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INSIGHTS IN PHARMACOGENETICS AND PHARMACOGENOMICS: 2021

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EDITORIAL
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Editorial: Insights in Pharmacogenetics and Pharmacogenomics: 2021

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Keywords: Pharmacogenetics (PGx), Pharmacogenomics (PGx), combinatorial & high-throughput methods, next generating sequencing, clinical practice, NAT1 acetylation, Bac-Mam system

Editorial on the Research Topic

Insights in Pharmacogenetics and Pharmacogenomics: 2021

The main aim of this Research Topic is to shed light on recent progress in the Pharmacogenetics and Pharmacogenomics field as well as current and future challenges, aiming to provide a thorough overview of the state of the art in this field.

The research topic comprises 10 articles to which 62 authors have contributed. Throughout the research topic, different aspects related to pharmacological treatment, effectiveness in drug response, and adverse drug events, are covered. Also, relevant aspects in the design of genetic tests are analyzed, regarding the different genetic variants that should be included in pharmacogenomics testing, taking into consideration the ethnicity of the patients. Also, the effects of genetic variants of a relevant enzyme such as the Arylamine N-acetyltransferase 1 (NAT1) in drug metabolism are reviewed, in two aspects: its enzyme activity and the putative influence on other genes that could have consequences beyond drug metabolism such as cancer risk. Finally, methods of expression of genetic variants for *in vitro* functional studies are also assessed.

The review of Tafazoli et al. focuses on the use of next-generation sequencing (NGS) in guiding drug treatment in clinical practice. It discusses both, the limitations of NGS platforms and putative solutions for solving these limitations. The reduced cost, one of the most important aspects, together with the capacity of simultaneously analyzing a large number of pharmacogenes, as well as the deep analysis it provides, makes this technology promising for routine clinical use.

The article by Sayer et al. analyzes the detection rate of currently available Combinatory Pharmacogenomics tests (tests covering multiple genes) (Wilke et al., 2005; Winner and Dechairo, 2015; Brown et al., 2017), which is widely used in recent years because of their economic utility, in this case, based on the cytochrome P450 gene variants they target. One interesting point in this study addresses the differences in the frequencies of genetic variants between different ethnic groups can make available tests less effective: The authors conclude that the detection rate of CPGx tests covering *CYP2C19*, *CYP2C9*, *CYP2D6*, and *CYP2B6*, show significant variation across ethnic groups, Sub-Saharian Africans and East Asians have a high rate of incorrect detection.

Two articles in this research topic addressed new clinical applications of pharmacogenetics. The work by Hongkaew et al. focuses on the treatment of autism spectrum disorder with risperidone. The authors created an algorithm to calculate the DRD2 genetic risk based on inferred protein expression. The article by Sales et al. is a systematic review assessing the utility of pharmacogenomics in the treatment of sickle cell anemia (SCA) with hydroxyurea. They analyzed more than 700 SNVs and identified 50 SNVs associated with fetal hemoglobin changes in patients with SCA treated with hydroxyurea.

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Two articles on this research topic put the focus on the NAT1 (arylamine N-acetyltransferase1) enzyme. The work by Carlisle et al. studied the effect of NAT1 expression levels on the expression of other genes. They found that nearly 3,900 genes were significantly associated with NAT1 activity in breast cancer cell lines modified to have increased, decreased, and null levels of NAT1. Thus, their work shows that NAT1 activity causes expression changes in many genes, thus raising the possibility that the role of NAT1 in cancer (Hein, 2000; Agundez, 2008; Carlisle et al., 2018) could be ultimately mediated by genes other than *NAT1*. The study by Doll and Hein analyzes the effects of SNVs in the *NAT1* coding exon on Michaelis-Menten kinetic constants for the carcinogen 4-aminobiphenyl and its N-hydroxylated metabolite, that are NAT1 substrates (Hein et al., 1993). They confirmed that some SNVs significantly reduced acetyltransferase activity. Also, they identified an SNV (rs4986782) that significantly reduced the apparent Km for these carcinogens.

The article by Muderrisoglu et al. analyzes the effects of SNVs in genes coding human nicotinic acetylcholine receptor subunits on nicotine addiction and the efficacy of smoking cessation therapy with varenicline, nicotine replacement therapy or bupropion. The study concludes that the response to smoking cessation treatments is independent of the nicotinic acetylcholine receptor α subunits genotype analyzed.

Giles et al. reviewed the applicability of several omic techniques (genomics, metagenomics, transcriptomics, proteomics, and metabolomics), in heparin-induced thrombocytopenia (HIT), an adverse drug event that has a high mortality (Jolicoeur et al., 2009). The study covers the relevance and current knowledge on omics in HIT, and it stresses the importance of multi-omics approaches to gain ground on the pathogenesis of this adverse drug event.

The aim of the study by Gloor et al. is to identify genetic risk factors for postoperative nausea and vomiting (PONV), aiming to improve the identification of at-risk patients. For this, they selected SNVs located around 13 different genes, including COMT, CHRM3, five 5-hydroxytryptamine receptors (HTR) subunits, OPRM1, DRD2, TACR1, FAAH1, ABCB1, as well as the inferred phenotypes for the cytochrome P450 enzymes CYP2D6, CYP3A, CYP2C9, CYP2C19, CYP1A2, and CYP2B6. Interestingly, they identified association or PONV recurrence with the CYP1A2 activity score and with *TACR1* and *HTR3* genotypes, and they developed a risk model based on these factors.

Finally, Miyauchi et al. analyzed the potential of the baculovirus-mammalian cell (Bac-Mam) expression system to analyze the effect of genetic variability in drug metabolism. This technique allows the transference of genes coding for drug-metabolizing enzymes into mammalian cells to obtain correct posttranslational modifications. The authors demonstrated that *CYP3A4*, *UGT1A1*, and *UGT2B7* can be efficiently transfected using this procedure. The Bac-Mam expression system, therefore, holds great promise for the functional analysis of the plethora of SNVs and allelic variants present in pharmacogenes, whose list is in continuous expansion as new genetic variations are described.

In sum, this Research Topic covered interesting findings and procedures that will help to gain ground in the development of Pharmacogenetics and Pharmacogenomics and their implementation in clinical practice.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Applying Next-Generation Sequencing Platforms for Pharmacogenomic Testing in Clinical Practice

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Pharmacogenomics (PGx) studies the use of genetic data to optimize drug therapy. Numerous clinical centers have commenced implementing pharmacogenetic tests in clinical routines. Next-generation sequencing (NGS) technologies are emerging as a more comprehensive and time- and cost-effective approach in PGx. This review presents the main considerations for applying NGS in guiding drug treatment in clinical practice. It discusses both the advantages and the challenges of implementing NGS-based tests in PGx. Moreover, the limitations of each NGS platform are revealed, and the solutions for setting up and management of these technologies in clinical practice are addressed.

Keywords: pharmacogenomics, clinical implementation, next generation sequencing, clinical practice, PGx testing

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INTRODUCTION

The Importance of Pharmacogenomics in Modern Medicine

Pharmacogenomics (PGx) utilizes individuals' genomic profiles to identify those who are at greater risk for adverse drug reactions or ineffectiveness. Many studies clearly indicate that drug-related genes, also referred to as "pharmacogenes," in the human genome contain extensive functional genetic variations (FGVs) and that different alleles are associated with diverse outcomes of drug treatments (Madian et al., 2012; Guchelaar, 2018; Suarez-Kurtz and Parra, 2018). Around 97–98% of people have at least one actionable FGV in their drug-related genes. In addition, the possibility of the presence of a genetic variant which could result in a loss of function (LOF) variant in pharmacogenes is 93% for every individual (Schärfe et al., 2017). Hence, the identification of the different genetic variants associated with the drug metabolism would impact on the prescription of medication, allowing for the selection of the right drug and dose, thereby reducing the potential adverse effects or

Abbreviations: ACMG, American College of Medical Genetics and Genomics; CADD, Combined Annotation-Dependent Depletion; CAP, College of American Pathologists; CLIA, Clinical Laboratory Improvement Amendments; CNV, Copy Number Variation; CYP, cytochrome P450; ExAC, Exome Aggregation Consortium; FDA, US Food and Drug Administration; GWAS, Genome-Wide Association Study; InDel, Insertion/Deletion; MAF, minor allele frequency; PROVEAN, Protein Variation Effect Analyzer; REVEL, Rare Exome Variant Ensemble Learner; SNP, Single Nucleotide Polymorphism; SNV, Single Nucleotide Variation; SV, structural variant; VCF, variant calling format; VEP, Variant Effect Predictor; VIP, Very Important Pharmacogenes.

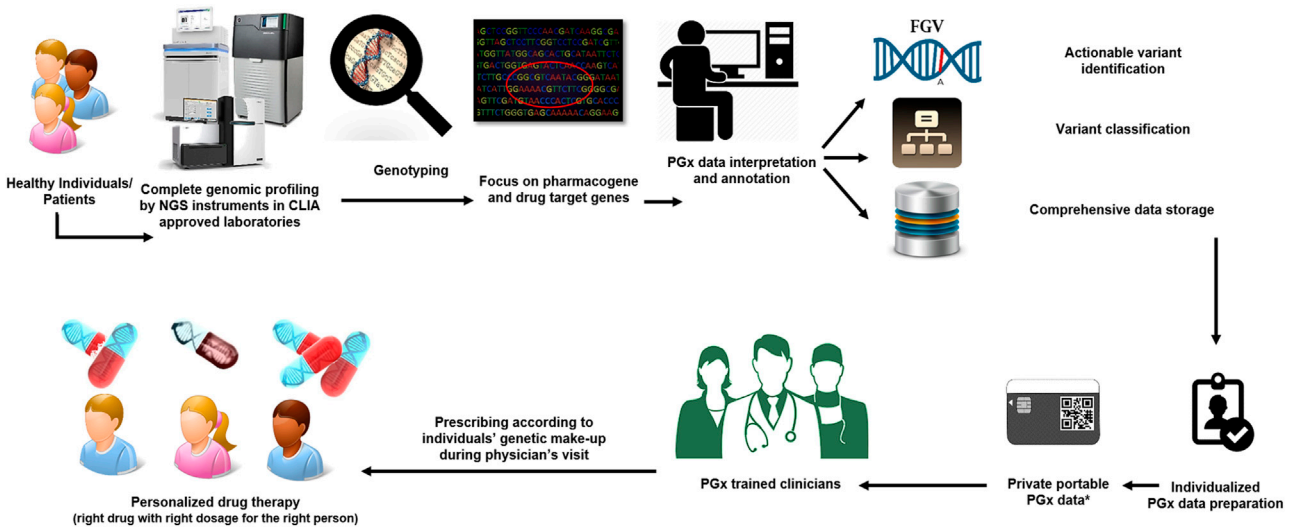

FIGURE 1 | A prospective view of the use of pharmacogenomics in modern medicine. Every person (sick or healthy individuals) will undergo comprehensive genomic screening before going to the physician/clinicians. The genetic variations in all pharmacogenes of an individual will be identified through data annotation and visualization by specific bioinformatics tools. The final report for each individual will be available through a private portable PGx electronic card. PGx trained clinicians will use an individual's genetic make-up report to tailor treatment to the patient's needs.

