# Why We Need to Take a Closer Look at Genetic Contributions to CYP3A Activity

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Cytochrome P450 3A (CYP3A) subfamily enzymes are involved in the metabolism of 40% of drugs in clinical use. Twin studies have indicated that 66% of the variability in CYP3A4 activity is hereditary. Yet, the complexity of the *CYP3A* locus and the lack of distinct drug metabolizer phenotypes has limited the identification and clinical application of CYP3A genetic variants compared to other Cytochrome P450 enzymes. In recent years evidence has emerged indicating that a substantial part of the missing heritability is caused by low frequency genetic variation. In this review, we outline the current pharmacogenomics knowledge of CYP3A activity and discuss potential future directions to improve our genetic knowledge and ability to explain CYP3A variability.

**Keywords: CYP3A locus, CYP3A4, CYP3A5, genetic variants, enzyme activity, missing heritability, pharmacogenomics**

## INTRODUCTION

The Cytochrome P450 CYP3A subgroup forms the largest proportion of CYP protein in the human liver and small intestine (Shimada et al., 1994; Rendic, 2002) and is involved in the metabolism of up to 60% of currently used drugs (Plant, 2007; Zanger et al., 2008). The *CYP3A* locus is located on Chromosome seven and consists of four distinct genes *CYP3A4*, *CYP3A5*, *CYP3A7*, *CYP3A43*, and three pseudogenes (Finta and Zaphiropoulos, 2000; Gellner et al., 2001). Notably, the four coding genes share more than 85% of their amino-acid sequence and have a partially overlapping substrate spectrum (Williams et al., 2002). However, the contribution to drug metabolism varies considerably between the *CYP3A* genes (Ozdemir et al., 2000; Lamba et al., 2002). Of the entire CYP3A enzyme family, the CYP3A4 and CYP3A5 enzymes are the most abundantly expressed proteins accounting for more than 95% of total *CYP3A* mRNA pool (Koch et al., 2002). CYP3A7, on the other hand, is most prominent in fetal liver cells and slowly diminishes after birth. CYP3A43 expression is almost negligible compared to the other enzymes (<5% of total mRNA).

CYP3A activity is characterized by substantial inter-individual heterogeneity. For instance, CYP3A4, the predominant isoform of CYP3A, shows a 10–100-fold inter-individual variability in enzyme activity (Perera, 2010). One of the causes of the variability in CYP3A activity is the presence of genetic variants in the genes encoding the CYP3A enzymes. The clinical importance of several of these variants has indeed been reported in many patients (Evans and Relling, 1999; Zanger et al., 2008; Zanger and Schwab, 2013). Interestingly, there are currently only two haplotypes that are used in clinical practice, the *CYP3A5\*3* allele resulting in a complete loss-of-function, and the *CYP3A4\*22* allele resulting in a decreased enzyme activity (Kuehl et al., 2001; Wang et al., 2011). By combining *CYP3A5\*3* and *CYP3A4\*22* more than 60% and 20% of the observed variability in tacrolimus and cyclosporine trough blood level could be explained respectively (Elens et al., 2011). Recommendations on how to apply *CYP3A5* genotyping results to optimize drug and dose selection

are included in clinical guidelines from the CPIC (Clinical Pharmacogenetic Implementation Consortium) (Birdwell et al., 2015) and for *CYP3A4* and *CYP3A5* by the DPWG (Dutch Pharmacogenomics Working Group) (Swen et al., 2011) (https://www.knmp.nl/index.php/media/1058).

Besides the genetic variants that form the *CYP3A5\*3* and *CYP3A4\*22* alleles, the *CYP3A* locus contains many more rare variants which are expected to play a role in enzyme activity and drug response. Using a repeated drug administration method Ozdemir et al. compared standard deviations for inter- and intra-person variation in the disposition of 10 different CYP3A4 substrates, including midazolam, and cyclosporine. Analyses of the disposition parameters of these orally administered substrates suggested that at least 60% of the variability in composite CYP3A4 activity is under genetic control (Ozdemir et al., 2000). Furthermore, substantial missing heritability in CYP3A pharmacogenomics has been reported (Klein and Zanger, 2013). A twin study compared the differences of metabolic similarity between monozygotic (MZ) and dizygotic (DZ) twins (Rahmioglu et al., 2011). In this cohort of 367 healthy twins, 66% (confidence interval: 50%–77%) of the induced CYP3A4 activity variation was found to be hereditary, while only ~20% was explained by current clinical PGx based on a GWAS study (Oetting et al., 2018). In this review, we outline the current pharmacogenetics knowledge of CYP3A activity and discuss potential future directions to improve our genetic knowledge and ability to explain CYP3A variability.

## Known *CYP3A4* and *CYP3A5* Haplotypes

There are 34 defined *-haplotypes for the *CYP3A4* and five in the *CYP3A5* gene designated by PharmVar (https://www.pharmvar.org/). Recently, three *CYP3A5* alleles (*CYP3A5\*2, \*4, and \*5*) have been reclassified as part of the *CYP3A5\*3* suballeles (Rodriguez-Antona et al., 2022). Among those haplotypes, the most widely studied genetic variant is the *CYP3A5\*3* allele (rs776746), characterized by a splice defect in intron 3. This loss-of-function variant generates a premature stop codon that causes a lower amount of functional protein (Kuehl et al., 2001). Notably, *CYP3A5\*3* allele is common but the allele frequency differs between ethnicities, with the frequency of approximately up to 0.92 in European Americans (EA) and fluctuating between 0.24 and 0.84 in non-European populations according to the PharmGKB frequency table (https://www.pharmgkb.org/page/cyp3a5RefMaterials). A *CYP3A5\*3/\*3* diplotype results in a 10–30 fold lower CYP3A5 expression level compared to *CYP3A5\*1/\*3* (Hustert et al., 2001a; Lin et al., 2002). In clinical practice, individuals are categorized into three distinct CYP3A5 activity groups: CYP3A5 expressors (carrying two *\*1* alleles), heterozygous expressors (carrying one *\*1* allele and one *\*3* allele) and non-expressors (carrying two *\*3* alleles) of which the last group is the most common. These predicted metabolizer phenotypes can help explain differences in drug metabolism. For example, the dose corrected tacrolimus concentration after transplantation in patients who carried at least one *CYP3A5\*1* allele was significantly lower compared to CYP3A5 non-expressor after the first month following transplantation (*1.49 ± 0.88* vs. *3.11 ± 4.27, p* = 0.01), which lasted for the first

