Title of the study: CYP2D6 polymorphism defining UM, IM, NM and PM status in unselected medically treated patients of general practice in Austria

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STUDY PROTOCOL

1 Background

The hepatic CYP2D6 enzyme metabolizes a significant number of drugs frequently prescribed in general practice/ family medicine. Various genetically different variants define if the patient is an ultra-rapid (UM), an extensive (EM) (the normal case), an intermediate (IM) or a poor metabolizer (PM). It is estimated that approximately 20- 25 % of frequently prescribed drugs are activated to more active or metabolized to ineffective or less effective drugs by CYP2D6. Substrates of CYP2D6 are mainly antidepressants, neuroleptics, opioids (e.g. codeine), beta-blockers, anti-arrhythmic drugs and various other single drugs. In case of an UM a drug can be metabolized too rapidly losing its therapeutic effect, requiring a higher dosage, or it can have a toxic effect, if it is converted too rapidly into the effective form (e.g. codeine). If metabolized too slowly (PM) it can accumulate and reach toxic levels.

Up to now data with respect to the frequency and distribution of the various types of metabolizers (UM, NM, IM, PM) in Austrian General Practice in patients prescribed drugs primarily metabolized by CYP2D6 are sparse.

2 Primary and secondary aims of the study

The primary aim of the study is to generate data on CYP2D6 polymorphism in patients of an average Austrian general practice, which allows to group patients according to their EM, UM, IM and PM status, which is of considerable practical relevance for deciding, which drug to choose. For this reason we describe the number and distribution of frequent and clinically relevant CYP2D6 alleles including copy number variations as well as the genotype-predicted phenotype in patients of the respective general practice.

In addition this study tries to investigate in how many patients the knowledge of the CYP2D6 metabolizing status would have been of clinical relevance with respect to the actually prescribed drug.

As a secondary aim, data relating to the total number of patients of the respective Austrian general practice having been prescribed one or more drugs within the last 3 years, mainly metabolized by CYP2D6 or being an inhibitor of it, are collected by extracting electronic records of the practice. In addition the average percentage of drugs metabolized by CYP2D6 in relation to the number of all prescribed drugs is registered by extracting the electronically stored prescriptions of the last 3 years.

3 Study design

This is an observational cohort cross-sectional study performed in an average Austrian general practice in 2017 and 2018 enrolling 290 unselected consecutive patients visiting the practice office for a routine blood test for various chronic medical conditions. Only in case a patient is actually taking or has been prescribed a drug metabolized by CYP2D6 (or taking a drug which is a strong inhibitor of CYP2D6) within the last 3 years, the genetic polymorphism of CYP2D6 is determined. No further genetic investigations or additional blood collections are performed.

For evaluating the number and characteristics of patients having been prescribed one or more specific drugs within the last 3 years, all relevant demographic and medical data are extracted from their electronic records and de-identified before further processing.

In addition the number of prescriptions (during the last 3 years) of all drugs metabolized by CYP2D6 (or which are strong inhibitors of CYP2D6) as identified by their ATC-code, in comparison to the total number of prescribed drugs of the practice is extracted from the electronic records

Patients who were considered to be eligible to participate in the study have to sign an informed consent after being informed also by orally given information about the aims of the study.

The study was approved by the Ethics Committee of Lower Austria.

4 Selection of patients

4.1 Inclusion criteria

In principle all patients who are capable of giving a written informed consent and who are visiting the practice due to various medical reasons for a routine blood sampling are suitable to be included into the study.

4.2 Exclusion criteria

Patients who are not capable to give a written consent or who are unable to understand the aim of the study are excluded from the study.

5 Blood sampling and processing of the specimens

A standardized blood sampling using a Vacutainer system with filling of an EDTA tube (4 ml) for the red and white blood count is performed. 300 microliters of the collected blood are used for further determination of the CYP2D6 polymorphism.

6 Isolation of DNA

DNA is extracted from EDTA blood in the practice laboratory by using the Spin Micro Extraction Kit® (ViennaLab,Vienna). The concentration and quality of DNA is measured using a Bio Photometer plus (Eppendorf). The extracted DNA is stored at -81°C in a ultra-low temperature deep freezer (U101-86, New Brunswick Scientific Co., Inc) without any further additives.

7 Real-time PCR for determination of copy number of the CYP2D6 gene

For the determination of the copy number of the CYP2D6 gene a real-time PCR in triplicates on an ABI StepOnePlus by using the CYP2D6 RealFast™ CNV Assay (ViennaLab) is performed in the practice laboratory.

