 genes [44]. In 2009, an international initiative to create pharmacogenetic guidelines was launched: the Clinical Pharmacogenetics Implementation

Consortium [45]. Recently CPIC published its first guideline that is publicly available in the Pharmacogenomics Knowledge Base [34,46]. There is an increasing call for new pharmacogenetic guidelines [56]. In addition to guidelines created by these consortia regulatory authorities such as the FDA and EMA are increasingly including pharmacogenetic information in drug labels. The FDA has a special genomics group that created a table of valid genomic biomarkers in the context of approved drug labels [57]. The table contains entries for over 20 drugs.

A fourth development is that genetic variation that has previously been associated with gene-drug interactions is increasingly being associated with disease and vice versa. This can be exemplified by the recently reported association between *CYP2C19* poor metabolizer status and significantly lower levels of depressive symptoms [58]. A second example is the reported association of *CYP2D6* ultrarapid metabolism with suicidal behavior [59,60]. An example of disease associated alleles with treatment outcome is provided in **Chapter 8**. In this chapter it is found that T2DM patients with a higher number of T2DM associated risk alleles also have an increased risk of non-response to treatment with sulfonylureas.

A fifth development is the integration of gene–drug and drug–drug interactions. To date, drug–drug interactions have been considered characteristic only for the drugs involved. However, in the light of current knowledge of pharmacogenetics, this might no longer be valid. For example, the interaction between a CYP2D6 inhibitor and a CYP2D6 substrate requires different management for CYP2D6 IMs than for CYP2D6 PMs. Therefore, the combination of gene–drug and drug–drug interactions may have major implications for drug prescribing and dispensing. Research in this field is only starting to evolve [61].

A sixth development is the significant technological advancement and associated price drops of genotyping cost. Figure 9.1.2 shows the development of the cost of sequencing a single genome. The sudden price drop at the beginning of 2008 represents the time when the sequencing centers transitioned from Sanger-based to 'next-generation' DNA sequencing technologies [62]. With a strong market-based competition and new approaches under development prices may even drop further to as low as a few hundred Euros within the next decade, bringing it close to the price of computed tomography and magnetic resonance imaging [63].

As a result of these developments, I foresee that pharmacogenetics will increasingly set foot inside routine clinical practice in the next 10 years. Dosing algorithms, including pharmacogenetics combined with traditional clinical variables will increasingly be developed. At first, most pharmacogenetic test will be applied to specific patient populations were stakes are high, such as transplantation patients or cancer patients but over time a wider clinical adoption will initiate. Over time, information concerning the genetic status of a patient will increasingly be considered a regular clinical parameter like renal function and body mass index. The main driver for this development is

the plummeting price of genotyping (Figure 9.1.2). The significant price drop will probably also limit the issue of cost-effectiveness since test costs are the major part of pharmacogenetics costs, and stimulate an increasing number of patients to purchase a direct-to-consumer genomewide profiling test. Although currently potential effects of this type of genetic testing are unknown [64], as a result clinicians may be increasingly confronted with patients with a known genotype. The increasing number of patients with a known genotype raises important issues and eliminates others.

First of all, the increasing preemptive availability of a patient's genotype will eliminate the hurdles associates with test logistics. A major issue that will remain is the discussion concerning the required evidence base for initial clinical implementation of pharmacogenetics. Some argue that pharmacogenetic interventions should not be implemented until they are proven superior over the existing standard of care by randomized clinical trials (RCTs) [65]. However, RCTs are very expensive and should be reserved for the most important medical interventions e.g. in the case of large public health burden. Many useful clinical applications of pharmacogenetics may never justify a RCT since the potential benefits of the test are simply too small. For example, the 5-hydroxytryptamine 3 receptor antagonists used to prevent nausea and vomiting are known to be metabolized by CYP2D6. As a result, CYP2D6 UM may be undertreated with a standard drug dose. However, the patient's clinician can easily increase the drug dose or switch to a different anti-emetic therapy in response of nausea. In this kind of situations, other levels of evidence such as one or two confirmatory studies reproducing evidence from the initial exploratory study combined with a strong biological rationale for a gene-drug interaction may be sufficient to justify clinical implementation of pharmacogenetic testing. A lower level of evidence may also be justified in situations where the genotype of a patient is already known. For example, if a patient has been previously genotyped and is reported to be a CYP2D6 PM should this patient be treated with the standard dose of haloperidol, a CYP2D6 substrate? There are multiple studies reporting lower dose requirements for CYP2D6 PMs [66-71]. Yet, no RCT has been published. Also there seems to be a different perception of the required evidence for clinical implementation of pharmacogenetic information compared to other, nongenetic clinically relevant information [56]. For example, the selective estrogen receptor modulator tamoxifen used for the treatment of estrogen receptor-positive breast cancer needs to be metabolically activated by CYP2D6 to its active component endoxifen that is 100 times more potent than the parent compound [72]. Both genetic variation resulting in reduced CYP2D6 activity and concomitant drugs that inhibit CYP2D6 activity are expected to result in a lower endoxifen concentration and consequent reduction in tamoxifen efficacy. Yet, it is generally accepted to avoid the use of strong CYP2D6 inhibitors (e.g. selective serotonin inhibitors) in patients treated with tamoxifen whilst there is no such acceptation regarding genotyping for *CYP2D6*.

A second major issue is to what extent genomic diversity can explain variability in drug response. To date, many pharmacogeneticists have focused mainly on single polymorphisms ignoring the combined effect of multiple polymorphisms and even more importantly the effect of epigenomics on drug response. In the near future whole genome sequencing will become increasingly available. Also microRNAs (miRNAs), short noncoding RNAs that negatively regulate the expression of target genes, may become increasingly important. Differences in miRNA expression could be an important factor contributing to variability in drug response and utilizing them opens a new era in the field of predicting drug response that that we will begin to explore [73-75].

Successfully bridging the gap from research to bedside seems within reach in the field of pharmacogenetics. In many cases, the risks associated with pharmacogenetics are limited whilst potential benefits are substantial. Challenges remain, but we should expect routine pharmacogenetic tests entering the process of drug prescribing and dispensing within the next decade.