year post transplantation (Zheng et al., 2004). For this reason, the *CYP3A5\*3* allele is included in the CPIC, DPWG, RNPGx (French National Network of Pharmacogenetics), and IATDMCT (International Association of Therapeutic Drug Monitoring and Clinical Toxicity) guidelines as being associated with tacrolimus metabolism leading to the recommendation to increase 1.5—2.5 fold initial dosage in *\*1* carriers (Swen et al., 2011; Birdwell et al., 2015; Picard et al., 2017). In addition, two other key haplotypes *CYP3A5\*6* (rs10264272, c.624G > A) and *CYP3A5\*7* (rs41303343, c.1035dup) are included in the guidelines (Birdwell et al., 2015). *CYP3A5\*6* causes a splicing defect, and *CYP3A5\*7* results in a frameshift. These two alleles have a frequency of 11%–19% in the African population but have not been observed or with extremely low frequency in non-African populations (https://www.pharmgkb.org/page/cyp3a5RefMaterials). Both haplotypes are associated with no CYP3A5 catalytic activity and contribute to tacrolimus pharmacokinetics variability (Campagne et al., 2018), explaining about 6% of the variability in tacrolimus trough concentrations in African American patients (Oetting et al., 2016).

Besides *CYP3A5\*3*, *CYP3A4\*22*, located in the most predominant isoform of *CYP3A*, is the second *-allele that is often used in clinical practice. The *CYP3A4\*22* allele (rs35599367) is characterized by a G > A substitution in intron 6, resulting in an increased formation of a truncated alternative splice variant *in vitro* (Wang and Sadee, 2016). The allele frequency in the Caucasian population ranges from 3% to –5%, which is higher than compared to other populations, for example less than 1% in the Asian population. Notably, patients carrying *CYP3A4\*22* had a 1.7 to five fold decreased CYP3A4 mRNA or protein expression level (Elens et al., 2011; Wang et al., 2011; Wang and Sadee, 2016), explaining 12% of CYP3A4 activity variability (Wang et al., 2011). Moreover, the reduced activity caused by the *CYP3A4\*22* allele was verified with several CYP3A substrates *in vivo* (Elens et al., 2012; Elens et al., 2013a; de Jonge et al., 2015), and its contribution to variability in CYP3A activity and its potential clinical usage were summarized by Elens et al. and Mulder et al. (Elens et al., 2013b; Mulder et al., 2021). While CYP3A4 is involved in the metabolism of many drugs, there are currently no clinical guidelines available that include *CYP3A4* genetic variants. This is potentially explained by the low frequency of the variants in combination with a relatively modest effect on enzyme function which makes it more difficult to ascertain a connection between genotypes and drug metabolism and clinical outcomes.

In addition to these two well-recognized haplotypes, there are 37 additional *-allele haplotypes defined for *CYP3A4* and *CYP3A5*. A recently published review presented a comprehensive summarization of all *CYP3A5* *-alleles (Rodriguez-Antona et al., 2022). Here we give an overview of all *CYP3A4* *-alleles. Notably, for the *CYP3A4*, most of *-alleles are characterized by core variants which are rare (Minor allele frequency (MAF) < 1%), or very rare (MAF <0.1%). The only exception is *CYP3A4\*36*, for which the global frequency is up to 0.42 based on the 1,000 Genomes. All *CYP3A4* alleles and *in vivo* evidence of their potential impact on enzyme activity are shown

**TABLE 1 |** Overview of core variants of *CYP3A4* haplotype[a].

| Allele | rs ID | Nucleotide changes (cDNA) | Amino acid changes | VEP annotation | *In vivo* evidence of function variability |
|---|---|---|---|---|---|
| *2 | rs55785340 | 664T > C | S222P | Missense | |
| *3 | rs4986910 | 1334T > C | M445T | Missense | |
| *4 | rs55951658 | 352A > G | I118V | Missense | |
| *5 | rs55901263 | 653C > G | P218R | Missense | |
| *6 | rs4646438 | 830_831 insA | D277fs | Frameshift | |
| *7 | rs56324128 | 167G > A | G56D | Missense | |
| *8 | rs72552799 | 389G > A | R130Q | Missense | Decreased CYP3A4 activity Apellániz-Ruiz et al. (2015b) |
| *9 | rs72552798 | 508G > A | V170I | Missense | |
| *10 | rs4986908 | 520G > C | D174H | Missense | |
| *11 | rs67784355 | 1088C > T | T163M | Missense | |
| *12 | rs12721629 | 1117C > T | L373F | Missense | |
| *13 | rs4986909 | 1247C > T | P416L | Missense | |
| *14 | rs12721634 | 44T > C | L15P | Missense | |
| *15 | rs4986907 | 485G > A | R162Q | Missense | |
| *16 | rs12721627 | 554C > G | T185S | Missense | |
| *17 | rs4987161 | 566T > C | F189S | Missense | |
| *18 | rs28371759 | 878T > C | L293P | Missense | Increased tacrolimus clearance Liu et al. (2017), increased cyclosporine clearance Xin et al. (2014) |
| *19 | rs4986913 | 1399C > T | P467S | Missense | |
| *20 | rs67666821 | 1461_1462 insA | P488fs | Frameshift | Decreased tacrolimus clearance Gómez-Bravo et al. (2018) |
| *21 | rs201821708 | 956A > G | Y319C | Missense | |
| *22 | rs35599367 | 522-191C > T | | Intron variant | Decreased enzyme activity[b] |
| *23 | rs57409622 | 484C > T | R162W | Missense | |
| *24 | rs113667357 | 600A > T | Q200H | Missense | |
| *26 | rs138105638 | 802C > T | R268X | Missense | Decreased tacrolimus clearance Werk et al. (2014) |
| *28 | rs570051168 | 64C > G | L22V | Missense | |
| *29 | rs1449865051 | 337T > A | F113I | Missense | |
| *30 | rs778013004 | 388C > T | R130X | Missense | |
| *31 | rs1303250043 | 972C > A | H324Q | Missense | |
| *32 | rs368296206 | 1004T > C | I335T | Missense | |
| *33 | rs756833413 | 1108G > T | A370S | Missense | |
| *34 | rs774109750 | 1279A > G | I427V | Missense | |
| *35 | rs188389063 | 7C > G | L3V | Missense | |
| *36 | rs2242480 | 1026 + 12G > A | | Intron variant | Substrate-dependent[b] |
| *37 | rs35599367 rs4986910 | 522-191C > T, 1334T > C | M445T | Intron variant, missense | |

