Potential of whole-genome sequencing-based pharmacogenetic profiling

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Pharmacogenetics represents a major driver of precision medicine, promising individualized drug selection and dosing. Traditionally, pharmacogenetic profiling has been performed using targeted genotyping that focuses on common/known variants. Recently, whole-genome sequencing (WGS) is emerging as a more comprehensive short-read next-generation sequencing approach, enabling both gene diagnostics and pharmacogenetic profiling, including rare/novel variants, in a single assay. Using the example of the pharmacogene *CYP2D6*, we demonstrate the potential of WGS-based pharmacogenetic profiling as well as emphasize the limitations of short-read next-generation sequencing. In the near future, we envision a shift toward long-read sequencing as the predominant method for gene diagnostics and pharmacogenetic profiling, providing unprecedented data quality and improving patient care.

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Current state of pharmacogenetics

The term pharmacogenetics (PGx) first appeared in scientific literature in 1959 [1] and describes the field of genetics that investigates the effect of genetic variability on individual drug response [2]. PGx includes germline variants which influence the pharmacokinetics and pharmacodynamics of pharmaceuticals as well as somatic tumor-driver variants that influence the response to cancer drug therapies [3].

At present, the Pharmacogenomics Knowledgebase (PharmGKB) lists 68 clinically important pharmacogenes, >100 gene-drug pairs and >700 annotated drugs (pharmgkb.org, accessed October 2020), integrating information

from various PGx resources, such as the Dutch Pharmacogenetics Working Group (DPWG, knmp.nl) and the Clinical Pharmacogenetics Implementation Consortium (CPIC, cpicpgx.org). Pharmacogenes may be classified according to their function into categories, such as encoding Phase I (e.g., CYP) and Phase II (e.g., UGTs) enzymes, transporters (e.g., *ABCB1*), drug targets (e.g., *VKORC1*), oncogenes (e.g., *EGFR*) or involved in the immune system (e.g., *HLA-B*) [4]. Detected (functional) variants are primarily described using the star (*) allele nomenclature in most pharmacogenes [5]. In PGx, differences in enzyme activity lead to varying phenotypes, according to which individuals are classified as poor metabolizer (PM), intermediate metabolizer, normal metabolizer (NM) or ultrarapid metabolizer [2].

The importance of PGx may be highlighted by several facts. For instance, adverse drug reactions are a substantial socio-economic burden, being responsible for up to ~12.8% of hospital admissions in the EU, while approximately 0.5% of adverse drug reactions are lethal [6]. Indeed, as almost every individual harbors at least one actionable sequence variant according to the DPWG or CPIC guidelines [7,8], PGx could be implemented for patient stratification in clinical care and clinical trials [7]. Nevertheless, the majority of drugs are currently prescribed without the integration of pharmacogenetic information.

Pre-emptive pharmacogenetic profiling offers better patient care, enabling the incorporation of PGx into clinical decision support (CDS). A plethora of approaches for pharmacogenetic profiling are available and recent technical advances in next-generation sequencing (NGS) enable the integration of PGx into clinical settings more than ever. Currently, however, the benefits of PGx from randomized controlled trials (RCTs) and meta-analyses are mostly limited to single drug-gene pairs and the genes/variants that should be tested for the optimal prediction of drug response are not standardized [4,9]. In addition, there is low inter-laboratory concordance of test results [9] and the abundance of options for the implementation of PGx can be overwhelming.

Here, we review the methods of PGx and explore the potential of pharmacogenetic profiling based on our whole-genome sequencing (WGS) pipeline [7], which enables the combination of comprehensive molecular diagnostics and pharmacogenetic profiling in a single assay. Moreover, using the example of the pharmacogene *CYP2D6*, we outline the challenges and opportunities of current pharmacogenetic profiling methods and discuss potential future directions.

Genotyping of pharmacogenetic variants

Traditional methods

After the first discovery of the biochemical basis of pharmacogenetic variation in the 1970s in the context of debrisoquine hydroxylation [10] and sparteine oxidation [11] by the enzyme later termed CYP2D6, the first pharmacogenetic test was made available in 1990 [12]. This test was developed to identify CYP2D6 PMs and was based on allele-specific PCR. Similarly, in the following years, more functional variants were identified in other pharmacogenes by means of PCR-based methods and Sanger sequencing. From the late 1990s, microarrays have enabled increased throughput and parallelized genotyping of common/known variants across several genes [4,13–16]. Meanwhile, a variety of molecular methods have been adopted for the research and diagnostic applications of PGx (Table 1).