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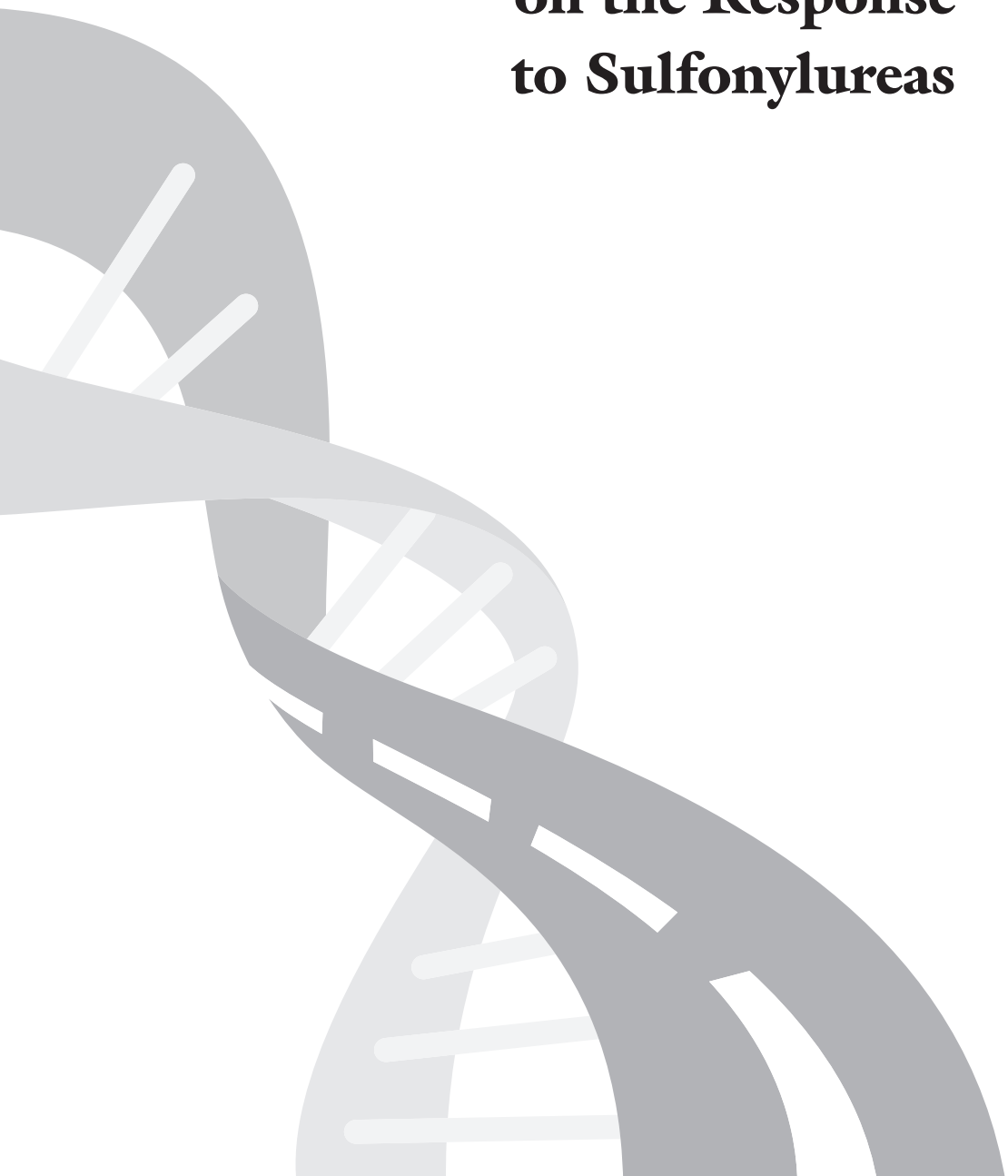
9.2 The Influence of Genetic Variation on the Response to Sulfonylureas



INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a common metabolic disease with an estimated prevalence of 171 million in 2000. The incidence of T2DM is increasing at an alarming rate and global prevalence estimates are up to 366 million for 2030 [1]. The therapeutic treatment of T2DM is aimed at the prevention or delay of long term microvascular and macrovascular complications by achieving the best possible glycemic control. For decades, sulfonylureas (SUs) have been an important part of treatment with oral antidiabetic drugs. Tolbutamide, glibenclamide (glyburide), gliclazide, and glimepiride are the most commonly used representatives of this group. These drugs act primarily by promoting pancreatic insulin secretion. They close the pancreatic β-cell potassium channels. This causes membrane depolarization, which triggers calcium influx and release of intracellular calcium stores, which in results in exocytosis of insulin containing vesicles and subsequent insulin release [2]. SUs are initiated at a low dose and escalated to the optimal dose with intervals of 2-4 weeks until the glycemic target (HbA1c < 7%) is achieved. However, there is significant interpatient variability in response to SUs, with approximately 10-20% of the patients experiencing primary failure (decrease in fasting glucose level < 1.1 mmol/L) and a similar percentage having an above average response (mean reduction HbA1c 1.5-2%) [3-5].

The influence of CYP2C9

Sulfonylureas are extensively metabolized in the liver primarily by CYP2C9 [6]. CYP2C9 genotype is known to have a significant effect on the pharmacokinetics of SU in healthy volunteers [7-12]. In a group of 207 incident sulfonylurea users treated in four university-affiliated primary care centers we found no statistically significant effect of the *CYP2C9*2* and *CYP2C9*3* alleles on the prescribed stable dose or time to stable SU dose (**Chapter 7**). However, a trend towards a lower stable dose for carriers of the *CYP2C9*3* allele was observed in the subgroup of patients treated with glimepiride. There also was no effect of the *CYP2C9*2* and *CYP2C9*3* alleles on decrease in fasting glucose levels for the relatively small subgroup of patients with fasting glucose level measurements available. Yet most studies on *CYP2C9* and SUs in T2DM patients report some significant effect (Table 9.2.1). However, in general the reported effect size is small, and due to publication bias there might also be more studies with negative results that failed to be published. Moreover, results are sometimes not replicated as is exemplified by the two papers of Holstein. In their initial 2005 paper [13] they reported a significant overrepresentation of carriers of the *CYP2C9*3* allele in a sample of 20 T2DM patients submitted to the emergency department with a severe hypoglycemia (symptomatic, requiring IV glucose, blood glucose measurement < 2.8 mmol/l) but no such finding was found in the 10-year follow up study [14]. Genotyping for the *CYP2C9*2* and *CYP2C9*3* allele currently seems to have no clinical implications for dosing of SU in primary care patients with T2DM.

The influence of genetic risk factors for type 2 diabetes mellitus

Genetic variation in other genes than *CYP2C9* might also contribute to interpatient variability in SU response. The pathogenesis of T2DM is not yet fully understood. Current theories include defects in insulin mediated glucose uptake in muscle, dysregulation of the adipocyte as a secretory organ, dysfunction of the pancreatic β-cell, and an impaired insulin action in the liver [15]. From 2007 onward an increasing number of genome wide association studies reporting associations between genetic variants and the risk for the development of T2DM have been published. These studies revealed the complex genetic background of T2DM that results in a single phenotypic disease. Probably as a consequence of this heterogeneity, interpatient variability of SU response cannot be explained by genetic variation in *CYP2C9* alone. Compared to pharmacogenetics affecting SU pharmacokinetics, variation in genes associated with the pharmacodynamics of SUs in T2DM patients has received even less attention. Genetic variants associated with SU response have been described for some monogenic forms of diabetes [16-18]. For polygenic T2DM, variants in the genes *KCNJ11*, *TCF7L2*, *ABCC8*, *IRS1*, and *NOS1AP* have been associated with SU response [19-22]. Of these, only the genes *KCNJ11* and *TCF7L2* were reported to contribute to an increased risk for T2DM in published GWAS. From the genome wide association studies a panel of 20 T2DM associated SNPs comprising 19 genes appears, that has been replicated in several studies [23-33]. We hypothesized that the multiple genetic background of T2DM might influence drug response (**Chapter 8**). Since the majority of the reported T2DM associated SNPs is involved in the process of insulin release from the pancreatic β-cells and SUs act by stimulating insulin secretion we investigated if response to SU treatment is influenced by the genetic variants. We found that patients with more than 17 T2DM risk alleles had a 1.7-fold reduced likelihood to achieve stable SU dose. These patients also show a marginally significant increased time to achieve stable SU dose compared to carriers of less than 17 risk alleles. However, the number of T2DM risk alleles does not seem to affect the prescribed average stable SU dose. Therefore, our data suggest that patients with a higher number of T2DM risk alleles have a decreased and delayed response to SU treatment.

CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH

Progression in the field of SU pharmacogenetic has been slow but significant. Our finding that patients with a higher number of T2DM risk alleles show a different response to SUs provides some "proof of principle" that the complex background of T2DM may ultimately result in the identification of different genetic subgroups of T2DM patients that require different pharmacotherapy. Yet we are only on the beginning of the understanding of what genetic variation determines response to SU and numerous challenges for future research remain. For example, the significant technological advancement in genotyping

Table 9.2.1 Studies investigating the effect of genetic variation in CYP2C9 on sulfonylurea pharmacokinetics and response

Author	Population	n	SU	Outcome	Effect	Ref
Kirchheiner et al.	Healthy volunteers	21	glyburide	PK	In homozygous carriers of *CYP2C9*3*, total oral clearance was less than half of that of *CYP2C9*1* ($p < 0.001$).	[7]
Yin et al.	Healthy volunteers	18	glyburide	PK	AUC(0-inf.) *CYP2C9*1/*3* 125% $p = 0.008$ vs. *CYP2C9*1/*1*.	[8]
Shon et al.	Healthy volunteers	18	tolbutamide	PK	In subjects heterozygous for the *CYP2C9*3* allele, C(max) and AUC of tolbutamide were significantly greater and the plasma half-life significantly longer than those in homozygous *CYP2C9*1* subjects.	[9]
Kirchheiner et al.	Healthy volunteers	23	tolbutamide	PK, Insulin + glucose response	Mean oral clearances of tolbutamide were 0.97 (95% CI 0.89-1.05), 0.86 (95% CI 0.79-0.93), 0.75 (95% CI 0.69-0.81), 0.56 (95% CI 0.51-0.61), 0.45 (95% CI 0.41-0.49) and 0.15 (95% CI 0.14-0.16) l/h in carriers of *CYP2C9* genotypes *1/*1*, **1/*2*, **2/*2*, **1/*3*, **2/*3* and **3/*3*, respectively.	[10]
Wang et al.	Healthy volunteers	19	glimepiride	PK	The AUC(0-inf.) was significantly greater in the *CYP2C9*1/*3* subjects than in *1 homozygotes ($p < 0.05$), with the **1/*3* and **3/*3* individuals demonstrating 1.3- and 1.4-fold increases in mean glimepiride AUC(0-inf.), respectively.	[11]
Suzuki et al.	T2DM patients	134	glimepiride	PK, HbA1c	The reduction in the HbA1c was significantly larger ($p < 0.05$) among the *CYP2C9*1/*3* subjects than among the *CYP2C9*1/*1* subjects. The pharmacokinetic study showed that the AUC for glimepiride in the *CYP2C9*1/*3* subjects was approximately 2.5-fold higher than that of the *CYP2C9*1/*1* subjects.	[35]
Ragia et al.	T2DM patients	176	sulfonylureas	Hypoglycemia	*CYP2C9*3* allele increased risk of mild hypoglycemia (OR 1.7).	[36]
Holstein et al.	T2DM patients	357	Sulfonylureas	Hypoglycemia	Genotypes *CYP2C9*3/*3* and **2/*3* were more common in the hypoglycemic group than in the comparison groups (10% vs. < 2%, respectively: OR 5.2; 95% CI 1.01-27).	[13]

Becker et al.	T2DM patients	475	Sulfonylureas	Fasting serum glucose levels, Required doses	*CYP2C9*1/*3* and **2/*3* treated with tolbutamide: • Dose increase between 1st and 10th prescription was 269 mg/day lower compared to **1/*1* (p = 0.009) • The fifference in prescribed daily dose between Rx 6 and 20, compared to the first Rx, was 316 mg lower (95% CI -497, -135, p = 0.0008) compared to **1/*1* • The adjusted decrease in FPG was 1,24 mmol/l larger (95% CI -2.75, 0.27, p = 0.11) compared to **1/*1* Other SUs: • No significant effect	[37]
Zhou et al.	T2DM patients	1,073	Sulfonylureas (80% gliclazide)	HbA1C < 7%	Patients with 2 copies of the *CYP2C9*2* or **3* allele were 3.4 times (p = 0.0009) more likely to achieve a HbA1c level <7% than patients with the */1*1 genotype. This corresponds to a 0.5% (p = 0.003) greater reduction in HbA1c concentration. • *2 and *3 allele carriers were less likely to experience treatment failure with SU monotherapy (p = 0.04; per-allele hazard ratio 0.79; 95% CI 0.63-0.99).	[38]
Holstein et al.	T2DM patients	203	sulfonylureas	Hypoglycemia (symptomatic, requiring IV glucose, blood glucose < 2.8 mmol/l)	• No overrepresentation of the *CYP2C9* PM genotypes in the hypoglycemic patients group. • Mean SU dose was lower in controls vs. hypoglycemic cases. (2.3, 1.8, and 1.7 vs. 2.5, 2.9, 2.8 EM, IM, PM controls vs EM, IM, PM hypoglycemic cases (p = 0.027).	[14]

PK, pharmacokinetics; AUC, area under the concentration-time curve; INF, infinity; T2DM, type 2 diabetes mellitus; CI, confidence interval; IV, intravenous; EM, extensive metabolizer; IM, intermediate metabolizer; PM, poor metabolizer; OR, odds ratio; Rx, prescription.

and associated price drop has permitted genome wide association studies. Within the field of diabetes, the GWAS technique has been mainly used to focus at disease but recently the first GWAS for glycemic response to metformin was reported. Since GWAS is hypothesis generating and does not rely on prior knowledge about underlying mechanisms these studies are bound to unravel new mechanisms in drug action. Indeed, the study reported an association between a SNP not previously associated with response to metformin [34]. Unfortunately this SNP only explained 2.5% of the variance in metformin response limiting its utility for treatment decisions. A comparable low explanatory value was found in our risk allele model (4.2%, **Chapter 8**, Table 8.3). This highlights a major issue that can be anticipated in SU pharmacogenetics; effect sizes of individual SNPs will be modest to small. In order to identify common SNPs with small to modest effect sizes, large cohorts with well characterized patients and outcome measures will be required. This presents a significant challenge since to date most SU pharmacogenetic studies have been of limited sample size. Once SNPs that predict response have been identified from GWAS, underlying pharmacological pathways explaining the observed effects will have to be elucidated and treatment algorithms incorporating these SNPs have to be developed and validated.