[a]Allele definitions are based on PharmVar. In vivo functionality is based on literature as activity is not reported in PharmVar. Therefore, we performed a literature search for each CYP3A4 *-allele in the table with Pubmed.
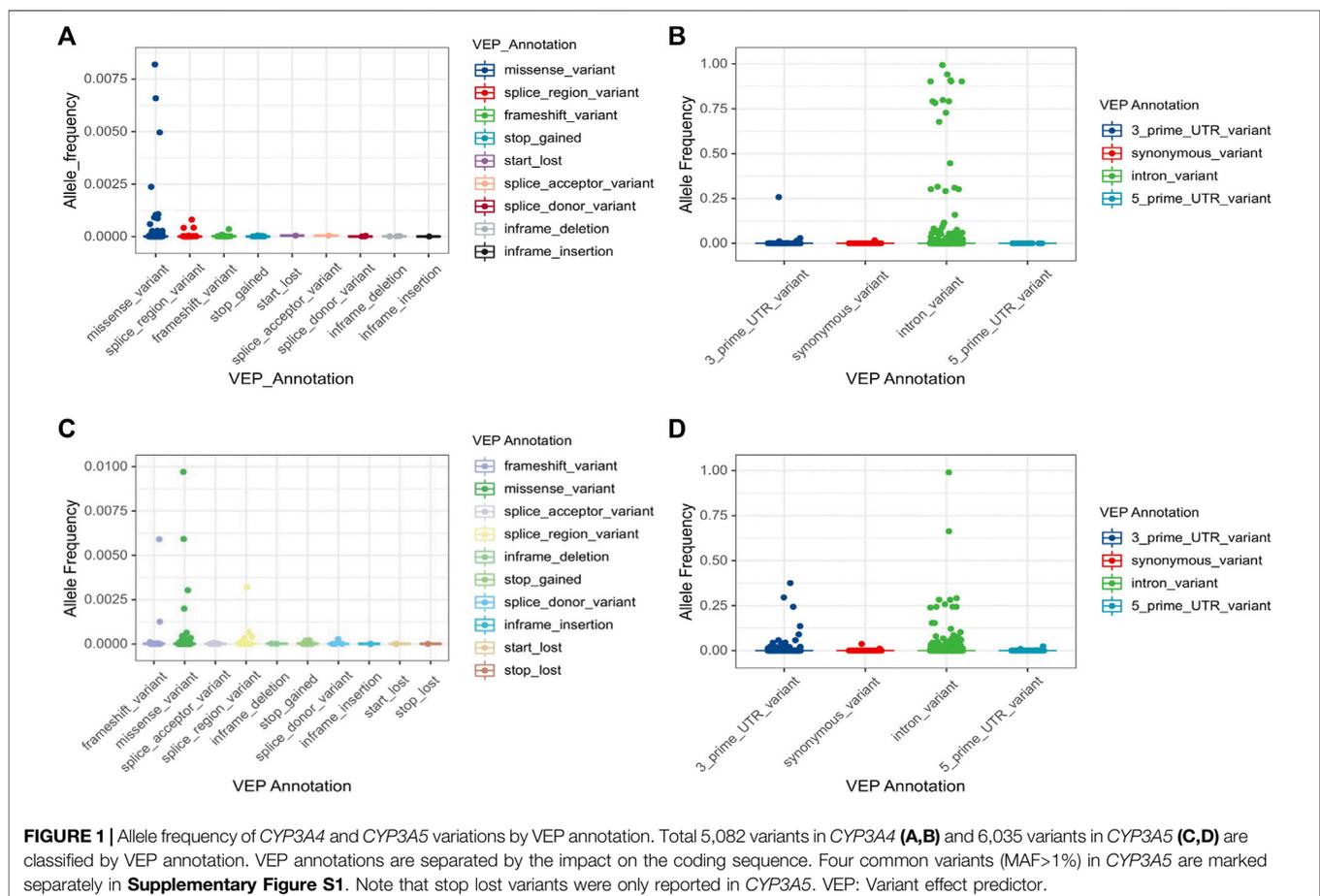[b]Explanations in detail were described in the tex.