the therapeutic inefficacy. For clinical interpretation of PGx tests, the Clinical Pharmacogenetics Implementation Consortium (CPIC) and the Dutch Pharmacogenetics Working Group (DPWG) guidelines are available as well as FDA drug-gene interaction recommendations. CPIC originally started as a shared project between PharmGKB and the Pharmacogenomics Research Network (PGRN) in 2009, and DPWG was launched in 2005 by the Royal Dutch Pharmacists Association. The two consortia have developed and published recommendations for numerous gene-drug interactions (JJ Swen et al., 2011; Relling and Klein, 2011). Both CPIC and DPWG provide updated, evidence-based, free access guidelines to facilitate and accelerate the establishment of a link between the results of PGx tests and specific dose recommendations. Nowadays, an increasing number of specified PGx tests are available in specialized CAP/CLIA approved clinical pharmacology/genome analysis centers around the world and can be found in the genetic testing registry (GTR, https://www.ncbi.nlm.nih.gov/gtr/) (Jiang and You, 2015).

The introduction of next-generation genome sequencing in PGx practice is an interesting and promising, albeit challenging, step. Currently, the field of PGx is moving from reactive testing of a single gene towards scanning an entire panel of genes involved in drug absorption, distribution, metabolism, and excretion (ADME) before prescribing (pre-emptive genotyping) by applying different types of next-generation sequencing (NGS) platforms (Bielinski et al., 2014; van Der Wouden et al., 2019). The results include all the PGx-related genetic variants in the genome which will be utilized to prepare drug dosing recommendations based on the predicted phenotype provided by the sequencing tests (**Figure 1**).

While the topic is highly popular and an overview of the current state of the NGS technologies for use in PGx testing has been offered in the literature previously (Schwarz et al., 2019), this article will discuss the challenges of detecting specific types of variants in PGx and interpreting such data in clinical practice. Solutions for the establishment and management of NGS devices in clinical practice are also addressed. A number of useful tables that provide detailed NGS-PGx-related information are also included. To aid with the terminology used throughout this manuscript, we included a concise glossary of NGS-related terminology in **Appendix 1**.

HOW CAN WE USE NGS FOR PGX ANALYSIS?

In this section, we firstly discuss the SNP-based PGx testing, which is currently the most frequently used test in the clinical PGx profiling of individuals, followed by targeted sequencing and whole-exome and whole-genome sequencing (WES/WGS).

SNP-Based PGx Testing in Clinical Practice

Fast, accurate, and inexpensive genotyping methods are key to the implementation of PGx in clinical practice. Currently, specific genotyping methods which mostly utilize different types of SNP-based genotyping approaches including real-time PCR with TaqMan probes and restriction fragment length polymorphism (RFLP) technique as well as gene panel-based genotyping methods such as ADME arrays are used in everyday clinical practice (Dorado et al., 2005; Johnson et al., 2012; Larsen and Rasmussen, 2017; Rasmussen-Torvik et al., 2017; Lemieux Perreault et al., 2018; Hippman and Nislow, 2019). In principle, genome-wide genotyping arrays such as Infinium Global Screening Array (GSA) could be used for routine PGx testing but are not yet commonly applied for

TABLE 1 | Summary of the recent studies that used the NGS technologies for functional PGx variant detection.

Study objective	n =	Applied NGS platform(s)	Covered drug-related genes	Identified variants	Result	Reference
Platform validation and variant discovery	3 × 96	Targeted sequencing	84	SNVs	A custom-designed panel (PGRNseq) could be an ideal platform for both the common and the rare PGx variants identification in large cohorts and suitable for the clinical tests	Gordon et al., 2016
Platform validation and variant discovery	376	Targeted sequencing	114	SNVs	Targeted sequencing panels are ready-to-use platforms for comprehensive pharmacogene profiling including common plus rare variants in ADME core genes towards the implementation of the personalized medicine	Han et al., 2017
Platform validation	2 (cell culture)	Targeted sequencing	3	SNVs CNVs InDels	Variants and haplotype detection of challenging ADME genes were successfully achieved	Ammar et al., 2015
Platform validation and variant discovery	235	Targeted sequencing	100	SNVs CNVs	Designed PGxSeq panel with high accuracy identified clinically relevant variants in 39 genes including CYP2D6 CNV and UGT1A1*28 TAA repeats in the promoter. The allele frequency and the homozygosity were also determined	Gulilat et al., 2019
Platform validation and variant discovery	150	Targeted sequencing	340	SNVs Small InDels	Panel-based NGS pipeline developed and revealed 7,273 novel variants in 340 ADME genes of 150 Caucasian liver donors with an accuracy of >99%. The functional prediction allowed for the prioritization of the variants for further analysis	Klein et al., 2019
Validation of known variants	60	Targeted sequencing	20	SNVs InDels	Prediction model of the atorvastatin plasmatic concentrations in healthy volunteers through the sequencing results explained well	Cruz-Correa et al., 2017
Platform validation	98	Targeted sequencing and WGS data	19	SNVs CNVs	The concordance between the two platforms estimated to >97% for identified variants. The CNVs concordance in CYP2D6 gene also demonstrated 90% of accuracy. 95 children had at least one clinically actionable pharmacogenetic variant	Cohn et al., 2017
Validation of known variants	1,583	Whole-exome sequencing data	11	SNVs	At least one actionable phenotype was present in 86% of individuals. Repurposing WES data can yield meaningful pharmacogenetic profiles for 7 of 11 important pharmacogenes, which can be used to guide the drug treatment	van der Lee et al., 2020a
Validation of known variants	94	Whole-exome sequencing	3	SNVs	Diagnostic genotyping identified PGx variants in CYP2C19, CYP2C9, and VKORC1 genes in 91% of all cases. Of this, 20% indicated potential immediate effects on the currently used medications	Cousin et al., 2017
Platform validation	36 + 12	Whole-exome sequencing	36	SNVs InDels	High concordance revealed through cross-comparison of WES and other platforms as well as the MiSeq amplicon sequencing data and the IPLEX ADME PGx panel. WES was introduced as a promising tool in PGx profiling with a low error rate of <1%	Wee Chua et al., 2016
Platform and discovery rate validation	2504 of WGS data + 59,898 of WES data	WGS and WES data	208	SNVs CNVs	The population-specific deletion and the duplications were revealed in 97% of the analyzed subjects and the related frequencies were reported and confirmed via Sanger sequencing	Santos et al., 2018
Platform validation and variant discovery	1,000 Genomes data	Whole-genome sequencing data	160	SNVs InDels	Putatively functional variants within known pharmacogenomics loci identified that could account for association signals and represent the missing causative variants underlying drug response phenotypes	Choi et al. (2019)
Variants validation and discovery	547 individuals from in-house cohort data + gnomAD data	Whole-genome sequencing data	11	SNVs InDels	For improved precision medicine, PGx testing should move towards WGS-based	Caspar et al. (2020)

(Continued on following page)

TABLE 1 | (*Continued*) Summary of the recent studies that used the NGS technologies for functional PGx variant detection.

Study objective	n =	Applied NGS platform(s)	Covered drug-related genes	Identified variants	Result	Reference
					approaches as a feasible and most comprehensive method	
Platform validation	44,000 biobank participants	WGS and WES data + microarray data	11	SNVs CNVs	WGS and microarray demonstrate more concordances for the obtained results. WES is not suitable for PGx preemptive predictions. However, the microarrays are more cost-effective than the sequencing platforms. Overall, the implementation of the PGx tests and the recommendations may affect at least 50 daily drug doses per 1,000 inhabitants	Reisberg et al. (2019)
Variant discovery	3	Targeted sequencing	16	SNVs	The functional alterations and variants with potential impact on anti-TNF drug response successfully introduced by rapid, sensitive, and cost-effective NGS-based pharmacogenetics methodology	Walczak et al., 2019
Variant discovery	392	Whole-exome sequencing	21,000	SNVs InDels	Exome sequencing revealed novel genetic loci with a strong association with on-treatment reactivity and hereditability of platelet and clopidogrel response	Price et al., 2012
Variant discovery	482 + 7	Whole-genome sequencing	231	SNVs InDels Tandem substitutions	17,733 ADME variants/individuals detected. In addition to known PGx markers, 1,012 novel variants with potential deleterious function identified in exons, introns, gene promoters, and proximal regulatory regions	Mizzi et al., 2014
Variant discovery	100	Whole-genome sequencing	437	SNVs	The analysis revealed 227 common and 466 rare population-specific potentially functional SNVs	Sivadas et al., 2017

this purpose. While the technology is still developing, the main limitation is that the identification of the structural PGx variants such as Copy Number Variations (CNVs) and hybrid genes as well as CYP2D6/7 is mostly ignored. Moreover, the variants in the pharmacogenes that are tested are limited to currently known and common alleles. Although several versions of arrays are being enriched with more specific PGx variants (thousands of drug-related biomarkers) (Arbitrio et al., 2016; Thermofisher.com/Pharmacoscan, 2018; Illumina, 2020), no phasing information will be obtained through these tests, which makes it more challenging to provide an accurate phenotype prediction.

Hence, the properties of NGS technologies make them an interesting approach to performing clinical PGx testing. In recent years, several investigators have explored different approaches utilizing NGS platforms, namely, targeted sequencing, WES, and WGS in pharmacogenomics. **Table 1** shows several studies stratified by different approaches.

Targeted Sequencing Panels

Research into PGx over the years has resulted in the identification of numerous genes which may play an essential role in drug metabolism, transport, and targeting in the human body. However, not all of them are strongly associated with drug response phenotypes and therefore CPIC and DPWG only provide clinical recommendations for specific variants in well-known pharmacogenes.