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Summary

Nederlandse Samenvatting

Curriculum Vitae

List of Publications

Nawoord

Summary

Introduction

In many patients drugs do not show the expected efficacy, whereas in other patients they cause toxic effects, sometimes even at low dose. Response rates to major classes of drugs range from 25 to 60 percent. For some patients, the reason for this variability may be explained by genetic variation. Pharmacogenetics is the study of variations in DNA sequence as related to drug response. The ultimate goal of pharmacogenetics is to predict and thereby improve drug response in the individual patient. The concept of interindividual differences in drug response was proposed as early as 1909. With the completion of the Human Genome Project in 2003 hope was raised that pharmacogenetics could be implemented in clinical practice in the near future. However, the expectations of the effect of a single nucleotide polymorphism (SNP) on drug response were unrealistically high and the clinical use of pharmacogenetic testing remained limited. Yet, the body of evidence supporting its usefulness is growing continuously. The research presented in this thesis aims to identify the reasons for the slow clinical translation of pharmacogenetics and to explore and expand possible solutions to address these obstacles. The thesis is divided into four parts. First, obstacles and possible solutions for the clinical implementation of pharmacogenetics are identified. In the second part, issues related to the quality control of pharmacogenetic testing are discussed. In the third part, the influence of genetic variation on the response to sulfonylureas (SUs), a class of commonly used oral antidiabetic drugs, is used as a case model to investigate the possibilities for pharmacogenetics in primary care. The fourth part contains the general discussion and a future outlook.

Clinical implementation of pharmacogenetics in primary care

In **Chapter 2** six challenges associated with the different phases in the translation process of the clinical implementation of pharmacogenetics are identified and solutions to overcome these obstacles are discussed. It is concluded that the major reason for the limited clinical translation of pharmacogenetics is the relative lack of scientific evidence for improved patient care by pharmacogenetic testing. Providing this scientific evidence presents a significant challenge. Other challenges for the implementation of pharmacogenetic testing include the selection of clinically relevant tests, providing data on diagnostic test criteria, providing information on cost-effectiveness and cost-consequences, to improve the acceptance among clinicians, and to develop guidelines directing the clinical interpretation of test results. The latter is essential since for most pharmacogenetic tests a detailed knowledge of pharmacology is a prerequisite for application in clinical practice, and both physicians and pharmacists might find it difficult to interpret the clinical value of pharmacogenetic test results. **Chapter 3** describes the initial development of such guidelines by the Dutch Pharmacogenetics Working Group. This multidisciplinary working group representing clinical pharmacists, physicians, clinical pharmacologists, clinical chemists, epidemiologists, and toxicologists was established in 2005 with

the objective to develop guidelines with pharmacogenetics-based therapeutic (dose) recommendations. After a systematic literature review that included 26 drugs therapeutic (dose) recommendations were composed for 17 drugs. The 26 drugs were substrates for CYP2D6 (n = 21), CYP2C19 (n = 1), CYP2C9 (n = 3), and UGT1A1 (n = 1). The guidelines do not call for prospective pharmacogenetic testing but are aimed at patients with a previously determined genotype. To assist drug prescribers and pharmacists the recommendations were included in an extensive electronic drug database (used by most computerized systems for drug prescription and automated medication surveillance in The Netherlands) since October 2006. In 2011 the number of pharmacogenetic guidelines is expanded from the initial 26 to 53 drugs and updates of the existing monographs are presented (Table 3.3). Assessed drugs were associated with genes coding for CYP2D6 (n = 25), CYP2C19 (n = 11), CYP2C9 (n = 7), thiopurine-S-methyltransferase (TPMT) (n = 3), dihydropyrimidine dehydrogenase (DPD) (n = 3), vitamin K epoxide reductase (VKORC1) (n = 2), uridine diphosphate glucuronosyltransferase-1A1 (UGT1A1), HLA-B44, HLA-B*5701, CYP3A5, and factor V Leiden (FVL) (all n = 1). Therapeutic (dose) recommendations were formulated for 39 (73.6%) of the drugs. In **Chapter 4** the results of a pilot experiment to investigate the feasibility of pharmacogenetic screening in primary care with respect to patient willingness to participate, quality of DNA collection with saliva kits, genotyping, and dispensing data retrieved from the pharmacy are described. A sample of polypharmacy patients, age > 60 years that used at least one drug metabolized by CYP2D6 or CYP2C19 was randomly selected. DNA was collected with saliva kits and genotyped for *CYP2D6* and *CYP2C19* with the AmpliChip. Out of the 93 invited patients, 54 patients (58.1%) provided informed consent. Nine saliva samples (16.7%) contained too little DNA. Call rates for *CYP2D6* and *CYP2C19* were 93.3% and 100% respectively. The frequencies of genotype-predicted-phenotype were 2.4%, 38.1%, 54.8%, and 4.8% for CYP2D6 poor-metabolizers (PM), intermediate-metabolizers (IM), extensive-metabolizers (EM), and ultrarapid-metabolizers (UM) respectively. For CYP2C19 genotype-predicted-phenotype frequencies were 2.2%, 15.6%, and 82.2% for PM, IM, and EM, respectively. From this study it is concluded that pharmacogenetic screening is feasible with regards to practical aspects and has potential clinical relevance for a primary care setting.

Quality control of pharmacogenetic testing

The application of pharmacogenetics in clinical practice may result in the adjustment of treatment of individual patients. Therefore, genotyping of patients in a routine clinical setting requires robust and reliable genotyping methods and good quality control is of great importance. In **Chapter 5** we surveyed the use of quality control samples in pharmacogenetic studies published from 2005 to 2007 in the two most prominent pharmacogenetic journals. We found that 86% of papers in which samples were genotyped

did not use or define quality controls. Most studies duplicated 5–10% of the samples as a quality control. This may serve as an internal control, but can not be regarded as an independent quality control sample to assure the validity of the pharmacogenetic test. To develop standardized quality controls, we cloned *TPMT*2*,**3B/C*; *CYP2D6*3*, **4*, **6*, **9*, **41*; *CYP2C9*2*, **3*; and *CYP2C19*2*, **3* in plasmids. These plasmids can be used for different pharmacogenetic assays and enable pharmacogeneticists to use standardized genotype controls for diagnostic testing.

A second quality related issue of importance for pharmacogenetics is the exclusion of SNPs because of poor genotyping. During one of our studies we experienced problems with genotyping rs757210, a SNP in the gene coding for hepatocyte nuclear factor 1β. Genotyping this SNP with a TaqMan assay failed to meet the pre-defined quality criteria of a call rate ≥ 95%. A literature search revealed that several studies reported similar problems in genotyping rs757210. In **Chapter 6**, the development of two alternative methods to genotype rs757210 is described. First we developed a pyrosequencing method, and since this methodology is not always available, also the feasibility of a high resolution melting method of small amplicons was investigated. The methods were tested in panels of type 2 diabetes mellitus patients (n=258) and healthy blood donors (n = 183). Results were confirmed by Sanger sequencing. Results for pyrosequencing and high resolution melting were in 99.6% concordance.