in **Table 1**. As the impact of these alleles are not assigned in PharmVar, the summarized impact of the alleles is based on available literature with *in vivo* evidence. *CYP3A4*8* was detected with whole-exome sequencing in a patient with a severe paclitaxel-induced peripheral neuropathy due to diminished CYP3A4 enzyme activity (Apellániz-Ruiz et al., 2015b). Notably, the *CYP3A4*18* haplotype is associated with a decreased midazolam metabolism but also results in a gain-of-function in the clearance of certain substrates such as sex steroids. These substrate specific effects may be attributed to structural changes in substrate recognition sites that results in catalytic activity variation (Kang et al., 2009). Besides, *CYP3A4*1G*, which has been redesignated as *CYP3A4*36*, has been reported a substate-dependent impact on CYP3A4 activity as well (Yuan et al., 2011; Dong et al., 2012; He et al., 2014). Notably, *CYP3A4*1G* has high linkage disequilibrium with *CYP3A5*3* and significantly related with a lncRNA, AC069294.1, that caused down-regulated CYP3A4 and CYP3A5 expression (Collins and Wang, 2022). Furthermore, the interaction of *CYP3A4*1G* and *CYP3A5*3* on drug pharmacokinetics, for instance, tacrolimus (Miura et al., 2011; Zuo et al., 2013) and sirolimus (Zhang et al., 2017) has been reported. For *CYP3A4*20*, a loss-of-function allele caused by a frameshift variant, it has been suggested that this variant causes an equal functional alteration as *CYP3A4*22*, given that in CYP3A5 non-expressors the phenotype of a heterozygous *CYP3A4*20* carrier was close to that of a CYP3A poor metabolizer (Lloberas et al., 2018). Moreover, *CYP3A4*20* presents a higher frequency and founder effect in the Spanish population, which highlights the contribution of rare CYP3A functional alleles in a specific population (Apellániz-Ruiz et al., 2015a). However, given the extremely low frequency of most of those additional *-alleles, there is not enough evidence for their functionality *in vivo* and for the clinical impact of these *-alleles. Several studies assess activities of these alleles *in vitro* (Fang et al., 2017; Xu et al., 2018; Li et al., 2019; Lin et al., 2019; Yang et al., 2019; Kumondai et al., 2021). Notably, their predicted

**TABLE 2 |** Summary of *CYP3A4* and *CYP3A5* allele number classified by VEP (variant effect predictor) annotation.

| VEP Annotation | Number of variants in CYP3A4 N (%) | | | Number variants of in CYP3A5 N (%) | | |
|---|---|---|---|---|---|---|
| | **All alleles** | **MAF<1%** | **MAF<0.1%** | **All alleles** | **MAF<1%** | **MAF<0.1%** |
| Frameshift variant | 20 (0.0057%) | 20 (0.143%) | 20 (0.474%) | 24 (0.3997%) | 23 (1.149%) | 21 (0.183%) |
| Intron variant | 4,472 (97.2661%) | 4,393 (89.829%) | 4,288 (87.122%) | 4,641 (69.1792%) | 4,559 (70.517%) | 4,445 (77.117%) |
| Missense variant | 218 (0.2206%) | 218 (5.531%) | 212 (4.541%) | 199 (0.2909%) | 199 (4.056%) | 195 (2.935%) |
| Splice acceptor variant | 1 (0.0004%) | 1 (0.009%) | 1 (0.030%) | 8 (7.9761%) | 7 (0.021%) | 7 (0.069%) |
| Splice donor variant | 3 (0.0004%) | 3 (0.010%) | 3 (0.034%) | 5 (0.0032%) | 5 (0.045%) | 5 (0.147%) |
| Splice region variant | 32 (0.0138%) | 32 (0.346%) | 32 (1.145%) | 45 (0.1997%) | 44 (0.872%) | 43 (1.245%) |
| Start lost | 1 (0.0004%)\ | 1 (0.009%) | 1 (0.030%) | 2 (0.0001%) | 2 (0.002%) | 2 (0.007%) |
| Stop gained | 10 (0.0012%) | 10 (0.030%) | 10 (0.101%) | 15 (0.0078%) | 15 (0.108%) | 15 (0.353%) |
| Stop lost | 0 | 0 | 0 | 2 (0.7525%) | 1 (0.001%) | 1 (0.003%) |
| 3′prime UTR variant | 216 (2.3511%) | 211 (3.402%) | 205 (4.243%) | 1027 (20.2284%) | 1000 (20.770%) | 968 (15.899%) |
| 5′prime UTR variant | 29 (0.0124%) | 29 (0.310%)\ | 29 (1.025%) | 108 (0.4166%) | 107 (2.147%) | 105 (1.033%) |
| Inframe deletion | 4 (0.0004%) | 4 (0.011%) | 4 (0.037%) | 3 (0.0004%) | 3 (0.006%) | 3 (0.020%) |
| Inframe insertion | 1 (0.00004%) | 1 (0.001%) | 1 (0.004%) | 1 (0.00007%) | 1 (0.001%) | 1 (0.003%) |
| Synonymous variant | 75 (0.1275%) | 74 (0.368%) | 74 (1.215%) | 71 (0.5452%) | 69 (0.303%) | 69 (0.987%) |
| Total | 5,082 (100%) | 4,997 (100%) | 4,880 (100%) | 6,151 (100%) | 6,035 (100%) | 5,880 (100%) |



**FIGURE 1 |** Allele frequency of *CYP3A4* and *CYP3A5* variations by VEP annotation. Total 5,082 variants in *CYP3A4* **(A,B)** and 6,035 variants in *CYP3A5* **(C,D)** are classified by VEP annotation. VEP annotations are separated by the impact on the coding sequence. Four common variants (MAF>1%) in *CYP3A5* are marked separately in **Supplementary Figure S1**. Note that stop lost variants were only reported in *CYP3A5*. VEP: Variant effect predictor.

function is shown to differ between substrates. For instance, the intrinsic clearance values of *CYP3A4*14* and *CYP3A4*15* haplotypes were higher compared to wild type for regorafenib (Li et al., 2019) and cabozantinib (Lin et al., 2019). By contrast, intrinsic clearance values were decreased for ibrutinib (Xu et al.,

2018). Moreover, Ketoconazole, a CYP3A4 inhibitor alters the function of *CYP3A4*14* and *CYP3A4*15* *in vitro* (Lin et al., 2019), which warranted drug-drug interaction plays a significant role in observed enzyme activity. Besides, the variability of enzyme activity caused by some of those *-alleles might be too

moderate to be identified, especially when influenced by diverse genetic and non-genetic factors *in vivo*. As a result, most of the currently reported haplotypes in *CYP3A4* and *CYP3A5* failed to be taken into account in clinical practice.