Gordon et al. developed the PGRNSeq panel as a balance between cost, throughput, and depth of coverage. The panel included clinically actionable CPIC genes as well as genes for which little was known, although a primary association with the PGx trait existed. It was concluded that the PGRNSeq panel is suitable for both the clinical investigations and the discovery studies. However, some non-coding parts and complex structural variants for specific pharmacogenes (including CYP2A6, CYP2D6, and HLA-B) alongside better computational resources for data interpretation remain to be developed. In a similar approach, Han et al. developed an unbiased and broad-range NGS panel and suggested that the utilization of such panels may be a valuable tool in the comprehensive study of PGx genes. The selection of genes for inclusion in the panel was based on the pharmaadme and Www.pharmaadme.org database (Gordon et al., 2016; Han et al., 2017).

Customized PGx panels can also serve as a highly accurate approach to variant detection in the clinical PGx testing. Gulilat et al. developed a targeted exome panel, named PGxSeq, for capturing both SNVs and CNVs in pharmacogenes. They demonstrated that PGxSeq could be employed as a reliable tool for common and novel SNVs alongside CNV detection in pharmacogenes in clinical use. However, a limitation of the work was that the validation was restricted to 39 loci in 16 genes in specific population samples. Moreover, pharmacogenetic variants in non-coding and regulatory parts were not included (Gulilat et al., 2019). A comprehensive PGx panel that includes all coding

regions, adjacent introns, and 5′ and 3′ UTRs in flanking sequences of 340 ADME genes has recently been developed by investigators in Germany. The identification of genes for inclusion in the panel was based on multiple sources including PharmaADME, PharmGKB, and ADME-related genes from the literature. Compared with other genotyping methods, accuracy was high, with >99% correct calls. The obtained data allowed for the covering of coding and functional non-coding parts and provided related data for both common and rare variants in addition to revealing novel associations. The detection of some limited InDels and integration of rare variants into PGx by the current computational predictors alongside the sample size were reported as limitations of the panel (Klein et al., 2019).

Long-Read Sequencing for Gene Panels

Several PGx genes involve complex variants such as tandem repeats, pseudogenes, and CNVs. Long-read sequencing approaches (on average over 10 kb in one single read) have been used previously in the profiling of different complex genomic loci and have been proposed for the identification of such challenging genomic areas in PGx (Ardui et al., 2017; Mantere et al., 2019; van der Lee et al., 2020a). In this field, Ammar et al. applied long-read sequencers to identify PGx variants and haplotypes in three challenging pharmacogenes: CYP2D6, HLA-A, and HLA-B. The constructed haplotypes were confirmed by HapMap data and statistically phased Complete Genomics (WGS data from the public 69 genomes project) and Sequenom genotypes (for 36 SNP, InDels, and CNVs for CYP2D6). The results demonstrated the potential of long-read sequencing in clinical PGx (Ammar et al., 2015). In addition to haplotyping, variant phasing is also a challenge in PGx. Long-read sequencing has also been employed to resolve phasing issues and provide a solution to the accurate genotyping of complex PGx genes. Yusmiati Liau et al. utilized the GridION platform for sequencing and haplotyping of the entire CYP2D6 gene. Known and new alleles and subvariants plus duplicated alleles were assigned accurately with correct phasing. The approach also demonstrated the capability of processing multiple samples simultaneously and appeared to be a time- and cost-effective method (Liau et al., 2019).

Whole-Exome Sequencing

More comprehensive methods such as WES and WGS identify high numbers of pharmacogenetic biomarkers. In addition, these sequencing approaches may facilitate the discovery of novel loci. While it is possible to reuse WES for PGx purposes for known variants, the application for novel variants is challenging as the investigators would need a confirmative study or extensive *in-vitro* research to attribute potential, newly identified variants in a particular gene to drug response. This is particularly true if it is not clear what functional effect the genetic variation exerts on protein function and/or expression. Van der Lee et al. investigated the feasibility of repurposing WES data for the extraction of a PGx panel of 42 variants in 11 pharmacogenes to provide a pharmacogenomic profile. Based on the Ubiquitous Pharmacogenomics (U-PGx; www.upgx.eu) panel which includes all the actionable genes and variants in the DPWG guidelines, the authors successfully extracted information regarding 39 variants out of the total 42. At least one actionable phenotype was present in 86% of the analyzed data from the included subjects. Although structural variants (SVs) and copy numbers in some pharmacogenes as well as CYP2C19, UGT1A1, CYP3A5, and CYP2D6 were not detected, and the study suffered from a small number of drug-related genes and a limited sample size, the authors concluded that the WES data can yield meaningful pharmacogenetic profiles for 7 out of 11 important pharmacogenes (van der Lee et al., 2020b). To assess the potential benefits and the limitations of using the clinical WES data for PGx analysis as a secondary finding, Cousin et al. analyzed the clinical WES data for the detection of any FGVs in three important pharmacogenes. PGx variants were extracted from the WES test results of patients and used in addition to their medical history data. A pharmacist interpreted the PGx data based on multiple resources including CPIC, UpToDate, Micromedex, and AskMayoExpert and used the information to perform a genotype-informed medication review. The authors concluded that PGx testing early in life would be helpful for prescribing physicians to make future prescribing decisions (Cousin et al., 2017). The accuracy and the concordance rate for the WES variant calling were also investigated by Wee Chua et al. The researchers performed a cross-comparison between the WES and MiSeq amplicon sequencing data in addition to the WES and iPLEX ADME PGx panel in 36 and 12 samples, respectively. The rate obtained for both comparisons was high (99%), which indicates that WES is a promising tool in PGx profiling of individuals with an estimated error rate of <1% (Chua et al., 2016). However, despite these positive results, an important limitation of WES is that several important PGx variants, including CYP2C19*17 and VKORC1, are located outside of the captured regions of routine whole-exome sequencing.

Whole-Genome Sequencing

Complete genomic variants (including PGx-related markers) for an individual would be available through the utilization of the WGS approach. Although the big data interpretation of such tests is still challenging, a decrease in sequencing costs alongside the comprehensiveness of WGS may result in the method becoming a standard platform for clinical PGx tests.

Through using the WGS data from phase 1 of the 1,000 Genomes project and subsequent annotation, 69,319 variants including SNVs (94%) and InDels (6%) were revealed in 160 pharmacogenes (127 CPIC genes and 64 VIP genes from PharmGKB). Minor allele frequency for the variants was >1%, of which 8,207 were in strong linkage disequilibrium (LD) (r^2 > 0.8) with known PGx variants. The alterations were distributed in various parts of the genome including intronic, coding, and 5′ upstream and 3′ downstream regions. In the end, the authors identified putatively functional variants within known pharmacogenomic loci underlying drug response phenotypes and suggested direct testing instead of relying on LD, which is going to be different among populations. A limited sample size and exclusion of rare variants (MAF <0.01) in addition to a lack of an experimental validation study were reported as the main

limitations of the investigation. However, the results from such PGx studies facilitate the translation of the findings of the genomic analysis into clinical practice (Choi et al., 2019). While the known PGx gene panels could be included in the WGS data and considered a source for clinical PGx and drug prescribing, the remainder of the information could still be useful for discovery studies.

The functional CNVs in ADME genes are distributed with significantly different frequencies across diverse populations (He et al., 2011; Martis et al., 2013). The NGS data could also be used for CNV calling in different ethnic backgrounds. The investigators used the integrated WGS and WES data from 1,000 Genome and ExAC repositories for CNV identification in 208 pharmacogenes. Novel CNVs (deletion in 84% and duplications in 91% of genes) across six different populations of non-Finnish Europeans, Africans, Finns, East Asians, South Asians, and admixed Americans were decoded successfully. The final result highlighted the necessity for the comprehensive NGS-based genotyping of the pharmacogenes for the CNV identification alongside their allele frequencies. The assessment of the contribution of such CNVs to the drug response outcomes is also possible through a population-specific analysis of rare variants (Santos et al., 2018). Applying NGS for recognizing the actionable variants in genomic profiles may lead to lifetime utilization of PGx information for related individuals. Furthermore, future bioinformatics tools could potentially be utilized for the NGS data re-analysis and the functional prediction of novel variants (Cousin et al., 2017).

As demonstrated, the targeted sequencing approaches are most suitable for genotyping of known PGx genes, including the low-frequency variants. For the discovery of novel pharmacogenes of interest, WGS and WES are considered better choices (Reisberg et al., 2019). WES and WGS also offer the possibility of data repurposing, which means that the clinicians can benefit from the existing clinical sequencing data to extract a PGx profile to inform drug treatment. Although the NGS data from different platforms offer many potential benefits, there are still several challenges and limitations which are discussed in the following sections.

CHALLENGES IN THE APPLICATION OF NGS PLATFORMS FOR THE DECODING OF PGX VARIANTS IN SPECIFIC PHARMACOGENES

From the studies presented above, it appears that most types of variants in the coding and non-coding or regulatory parts of drug-related genes including SNVs, InDels, CNVs, and some structural alterations such as tandem substitutions could be identified with NGS, particularly with long-read sequencers and WGS. However, some well-known clinically actionable pharmacogenetic variants still pose a challenge for the NGS methods. Challenging genes include some core ADME genes, such as CYP2D6 which contains many different known (>100 * alleles, www.pharmvar.org) variants in different populations. Moreover, high sequence similarity and genetic recombination between real genes and close pseudogenes, such as CYP2D7 and CYP2D8, structural rearrangement complexities, and high CNVs among individuals present substantial challenges. Here, the routine short-read NGS approaches will not clarify the genetic profile of an individual and offer proper phenotype prediction. Furthermore, difficulties in the alignment procedures make interpretation and translation into clinical use complicated. Although some of these problems can be resolved by high-resolution techniques, including long-read sequencing, such sequencers with lower error rates (as well as PacBio Sequel HiFi II) are only available through highly specialized centers and are not yet applied in routine clinical practice (Yang et al., 2017). In addition, the technology is currently not being considered for the large-scale genome analysis in the PGx studies (van der Lee et al., 2020a).