The influence of genetic variation on the response to sulfonylureas

The third part of this thesis is dedicated to the investigation of the influence of genetic variation on the response to SUs. SUs are part of the mainstay of treatment of type 2 diabetes mellitus (T2DM) with oral antidiabetic drugs. We selected SU treatment as a case model to investigate the potential role of pharmacogenetics because most T2DM patients are treated in primary care. Secondly, there is significant interpatient variability in response to SUs, with approximately 10–20% of the patients experiencing primary failure. Thirdly, SUs are metabolized by the polymorphic enzyme CYP2C9 and this enzyme also plays an important role in the metabolism of many other drugs frequently used in primary care. Two allelic variants, *CYP2C9*2* and *CYP2C9*3*, have been associated with a decreased metabolic capacity and elevated sulfonylurea serum levels in pharmacokinetic analyses performed in healthy individuals. In **Chapter 7** we investigated the effect of the *CYP2C9*2* and *CYP2C9*3* alleles on prescribed SU dose and time-to-stable SU dose. A group of 207 incident sulfonylurea users treated in four university affiliated primary care centers was identified. No significant effects of the *CYP2C9*2* and *CYP2C9*3* alleles were found. However, a trend towards a lower stable glimepiride dose for carriers of the *CYP2C9*3* allele was observed. We concluded that genotyping for the *CYP2C9*2* and *CYP2C9*3* alleles currently appears to have no clinical implications for dosing of SUs

in primary care patients with T2DM. In our search for genetic variation that might help to explain the interpatient variability in response to SUs we then hypothesized that a panel of 20 associated T2DM risk alleles might influence response to SU treatment. The rationale for this hypothesis was that the majority of the panel of T2DM risk associated SNPs is involved in the process of insulin release from the pancreatic beta-cells. Two SNPs, in the genes *KCNJ11* and *TCF7L2* had previously been correlated with variation in SU response. Furthermore, in subjects analyzed for genetic variation in the genes *TCF7L2*, *PPARG*, *FTO*, *KCNJ11*, *NOTCH2*, *WFS1*, *CDKAL1*, *IGF2BP2*, *SLC30A8*, *JAZF1*, and *HHEX*, it was reported that subjects with 12 or more T2DM risk alleles did not increase their insulin secretion to compensate for the increased insulin resistance as efficiently as those with 8 or less risk alleles. In **Chapter 8** we analyzed the effect of the panel of 20 repeatedly associated T2DM risk alleles on the response to SU treatment in 207 T2DM patients of the cohort described in Chapter 7. For each patient a genetic risk score was calculated based on the number of risk-alleles. With this score, patients were categorized into three predefined genetic risk groups. The effect of the genetic risk group on the achievement of stable SU dose, prescribed stable SU dose, and time to stable SU dose was analyzed. Patients with more than 17 T2DM risk alleles had a 1.7-fold reduced likelihood to achieve stable SU dose ($p = 0.044$). No significant effect of the number of T2DM risk alleles on prescribed dose was found. The patients with more than 17 T2DM risk alleles showed a marginally significant increased time to stable dose (hazard ratio: 0.81; 95% confidence interval, 0.75–1.01, $p = 0.058$). We concluded that T2DM risk alleles are associated with response to SUs in primary care T2DM patients. This suggests that individualization of T2DM treatment according to genetic profile may be an opportunity to improve clinical outcome.

General discussion

In **Chapter 9** the results presented in this thesis are discussed and a future perspective of the implementation of pharmacogenetics in clinical practice is outlined. **Chapter 9.1** first discusses the translation of pharmacogenetics from concept to clinic. In the upcoming years, we foresee six important developments. First, dosing algorithms that include pharmacogenetic as well as more traditional clinical variables such as age, renal function and bodyweight will be developed. Secondly, pharmacogenetics will increasingly be applied to prevent idiosyncratic drug reactions such as the hypersensitivity reaction to abacavir. Thirdly, we will see a sharp increase in the number of available pharmacogenetic guidelines created by groups such as the Dutch Pharmacogenetics Working Group or the USA Clinical Pharmacogenetics Implementation Consortium. In addition, regulatory authorities such as the EMA and FDA will increasingly include pharmacogenetic information in drug labels. Fourthly, genetic variation that has previously been associated with disease will increasingly be associated with gene-drug interactions and vice versa. Fifthly, we will

see the beginning of the integration of gene–drug and drug–drug interactions. A sixth and final development will be the significant technological advancement and associated price drops of genotyping cost. As a result of these developments, pharmacogenetics will increasingly set foot inside routine clinical practice in the next 10 years. A major issue will be the required evidence base for initial clinical implementation of pharmacogenetics e.g. is a RCT required for each gene-drug interaction prior to clinical implementation? In some situations, other levels of evidence such as one or two confirmatory studies reproducing evidence from the initial exploratory study combined with a strong biological rationale for a gene-drug interaction may be sufficient to justify initial clinical implementation of pharmacogenetic testing. A second major issue is to what extent genomic diversity can explain variability in drug response. Pharmacoepigenomics might open a new era in the field of predicting drug response.

In **Chapter 9.2** the results from the two SU studies are discussed and a future outlook is presented. The finding that patients with a higher number of T2DM risk alleles show a different response to SUs provides some "proof of principle" that the complex background of T2DM may ultimately result in the identification of different genetic subgroups of T2DM patients that require different pharmacotherapy. Yet we are only on the beginning of the understanding of what genetic variation determines response to SUs and numerous challenges for future research remain. A major issue that can be anticipated in SU pharmacogenetics is that effect sizes of individual SNPs will be modest to small. In order to identify common SNPs with small to modest effect sizes, large cohorts with well characterized patients and outcome measures will be required. This presents a significant challenge since to date most SU pharmacogenetic studies have been of limited sample size. Once SNPs that predict response have been identified, underlying pharmacological pathways explaining the observed effects will have to be elucidated and treatment algorithms incorporating these SNPs have to be developed and validated.

Nederlandse Samenvatting

Inleiding

De reactie op een geneesmiddel kan sterk verschillen tussen patiënten. Zo is wel gesuggereerd dat het percentage patiënten dat goed reageert op een behandeling met een geneesmiddel varieert van 25 tot 60 procent. Farmacogenetica onderzoekt in welke mate verschillen in het genetisch profiel van mensen een verklaring vormen voor de interindividuele verschillen in effectiviteit en bijwerkingen van geneesmiddelen. Het uiteindelijke doel van farmacogenetisch onderzoek is om te komen tot een geneesmiddelentherapie op maat voor de individuele patiënt.