In the absence of regulation and clear guidelines, the applied methods and interrogated genes/variants may vary among testing laboratories, leading to inconsistent results [9]. Typically, the tested variants are selected according to the indication or the drug class, such as antihypertensives, psychotropics or anticoagulants [4]. From an economic perspective and particularly considering possible re-use of results [24], pre-emptive testing of multiple variants is superior to a single gene-drug approach [9]. Due to their cost efficiency, scalability, short turnaround time and high clinical utility, PCR- and microarray-based targeted genotyping (hereafter collectively referred to as targeted genotyping) has frequently been applied for pharmacogenetic profiling. For instance, in the ubiquitous pharmacogenomics (U-PGx) program, which aims to provide evidence for the clinical utility of pre-emptive pharmacogenetic profiling, a PCR-based assay is being applied for pharmacogenetic profiling [25]. Modern microarrays may be limited to pharmacogenetic variants (e.g., AmpliChip CYP450 [Roche, Basel, BS, Switzerland], PharmacoScan [ThermoFisher, MA, USA]), or interrogate close to 1 million variants (e.g., Infinium Global Screening Assay 3.0 [Illumina, CA, USA], Genome-Wide Human SNP Array 6.0 [Affymetrix, CA, USA]) and may be leveraged for pharmacogenetic profiling of large-scale biobanks [8,26].

A major caveat of targeted pharmacogenetic profiling is the subset of interrogated variants. The selection of variants to be tested may be decisive for treatment outcome, as illustrated by the conflicting results of several studies aiming to assess the efficacy of PGx-guided compared with clinically guided dosing of the anticoagulant warfarin.

Table 1. Comparison of methods for pharmacogenetic profiling.

	Advantages	Disadvantages	Ref.
Traditional methods	<ul style="list-style-type: none"> Low costs, short turnaround time, interpretation of results 	<ul style="list-style-type: none"> Limited to the subset of tested variants/fragments 	
PCR (e.g., Luminex xTAG CYP2D19/CYP2D6, LGC SNPLINE)	<ul style="list-style-type: none"> High sensitivity Variety of available PCR-based methods (TaqMan, long-range PCR, quantitative real-time PCR, digital PCR) 	<ul style="list-style-type: none"> Low/limited throughput 	[15]
Sanger sequencing	<ul style="list-style-type: none"> Allows sequencing with single-base-pair resolution Combination with long-range PCR for allele specificity 	<ul style="list-style-type: none"> Low/limited throughput Laborious 	[15]
Multiplex ligation-dependent probe amplification (MLPA)	<ul style="list-style-type: none"> Semi-quantitative detection of CNVs 	<ul style="list-style-type: none"> Low/limited throughput and limited to probe-binding sites Laborious, sensitive to handling 	[17]
Mass spectrometry (e.g., MALDI-TOF)	<ul style="list-style-type: none"> High throughput (option for automation) High sensitivity 	<ul style="list-style-type: none"> Expensive equipment, no US FDA-approved platform 	[18]
Microrrays (e.g., ThermoFisher PharmacoScan, Affymetrix Genome-Wide Human SNP Array)	<ul style="list-style-type: none"> High throughput (option for automation) 	<ul style="list-style-type: none"> Complex structural variants remain undetected (e.g., CYP2D6*13,*68) 	
Short-read NGS (e.g., Illumina, Ion Torrent, BGI Genomics)	<ul style="list-style-type: none"> Detection of rare/novel variants 	<ul style="list-style-type: none"> Turnaround time, interpretation of results 	
Targeted sequencing (e.g., Ion AmpliSeq Pharmacogenomics Research Panel, PGRNseq)	<ul style="list-style-type: none"> Cost efficient Existing PGx-specific analysis tools (for PGRNseq) 	<ul style="list-style-type: none"> Separate assays for diagnostics and pharmacogenetic profiling Enrichment laborious 	[19]
WES	<ul style="list-style-type: none"> Increased throughput Cost efficient (molecular diagnosis and pharmacogenetic profiling in one assay) 	<ul style="list-style-type: none"> Incomplete coverage of exome Non-exonic variants are not interrogated (e.g., CYP2C19*17, CYP3A5*3, UGT1A1*28/*37 and VKORC1*2) 	[7]
WGS	<ul style="list-style-type: none"> Existing PGx-specific analysis tools Molecular diagnosis and pharmacogenetic profiling in one assay 	<ul style="list-style-type: none"> Read alignment and variant calling in homologous/repetitive regions (e.g., CYP and UGT genes) 	[7]
Long-read NGS (e.g., PacBio, ONT)	<ul style="list-style-type: none"> Phasing, variant detection in homologous/repetitive regions 	<ul style="list-style-type: none"> Raw-read error rate (ONT), costs, input material (PacBio) 	
Targeted sequencing and WES	<ul style="list-style-type: none"> Variety of available target enrichment methods (e.g., PCR-based, hybridization-based, computational enrichment by ONT) 	<ul style="list-style-type: none"> Target enrichment may be laborious 	[20–22]
WGS	<ul style="list-style-type: none"> Molecular diagnosis and pharmacogenetic profiling in one assay 	<ul style="list-style-type: none"> Unavailability of PGx-specific analysis tools for long reads 	[23]

CNV: Copy number variation; NGS: Next-generation sequencing; ONT: Oxford Nanopore Technologies; PacBio: Pacific Biosciences; PGRN: Pharmacogenomics Research Network; PGx: Pharmacogenetics; WES: Whole-exome sequencing; WGS: Whole-genome sequencing.