Another example of a challenging pharmacogene is UGT1A1, with some important variants in the non-coding parts of the gene (TA repeats in the promoter of the gene, particularly UGT1A1*28, which affect the gene transcription and hence enzyme activity) (Bosma et al., 1995; Dalén et al., 1998; Numanagić et al., 2015). The gene harbors more than 113 functionally relevant variants, most of which reduce or enhance enzyme function, in addition to many other variants with unknown significance. The allele frequency is heavily population-specific, too. However, most of the panels focus on commonly known genotypes and could easily miss predictive variants in particular cases. By way of illustration, FDA approved the test for *28 allele but not *6 allele for irinotecan, although the latter is the main cause of the altered activity of the UGT1A enzyme in the Asian populations (Ikediobi et al., 2009). Also, the utilization of more comprehensive platforms such as WES is accompanied by poor and insufficient coverage for non-coding parts, which may result in the lower concordance and weak diplotype and CNV calls for the UGT1A1 gene (van der Lee et al., 2020b).

A third challenging region is the HLA genes. They are characterized by high sequence homology and prone to error in the capturing procedure and possible misalignment in the mapping processes. In addition, more than 21,000 known alleles and several pseudogenes and some InDels in the intronic regions of HLA class I and class II genes require the utilization of a proper platform, and more advanced IT infrastructure for the bioinformatics analysis and the identification of various potential predictive PGx markers, particularly in the newly studied populations (Klasberg et al., 2019). HLA alleles are important not only in PGx but also in other medical fields, including the genomic evaluation of multifactorial disorders and organ transplantation. Unfortunately, most of the HLA variants are rare and population-specific and are not included in routine clinical PGx testing (Nakkam et al., 2018). Today, many bioinformatics tools and algorithms available for HLA variant calling and haplotype phasing based on the WGS, WES, and targeted sequencing results. However, the high coverage of the genomic region is preferred as input for the allelic imputation by most software (Karnes et al., 2017). The available tools and their pros and cons have been discussed

TABLE 2 | Pharmacogenes with the associated challenges that render them difficult to genotype.

Gene	Challenge(s)	Reference
CYP2D6	–Structural variants and gene rearrangements –Pseudogenes –Copy Number Variations –Presence of novel variants –Highly polymorphic region –Substrate-specific effects of some alleles	Taylor et al. (2020) PharmVar structural variations CYP2D6
UGT1A1	–Rare population-specific variants –Variants in non-coding parts of the gene –Independent haplotypes with less linkage disequilibrium	Barbarino et al. (2014) Marques and Ikediobi, 2010
VKORC1	–Important variants in non-coding parts of the gene	Saminathan et al. (2010) Owen et al., 2010
HLA	–Rare population-specific variants –Highly polymorphic regions	Illing et al. (2017) Klasberg et al., 2019
SLC6A4	–Rare population-specific variants	Lam (2013)

comprehensively in the literature (Ka et al., 2017; Kawaguchi et al., 2017; Xie et al., 2017). In general, to overcome the challenges of decoding PGx variants in specific genes, up-to-date knowledge of PGx-related genomics for physicians requesting the test in addition to the selection and utilization of an appropriate platform and interpretation tools for each situation by PGx test centers is required. This may also include previous knowledge of some particular PGx alleles with substrate-specific effects. For example, CYP2D6*17 encodes an enzyme with an increased capacity to metabolize haloperidol but an impaired ability to metabolize codeine (Oscarson et al., 1997; Wennerholm et al., 2002). In addition, occasional discrepancies between guidelines on the classification of genotypes into metabolic groups (which is key to formulating corresponding therapeutic recommendations) must also be considered (Caudle et al., 2020). **Table 2** summarizes some challenging pharmacogenes and their main features that need to be taken into consideration during sequencing or panel design.

CHALLENGES AND OPPORTUNITIES FOR DATA ACQUISITION AND INTERPRETATION

The NGS data annotation, in the form of PGx phenotype prediction, is a highly specialized task that requires both molecular knowledge and clinical knowledge. The extraction of actionable, putative, or likely pathogenic variants from large, sophisticated raw data requires considerable time and effort as well as accurate validation methods. The current approaches include newly developed PGx dedicated tools for star allele calling in pharmacogenes (discussed in the following sections). Here, we address the key considerations, discuss some features of the common PGx-related tools, and propose solutions for managing the challenges.

Targeted Sequencing Panels

Unlike with other genotyping approaches, performing a sequencing run always offers the possibility of decoding novel variants in the sequenced part(s). This has also been observed in the targeted sequencing panels of known pharmacogenes, where novel variations appeared in addition to common markers (Gulilat et al., 2019). Indeed, the variants with unknown clinical significance (VUS) in the NGS data and with no clear connection to pharmacogenetics present a real challenge as far as the implementation of such technologies in clinical practice is concerned. Nevertheless, handling VUS as potentially important identified variants is essential since if appropriate approaches to the correct interpretation were not available, the real functional alleles might simply be introduced as non-actionable. Therefore, a prediction is not feasible easily on the functionality of VUS to interpret the potential effects on the drug responses in a patient. However, because of the lower number of such findings in panels, replication and validation studies using other orthogonal genotyping methods, in silico algorithms, genetic screening for first degree relatives of the proband, and use of GWAS, HapMap, or gnomAD datasets for meta-analysis will be faster and more easier with regard to predicting and confirming the negative or neutral functionality of variants and demonstrating the phenotype associations in the targeted sequencing approaches (Svidnicki et al., 2020).

Whole-Exome and Whole-Genome Sequencing

As expected, VUS are more common in WES and WGS. The situation becomes even more complicated when the results involve novel PGx genes. Online tools such as SIFT and PolyPhen2 as well as other algorithms, including CADD and PROVEAN, plus Ensembl based sources with multiple integrated tools like VEP and REVEL, are available for the prediction of the damaging effects of a large number of variants. However, these tools rely primarily on evolutionary conservation and utilize amino acid or nucleotide sequence alignment, which is less applicable to pharmacogenes. Also, low predictive value of these tools has recently been demonstrated (Lee et al., 2019; Zhou et al., 2019).

Furthermore, incidental findings (IFs), referred to as secondary findings in the ACMG recommendations (Kalia et al., 2017), can be expected in different types of high

TABLE 3 | Key features of the PGx dedicated variant functional prediction tools.

Tool/Algorithm	Main features	Reference
Stargazer	Stargazer calls the star alleles from the NGS data by detecting SNVs, InDels, and structural variants. Stargazer detects variations with structural changes including gene duplications, deletions, and conversions by calculating the paralog-specific copy numbers from read depth	Lee et al. (2019)
PharmCAT	Pharmacogenomics Clinical Annotation Tool (PharmCAT) captures the variants indicated in guidelines from a genomic data set derived from sequencing or genotyping technologies (i.e., VCF), infers haplotypes and diplotypes, and generates a report containing genotype/diplotype-based annotations, as well as guidelines and recommendations according to CPIC guidelines	Sangkuhl et al. (2020)
Aldy	Aldy is a computational tool that performs allelic decomposition of highly polymorphic, multi-copy genes through the use of the whole or targeted genome sequencing data and identifies multiple rare and novel alleles for several important pharmacogenes	Numanagić et al. (2018)
Astrolabe	Astrolabe (former Constellation) is a computational method and probabilistic scoring system that enables automated ascertainment of CYP2D6 and CYP2D19 activity scores from the unphased NGS data, aligned with the catalog of pharmacogenetic alleles with high percentage of analytic sensitivity and specificity	Twist et al. (2017)
Cypripi	Cypripi is an algorithm that computationally assumes CYP2D6 genotype at base-pair resolution from the high throughput sequencing data. It can resolve complex genotypes, including the alleles that are the product of the duplication, deletion, and fusion events involving CYP2D6 and its related pseudogene, CYP2D7	Numanagić et al. (2015)
g-Nomic	g-Nomic is PGx interpretation software that provides recommendations on the suitability of a given combination of drugs for each patient according to their genes and polymedication	Sabater et al. (2019)
PHARMIP	PHARMIP uses drug modeled structure and up-to-date bioinformatics tools and/or databases to understand the genetic factors that cause drug-related adverse reactions	Zidan et al. (2020)
Cyrius	Superior, accurate genotyping of CYP2D6 compared to other existing methods as well as Aldy and Stargazer. All types of variants and haplotype calling in addition to the structural and homology analysis will be covered for both GRCh38 and 37 genome builds	Chen et al. (2021)

throughput sequencing and genotype screening methods. They are mostly defined as annotated functional variants in major drug-related genes which were not expected in the specified assessment but may be either related or unrelated to the particular medication taken by the patient. This adds to the complexity of reporting findings from PGx profiling, where the DNA variants may alter the drug efficacy or increase the risk of serious adverse drug reactions. Such findings could be reported as variants with potential usage in guiding therapy if they are managed properly through appropriate clinical genomic assays, vigorous genotype-phenotype correlation studies, and utilization of PGx-related sources for data interpretation and variant scoring (Lee et al., 2016). However, the existence of secondary findings would also be associated with some technical issues in the employed NGS platform. These issues include the percentage of coverage and type of sequencing methods as well as the number of evaluated individuals, evaluation of family members or randomly selected patients (Westbrook et al., 2013). Yet, not all secondary findings that are identified need to be reported in the result of a clinical test. The ACMG also declared a policy statement for reporting particular secondary findings in the clinical setting (L Blackburn et al., 2015; Miller et al., 2021). However, the statement is related to non-PGx secondary findings. Moreover, many pharmacogenetic variants are not disease-causing. Therefore, the relevance of reporting secondary findings may not be obvious at the time of submitting the report, particularly when only a specific set of pharmacogenes is tested. For the pharmacogenes connected with disease risk, the secondary findings may be handled in accordance with the current ACMG recommendations; that is, it is not necessary to provide a separate set of recommendations for those genes. Nevertheless, while the purpose of PGx testing is to exhaustively (and pre-emptively) profile genes that may potentially alter the drug response, curating and storing the information relevant to the future drug therapy may indicate that no findings should be considered "secondary," particularly when untargeted methods as well as WES and WGS are employed.