De gedachte dat de verschillen in de reactie op een geneesmiddel tussen mensen erfelijk kunnen zijn is voor het eerst beschreven in 1909. In 2003 werd het internationale humane genoomproject voltooid, waarbij voor het eerst de sequentie van het DNA tot in detail in kaart werd gebracht. Na de voltooiing van het humane genoomproject werd farmacogentica door velen gezien als een van de eerste praktische toepassingen die uit dit project zou voortvloeien. Deze ontwikkeling is echter veel minder snel gegaan dan verwacht. De mate waarin één kleine verandering in het DNA (SNP) in staat was om de reactie op een geneesmiddel te verklaren, bleek kleiner dan verwacht. Voor veel behandelaren is farmacogenetica (nog) geen routinematig toegepaste methode om farmacotherapie te individualiseren. Toch neemt het beschikbare wetenschappelijke bewijs voor toepassing van farmacogenetica dagelijks toe. Dit proefschrift, getiteld 'Het toepasbaar maken van farmacogenetica in de eerstelijnsgezondheidszorg', heeft als doel om de oorzaken voor de beperkte toepassing van farmacogenetica in kaart te brengen en om mogelijke oplossingen om deze oorzaken weg te nemen te verkennen. Het proefschrift bestaat uit vier delen. Het eerste deel bestaat uit een analyse van de mogelijke oorzaken voor de beperkte toepassing van farmacogenetica en draagt mogelijke oplossingen aan om de toepassing te verbeteren. Het tweede deel beschrijft onderzoek naar de kwaliteitsborging van farmacogenetische testen. Het derde deel beschrijft onderzoek naar de toepassingsmogelijkheden van farmacogenetica in de eerstelijnsgezondheidszorg, waarbij het effect van farmacogenetica op de respons op de behandeling van type 2 diabetes met sulfonylureumderivaten als een model wordt gebruikt. Het vierde deel bevat een discussie van het in dit proefschrift beschreven werk en een beschouwing van toekomstige ontwikkelingen.

Farmacogenetica: van concept naar klinische praktijk

In **hoofdstuk 2** worden zes barrières en bijbehorende oplossingen voor de klinische toepassing van farmacogenetica in kaart gebracht. De belangrijkste barrière is het beperkte wetenschappelijke bewijs dat het toepassen van farmacogenetica ook daadwerkelijk leidt tot een verbeterde behandeluitkomst voor de patiënt. Andere barrières zijn de selectie van klinisch relevante testen, het ontbreken van gegevens over de diagnostische waarde

van farmacogenetische testen, het ontbreken van studies naar de kosteneffectiviteit, het gebrek aan kennis van en scholing over farmacogenetica onder artsen, apothekers en patiënten, en het ontbreken van richtlijnen die helpen bij de interpretatie van een farmacogenetische test. Het ontwikkelen van dergelijke richtlijnen is essentieel omdat voor die interpretatie vaak gedetailleerde kennis van het werkingsmechanisme van geneesmiddelen nodig is. **Hoofdstuk 3** beschrijft de ontwikkeling van dergelijke richtlijnen door de farmacogeneticawerkgroep. Deze multidisciplinaire werkgroep is in 2005 opgericht door het geneesmiddelinformatiecentrum van de Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie met als doelstelling om arts en apotheker te helpen bij het in de praktijk toepassen van farmacogenetica. Na literatuuronderzoek werden in eerste instantie voor 17 van de 26 onderzochte geneesmiddelen adviezen opgesteld. De onderzochte geneesmiddelen waren substraat voor de enzymen CYP2D6 (n = 21), CYP2C19 (n = 1), CYP2C9 (n = 3), and UGT1A1 (n = 1). Adviezen bestonden uit dosisaanpassing, selectie van een alternatief middel, bloedspiegelbepaling, waarschuwing voor bijwerking, etc. De adviezen zijn geen oproep om prospectief te genotyperen, maar zijn bedoeld om de kennis van farmacogenetica toe te passen bij patiënten van wie het genotype om wat voor reden dan ook bekend is. Sinds oktober 2006 worden deze adviezen voor apothekers en voorschrijvers als onderdeel van de G-standaard (een elektronische bestand met informatie over geneesmiddelen) verspreid, waardoor deze elektronisch beschikbaar zijn tijdens het voorschrijven en bij de medicatiebewaking. In 2011 werd het aantal richtlijnen uitgebreid van 26 naar 53 geneesmiddelen (Tabel 3.3). De onderzochte geneesmiddelen waren substraat voor CYP2D6 (n = 25), CYP2C19 (n = 11), CYP2C9 (n = 7), thiopurine-S-methyltransferase (n = 3), dihydropyrimidine dehydrogenase (n = 3), vitamin K epoxide reductase (n = 2), uridine diphosphate glucuronosyltransferase-1A1, HLA-B44, HLA-B*5701, CYP3A5, en factor V Leiden (allen n = 1). Voor 39 (73,6%) van de onderzochte geneesmiddelen werden dosisaanpassingen geadviseerd.

Hoofdstuk 4 beschrijft de resultaten van een pilot-onderzoek naar de uitvoerbaarheid van een farmacogenetische screening in de eerste lijn. Hierbij is vooral gekeken naar praktische aspecten zoals de bereidwilligheid van patiënten om deel te nemen aan het onderzoek, de kwaliteit van het met speekselmonsters verzamelde DNA, kwaliteit van het genotyperen en de kwaliteit van de bij de apotheek verzamelde voorschrijfgegevens. Voor dit onderzoek werden patiënten van ouder dan 60 jaar die meer dan vijf geneesmiddelen gebruikten waarvan er minimaal één door het enzym CYP2D6 of het enzym CYP2C19 werd afgebroken geselecteerd. DNA werd verzameld met behulp van een speekselmonster. Het genotype voor *CYP2D6* en *CYP2C19* werd bepaald met de AmpliChip. In totaal werden 93 patiënten benaderd met de vraag of ze aan het onderzoek deel wilden nemen. Hiervan stemden er 54 (58,1%) in met deelname. Negen speekselmonsters bevatten te weinig DNA om het genotype vast te kunnen stellen.

Voor de resterende monsters slaagde het genotyperen in 93,3% van de monsters voor *CYP2D6* en 100% van de monsters voor *CYP2C19*. Van de gegenotypeerde patiënten was 2,4% langzame metaboliseerder (PM), 38,1% had een vertraagd metabolisme (IM), 54,8% een normaal metabolisme (EM) en 4,8% was een snelle metaboliseerder (UM) voor CYP2D6. Voor CYP2C19 was dit 2,2% PM, 15,6% IM en 82,2% EM. Op basis van de resultaten in hoofdstuk 6 wordt geconcludeerd dat farmacogenetische screening in de eerste lijn uitvoerbaar is in, en mogelijk klinisch relevant is voor, de eerste lijn.