Indeed, the 2013 European Pharmacogenetics of Anticoagulant Therapy (EU-PACT) [27] and the 2017 Genetic Informatics Trial (GIFT) [28] RCTs as well as a retrospective analysis [29] and a matched control study [30] showed clinical benefits of PGx-guided dosing, whereas in the 2013 Clarification of Optimal Anticoagulation through Genetics (COAG) RCT [31] PGx-guided dosing lead to no significant improvement. These conflicting findings are potentially explained by the fact that in the COAG RCT, ~35% of the participants were of African descent. Since only the variants *CYP2C9**2,*3 and *VKORC1**2 were tested, but not the PM variants *CYP2C9**5,*6,*8,*11, which are frequent in Africans, carriers of these variants were erroneously assigned the *1 (i.e., wild-type) allele and, thus, were potentially misdosed due to incomplete genetic data.

Moreover, in recent years, several studies have explored the pharmacogenetic landscape on population scale [7,8,32–35]. These studies showed that rare variants comprise roughly 30–40% of the variation in pharmacogenes and in some gene families even >90% of the PGx variation may be attributed to rare variants. Although there were differences in the results of these studies, they may largely be attributed to the selected genes, the size of studied cohort and whether the study considered all variants or exclusively focused on selected common/functional variants. In general, however, all rare variants in pharmacogenes (i.e., including nonselected ones) can play a role in drug response and, hence, be of clinical relevance. As targeted genotyping cannot detect rare (i.e., non-targeted) variants and the cost per base of NGS decreases, NGS-based pharmacogenetic profiling seems like the logical successor of traditional methods.

NGS-based pharmacogenetic profiling

The advent of NGS has transformed the field of genetics by enabling the parallel sequencing of multiple genes using targeted sequencing, the exonic regions of the genome using whole-exome sequencing (WES) or the entire genome using WGS to a large extent [36]. These techniques have been increasingly used not only in the gene diagnostics of rare diseases and in oncology but are also starting to be incorporated into the field of PGx. NGS-based profiling of pharmacogenes enables to expand pharmacogenetic testing from selected variants to entire genes, allowing the detection of rare variants and the identification of novel variant/gene drug associations (Table 1).

Current NGS-based targeted PGx panels include the AmpliSeq Pharmacogenomics Research Panel (thermofisher.com) or PGRNseq [19], enabling deep sequencing of the most frequently affected pharmacogenes. The ongoing eMERGE-PGx project [37] deploys PGRNseq across nine institutions in the USA with the aim to assess pharmacogenetic variation in ~9000 participants, particularly rare variants, and to generate a large variant repository called SPHINX (emergesphinx.org), partially linked to phenotypic data, allowing the investigation of novel pharmacogenetic variation. Accordingly, this study tackles one of the major barriers of PGx and aims to expand the scope of PGx. Ultimately, both targeted genotyping and targeted sequencing-based pharmacogenetic profiling arguably have the limitation that they need to be performed as a dedicated test for PGx, whereas WGS, rather than WES, offers the possibility of enabling both gene diagnostics and pharmacogenetic profiling in a single test [7].

Indeed, although WES is one of the most widely used and cost-effective NGS technologies allowing the sequencing of the exome, including pharmacogenes, we and others have shown that (i) WES *de facto* does not enable the sequencing of the entire exome due to missing capture probes and its sensitivity to guanine-cytosine (GC) content [38–40]; and (ii) depending on the applied exome capturing kit, several well-known but non-exonic (i.e., non-captured) pharmacogenetic variants are not interrogated using WES, including *CYP2C19**17, *CYP3A5**3, *UGT1A1**28/*37 and *VKORC1**2 [7,26]. In the worst case and without appropriate consideration, these limitations may lead to incomplete or false-negative diagnoses and/or to incorrect pharmacogenetic recommendations.

Based on current progress in NGS technologies, it is foreseeable that WGS will over time increasingly replace WES not only in research but also in clinical applications. WGS has the advantage that it overcomes the limitations of WES, in other words, WGS (60× PCR-free, PE150) not only allows better (more accurate) sequencing of the exome at appropriate coverage, but also enables sequencing of non-coding regions [36,38,40]. Moreover, the continuous and uniform coverage of PCR-free WGS facilitates detection of copy number variations [36,39,40]. As short-read WGS represents the most comprehensive short-read sequencing (SRS) approach, it constitutes a platform for both gene diagnostics and pharmacogenetic profiling in one assay, allowing the identification of both common/known and rare/novel pharmacogenetic variants [7,41]. Indeed, we have recently demonstrated how pharmacogenetic profiling may be integrated into a diagnostic workflow [7]. Briefly, after extracting the genotypes of the current 46 U-PGx variants from the WGS data using dedicated bioinformatics tools, they are interpreted according to the DPWG guidelines, producing a pharmacogenetic report as well as the Medication Safety Card in credit card format [7,9,42]. Likewise, the open-source Pharmacogenomics Clinical Annotation Tool represents a similar approach, which accepts WGS (genomic) Variant Call Format ([g]VCF) and Binary Alignment Map (BAM) files as input and generates pharmacogenetic reports according to the CPIC guidelines [43].