The test is based on the fluorogenic 5’ nuclease assay, also known as TaqMan® assay. Each reaction contains gene-specific primer pairs for amplification of CYP2D6 and endogenous control (EC) gene fragments with 141 bp each. Further components are two dual-labeled, gene-specific hydrolysis probes, the FAM-labeled CYP2D6 probe and the HEX-labeled EC probe, which hybridize to an internal sequence of the amplified fragments. The proximity of the 5’-fluorescent reporter and 3’-quencher dye on intact probes prevents the reporter from fluorescing. During the extension phase of PCR the 5’ - 3’ exonuclease activity of Taq DNA polymerase cleaves the 5’-fluorescent reporter from the hybridized probe. The physical separation of the fluorophore from the quencher dye generates a fluorescent signal in real-time, which is proportional to the accumulated PCR product. The CYP2D6 RealFast™ CNV Assay is a relative quantitation assay and compares the amount of both nucleic acid targets (CYP2D6 and EC) in relation to the CYP2D6 CNV Calibrator. The EC gene is used to

normalize fluorescence signals between different samples and serves as a PCR positive control.

For normalization of data ROX dye to a final concentration of 1 microliter to the 2 x Probe Mix is added.

RT- cycling conditions for the ABI StepOneplus cycler: Initial denaturation: 95°C 10 min 1 cycle; denaturation 95°C 15 sec 40 cycles; annealing /extension 60°C 1 min.

8 PCR and hybridisation for CYP2D6 allele determination by the PGX-CYP2D StripAssay™

This assay is used for a subset of samples in this study and covers only 3 polymorphic loci: 1795delT (2D6*6), 1934G>A (2D6*4) and 2637delA (2D6*3). The test principle and procedure are similar to the PGX-CYP2D6 XL StripAssay® as described below but using different cycling conditions for the Palm-Cycler (Corbett Life Science): pre-PCR: 94°C/2 min; thermocycling: 94°C/15 sec.- 58°C/ 30 sec.-72°C/30 sec (35 cycles); final extension: 72°C/3 min.

9 PCR and hybridisation for CYP2D6 allele determination by the PGX-CYP2D6 XL StripAssay®

For the determination of 19 clinically relevant CYP2D6 alleles (*1; *2 A, *2 B-M; *3, *4A-H, K, L or P, *4J or N, *4M; *5; *6 A, B or D, *6C; *7, *8; *9,; *10A or B, *10 C or D; *11; *12; *14; *15; *17; *29; *35; *39; *40 or *58; *41) a PCR amplification on a Palm-Cycler (Corbett Life Science, Eight Mile Plains, QLD 4113, Australia) using biotinylated primers is first performed. The amplification products are further hybridized to a test strip containing allele-specific oligonucleotide probes immobilized as an array of parallel lines. Bound biotinylated sequences are detected using streptavidin-alkaline phosphatase and color substrates. The evaluation of this reaction is done manually by using the StripAssay®-Evaluator (ViennaLab,Vienna), a proprietary PC program to determine the homozygous or heterozygous genotype.

Cycling conditions for the Palm-Cycler (Corbett Life Science): pre-PCR: 95°C/4 min; thermocycling: 95°C/25 sec.- 60°C/ 45sec.-72°C/1min (36 cycles); final extension: 72°C/3 min.

According to the manufacturer the StripAssay and RealFastCNV Assay results were 100% concordant with genotypes and copy numbers obtained by reference methods and specimens, such as Sanger sequencing, Coriell reference DNA or long-range PCR. The PGX-CYP2D6 XL StripAssay® and the CYP2D6 RealFast™ CNV Assay have been successfully validated on 118 and 98 samples, respectively.

For internal evaluation of validity, sensitivity and specifity the study-laboratory participated successfully in the PGX-CYP2D6XL StripAssay® and CYP2D6 RealFast™ CNV Assay Confirmation Program.

10 Statistics

Statistical Analysis Plan

Statistics

Statistical Analysis

The data are recorded and analyzed using Microsoft Excel Version 10 and the R Language and Environment for Statistical Computing and Graphics, Version 2.9. Standard methods are used for the description of data (frequencies and percentages for categorical data, mean and standard deviation for continuous data). To compare frequencies of CYP2D6-specific drugs prescribed in the single practice with the total number of the respective prescriptions in

Lower Austria, Spearman`s rank correlation-coefficient is calculated. Differences between groups were calculated by paired t-test. A p-value < 0.05 was considered to indicate statistical significance.

The Hardy-Weinberg- equilibrium is calculated using Fisher´s Exact Chi-Square test due to the expected small numbers of genotypes.