Recently Developed Bioinformatics Algorithms for PGx Variant Calling

Concentrated efforts have been undertaken to design and develop specific PGx tools for the identification of SNVs, CNVs, structural rearrangements, gene deletion, gene duplication/multiplication, haplotype phasing, diplotype calling, and phenotype prediction out of the NGS data in the clinical setting. The tools as well as Stargazer, PharmCAT, Astrolabe, Aldy, Cypripi, include special algorithms, which were designed for the interpretation of the PGx variants (Numanagić et al., 2015; Twist et al., 2016; Klein and Ritchie, 2018; Numanagić et al., 2018; Lee et al., 2019). Furthermore, some other tools including g-Nomic and PHARMIP were developed for providing recommendations based on the general information obtained from a PGx test (Sabater et al., 2019; Zidan et al., 2020). The advantages and the disadvantages of each of the tools have been demonstrated previously in the literature (Twesigomwe et al., 2020). **Table 3** provides a concise overview of the key features of these tools. Stargazer, Astrolabe, and Aldy have been fully analyzed and are widely used in the field. Twesigomwe and colleagues have recently performed a comprehensive and

systematic comparison of the functions of these three tools in calling different CYP2D6 variants. The results of the study demonstrate that Aldy and Astrolabe are better common and rare SNV callers compared to Stargazer. Yet, Stargazer outperformed the other tools in rare homozygous allele phasing due to its in-built supplementary algorithm. Calling InDel star alleles in the short-read NGS data and the hybrid rearrangements was challenging for all three algorithms. For other structural variants, gene deletion, duplication, and multiplications, Aldy demonstrated higher concordance in comparison to Stargazer and Astrolabe, respectively. Noticeably, Astrolabe performed weak structural variant calling in comparison to the other two tools. Although Stargazer displayed better performance in CNV calling and the identification of hybrid rearrangements, it simultaneously revealed the highest number of non-genotyped diplotypes for the samples including structural variants. Unfortunately, all three tools had difficulty calling diplotypes with high copy numbers. While these genotypes are very rare, they may still be considered an important variant in some isolated populations. The phenotype prediction and the clinical accuracy of Aldy, Astrolabe, and Stargazer were also evaluated. Remarkably, the concordances were higher than the diplotype concordances as the activity scoring systems may assign the same values as the true function of the wrongly genotyped samples. The impact of the sequencing coverage and the misalignment of InDels on genotyping accuracy was also investigated. The study, however, had some limitations. It used simulated data for most rare and structural variants, did not compare the performances of the three tools across the NGS data from the targeted custom-capture panels, and did not compare the impacts of different aligners on the variant calling processes. Novel SNVs calling was also not analyzed in the study and reliable validation studies were not included (Twesigomwe et al., 2020). Aldy and Stargazer may also result in false-positive/false-negative results in small variant calling, since they rely on initial read alignments. Another major obstacle is that two of the three tools does not support the GRCh38 genome assembly and that the investigators may need to lift their alignments to GRCh37 (i.e., https://genome.ucsc.edu/cgi-bin/hgLiftOver). To address these challenges, Chen et al. developed Cyrius, a novel bioinformatics method for all classes of variants and haplotype calling from CYP2D6 in the WGS data (also included in **Table 3**). The tool can overcome CYP2D6 and CYP2D7 homology challenges and work with both GRCh37 and 38 to accurately genotype CYP2D6 with a higher overall concordance rate with true genotypes (99.3%). Compared to Aldy and Stargazer, superior genotyping was demonstrated for both GeT-RM and long-read data, and the application of the method led to improved understanding of CYP2D6 genetic diversity within five ethnic groups. The authors are currently extending the method to genotype other pharmacogenes with a paralog, CYP2A6 and CYP2B6, and plan to apply it to more genes in the future (Chen et al., 2021). Overall, it is useful to be aware of the specifications and the features of each of the tools in order to increase their utility while applying such algorithms to calling different PGx variants out of high throughput sequencing results.

Solutions for the Management of Challenges in Applying the NGS-PGx Tests in the Clinic

Here, we present three main problems which may arise during clinical NGS testing for PGx in everyday practice and discuss solutions.

Firstly, based on the type of panel or other selected approaches, the setup and the initiation of NGS tests (covering PGx markers) in every clinic will require a substantial investment and reimbursement by insurance companies, bioinformatics infrastructure, specific software and computational tools, and professional clinical experts for data interpretation. In addition, validation studies to determine and improve the clinical utility and the validity are essential. Once a positive evaluation has been performed by public and private payers, relevant NGS-derived PGx tests could be considered for implementation in routine clinical practice. Estimated costs of PGx profiling may vary substantially depending on the type of test applied. Is the PGx assessment a pre-emptive NGS test or repurposed findings from diagnostic WES/WGS? Currently, the test coverage and reimbursement are still considered major barriers to routine clinical use. Enhancing physicians' awareness of the type of test to be requested, gaining third-party support, increasing the number of clients through direct-to-consumer genetic testing companies, and decreasing the cost of tests due to advances in diagnostic technologies may play an essential role in bringing the clinical utility of PGx tests to the attention of insurance companies (L Rogers et al., 2020). While many related services are currently limited to reactive single-gene testing, some clinical centers offer routine pre-emptive PGx tests. For example, all patients treated for an active disease at St. Jude Research Hospital are offered PGx testing (www.stjude.org/pg4kds). Recently, Anderson et al. performed a large-scale study in the United States and demonstrated that only a few core pharmacogenes, including CYP2C19, CYP2D6, CYP2C9, VKORC1, UGT1A1, and HLA class I, were covered by the patients' insurance (Anderson et al., 2020).

Secondly, as mentioned previously, the evolutionary conservation is less applicable to the drug-related genes and therefore the conventional computational algorithms have low predictive accuracy when applied to the pharmacogenetic variants. The difficulties with novel and big data interpretation could be overcome by applying combined and optimized calculation tools and algorithms (at least 6-7 of such bioinformatics tools) for allele imputation (see **Appendix 1**) of PGx single- or multi-marker signatures, as well as confirming such genetic variants as predictive for the drug response with more accuracy (Zhou et al., 2019; Tafazoli et al., 2021). However, not all pharmacogenes have this limitation. Indeed, some genes appear relatively free of evolutionary constraints and are highly similar to other genes. This is particularly true for the genes that are involved in the transfer of endogenous substances (i.e., OTC1). Whenever a novel PGx variant is identified in evolutionarily conserved positions, such genes may still benefit

TABLE 4 | Useful databases for PGx results interpretation in the clinical practice.

Database	Main Activities and Features	Link	Reference
PharmGKB	The Pharmacogenomics Knowledgebase is a truly comprehensive and publicly available, online knowledgebase responsible for the aggregation, curation, integration, and dissemination of the knowledge regarding the impact of the human genetic variation on the drug response	https://www.pharmgkb.org/index.jsp	Barbarino et al. (2018)
CPIC	The Clinical Pharmacogenetics Implementation Consortium (CPIC®) is an international consortium to address the clinical implementation of the pharmacogenetic tests by creating, curating, and posting freely available, peer-reviewed, evidence-based, updatable, and detailed gene/drug clinical practice guidelines	https://cpicpgx.org/	Relling and Klein (2011)
DPWG	The Dutch Pharmacogenetics Working Group includes clinical pharmacists, physicians, clinical pharmacologists, clinical chemists, epidemiologists, and toxicologists to develop pharmacogenetics-based therapeutic (dose) recommendations and assist the drug prescribers and the pharmacists by integrating the recommendations into computerized systems for drug prescription and automated medication surveillance	https://www.pharmgkb.org/page/dpwg	JJ Swen et al. (2011)
PharmVar	The Pharmacogene Variation (PharmVar) Consortium is a central repository for the pharmacogene (PGx) variation that focuses on the haplotype structure and the allelic variation. The information in this resource facilitates the interpretation of the pharmacogenetic test results to guide the precision medicine	https://www.pharmvar.org/	Gaedigk et al. (2018)
PMKB	The Precision Medicine Knowledgebase (PMKB) is a project of the Institute of Precision Medicine (IPM) at Weill Cornell Medicine, which is organized to provide information about the clinical cancer variants and the interpretations in a structured way as well as allowing the users to submit and edit the existing entries for the continued growth of the knowledgebase. All changes are reviewed by cancer pathologists	https://pmkb.weill.cornell.edu	Huang et al. (2017)
PharmaADME	An industry-initiated effort launched to develop a consensus, "Core List" of standardized "evidence-based" drug metabolizing (ADME) genetic biomarkers that are broadly applicable to many pharmaceutical clinical trials and FDA drug submissions	http://www.pharmaadme.org/joomla/	pharmaadme and www.pharmaadme.org
Flockhart Table	The website provides a table designed as a hypothesis testing, teaching, and reference tool for the physicians and researchers interested in the drug interactions that are the result of the competition for or effects on the human cytochrome P450 system. The table contains lists of drugs in columns under the designation of specific cytochrome P450 isoforms	https://drug-interactions.medicine.iu.edu/MainTable.aspx	Flockhart and Oesterheld (2000)
SEAPharm	The Southeast Asian Pharmacogenomics Research Network (SEAPharm) established in Asia to enable and strengthen the PGx research among various PGx communities within but not limited to countries in SEA, with the ultimate goal of supporting PGx implementation in the region	–	Chumnumwat et al. (2019)
PGRN	The Pharmacogenomics Research Network, PGRN I–III, was funded from 2000 through 2015 by multiple Institutes and Centers of the NIH. The network catalyzed pharmacogenomics discoveries both nationally and internationally through the conduct of collaborative research focused on the discovery and the translation of the the genetic determinants of the drug response, to enable safer and more effective drug therapies	https://www.pgrn.org/	–
SuperCYP	A comprehensive database on cytochrome P450 enzymes including a tool for analysis of the CYP-drug interactions	https://bioinformatics.charite.de/supercyp/	Preissner et al. (2010)

(Continued on following page)

TABLE 4 | (*Continued*) Useful databases for PGx results interpretation in the clinical practice.

Database	Main Activities and Features	Link	Reference
FDA-Pharmacogenomic	Table of pharmacogenomic biomarkers in drug labeling	https://www.fda.gov/drugs/science-and-research-drugs/table-pharmacogenomic-biomarkers-drug-labeling	–

from routine predictor tools to indicate their functional impact (Shu et al., 2003). However, in the absence of distinct clinical data, both computational and laboratory models are needed for the genotype-guided drug therapy based on previously unreported genomic variants (Shrestha et al., 2018).