## Rare Variants in the *CYP3A* Locus

As mentioned, genetic variants in the *CYP3A* genes are related to CYP3A enzyme activity and, thereby drug response. However, currently used PGx approaches focusing on the well-known *\*-alleles, explain only a small proportion of the overall variability in the pharmacokinetics of CYP3A substrates. A recently published twin study revealed that up to 73% of the variability in CYP3A activity is attributed to genetic factors but only a part of this could be explained by PGx (Matthaei et al., 2020). This missing heritability suggests a role of more impactful variants inside and outside the *CYP3A* locus beyond the currently known and used haplotypes. For instance, a study showed that rare variants in *CYP3A4* may account for up to 99% of the functional variability (Kozyra et al., 2017). This proportion is much higher than some other pharmacogenes, in which rare variants contribute to 30%–40% of the variability.

Numerous genetic variants have been detected in the *CYP3A* locus, as shown in the data from The Genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org/). More than 90% of genetic variants in this locus are rare, with a MAF of less than 1% (**Table 2**). In *CYP3A4*, a total of 5,082 variants are identified. Of these, only 85 are common (MAF >1%) (**Figures 1A,B**). All common variants are either located in non-coding regions or are synonymous variants, which do not result in amino acid changes. By contrast, all 285 non-synonymous variants are rare (MAF <1%) of these 218 are missense variants. In addition, there are 32 and 20 splice region variants and frameshift variants, respectively, which is more than the rest of the Variant Effect Predictor (VEP) annotation groups. Finally, there are over four thousand rare intronic variants. For *CYP3A5* (**Figures 1A,C,D**), a total of 6,151 variants are identified, of which 116 are common. Those common variants were mostly intronic or in the 3′untranslated region (3′-UTR) except for four variants which were non-synonymous (**Figure 1D**; **Supplemental Figure S1**). Moreover, 199 missense variants were detected in *CYP3A5*, all of which are rare, which is much more than other variant types. Notably, over 51% of all rare variants in the *CPY3A4* and *CYP3A5* genes were detected only in non-European populations. On the other hand, around 31% of all rare variants are limited to the European (non-Finnish) population [1576 (31.5%) and 1873 (31.0%) for *CYP3A4* and *CYP3A5* respectively]. These findings highlight the differences between ethnicities (https://gnomad.broadinstitute.org/).

For both *CYP3A4* and *CYP3A5*, intronic variants are the most common type of variants, accounting for 97% and 69% of the single nucleotide variants (SNVs), respectively (**Table 2**). Although an intron is not translated, intronic variants are still of importance as they can influence splicing. For example, the core variant of the *CYP3A4\*22* allele is a deleterious intronic variant. Furthermore, rs4646450, located in the *CYP3A5* intron region, was associated with decreased protein expression and CYP3A4 activity, explaining 3%–5% of the variability (Klein et al.,

2012). By contrast, most of common variants do not seem to cause alterations which can influence CYP3A activity. Moreover, a genome-wide associated study failed to identify any common variants related to induced CYP3A4 activity within 310 twins (Rahmioglu et al., 2013). Therefore, in the *CYP3A* locus, rare variants are the most likely to contribute profoundly to the variability of CYP3A enzyme activity. Notably, the log-transformed distribution of midazolam clearance showed a unimodal pattern (Lin et al., 2001), which suggests that the genetic impact on CYP3A activity could be attributed to a large number of small impact variants rather than only a few high impact ones.

## Genetic Factors Outside the CYP3A Locus

While variants within the genes in the *CYP3A* locus can influence CYP3A enzyme activity, variability in expression can also play a role. Transcriptional regulation of CYP3A expression has been reported in many studies (Yuan et al., 2020). Among those transcriptional factors, two predominant nuclear receptors, pregnane X receptor (*PXR, NR1I2*) and constitutive androstane receptor (*CAR, NR1I3*), have been repeatedly associated with altered CYP3A activity (Lolodi et al., 2017). These are ligand-activated nuclear receptors which, after being activated by specific ligands, heterodimerize with the retinoic acid receptor (*RXR; NR2B1*). Subsequently, they bind to the 5′ regulation region of target genes at the hormone-responsive elements (HREs) motif (Chen et al., 2012; Banerjee and Chen, 2013; Tebbens et al., 2018). Several HREs share the typical nuclear structure, implying the overlapping of downstream genes. But the expression regulation effect varies between target genes (Timsit and Negishi, 2007). Studies have shown that *CYP3A* isoforms are mainly regulated by *PXR* (Mbatchi et al., 2018). More functional nuclear receptor SNVs related to CYP3A activity and CYP3A substrate metabolism are summarized in **Table 3**. Moreover, the interaction between nuclear receptors also plays a role in CYP3A activity variation. Hepatocyte nuclear factor-4α (HNF4α) is a liver-enriched nuclear receptor and is associated with CYP3A4 and CYP3A5 expression (Jover et al., 2001). *HNF4α* not only regulates *CYP3A4* transcription directly by binding to two *CYP3A4* 5′ upstream regions featured with direct repeat (DR) 1-type motifs (Tegude et al., 2007) but also serves as a coactivator that interacts with the other two CYP3A4 regulators *PXR* and *CAR*, resulting in CYP3A4 expression variability. One of the *HNF-4α* polymorphisms (rs2071197), combined with *PXR\*1B*, was related to the concentration-to-dose (C/D) ratios of carbamazepine, wild type *HNF-4α* carriers had higher C/D ratios in *PXR\*1B* genotype rather than *PXR\*1B* non-carriers (Saruwatari et al., 2014). Furthermore, the *CYP3A4* mRNA expression in pediatric livers could be better predicted with the model, including the expression level of *HNF-4α*, *PXR*, *CAR*, and their heterodimer partner *RXRα* (Vyhlidal et al., 2006). The interaction of transcriptional factors implies that the expression of CYP3A is the combined effect of multiple transcription factors. More *CYP3A* transcription regulators were reported recently. For instance, the *TSPYL* family suppressed the CYP3A4 expression and one SNV in *TSPYL1*, rs3828743, reversed the suppression effect (Qin et al., 2018). In