Despite being the predominant NGS approach, SRS is subject to inherent limitations due to its read length of typically ~150 bp. These short reads may not be unambiguously mapped to the reference genome, particularly in repetitive and/or homologous regions longer than the read length [36]. These regions are collectively termed the 'dead zone' and comprise ~17% of the reference genome [36], including several pharmacogenes, such as members of the *UGT* and *CYP* gene families. A notable example is *CYP2D6*, which is challenging to accurately genotype due to its homologous pseudogenes (*CYP2D7* and *CYP2D8*), numerous star alleles and complex structural variation [16].

WGS-based pharmacogenetic profiling of *CYP2D6*

Using a subset of the GeT-RM 2019 consensus genotypes for *CYP2D6* [44], we have previously benchmarked the bioinformatic tools Astrolabe [45], Aldy [46] and Stargazer [47], which enable *CYP2D6* genotyping using SRS data. We and others noticed that Aldy reached the highest accuracy in the WGS-based genotyping of *CYP2D6* [7,48]. Recently, a novel approach named Cyrius [49] has been introduced, promising improved accuracy. Here, we expand our previous benchmark with the results of Cyrius, which, however, may be biased in its favor as Cyrius was optimized/trained using the GeT-RM *CYP2D6* genotypes [44,49] (Supplementary Table 1). For our expanded benchmarking of WGS-based *CYP2D6* variant detection, we assessed the *CYP2D6* genotypes and predicted PGx

Figure 1. Comparison of Aldy and Cyrius using 565 clinical whole genomes. (A) Relative frequencies of *CYP2D6* functional phenotypes predicted by the software tools Aldy and Cyrius using the whole-genome sequencing (60× PCR-free, PE150) data of 565 unrelated samples. Unknown Function: Diplotypes of which the function of at least one allele is unknown. No Prediction: Samples for which the respective tool could not call a *CYP2D6* genotype. Error bars indicate 95% CIs calculated using vassarstats.net/prop1.html (including continuity correction). **(B)** Number of *CYP2D6* genotypes predicted by Aldy and/or Cyrius as well as reason for discordant predictions. Concordant: 534 samples for which Aldy and Cyrius predicted equal/concordant genotypes using Isaac BAM files. Concordant (BWA): 5 of the 31 samples with different/discordant genotypes for which the predictions were identical/concordant using input BAM files aligned with BWA instead of Isaac. No Prediction: Five samples for which neither Cyrius nor Aldy could call a *CYP2D6* genotype. The allele *CYP2D6**4.013 (*4N) is only predicted by Cyrius in seven samples. SNV: Single nucleotide variant; SV: Structural variant.

phenotypes in 565 primarily Caucasian short-read WGS samples (i) using Aldy [46] compared with Cyrius [49]; (ii) in addition considering rare PGx variants; (iii) exclusively considering the currently implemented U-PGx *CYP2D6* alleles.

For (i), WGS (PCR-free, 60× PE150) of 565 unrelated individuals was performed as previously described [40]. Subsequently, FASTQ files were aligned by using Isaac (v.01.15.02.08 or v.04.18.11.09), which is >5× faster than the native gold-standard aligner Burrows-Wheeler Aligner (BWA) [50], generating the required input BAM files for Aldy (v2.2.6) and Cyrius (v1.0). Of the 565 analyzed samples, the genotypes and phenotypes were identically predicted by both tools in 534 and 553 samples, which equates to an identical genotype and phenotype detection rate of 94.5 and 97.9%, respectively (Figure 1). We recognize the caveat that the genotypes were mostly not confirmed by an additional method, in other words, we cannot exclude identical genotyping errors of Aldy and Cyrius. In the 534 samples with identical predictions, we identified 24 *CYP2D6* haplotypes (Supplementary Table 2) which comprise 76 unique diplotypes (Supplementary Table 3). Of these concordant 534 samples, ~56.0, ~34.6, ~5.1 and ~2.6% were classified according to the DPWG guidelines as NM, intermediate metabolizer, PM and ultrarapid metabolizer, respectively (Figure 1). These functional predictions are largely in line with previous estimates for Caucasians [8,26,51,52], but provide additional evidence that complex structural variants (SVs), such as *68+*4 (relative frequency 6.5%), are more common than previously estimated [49].