De kwaliteitsborging van farmacogenetische testen

Omdat de klinische toepassing van farmacogenetica mogelijk leidt tot aanpassingen aan de behandeling met geneesmiddelen van patiënten is het van belang om betrouwbare methoden te hebben om het genotype vast te stellen. Hiervoor is het van belang om goede kwaliteitscontrolemonsters te hebben. **Hoofdstuk 5** beschrijft de resultaten van een onderzoek naar het gebruik van kwaliteitscontroles in farmacogenetische analysemethoden die waren toegepast in de periode 2005-2007 en gepubliceerd in twee toonaangevende farmacogenetische tijdschriften. Uit dit onderzoek bleek dat in 86% van de onderzochte publicaties geen kwaliteitscontroles werden beschreven. De meeste onderzoekers voerden bij 5-10% van de monsters een duplobepaling uit als kwaliteitscontrole. Dit is op zich een goede interne controle, maar het is geen vervanging van een onafhankelijke kwaliteitscontrole om de validiteit van een farmacogenetische analysemethode vast te stellen. Door stukjes DNA met daarin de genetische varianten van *TPMT*2*, **3B/C*; *CYP2D6*3*, **4*, **6*, **9*, **41*; *CYP2C9*2*, **3* en *CYP2C19*2*, **3* in een plasmide te plakken is het gelukt om voor deze veel bepaalde genetische varianten gestandaardiseerde controles te maken. In **hoofdstuk 6** wordt een tweede probleem met betrekking tot kwaliteitszorg beschreven, namelijk het uitsluiten van SNP's in de analyse vanwege slechte resultaten van het genotyperen. Tijdens één van de in dit proefschrift beschreven onderzoeken ontstonden problemen met het genotyperen van rs75210, een SNP in het gen dat codeert voor HNF1β. Met de gebruikte TaqMan-analyse lukte het niet om van meer dan 95% van de monsters het genotype te bepalen. Om dit probleem te verhelpen zijn twee alternatieve bepalingsmethoden ontwikkeld. Eerst werd een pyrosequencing-methode ontwikkeld en vervolgens een smeltmethode ("high resolution melting") omdat in veel laboratoria de pyrosequencing-techniek niet beschikbaar is. Beide methoden werden getest op DNA van een groep van 258 type 2 diabetes mellitus-patiënten en een groep van 183 gezonde bloeddonoren. De resultaten werden bevestigd met een 'gouden standaard'-methode van genotyperen (Sanger sequencing) en waren in 99,6% van de gevallen gelijk tussen de verschillende methoden.

Het effect van genetische variatie op de respons op behandeling met sulfonyureumderivaten

Het derde deel van dit proefschrift beschrijft het onderzoek naar de invloed van genetische variatie op de respons op de behandeling met sulfonylureumderivaten. Deze geneesmiddelen worden toegepast bij de behandeling van patiënten met diabetes mellitus type 2 (T2DM). De dosering van sulfonylureumderivaten wordt per patiënt vastgesteld. Dit gebeurt door middel van dosistitratie waarbij iedere 2-4 weken op geleide van effect de dosering wordt opgehoogd. Bij circa 10-20% van de patiënten werkt behandeling met sulfonylureumderivaten onvoldoende (daling in nuchter bloedglucose < 1,1 mmol/L) en een vergelijkbaar percentage reageert beter dan gemiddeld. Deze behandeling is om drie redenen als model gekozen om een mogelijke rol van farmacogenetica in de eerste lijn te onderzoeken. Ten eerste worden de meeste T2DM-patiënten in de eerste lijn behandeld. Ten tweede vertoont circa 10-20% van de patiënten onvoldoende respons op de behandeling met sulfonylureumderivaten. Ten derde worden sulfonylureumderivaten afgebroken door het enzym CYP2C9, een enzym dat betrokken is bij het metabolisme van veel geneesmiddelen die in de eerste lijn worden toegepast. Van het gen dat codeert voor CYP2C9 zijn meer dan 25 variantallelen beschreven. Twee van deze varianten, *CYP2C9*2* en *CYP2C9*3*, komen frequent voor in de Kaukasische populatie en coderen voor verminderd functioneel enzym. In onderzoek met gezonde vrijwilligers die een eenmalige dosis van een sulfonylureumderivaat kregen is aangetoond dat dragerschap van het *CYP2C9*2* of *CYP2C9*3* allel resulteert in een verminderde afbraak en dus een hogere blootstelling. **Hoofdstuk 7** beschrijft een studie naar het effect van *CYP2C9*2* en *CYP2C9*3* op de hoogte van de voorgeschreven dosis waarop T2DM-patiënten stabiel werden ingesteld. Daarnaast werd ook gekeken naar het effect op de benodigde titratietijd tot het bereiken van deze stabiele dosis. In totaal werd DNA van 207 T2DM-patiënten verzameld. Er werd geen statistisch significant effect van het *CYP2C9*2* en/of *CYP2C9*3* allel op de ingestelde dosis of de benodigde titratietijd gevonden. In de subgroep van glimepiride (één van de sulfonylureumderivaten) gebruikers werd wel een trend naar een lagere dosis voor dragers van een *CYP2C9*3* allel waargenomen. Op basis van dit onderzoek werd geconcludeerd dat genotyperen voor *CYP2C9*2* en *CYP2C9*3* op dit moment geen meerwaarde heeft voor het doseren van sulfonylureumderivaten in de eerste lijn. Sinds 2007 is er door middel van genoombrede associatieonderzoeken steeds meer bekend geworden over erfelijke risicofactoren voor het ontwikkelen van T2DM. Uit deze onderzoeken komt een set van 20 risico-allelen naar voren die consequent geassocieerd zijn met een verhoogd risico op T2DM. De meerderheid van de allelen in deze set is geassocieerd met genen die een rol spelen bij de insuline-afgifte door de β-cellen in het pancreas. De genen *KCNJ11* en *TCF7L2* waren in eerdere onderzoeken ook geassocieerd met variatie in de respons op sulfonylureumderivaten. Ook was al beschreven dat individuen met 12 of meer risico-allelen voor de genen *TCF7L2*, *PPARG*, *FTO*, *KCNJ11*,

NOTCH2, *WFS1*, *CDKAL1*, *IGF2BP2*, *SLC30A8*, *JAZF1* en *HHEX* minder goed in staat waren om hun insuline-afgifte aan te passen aan toenemende insulineresistentie in vergelijking met individuen met acht of minder risico-allelen. Deze gegevens leiden tot de hypothese dat patiënten met een groter aantal T2DM risico-allelen minder goed reageren op de behandeling met een sulfonylureumderivaat. **Hoofdstuk 8** beschrijft een onderzoek naar het effect van het panel van 20 risico-allelen op de behandeling met sulfonylureumderivaten in het cohort van 207 T2DM-patiënten beschreven in hoofdstuk 7. Per patiënt werd een genetische risicoscore berekend gebaseerd op het aantal T2DM risico-allelen. Met deze risicoscore werden patiënten ingedeeld in drie vooraf gedefinieerde risicogroepen. Vervolgens werd het effect van de genetische riscogroep op het bereiken van een stabiele dosering, de hoogte van de stabiele dosering en de benodigde titratietijd onderzocht. Patiënten met meer dan 17 risico-allelen hadden een 1,7 keer kleinere kans om stabiel ingesteld te worden op een sulfonylureumderivaat. Ook lijkt voor deze patiënten een langere titratietijd nodig, hoewel dit net niet statistisch significant was. Het aantal risico-allelen had geen relatie met de hoogte van de stabiele dosis. Op basis van deze studie werd geconcludeerd dat allelen die een verhoogd risico op T2DM geven ook geassocieerd zijn met de respons op de behandeling met sulfonylureumderivaten. Dit suggereert dat in de toekomst mogelijk de behandeling van T2DM kan worden verbeterd door rekening te houden met het individuele genetische profiel van de patiënt.