Other PGx specific computational models and algorithms with a high sensitivity and specificity have also been developed for the prediction of the loss of function and/or the functionally neutral variations. The scores obtained with the models could provide quantitative estimation of the impact of different variants on the gene function. A comprehensive analysis of the computational prediction methods and evaluation of the recent progress in the functional interpretation of non-coding variants for drug-metabolizing enzymes and transporters is provided by Zhou and colleagues (Zhou et al., 2018). Once the functionality of a variant is known, the effect on drug pharmacology needs to be estimated. For this, pathway analysis databases as well as DAVID, Human Metabolome Database, String-db, and KEGG could be used to identify the molecular connections between the altered allele(s) in specific genes and the other related genes in the cell. Moreover, newly developed PGx specific tools such as Aldy, Stargazer, Astrolabe, and Cyrius can also help with NGS data processing in the PGx analysis (Klein and Ritchie, 2018; Lee et al., 2019). **Table 4** lists some databases which are useful in interpreting the results of the clinical PGx analysis. We have also recently reviewed the software and the algorithms dedicated to the functional prediction alongside the related mechanism of action in such tools while using the PGx functional analysis (Tafazoli et al., 2021). After finding a potentially strong relationship between the identified variant(s) and the drug response, particular *in-vitro* assessments as well as cell line modifications may be considered for exploring the functional consequences of the altered alleles and diplotypes on the activity of the related protein. However, the latter is not appropriate in clinical use as it increases the turnaround time considerably. As the final step, the clinical association analysis will confirm the connection between the novel variants and the drug response phenotypes in the patients. Needless to say, it is suitable solely for the patient data analysis and not pre-emptive PGx profiling of a healthy individual with no clinically observable phenotype (Ji et al., 2013).

Finally, while well-known and annotated PGx variant(s) can be used immediately in patient care, the clinical translation and utilization of newly introduced variants requires substantial evidence and records of gene-drug interaction as well as phenotyping data. Nevertheless, such data would be stored primarily for the research purposes and the patient may be recontacted for further investigations. Since the prediction of an individual's metabolic status is very important for drug dosage modifications in a clinic, the translation of the sequencing results into phenotype assignment must follow the universal standardized test interpretation approaches. A gene continuum activity score system has been introduced to deal with such situations and may be accepted by reference laboratories and medical centers for converting the genotype data to the clinically actionable recommendations (Hicks et al., 2014). However, to facilitate the incorporation of the high throughput derived PGx reports in the clinical setting, it is necessary to provide the healthcare professionals with more applicable, evidence-based results and employ standardized and updated cohort and case reports (Giri et al., 2019; Krebs and Milani, 2019).

CONCLUSION

The NGS technologies have been used in the PGx research studies for a decade. The rapid development in accessories and supporting bioinformatics tools in addition to the reduced cost and the technological advancement that will allow for testing of a larger number of drug-related genes and biomarkers will result in the widespread use of such methods in various clinical settings. The main challenges are management of identified VUS, a lack of specific variant caller software, poor haplotype phasing, insufficient coverage of some parts of the genome by different platforms, limited capacity to assess variant functionality *in-vitro*, and limited ability to assess functionality through computational approaches. Nevertheless, the application of NGS in PGx testing in the clinical practice is continually increasing, paving the way for new PGx variant discovery and a bright future for pharmacogenomics-guided drug treatment.

AUTHOR CONTRIBUTIONS

AT designed the study, conducted the search for literature, and wrote the entire manuscript. H-JG and JS supervised the study thoroughly and revised and edited the manuscript. WM performed the search for literature and modified the text as well. AK provided the idea and introduced the topic for the manuscript. All authors have read and agreed to the current version of the manuscript.

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APPENDIX 1: MINI-GLOSSARY OF THE NGS TERMINOLOGIES USED IN THIS ARTICLE

Targeted sequencing	Sequencing of specific parts of the genome or sets of genes (multiple genes) at once
Whole-exome sequencing	Sequencing of all exonic (protein-coding) regions of the genome. It also includes some important flanking sequences
Whole-genome sequencing	Sequencing of the entire genome of an organism, including all the non-coding and coding parts in addition to the mitochondrial DNAs. It is the most comprehensive sequencing method among others
Long-read sequencing	Newer sequencing methods (also called third-generation technologies) with the ability to read and produce long sequences of DNA between 10,000 and 100,000 base pairs at one runtime. The method is useful, particularly for the structural variant detection and haplotype phasing
Gene panel	A specific set of genes selected for particular analysis purposes as well as sequencing methods or disease-specific gene profiling
PGx panel	A specific set of pharmacogenes or drug target genes selected for particular analysis purposes
Coverage	The number of times a portion of the genome is sequenced in a sequencing reaction. Frequently expressed as “depth of coverage” and numerically as 1X, 2X, 3X, etc.
Depth of coverage	See above
VCF file	Variant calling format is a standard variant reporting format which was invented during the 1,000 Genomes project. Such files display the genomic variants with their coordinates in the NGS results
Secondary findings	Unrelated genomic variants to the primary purpose of the test revealed during a sequencing run
Haplotype phasing	Determination of paternal or maternal origins (inheritance) of each chromosome while putting into haplotypes. In this way, the researchers can assign the alleles to the paternal and maternal chromosomes and obtain a comprehensive picture of genomic variants for the specific haplotype Statistical estimation of the haplotypes from the genotyping data is also called haplotype phasing
Allele imputation	A statistical method for inferring the genotypes that are not directly measured. Estimation of unobserved genotype, including genetic markers from known haplotype or reference genotype. Particularly beneficial in GWAS studies
VUS	A genetic variant for which the association with a specific phenotype cannot be determined definitively

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BRIEF RESEARCH REPORT
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Clinical Implications of Combinatorial Pharmacogenomic Tests Based on Cytochrome P450 Variant Selection

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Despite the potential to improve patient outcomes, the application of pharmacogenomics (PGx) is yet to be routine. A growing number of PGx implementers are leaning toward using combinatorial PGx (CPGx) tests (i.e., multigene tests) that are reusable over patients' lifetimes. However, selecting a single best available CPGx test is challenging owing to many patient- and population-specific factors, including variant frequency differences across ethnic groups. The primary objective of this study was to evaluate the detection rate of currently available CPGx tests based on the cytochrome P450 (CYP) gene variants they target. The detection rate was defined as the percentage of a given population with an "altered metabolizer" genotype predicted phenotype, where a CPGx test targeted both gene variants a prospective diplotypes. A potential genotype predicted phenotype was considered an altered metabolizer when it resulted in medication therapy modification based on Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines. Targeted variant CPGx tests found in the Genetic Testing Registry (GTR), gene selection information, and diplotype frequency data from the Pharmacogenomics Knowledge Base (PharmGKB) were used to determine the detection rate of each CPGx test. Our results indicated that the detection rate of CPGx tests covering CYP2C19, CYP2C9, CYP2D6, and CYP2B6 show significant variation across ethnic groups. Specifically, the Sub-Saharan Africans have 63.9% and 77.9% average detection rates for CYP2B6 and CYP2C19 assays analyzed, respectively. In addition, East Asians (EAs) have an average detection rate of 55.1% for CYP2C9 assays. Therefore, the patient's ethnic background should be carefully considered in selecting CPGx tests.

Keywords: pharmacogenomics, pharmacogenomic tests, variant selection, detection rate, cytochrome P450 enzymes

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INTRODUCTION

Drug-related morbidity and mortality owing to unoptimized medication therapy are estimated to cost $528.4 billion annually in the United States alone (Watanabe et al., 2018). Pharmacogenomics (PGx) – the study of the role of an individual's genetic makeup in drug response – has the potential to reduce adverse reactions to medications and lower medical costs by individualizing

treatments based on genetic makeup. In a study of Medicare and Medicaid patients examining over 70 million patient records, over half of the patient population received at least one drug with PGx implications (Samwald et al., 2016). Studies comparing PGx guided therapies vs. non-PGx guided therapies in psychiatric patients show both improved therapy outcomes and significant cost savings (Hornberger et al., 2015; Tanner et al., 2019). Clinical PGx practice has the potential to be relevant to a large patient population, bring cost savings, and improve therapy outcomes.

Critical to PGx implementation is the availability of genetic test results having relevant genes to guide therapy decisions. Reactionary approaches to PGx practice, meaning individual genes are tested when there is a suspected need, is inefficient with respect to cost and time. As clinical decision support systems grow, preemptive PGx testing approaches are being utilized, allowing genotyping results to be available prior to prescribing decisions and in the planning of therapy (Dunnenberger et al., 2015). Combinatorial PGx tests (CPGx) are critical to the development of these programs because they offer genotyping results of several different genes simultaneously. While utilizing CPGx tests preemptively helps overcome barriers to PGx implementation, it is important to ensure the genotyping results they provide are reliable. If CPGx tests utilized preemptively have limitations, they can cause sub-optimal outcomes for subsequent therapeutic decisions.

Cytochrome P450 (CYP) enzyme genotypes are relevant to PGx practice for a variety of reasons. CYP enzymes play a role in the metabolism of over 90% of available prescription medications (Lynch and Price, 2007; McDonnell and Dang, 2013). In addition to their critical role in drug metabolism, the prevalence of genetic polymorphisms of CYP enzymes is well documented in diverse patient populations. In a study of nearly 10,000 patients screened for common CYP enzyme variants, 91% of them had at least one variant linked to changed metabolic status (Van Driest et al., 2014). CYP Enzyme genotyping plays a central role in PGx practice for these reasons. Given this, the success of a preemptive PGx testing program with CPGx tests can be greatly influenced by the extent CYP enzyme genotypes are accurately characterized.

Gene variant selection of CPGx tests is an essential factor to consider that can influence therapeutic decisions (Petry et al., 2021). CPGx test performance can vary because laboratories providing PGx tests use targeted genotyping technologies to screen for specific variants with well-characterized drug-gene interactions (Guo et al., 2019). Currently, all target variant CPGx tests that do not find putative variants included in the test report the gene as a normal (*1) variant by default (Mukerjee et al., 2018). In a study of CYP2C9 genotype-guided warfarin dosing vs. standard clinical dosing in 2013, African American patients were in the therapeutic range significantly less with genotype-guided doses (Kimmel et al., 2013). Subsequent studies incorporating more relevant variants showed significantly improved outcomes (Limdi et al., 2015). Determining appropriate CPGx tests to be used for each patient based on relevant gene variants is a potential barrier providers face in implementing PGx testing services.

One of the challenges in determining an appropriate CPGx test is the lack of publicly available information describing them. In a study of direct-to-consumer genetic tests in 2016, less than one-third of tests identified had specific gene variant selection information available (Hall et al., 2017). The National Institutes of Health (NIH) created the Genetic Testing Registry (GTR) in 2010 to collect genetic test information and to enhance their availability, validity, and usefulness (Rubinstein et al., 2013). Information about the tests is voluntarily reported by the commercial clinical laboratories who developed them and is updated regularly. The GTR is considered one of the most valuable genetic testing repositories and is often cited in PGx guidelines.