In addition, for the 31 samples with discordant *CYP2D6* genotypes we re-aligned the FASTQ files using BWA v0.7.17 (accelerated with Sentieon [53]), manually evaluated the resulting BAM files using the Integrative Genomics Viewer [54] and re-ran Aldy and Cyrius (Supplementary Table 4). Thereby, five of the 31 discordant samples could be 'rescued' (i.e., the predictions of Aldy and Cyrius were identical/concordant); four cases were due to a novel result of Aldy and one due to a novel result of both, Aldy and Cyrius, indicating the higher sensitivity of Aldy to the alignment tool used for the input BAM file. An important difference between Isaac and BWA is the handling of reads which can be aligned/mapped to multiple positions. By default, Isaac (v.01) aligns such reads to the first possible genomic location [50], whereas BWA aligns such reads randomly [55], which may account for certain

differences in variant calling and is observable by the number of reads aligned to REP6 and REP7 (Supplementary Figure 1). The most frequent reasons for discordances were because of the allele *CYP2D6**4.013 (previously known as *4N) is not covered by Aldy, and therefore, reported as *4 in seven samples as well as due to differences in variant phasing in ten samples (Supplementary Table 4). For example, in one sample Aldy called the diplotype *27/*41, while Cyrius called *1/*32. Combined, these alleles comprise the same variants but in different phase. Moreover, the tandem rearrangement *68+*4 was called with a concordance close to 100%, whereas in other rearrangements, including *10, *13 and *36, the same variants were detected, but on different alleles. Analyzing such discordant results is challenging solely using SRS data and hence orthogonal methods, long-read data or data of family members would be necessary for clarification. However, these discordant genotypes provide important insights, pinpointing which types of variants might be miscalled using such tools.

Both Aldy and Cyrius are easy to use, provide results in less than a minute for WGS input data, do not require high-end hardware, and appear to perform highly accurate and concordant in most cases. Aldy currently supports ten pharmacogenes, while Cyrius is only applicable to *CYP2D6*. However, Cyrius has the advantages over Aldy of being less sensitive to the alignment software, supporting GRCh38/hg38 in addition to GRCh37/hg19, and being slightly more up-to-date regarding allele designations.

For (ii), in the 534 WGS samples with identical predictions we identified additional *CYP2D6* sequence variants using a previously described method [7]. Thereby, we detected 17 coding variants with unknown PGx phenotype (according to pharmvar.org/gene/CYP2D6). One of these variants, NM_000106.5:c.1353G>A p.(Met451Ile), occurred heterozygous only in one sample and was classified as likely deleterious using a PGx-specific variant prediction framework [56] and thus may alter the *CYP2D6* activity predicted by Aldy and Cyrius (more details in Supplementary Table 5). Aldy additionally detected possibly functional variants, which are not part of a star allele in the form of appending the suffix '-like' to the identified allele (e.g., *1/*2-like), whereas Cyrius does not report additional detected variants.

For (iii), we limited the analysis of the 534 WGS samples with identical predictions to the 11 *CYP2D6* alleles currently tested in the U-PGx project and as recently proposed (*3, *4, *5 (del), *6, *8, *9, *10, *14, *17, *41, xN) [9,25]. In the absence of these variants, the wild-type allele (*1) was assigned. Accordingly, in the 534 samples we detected 33 unique diplotypes, while 53 of the 76 diplotypes detected by Aldy/Cyrius were incompletely assigned by exclusively considering the current U-PGx alleles (Supplementary Table 3). However, as common but normal functioning alleles including *CYP2D6**2, *33, *35 comprise the majority of non-U-PGx alleles, the predicted metabolizer status only differs in a total of eight diplotypes of 12 samples (Supplementary Table 3). Notably, the classification according to the U-PGx system does not enable direct variant phasing. Thus, in the case of duplications, it is not known whether a functional or a nonfunctional allele is duplicated and the system outputs the metabolizer status assuming the most severe phenotype, which represents a major source of discordant phenotype predictions (Supplementary Table 3).

Our data underline that integrating pharmacogenetic profiling into a diagnostic workflow based on WGS is not only feasible and cost efficient, but also largely allows accurate prediction of *CYP2D6* genotype and phenotype using appropriate software tools. Moreover, for the example of *CYP2D6*, our data corroborate the notion that restricting the analysis to the most common and most well-known variants seems to be a useful approach for the majority of individuals. However, similarly as integrating SVs for *CYP2D6* phenotype prediction [51], including all known star alleles provides an additional benefit for certain/rare cases. Furthermore, the incorporation of rare non-star-allele variants into pharmacogenetic profiling is at least partly achievable. While loss-of-function (LOF) variants in pharmacogenes, such as nonsense, frameshift or splicing mutations leading to haploinsufficiency (i.e., inactive alleles), may be rather easily interpreted using standard NGS interpretation tools, other variants are more challenging, requiring specialized tools, such as the here applied PGx-specific framework [56], or functional assays. Moreover, variant phasing may be decisive for phenotype prediction but, without additional evidence, it is not possible directly from SRS or targeted genotyping data. Although it is difficult to extrapolate from *CYP2D6* to other genes, we assume that the results reported here are likely also applicable to other pharmacogenes to some extent.