Algemene discussie

In **hoofdstuk 9** worden de resultaten van het in dit proefschrift beschreven onderzoek besproken en wordt een toekomstvisie over de implementatie van farmacogenetica in de eerste lijn gepresenteerd. **Hoofdstuk 9.1** beschrijft zes belangrijke ontwikkelingen voor de implementatie van farmacogenetica in de eerste lijn. De eerste ontwikkeling is dat er doseeralgoritmen ontwikkeld gaan worden die naast traditionele klinische variabelen zoals leeftijd, nierfunctie en gewicht ook rekening houden met farmacogenetica. De tweede ontwikkeling is dat farmacogenetica in toenemende mate zal worden toegepast ter verklaring en preventie van geneesmiddelengeïnduceerde bijwerkingen zoals de overgevoeligheidsreactie op abacavir. De derde ontwikkeling is een sterke toename van het aantal beschikbare richtlijnen voor het interpreteren en toepassen van de resultaten van een farmacogenetische test zoals deze ontwikkeld zijn door de Nederlandse farmacogeneticawerkgroep en het Amerikaanse Clinical Pharmacogenetics Implementation Consortium. Ook zullen registratieautoriteiten zoals de EMA en de FDA in toenemende mate informatie over farmacogenetica op laten nemen in de productinformatie van geneesmiddelen. Een vierde ontwikkeling is dat genetische variatie die was geassocieerd met ziekte zal worden geassocieerd met de respons op geneesmiddeltherapie en vice versa. Een vijfde ontwikkeling is het integreren van de kennis van gen–geneesmiddelinteracties

met geneesmiddel–geneesmiddelinteracties. Een zesde en laatste ontwikkeling is de enorme technologische vooruitgang en de bijbehorende prijsdaling die dit veroorzaakt op het gebied van genotyperen.

Het gevolg van deze zes ontwikkelingen is dat farmacogenetische kennis in de komende 10 jaar steeds vaker klinisch zal worden toegepast. Een van de vraagstukken die beantwoord zal moeten worden is welke mate van bewijs nodig is voordat een farmacogenetische test klinisch toegepast kan worden. Is bijvoorbeeld altijd een gerandomiseerd dubbelblind onderzoek noodzakelijk, of voldoen andere vormen van bewijs? In sommige situaties zijn één of twee studies die eerdere resultaten bevestigen in combinatie met een goed onderbouwd onderliggend mechanisme voldoende voor klinische implementatie. Een tweede belangrijk vraagstuk is in welke mate genetische variatie verschillen in de respons op geneesmiddelen kan verklaren. Mogelijk gaat de farmacoepigenetica hier een belangrijke rol in spelen.

Hoofdstuk 9.2 bespreekt de resultaten van de twee onderzoeken naar het effect van genetische variatie op de respons op een behandeling met sulfonylureumderivaten. De bevinding dat patiënten met een groter aantal T2DM risico-allelen een andere respons op de behandeling met sulfonylureumderivaten vertonen is een “proof-of-principle” van het concept dat de multigenetische oorsprong van T2DM uiteindelijk kan leiden tot het identificeren van subgroepen patiënten met T2DM die een andere behandeling nodig hebben. Toch staan we nog slechts aan het begin van het ontrafelen van de invloed van genetische variatie op de respons op sulfonylureumderivaten. Een belangrijke uitdaging is dat de effecten van individuele SNP’s op de variatie in respons waarschijnlijk relatief klein zullen zijn. Om zulke effecten te kunnen vinden zijn grote groepen goed gedefinieerde patiënten nodig terwijl de meeste studies naar de farmacogenetica van sulfonylureumderivaten tot nu toe klein waren. Zodra SNP’s zijn gevonden die respons kunnen voorspellen en het onderliggende farmacologische mechanisme is opgehelderd, moeten er algoritmes worden ontwikkeld en gevalideerd die deze kennis klinisch toepasbaar maken.

Curriculum Vitae

Joachim Jesse Swen was born in Alkmaar, The Netherlands on March 5th 1978. After finishing secondary school (VWO) at the Han Fortmann College in Heerhugowaard in 1996, he started his study Pharmacy at the Utrecht University. In 2002 he obtained his doctoral exam, followed by his PharmD exam, in 2004. As part of his doctoral program he worked for nine months on a research project aimed at the development of a model to predict the lymphatic drug transport of testosterone esters. This work was performed at the Department of Pharmaceutics of NV Organon in Oss (supervisors Prof. dr. H. Vromans, Dr. W.A. Faassen). From May 2004 to January 2006, he was involved in the implementation of PeopleSoft ERP software in the Department of Clinical Pharmacy & Toxicology of the Leiden University Medical Center. In 2006 he started his training as clinical pharmacist which was completed in September 2011 (supervisor Prof. dr. H-J Guchelaar). At the same time he started working on the studies presented in this PhD thesis (supervisors Prof. dr. H-J Guchelaar, Prof. dr. W.J.J. Assendelft, Dr. J.A.M. Wessels). As part of his PhD project he joined the Helix group at the Department of Genetics, Stanford University, Palo Alto, CA, USA, for a period of three months in the fall of 2011 (supervisor Prof. dr. R. Altman). After completion of his PhD project he will continue his career as clinical researcher / pharmacist in the field of pharmacogenetics at the Leiden University Medical Center.

Jesse Swen lives in Amsterdam and is married to Charlotte Gubbels.

List of Publications

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Swen JJ, Huizinga TW, Gelderblom H et al. Translating pharmacogenomics: challenges on the road to the clinic. *PLoS Med* 2007;4(8):e209.

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Van der Molen H, de Raadt W, Swen JJ. Van specifiek naar algemeen. ERP in het LUMC. *ICTzorg* 2007;2(8):10-12 (article in Dutch).

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Nawoord

Onderzoek kan je letterlijk en figuurlijk ver brengen; terwijl ik dit schrijf zit ik circa 9.000 km van Nederland in de Californische oktoberzon.

De totstandkoming van dit proefschrift laat zich vergelijken met een klein sneeuwvlokje dat in december 2004 op de top van een berg terecht kwam en in de jaren daarna al naar beneden rollend steeds in omvang is toegenomen. Op de tocht naar beneden zijn er vele splitsingen gepasseerd, waarbij vooraf niet altijd duidelijk was waar we aan de voet van de berg uit zouden gaan komen. Onderweg was de helling soms zo vlak, dat er, om te blijven rollen, een duwtje nodig was. Graag wil ik iedereen die hier aan heeft bijgedragen bedanken.

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Lieve, lieve Cleo, sommige dingen zijn niet in woorden te vatten...