There are additional resources that support PGx implementation. The Clinical Pharmacogenomics Implementation Consortium (CPIC) helps by creating, curating, and posting freely available, peer-reviewed, evidence-based, updatable, and detailed gene/drug clinical practice guidelines (Relling and Klein, 2011). The Pharmacogenomics Knowledge Base (PharmGKB) is another resource that curates and disseminates knowledge about clinically actionable gene-drug associations, genotype-phenotype relationships, and gene frequency data (Whirl-Carrillo et al., 2012). Finally, specifically for variant selection guidance, the Association for Molecular Pathology (AMP) has established a two-tier evidence-based recommendation system to help laboratory professionals select appropriate gene variants in genotyping assays for CYP2C9 and CYP2C19 (Pratt et al., 2018, 2019). These resources can aid clinicians to know what variants would be appropriate for their patients in CPGx tests.

In our study, we specifically evaluate CPGx tests and their variant selection practices with respect to CYP enzymes. This is due to the critical role CPGx tests and CYP enzymes play in the implementation of preemptive CPGx testing programs. We leverage valuable resources (GTR, CPIC, PharmGKB, and AMP) to perform our evaluation of the current landscape of detection rates of available CPGx tests based on known variant frequencies across various ethnic populations. A list of potential CPGx tests were identified utilizing the GTR, which was subsequently filtered to only include CYP relevant tests. For subsequent analysis, published CPIC guidelines were utilized to identify CYP enzyme phenotypes of interest. These in combination with PharmGKB gene frequency data allowed us to determine the extent genotype predicted phenotypes of interest occur in diverse populations and how well CPGx tests identify them. In our evaluation, gene coverage percentages were determined and the detection rate of CPGx tests were calculated covering five CYP enzymes across various ethnic groups.

MATERIALS AND METHODS

Identification of PGx Tests

The GTR was used to identify PGx tests from 02/23/2021 to 02/26/2021. As keywords, "pharmacogenetic" or "pharmacogenetics" or "pharmacogenomics" or "pharmacogenomic" were used for the search. The resulting PGx test list was filtered based on the

following inclusion criteria: assays that used "targeted variant analysis" as their test methodology, CPGx tests including at least two genes, and publicly available gene variant selection information (**Supplementary Figure 1**). Exclusion criteria for potential CPGx tests included assays using alternative sequencing techniques (whole genome or exome sequencing), assays only including one gene, and CPGx tests without publicly available variant selection information. Gene and variant coverage for the CYP enzymes, specifically, CYP2B6, CYP2D6, CYP2C9, CYP2C19, and CYP3A5, were investigated due to their well-studied gene frequencies and role in PGx practice. Variants considered for analysis of gene selection, gene coverage percentage, and detection rate were those included in PharmGKB gene frequency tables (See **Supplementary Table 1** for detailed list).

Calculation of CPGx Test Coverage Percentage

To illustrate the extent of the number of variants included in PGx tests relative to the number of known variants, a variant coverage percentage was calculated for each CPGx test. Gene coverage percentage is the total number of variants targeted by a CPGx test divided by the total known variants (Eq. 1). Total known variants were the number of listed variants within PharmGKB gene frequency tables.

$$\text{CPGx test coverage percentage} = \frac{\text{Number of variants targeted}}{\text{Total number of known variants}} \text{ x } 100$$

Equation 1: Calculation of CPGx test coverage percentage. The number of variants targeted was found by identifying which variants each CPGx test selected from a search using the GTR and other resources. The total number of chosen variants were then summed, in which the total number of known variants was the number of listed variants within the PharmGKB gene frequency tables.

Calculation of Detection Rate

Diplotype to phenotype translation and diplotype frequencies were obtained from the PharmGKB database for CYP2B6, CYP2D6, CYP2C9, CYP2C19, and CYP3A5. These reported frequencies include nine different ethnic groups. The diplotypes included in PharmGKB lists are based on the Pharmacogene Variation (PharmVar) database and the resulting diplotypes from their listed core variants (Gaedigk et al., 2018). These resources, combined with PGx test information from the GTR, were used to calculate the detection rate of CPGx tests in the different ethnic groups.

Potential diplotypes for CYP enzyme subclasses were filtered to only include those that predict "altered metabolizer" phenotypes. A genotype-predicted phenotype is considered an "altered metabolizer" when it results in a required alteration of medication therapy to dosage or medication choice according to published CPIC guidelines (Birdwell et al., 2015; Hicks et al., 2015; Bell et al., 2017; Hicks et al., 2017; Desta et al., 2019; Theken et al., 2020; Crews et al., 2021; Karnes et al., 2021; Lima et al., 2021). Genotype-predicted phenotypes leading to potential altered metabolizer status are unique to each CYP enzyme sub-group (**Table 1**). Even though, predicted phenotype does not guarantee adverse drug reactions or suboptimal outcomes, in a pre-emptive approach the resulting predicted phenotypes would influence future therapy decisions. Thus, for our study, we rely on these genotype-predicted phenotypes to define "altered metabolizers."

Population altered metabolizer frequency is the sum of all altered metabolizer diplotype frequencies for each ethnic group. Detectable altered metabolizer frequency represents the total frequency of altered metabolizers, where a given CPGx test contains both gene variants within its diplotype (detectable diplotypes). Detection rate is the proportion of individuals with altered metabolizing genotype predicted phenotypes that have detectable diplotypes. Therefore, it is calculated by dividing the detectable altered metabolizer frequency by the population altered metabolizer frequency (Eq. 2). Each ethnic group represented in gene frequency tables has a detection rate value calculated for each CPGx test.

$$\text{Detection Rate} = \frac{\text{Detectable altered metabolizer frequency}}{\text{Population altered metabolizer frequency}} \text{ x } 100$$

Equation 2: Calculation of detection rate. Altered metabolizer phenotypes are defined as genotype predicted phenotypes resulting in an alteration of medication therapy. Detectable altered metabolizer frequency is the sum of all altered metabolizer diplotypes frequencies, where both gene variants are included in the CPGx test. Population altered metabolizer frequency is the sum of all altered metabolizer diplotypes frequencies.

TABLE 1 | Genotype-predicted phenotypes for each CYP enzyme that cause alteration of medication therapy (altered metabolizing status) based on Clinical Pharmacogenomics Implementation Consortium (CPIC) guidelines.

CYP enzyme	Genotype-predicted phenotypes considered an altered metabolizer status	CPIC Guidelines Referenced
CYP2B6	Intermediate and poor metabolizers	Efavirenz and efavirenz-Containing Antiretroviral Therapy
CYP2C19	Ultra-rapid, rapid, likely intermediate, intermediate, likely poor, and poor metabolizers	Proton Pump Inhibitors
		Selective Serotonin Reuptake Inhibitors
		Tricyclic Antidepressants
		Clopidogrel
CYP2C9	Activity scores less than 2	Phenytoin
		Fosphenytoin
		NSAIDs
CYP2D6	Activity scores less than 1.25 and greater than 2.25	Opioids
		Selective Serotonin Reuptake Inhibitors
		Tricyclic Antidepressants
		Atomoxetine
		Ondansetron
		Tropisetron
CYP3A5	Intermediate and extensive metabolizers	Tacrolimus

Association for Molecular Pathology Tier 1 and Tier 2 recommendations were evaluated for CYP2C9 and CYP2C19. Detection rate was calculated for each tier in the same way it was calculated for prospective CPGx tests. These results show the extent these recommended tiers are representative of diverse ethnic groups and allow for comparison of current PGx tests against AMP recommendations. Since only publicly available datasets were used and no animal study was conducted, this study did not require IRB or IACUC approval. All analysis codes are available at https://github.com/sayer108/CPGx_test_evaluation.

RESULTS

Genetic Testing Registry Search Results

A search of the GTR showed 178 potential PGx tests, 56 of them being CPGx tests (**Figure 1A**). Gene selection information was publicly available for 25% of CPGx tests, only 14 of 56 of them. CYP2C9, CYP2C19, and CYP2D6 were selected by 11 CPGx tests; only six CPGx tests covered CYP2B6. The most commonly selected gene targets in CPGx tests were CYP enzymes, with CYP2D6 and CYP2C19 being selected by 50 different tests (**Figure 1B**; Diagnostics R, 2021; Genetics C, 2021; Genetics H, 2021; Genomics A, 2021; Health A, 2021a,b; Incorporated CH, 2021; Indiana University School of Medicine DoDG-PL, 2021; Invitae, 2021; Laboratories KD, 2021; Lineagen, 2021; OneOme, 2021; Services PML, 2021).

Prevalence of Altered Metabolizing CYP Phenotypes

Altered metabolizer genotype predicted phenotypes make up a significant proportion of population for all ethnic groups analyzed within each CYP enzyme subclass (**Figure 2**). The average altered metabolizer frequencies (range) across ethnic groups for each CYP enzyme subclass were CYP2B6 67% (50–87%), CYP2C19 62% (37–96%), CYP2C9 26% (9–40%), CYP2D6 23% (32–57%), and CYP3A5 44% (14–73%).

Coverage Percentage and Detection Rate of Pharmacogenomic Tests

The detection rate of PGx tests was calculated for 14 CPGx tests that covered one or more of the following CYPs: CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A5. The detection rate of CPGx tests varied significantly, with values ranging from 24 to 100%. The average overall detection rate and range of values of detection rate of the CPGx tests for all ethnic groups within each CYP enzyme sub-group were CYP2B6 (77.6%, 43–100%), CYP2C9 (88.4%, 24–100%), CYP2C19 (92.3%, 32–100%), CYP2D6 (81.4, 56–100%), and CYP3A5 (100%, 100–100%; **Supplementary Tables 2–8**; **Supplementary Figure 2**).