Pharmacogenetic profiling using long-read sequencing

Although SRS is powerful, it comes with inherent limitations. In contrast, long-read sequencing (LRS; read lengths >1 kilobases) for example, by Pacific Biosciences (pacb.com; PacBio) and Oxford Nanopore Technologies (nanoporetech.com; ONT) has the potential to overcome these limitations, but has often been dismissed for being

too expensive and having raw-read error rates of $\sim 10\%$, compared with $<1\%$ using SRS [36,57]. However, along with other recent seminal studies [23,58,59], the recently published precisionFDA Truth Challenge V2, which aimed to call sequence variants in difficult-to-map regions, emphasized that LRS alone or in combination with SRS is the superior tool for variant calling, phasing and resolving complex or repetitive genomic regions, including several pharmacogenes [60].

Indeed, in the recent years, further technical advances in the form of the PacBio Sequel II (and upcoming IIe) device and the single-molecule real-time (SMRT) Cell 8M allows increased throughput and improved raw-read accuracy. Specifically, by generating and reading circular template DNA molecules multiple times (circular consensus sequencing), high-fidelity reads with an accuracy of 99.8% can be generated. This is similarly accurate as SRS by Illumina, but with an average read length of ~ 15 kilobases, which is sufficient for reads to span the entire *CYP2D6* gene and resolve the *HLA* locus (with read depth $\sim 30\times$) [16,23,58]. The improved raw-read accuracy enables highly contiguous (N50 > 15 Mb) *de novo* human genome assembly, accelerating the process by 10–100-fold, improves variant phasing, and enables accurate single nucleotide variant, indel and SV detection at a read depth of $\sim 15\times$ coverage [23]. Thereby, a full human genome can be sequenced by high-fidelity sequencing with as few as 2–3 SMRT cells 8M, decreasing sequencing costs.

The competitor of PacBio, ONT, provides flexible sequencing at various scales, from the smaller Flongle designed for microbiology, to the GridION or PromethION, enabling WGS and up to population-scale sequencing. Indeed, a recent population-scale study has shown that the sequencing of human genomes at $> 10\times$ read depth of coverage with accurate SV detection is possible using a single PromethION flow cell, which contains ~ 3000 nanopore channels compared with ~ 500 on MinION/GridION flow cells [61,62]. Furthermore, the release of ONTs most recent nanopore (R10.3) promises improved raw-read accuracy [63].

For LRS, currently there is no gold standard comparable with BWA/GATK for alignment and variant calling in SRS. Genome assemblies from LRS may be generated using one of the many available assemblers, including FALCON [64], Canu [65], Flye [66], wtdbg2 [67] and Shasta [59]. Consensus accuracy may be further increased by using polishing tools, such as Nanopolish [68], Racon [69] Medaka [70] or Arrow [71], resulting in assemblies similarly accurate as such generated by SRS, but with substantially longer contiguity [72].

In the field of PGx, both PacBio and ONT have been applied for the sequencing of long-range PCR amplicons of the homology-burdened genes *CYP2D6*, *HLA-A* and *HLA-B* to accurately detect and phase haplotypes [73–75]. Moreover, to reduce sequencing costs and increase sequencing read depth, similarly as in SRS, samples may be multiplexed and LRS may be targeted to pharmacogenes. In addition to long-range PCR, pharmacogenes may also be enriched using CRISPR/Cas9-based methods (e.g., [76]) or by hybridization capture [20]. In addition, two recent approaches enable purely computational targeting of genomic regions using ONT, based on real-time selection of DNA molecules of interest using high-end central processing units (CPUs) [21] or graphics processing units (GPUs) [22].

Taken together, the paradigm of accurate SRS and inaccurate LRS reads seems to become increasingly outdated. Although SRS-based *CYP2D6* variant callers, such as Aldy and Cyrius, are currently not compatible with LRS, the field of LRS is rapidly growing, driven by the exploration of novel applications and bioinformatic analysis tools of PacBio and ONT LRS by the scientific community (e.g., [58,77,78]).