**TABLE 3 |** Summary of single nucleotide variants in CYP3A4 transcriptional factors associated with variability of CYP3A4 activity.

| Gene | rsID | MAF (%) | Location | Nucleotide changes | Effect | | | Sample size | References |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Transcriptional regulator | CYP3A activity | Substrate metabolism | | |
| NR1I2 (PXR) | rs3814058 | 21.1 | 3′UTR | T > C | Decreased expression | decreased | Decreased repaglinide clearance | 300 | Du et al., (2013) |
| | rs2276706 | 38.3 | Intron1 | G > A | Decreased expression | decreased | No effect on everolimus metabolis* | 300 53* | Moes et al. (2012); Du et al. (2013) |
| | rs1523127 | 58.9 | 5′UTR | C > A | No change in RNA expression | Increased CYP3A4 mRNA expression* | No effect on tacrolimus clearance | 52* 336 | King et al. (2007); Wang et al. (2016) |
| | rs3814055 | 38.3 | 5′UTR | C > T | Higher PXR promotor activity (in vitro) | | No effect or decreased tacrolimus clearance* | 35 32* 240* | Lamba et al. (2008); Benkali et al. (2009); López-Montenegro Soria et al. (2010); Kurzawski et al. (2017); Rana et al. (2017) |
| | rs2472677 | 44.9 | Intron 1 | C > T | Higher mRNA level | Increased* | Increased Atazanavir apparent clearance[#] | 16 45* 109[#] | Hustert et al. (2001b); Lamba et al. (2008); Siccardi et al. (2008) |
| | rs1523130 | 58.2 | 5′UTR | T > C | | Decreased | | 128 | Lamba et al. (2008); Lamba et al. (2010) |
| | rs13085558 | 14.1 | Intron 1 | T > C | | Increased (Female) | | 17 | Lamba et al. (2008) |
| | rs3732359 | 73.8 | 3′UTR | G > A | | Increased | Increased midazolam clearance | 53 | Oleson et al. (2010) |
| | rs3732360 | 71.3 | 3′UTR | C > T/C > G | | Increased | Increased midazolam clearance | 53 | Oleson et al. (2010) |
| | rs3814057 | 19.6 | 3′UTR | A > C/A > T | Increased PXR mRNA expression* | | Affected the concentration of Voriconazole | 44* 172 | Oleson et al. (2010); Zeng et al. (2020) |
| | rs7643645 | 34.9 | Intron 1 | A > G | Decreased PXR mRNA expression | Decreased CYP3A4 mRNA level | | 16 | Hustert et al. (2001b); Lamba et al. (2008) |
| | rs6785049 | 58.3 | Intron 5 | G > A | | 2-fold higher CYP3A4 expression | Decreased Tacrolimus clearance | 83 | Stifft et al. (2018) |
| NR1I3 (CAR) | rs11265572 | 1.0 | 5′flanking region | G > T | | | Increased log-transformed tacrolimus C/D ratios | 96 | Chen et al. (2014) |
| | rs201406656 | 0.006 | Exon7 | A > G | Lower CYP3A4 transactivation effect (in vitro) | | | | Ikeda et al. (2005) |
| | 1–161229921-A-G[a] | 0.0007 | Exon9 | A > G | Lower CYP3A4 transactivation effect (in vitro) | | | | Ikeda et al. (2005) |
| NR1I1 (VDR) | rs1544410 | 38.8 | Intron9 | G > A/G > T/ G > C | | Decreased intestine CYP3A4 expression | | 30 | Thirumaran et al. (2012) |
| | rs4516035 | 39.9 | Intron1 | A > G | | Higher CYP3A4 expression and activity | | 210 | Thirumaran et al. (2012); Nylén et al. (2014) |
| | rs11568820 | 28.1 | Intron1 | G > A | Decreased transactivation of VDR promoter | Higher CYP3A4 expression | | 30 | Thirumaran et al. (2012) |

(Continued on following page)

**TABLE 3 |** (Continued) Summary of single nucleotide variants in CYP3A4 transcriptional factors associated with variability of CYP3A4 activity.

| Gene | rsID | MAF (%) | Location | Nucleotide changes | Effect | | | Sample size | References |
|------|------|---------|----------|--------------------|--------|---|---|-------------|------------|
| | | | | | Transcriptional regulator | CYP3A activity | Substrate metabolism | | |
| | rs731236 | 38.7 | Exon 9 | A > G | Decreased 60% VDR gene expression | No association with mRNA expression of CYP3A4 | | 114 | Betts et al. (2015) |
| HNF4a | rs2071197 | 10.8 | Intron 1 | G > A | G allele may diminish HNF-4α expression | | Associated with CBZ concentrations stratified by PXR*1B | 168 | Saruwatari et al. (2014) |
| NR3C1 (GR) | rs258747 | 51.4 | 3′flanking region | A > G | | Lower CYP3A4 activity | | 148 | Klein et al. (2012) |
| PPARA | rs4253728 | 25.1 | Intron3 | G > A | AA homozygotes had 1.6-fold lower PPARA protein level* | Explained 5% CYP3A4 activity variation | Lower 2-OH-atorvastatin/ atorvastatin $AUC_{0-\infty}$ ratio in vivo | 46* 148 | Klein et al. (2012); de Keyser et al. (2013) |
| | rs4823613 | 28.2 | Intron3 | A > C/A > G | | Explained 9% CYP3A4 activity variation | | 148 | Klein et al. (2012) |
| ARNT | rs2134688 | 89.1 | Intron4 | G > A | | Lower CYP3A4 mRNA and protein expression | | 150 | Klein et al. (2012) |
| TSPYL1 | rs3828743 | 24.1 | Exon1 | G > A/G > T | Increased CYP3A4 expression (In vitro) | | Increased abiraterone clearance (in vitro) and decrease the response rate of abiraterone* (in vivo) | 89* | Qin et al. (2018) |
| PGRMC2 | rs3733260 | 18.8 | Intron1 | G > T | | Decreased CYP3A4 activity | | 147 | Klein et al. (2012) |