Interpretation of genetic variation into actionable guidelines: the final frontier of PGx

While in the foreseeable future accurate detection and phasing of all sequence variants in an individual's genome is within reach, we and others expect that variant interpretation will remain a challenge for a considerable time to come [79]. Following the sequencing of the first human genome, enormous progress has been made in the *in silico* interpretation of sequence variants. However, the most efforts of variant effect prediction have focused on the identification of damaging/deleterious variants, in other words, the distinction from neutral variants. *In silico* algorithms are either based on physiochemical differences among amino acids (e.g., Grantham score [80]), phylogenetic conservation (e.g., GERP, SiPhy) and/or effect on protein structure (e.g., Polyphen-2, SIFT) or ensemble tools combining various existing tools into a single score (e.g., CADD, DANN, Eigen, REVEL) [57]. These algorithms, however, are not optimized for often evolutionary diverse pharmacogenes. Moreover, given that most of these tools have been developed to identify LOF variants, their accuracy for gain-of-function variants is limited [81]. To address these challenges and to more accurately identify functional variants in pharmacogenes, a PGx-specialized framework has recently been developed by combining and adapting the thresholds of several common prediction algorithms [56].

These prediction scores, which we also applied to classify functional variants in *CYP2D6* (Supplementary Table 5), are designed to classify single nucleotide variants. However, star alleles often comprised several core variants and, for instance, ~50% of the current *CYP2D6* star alleles have an unknown function [79]. Moreover, using the star allele nomenclature and the activity score system, a considerable amount of variability in *CYP2D6*-mediated drug metabolism remains unexplained [82]. Two recent studies addressed these challenges, both applied deep learning models to predict *CYP2D6* function, incorporating and weighing the effects of multiple variants on the same haplotype and confirmed the results with *in vitro* data [83,84]. In these studies, the models predict enzyme activity on a continuous scale and propose to omit the star allele system for *CYP2D6* [84]. Indeed, with more sequencing data available, detected variants in the polymorphic *CYP2D6* gene will continue to grow, potentially giving rise to increasingly complex star alleles [84,85]. One could argue that the star allele nomenclature, originally developed for a more manageable number of alleles, could be replaced by systems based on continuous scales, which are perhaps more suitable for the myriad of NGS-detected variants.

Current limitations of PGx

PGx has long been anticipated to be a major driver of improved drug safety and/or efficacy and thereby cost reductions for healthcare systems [14]. Although the success of PGx arguably does not yet have a transformative impact on therapeutic medicine as predicted in the early 2000s [86], scientific evidence is accumulating, indicating that incorporation of PGx into CDS may improve safety and efficacy as well as reduce costs and hospitalization compared with standard treatment [87]. Despite this accumulating evidence, several barriers remain for PGx-based precision medicine in clinical routine. Recent surveys of healthcare professionals showed that, in general, the acceptance of the premise and benefits of PGx is high, while perceived knowledge, lack of clear guidelines and the costs of pharmacogenetic testing still represent barriers [87–91]. Thus, educational resources, such as PharmGKB or the U-PGx e-learning platform [25,92] are highly warranted.

In spite of largely accepted scientific evidence for PGx, some points of discussion still exist, most of which originate from the fact that, currently, PGx fails to explain 100% of variability in drug response. This could partly be explained by other factors affecting drug response, such as age, sex, co-medication, disease state, kidney and liver function, which may explain up to 60% of the interindividual variability in drug response [93]. In addition, our current understanding of PGx is still incomplete. For instance, in addition to rare variants, *cis*- and *trans*-regulating variants have been identified in pharmacogenes, adding an additional layer of complexity to genotype to phenotype translation [15].

The final step of the implementation of PGx into CDS is a system that informs healthcare professionals either when a PGx test is indicated (pre-test alert) or when an actionable variant has been detected (post-test alert). Ideally, CDS systems should be electronic, easily accessible and provide intuitive guidelines on drug selection and dosing [87]. Several efforts are underway, including approaches to integrate CDS into electronic health records [37,94] or the platform-independent Medication Safety Code initiative [25,42]. The broad establishment of standardized pharmacogenetic testing will be challenging [4] and stakeholders will need to rely on well-founded research to enable its implementation.

Conclusion

Since the beginning of its implementation, PGx has made substantial progress. Nevertheless, its current real-life applications are imperfect solutions and the impact, optimal platform and evidence of the benefits of PGx remain points of discussion in the scientific community [9,86,95,96]. To date, focusing on the most common variants with functional prediction, targeted genotyping still represents the most widely adapted pharmacogenetic profiling approach. However, harboring the limitation that rare but clinically (highly) relevant variants remain undetected, targeted genotyping will continuously be replaced by NGS-based approaches. As the interpretation of NGS-detected rare variants in pharmacogenes is challenging, we advocate that NGS data may *in silico* be reduced to a subset of variants with existing clinical guidelines. LOF variants as well as rare and putatively clinically relevant variants may be assessed in a research setting and ideally stored in publicly available database(s) [7,9,37,97]. To enable the goal of CDS [87], integration of pharmacogenetic information into electronic health records with appropriate alerts or platform-independent systems will be highly valuable.

In the context of rare diseases, diagnostic NGS is routinely performed for screening of disease-causing mutations, irrespective of a suspected pharmacogenetic predisposition. WGS not only generates the most comprehensive sequencing data for mutation screening, but also provides an added value because the data may be used for

pharmacogenetic profiling as well. Such an approach represents an efficient two-in-one assay without generating significant additional costs. However, the limitations of SRS, particularly inaccessible genomic regions and variant phasing, also apply to PGx. Consequently, LRS either alone or in combination with SRS will not only continue to find application in both research and clinical testing but also in PGx.

Future perspective

To avoid a further gap between technical advances and their translation into clinical care, well-designed prospective clinical studies (such as the U-PGx PREPARE trial) will provide further evidence for the efficacy of pre-emptive pharmacogenetic testing as a valuable tool of precision medicine. Further technical advances will lead to the abundance of detected sequence variants in pharmacogenes, including such with unknown function/significance. For phenotyping of pharmacogenes, artificial intelligence-based solutions may be coupled and evaluated with high-throughput *in vitro* functional assays, based on patient-derived, inverse PCR- or CRISPR/Cas9-generated libraries, which has already been demonstrated for *CYP2D6* on a smaller scale [83,84,98]. Finally, information on PGx in clinical trials is currently limited to oncology [3]. As anticipated in the early 2000s, PGx information may be leveraged for stratification of clinical trials and potentially also for the rescue of dismissed drugs due to previously non-significant results [14].

In addition, the shift of NGS and PGx toward point-of-care testing can be expected, or even toward portable sequencing, for example, using ONT's smartphone-connectable SmidgION [99]. We envision that in the coming years, LRS will be sufficiently mature to usher in the era of long-read genomics and that millions of patients worldwide will have access to high quality sequencing data, furthering the implementation of precision medicine.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/pgs-2020-0155

Author contributions

SM Caspar, J Meienberg and G Matyas contributed toward conceptualization. SM Caspar, T Schneider, P Stoll and J Meienberg contributed toward data curation. G Matyas contributed toward funding acquisition. SM Caspar, T Schneider, P Stoll, J Meienberg and G Matyas contributed toward methodology. SM Caspar, T Schneider and P Stoll contributed toward software. G Matyas contributed toward supervision. SM Caspar, J Meienberg and G Matyas contributed toward validation. SM Caspar and T Schneider contributed toward visualization. Original draft preparation contributed by SM Caspar. Review and editing of manuscript contributed by SM Caspar, T Schneider, P Stoll, J Meienberg and G Matyas. All authors have read and agreed to the published version of the manuscript.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

For investigations involving genetic data of human subjects, informed consent has been obtained from the participants involved.

Executive summary

Current state of pharmacogenetics

- Pharmacogenetics (PGx) represents a major driver of precision medicine, with the promise of enabling medical treatments tailored to an individual's pharmacogenetic predisposition. Thereby, treatment outcomes may be improved and costs reduced.
- Pharmacogenetic profiling is currently neither the standard in clinical care nor in the stratification of clinical trials.

Approaches for pharmacogenetic profiling: navigating through a labyrinth of possibilities

- A plethora of methods for the genotyping of pharmacogenes exist, but are largely unstandardized. Most providers only interrogate a subset of common/well known variants for which international guidelines exist.
- Rare variants account for ~30–40% of interindividual variation in pharmacogenes and thereby likely contribute to the variation of individual drug response; however, the functional effects of these variants remain challenging to interpret.
- Next-generation sequencing, such as targeted sequencing of pharmacogenes enables a more comprehensive approach to identify common and rare variants.

Whole-genome sequencing as a platform for pharmacogenetic profiling

- Whole-exome sequencing has the drawback that non-coding but relevant variants, including *CYP2C19*17* and *CYP3A5*3*, are potentially not detected as well as structural variants may be missed.
- We have recently demonstrated that whole-genome sequencing (WGS) may be used for gene diagnostics and PGx in a single assay. Comparing two dedicated bioinformatics tools, in this perspective, we show that short-read sequencing data are largely sufficient for accurate genotyping of the polymorphic *CYP2D6* gene.

The future of genetics belongs to long-read sequencing

- As caveats of short-read sequencing include variant phasing and genomic regions with mappability <1, long-read sequencing (LRS) provides an alternative which may overcome these challenges.
- Further technical developments of PacBio and Oxford Nanopore Technologies render the paradigm of 'inaccurate LRS' increasingly outdated.

Conclusion & future perspective

- Although several barriers still exist, PGx has made substantial progress and the results of large-scale studies, such as the ubiquitous pharmacogenomics PREPARE trial, will further promote the field.
- We advocate that if WGS is performed for gene diagnostics, pharmacogenetic profiling may be performed using the existing WGS data without generating significant additional costs.
- We envision that within the next years, LRS will emerge as the predominant sequencing technology and will enable more comprehensive gene diagnostics and PGx.
- Interpretation of the sequence variants will likely remain challenging. Artificial intelligence- or high-throughput *in vitro* assays will be valuable to overcome this final barrier.

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