[a]There is no rsID available for this variants in the database.

addition to transcriptional regulation, the CYPP450 oxidoreductase (POR) is reported to influence CYP3A activity by participating in electron transfer to the CYP3A enzyme (Masters, 2005). Indeed several studies showed that a common variant POR*28 (rs1057868) was associated with lower C/D ratios and higher dose requirement of tacrolimus (de Jonge et al., 2011; Suetsugu et al., 2019; Nakamura et al., 2020) and cyclosporin A (Cvetković et al., 2017). In addition, epigenetic regulation of the CYP3A enzymes is an emerging research field (Tang and Chen, 2015). Several mechanisms have been revealed particularly in microRNA (miRNA) involved in transcriptional and post-transcriptional regulation (Wei et al., 2014; Ekström et al., 2015). Notably, miRNA not only targets the 3′-untranslated region (3′UTR) of CYP3A4 directly (Pan et al., 2009) but also impacts the function of CYP3A4 transcriptional factors, for instance, HNF4α (Takagi et al., 2010) and VDR (Pan et al., 2009). More mechanisms of epigenetic regulation and related miRNA have been reviewed but a detailed description is outside the scope of this manuscript (Dluzen and Lazarus, 2015; Lolodi

et al., 2017). Overall, while some genetic factors outside the CYP3A locus have been linked to the variability of CYP3A4 expression or CYP3A activity, conflicting data are present and for none of the variants there is sufficient evidence to support clinical application.

# Opportunities for Pharmacogenomics Studies on CYP3A Missing Heritability

Based on a genome-wide association study (GWAS) consisting of 1,446 kidney transplant recipients, 12.5% of tacrolimus trough concentration variability can be explained by CYP3A5*3 variants, including both donor factors and recipient factors. The explained variability increases to 16.9% after taking CYP3A4*22 into account (Oetting et al., 2018). This study combined with the two twin studies mentioned above encourages further exploration of the missing heritability in CYP3A4. And as discussed in this review, many of the variants in the CYP3A locus are of unknown impact and are not included in the clinical *-nomenclature.

**FIGURE 2 |** A Framework of future strategy for the CYP3A enzyme activity prediction.
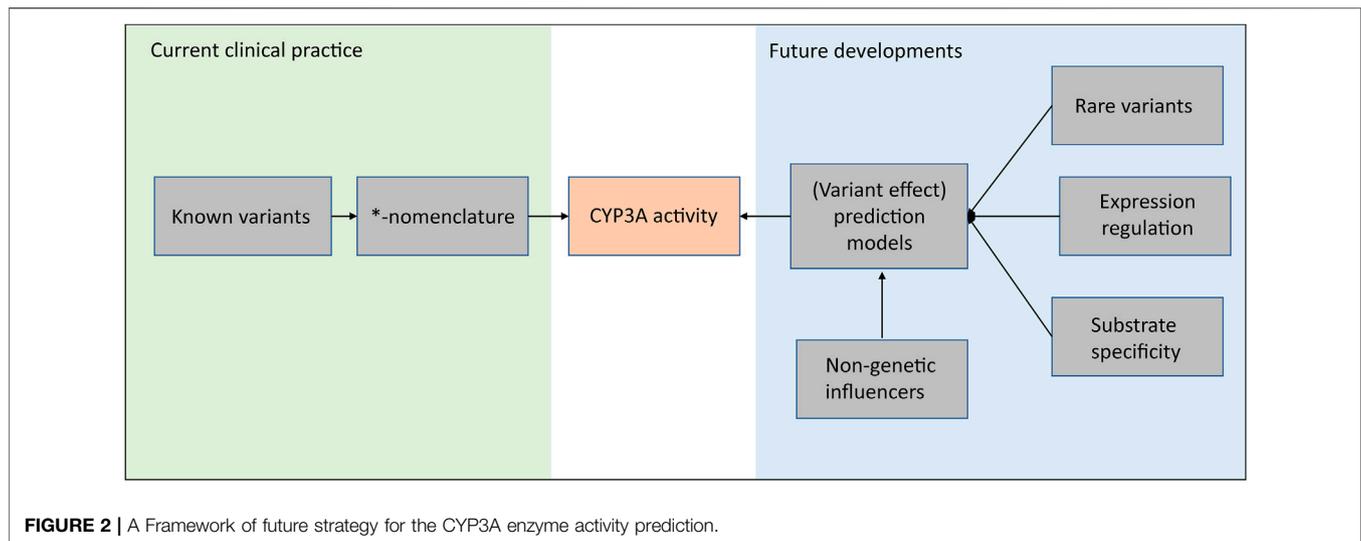
Moreover, not for all known *-haplotypes the impact is known. This limits the implementation of these variants and haplotypes in clinical practice. As shown in **Figure 2**, in our opinion, these future developments should focus on a unifying approach that incorporates all (genetic) factors which can influence CYP3A activity. For example, advanced prediction models, e.g., neural network models, which incorporate rare variants, expression regulation and non-genetic contributions, are expecting as a promising strategy for future developments.

However, currently studies in genetic variants detection and interpretation have inevitable limitations. GWAS analysis identified some genetic variants associated with the variability of CYP3A4 activity. However, by design GWAS analyses exclude rare variants and sometimes even low-frequency variants (MAF<5%) for reasons of quality control. As a result, GWAS is incapable to identify rare variants associated with clinical outcomes. Moreover, GWAS analysis generally relies on SNV screening array, which has difficulty detecting variants in complex genes and cannot detect novel variants. However, these types of variants are abundantly present in the CYP3A locus.

The complexity of the CYP3A locus encourages the application of advanced sequencing technologies. First of all, next-generation sequencing (NGS) provides us with almost all of the SNVs in the CYP3A locus. However, NGS generally relies on short reads (100–200 bp), which makes it limited in characterizing complex regions extending that length. Moreover, short-read sequencing still has limitations in exploring the structural variation and providing phasing information directly (Russell and Schwarz, 2020; van der Lee et al., 2020). Besides NGS, long-read sequencing is a promising solution for comprehensive genetic information collection (Ameur et al., 2019). With long-read sequencing, all single-nucleotide variants and most of the structural variants can be detected and those variants can often be phased directly, which is crucial for phenotype prediction (van der Lee et al., 2020). The accuracy and size of phased haploblocks of long-read sequencing

seems most prominent in the complex pharmacogenes. This information can also be used to optimize models to predict drug response by taking phasing and full gene sequencing into account. Indeed, recently a continuous scale model based on full gene variants data explored by long-read sequencing and neural network improved the explanation of CYP2D6 activity variability from 54% to 79% compared with the conventional phenotype classification (van der Lee et al., 2021).

Nonetheless, these sequencing technologies come with higher costs and significantly more data to analyze, resulting in consideration of cost-benefit balance and the challenging of novel variant interpretation. To assess the individual impact of a large number of low frequency variants, and in the absence of in vivo data, several in silico tools, including SIFT, PolyPhen-2, and CADD have been developed. However, the predicted effect of variants differed among in silico tools (Gulilat et al., 2019). Moreover, compared with in vitro models, the accuracy of those in silico prediction only reaches up to 80% for pharmacogenetic purposes (Han et al., 2017). SIFT, PolyPhen-2 show a higher false-negative rate in predicting gain-of-function variants compared to loss-of-function (Flanagan et al., 2010). Remarkably, neural network models have been developed to predict the unknown function *-alleles in CYP2D6. This model explains 47.5% of unknown function variants in *-alleles with 88% accuracy (McInnes et al., 2020), which encouraged the further implementation of neural network based approaches. By contrast, in vitro models provide us a better understanding of the effects of variants, especially for rare variants (Kumondai et al., 2021). However, it is a costly and time-consuming process to establish in vitro models for each individual CYP3A variant, which can probably never be realized and hampers their clinical implementation. Furthermore, by-design an in vitro system is not useful to assess the effect of variants located in non-coding regions, which occur frequently in the CYP3A locus.

Given the broad substrate spectrum of drugs affected by the CYP3A enzymes and the presence of substrate-specific effects, functional prediction based on in vitro data with only a limited

number of substrates are controversial. Therefore, *in vivo* evidence is considered the gold-standard to establish if variants have a significant effect on enzyme activity, especially for non-coding and synonymous SNVs. For novel missense SNVs, drug-related clinical information is highly recommended. However, particularly for the frequently occurring low-frequency non-synonymous variants in the CYP3A locus, this evidence is hard to generate. Indeed, quite a few studies failed to detect functional variants or identify their associations with clinical outcomes (Belmonte et al., 2018; Riera et al., 2018). Moreover, lacking of clinical significance prevent their further study only based on *in vitro* evidence.

Conventional drug metabolizer phenotypes distinguish three or four metabolizer phenotype groups based on a limited number of variants. This classification assumes a fixed effect of each variant, where the predicted phenotype depends on the combined effects of those several well-known variants. This same method is used for almost all CYP-enzymes with in the end only five phenotype categories (poor-, intermediate-, normal-, rapid- and ultra-rapid metabolizers) (Caudle et al., 2017). However, it has been demonstrated that enzyme activity is not categorical but continuous. As a result, the conventional variant to metabolizer phenotype interpretation which only includes a limited number of well-known variants with fixed effects inevitably leads to missing information. Previous studies have shown that, at least for CYP2D6, a continuous model is able to better explain the enzyme activity (McInnes et al., 2020; van der Lee et al., 2021). It can be expected that the same principle holds true for CYP3A as well. Furthermore, in the CYP3A locus more than one coding isoforms with considerable overlapped substrate specificity contributes to the overall CYP3Aenzyme activity, complicating the predictions even more. As mentioned previously, CYP3A mediated clearance shows a unimodal distribution which does not fit the current categorical phenotype system. In the current system, the function of variants that cause slight or moderate CYP3A activity variability could be obscured by variants that have predominant effects, which further complicates the analysis of the contribution of every variant in every *CYP3A* gene. To deal with the complexity of the *CYP3A* locus, the use of artificial intelligence (AI) is an opportunity. AI models could include all variants detected to predict both variant effect as well as overall enzyme activity without the use of the categorical models (Zou et al., 2019).

Besides genetic variation, environmental factors also contribute to variability in CYP3A activity. For instance, 20% of induced CYP3A4 activity variation was attributed to BMI, alcohol use, and smoking (Rahmioglu et al., 2011). Also, inflammation has been shown to affect CYP3A activity through modulation of the expression (Vet et al., 2016). Moreover, some clinical variables, including transplant recipient age, glomerular filtration rate, anti-cytomegalovirus drug use, simultaneous pancreas-kidney transplant and antibody induction, account for 19.8% of the variability in tacrolimus trough concentrations (Oetting et al., 2016). Finally, CYP3A inducers and inhibitors have been shown to cause a ~400-fold fluctuation in CYP3A activity and drug clearance (Hohmann et al., 2016). Zanger et al. summarized these and other non-genetic factors which are beyond the scope of this review (Zanger and Schwab, 2013). In summary, genetic variability in the *CYP3A* locus has been consistently linked with the inter-individual variability of drug metabolism and differences in drug response. Despite indications for clinical relevance, only a limited number of variants are included in CYP3A metabolizer phenotype prediction and clinical practice. The abundance of rare variants in the *CYP3A* locus and possible multi-genic regulations of CYP3A expression combined with the rapid technological advances in sequencing technologies present an opportunity for future investigations and offer a potential explanation for the observed missing heritability.

# AUTHOR CONTRIBUTIONS

Writing—original draft preparation, QZ and ML; Writing—review and editing, JS and TG. All authors have read and agreed to the published version of the manuscript.

# SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.912618/full#supplementary-material

**Supplementary Figure S1 |** The allele frequency of four common *CYP3A5* variants with potential impact VEP annotation.3.

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