The PharmGKB lists 61 potential variants for CYP2C9; gene coverage percentage ranged from 4.9 to 23% for CPGx tests covering CYP2C9. The detection rate for the East Asian (EA) population was lower than the rest of the ethnic groups, with an average of 55% across CPGx tests. All other ethnic groups had an average detection rate of 70% or higher. AMP Tier 1 and Tier 2 recommendations also had very low detection rates for the EA population, 51 and 55%, respectively. Both tiers had detection rates of 95% or higher for all other ethnic groups (**Figure 3A**; **Supplementary Tables 1, 3,** and **8**). The highest performing CPGx test among East Asians was the RPRD assay, with a detection rate of 92%. However, for other ethnic groups like Sub-Saharan African (SSA) and African-American Afro-Caribbean (AAAC) populations the CPGx test with the highest detection rate was the Admera assay with a detection rate of 99% (**Supplementary Table 8**). Similarly, the PharmGKB lists 137 potential variants for CYP2D6 and tests analyzed have gene coverage percentage ranging from 15 to 54%. Average detection rates for all CPGx tests with respect to all ethnic groups ranged from 70 to 90%, with no obvious outliers (**Figure 3B**; **Supplementary Tables 1** and **4**). All ethnic groups had the highest detection rate with the RPRD assay except for East Asian populations, where the OneOme assay had the highest detection rate. PharmGKB lists 32 potential variants for CYP2C19 with gene coverage percentages ranging from 5.7 to 75%. The detection rate for the SSA and AAAC populations was lower than the rest of the ethnic groups, 77, and 85%, respectively. The rest of the ethnic groups had average detection rates of 95% or higher for all CPGx tests. RPRD had the highest detection rate for AAAC and SSA populations at 100%. Many of the Ethnic groups had 100% detection rates with several different assays studied. AMP recommended Tier 1 and Tier 2 variants had detection rate values of 95% or higher for all ethnic groups except the SSA and AAAC populations. Tier 1 had a detection rate value of 75% for the SSA population, much lower than Tier 2 at 93% (**Figure 3C**; **Supplementary Tables 1, 5,** and **8**). The PharmGKB lists 38 potential variants for CYP2B6; gene coverage percentage ranged from 7 to 26.3%. The SSA population had an average detection rate of 63.9% for all CPGx tests, the lowest compared to all ethnic groups. The rest of the groups had average detection rates greater than 70% for all CPGx tests (**Figure 3D**; **Supplementary Tables 1** and **6**). The RPRD assay performed best for the SSA population with a detection rate of 100%. Results for CYP3A5 showed a 100% detection rate for all CPGx tests (**Supplementary Tables 2, 7,** and **8**).

DISCUSSION

In this study, the ranges of detection rates were evaluated for currently available CPGx tests to demonstrate the variability that can occur depending on ethnic background and PGx test selection. Our results showed some ethnic groups clearly have higher and more consistent detection rate scores across CYP enzymes compared to others. This demonstrates that variant selection for the chosen assay can favor some populations more than others. Additionally, even patients of the same ethnic background can receive CPGx tests with drastically different detection rates due to variant selection.

The calculated detection rate of CPGx tests reflected that certain ethnic groups had diminished detection rate values for almost all the CPGx tests analyzed. For instance, if providers

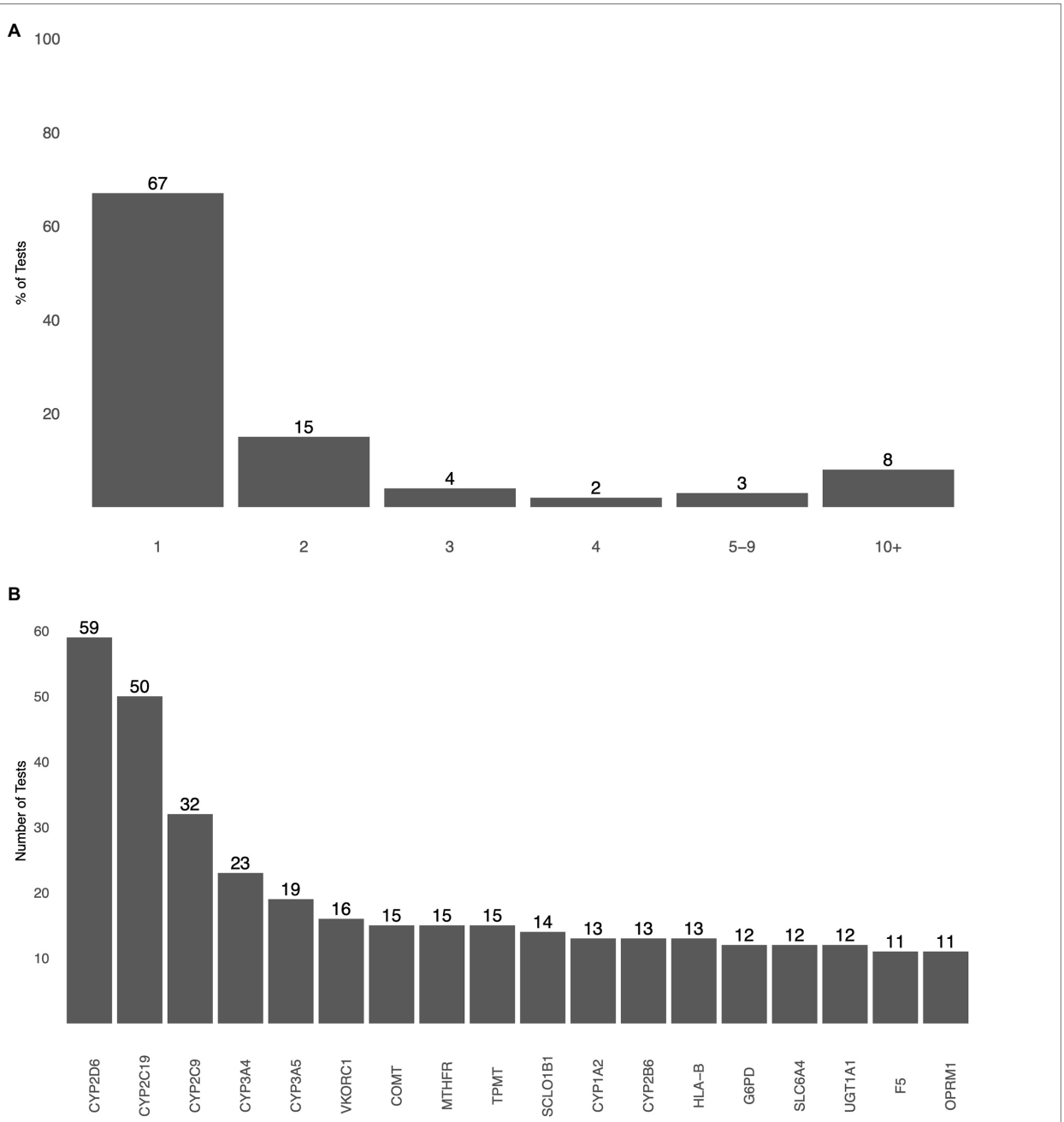

FIGURE 1 | (A) The total number of genes selected by pharmacogenomics (PGx) tests as detailed by the test information included in the Genetic Testing Registry (GTR). **(B)** Most commonly selected genes by PGx tests based on gene selection information included in GTR.

seeking to test patients of East Asian descent for CYP2C9 metabolizing status utilized any one of the CPGx tests, the patients metabolizing status would likely be mischaracterized. Additionally, our results show a large variance in PGx test performance, meaning clinicians basing decisions on current test results could be depending on incorrectly characterized phenotypes. The greatest example of this variance was observed with respect to the Oceanian (Oc) population and CYP2D6. While average detection rate was greater than 80% for CPGx tests, there were multiple tests with detection rates between 30 and 40%. If clinicians are aware of the limitations of selective CPGx tests with respect to different ethnic groups and their variability, they can select an alternative; thus, patients will be less likely to experience sub-optimal therapeutic outcomes.

Evaluation of AMP recommended Tier 1 and Tier 2 alleles showed consistent detection rates of 90% or higher across ethnic groups with a few notable exceptions. Specifically, East Asian and SSA populations had significantly lower detection rates for Tier 1 and 2 recommendations for CYP2C9 and CYP2C19, respectively. In these cases, there are a significantly

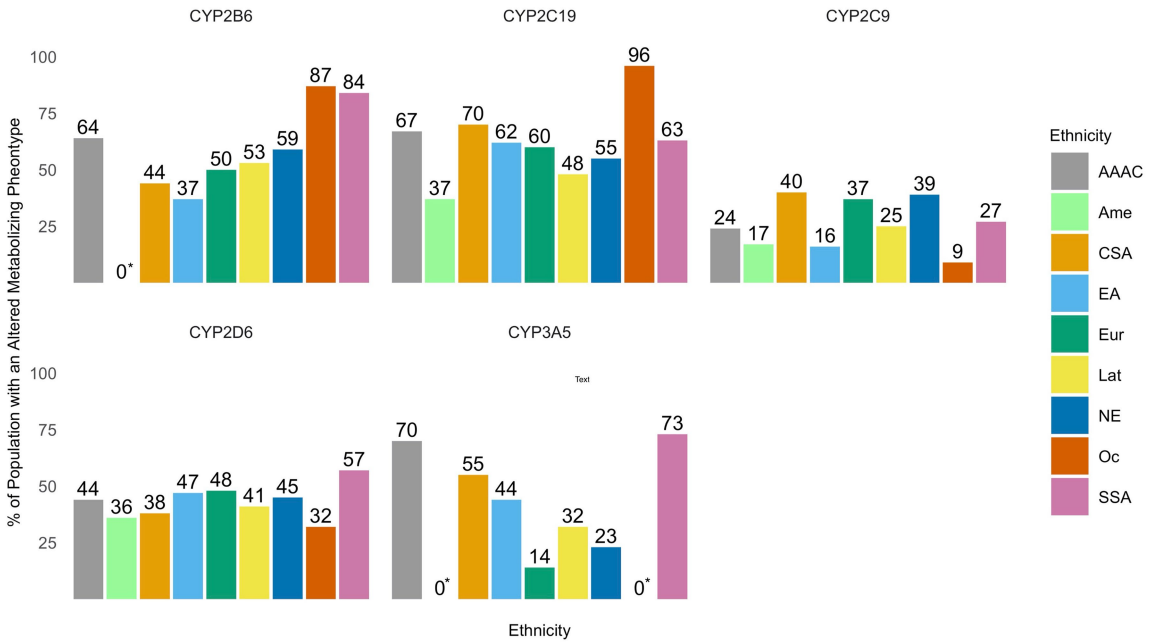

FIGURE 2 | The prevalence of altered metabolizing phenotypes of cytochrome P450 (CYP) enzymes across various ethnic groups. Abbreviations for ethnic groups are as follows: AAAC, African American/Afro-Caribbean; Amer, American; CSA, Central/South Asian; EA, East Asian; Eur, European; Lat, Latino; NE, Near Eastern; Oc, Oceanian; and SSA, Sub-Saharan African. *Values of 0 are due to the lack of variant frequencies documented in the Pharmacogenomics Knowledge Base (PharmGKB) database for a specific ethnic group.

higher number of relevant gene variants compared to the other populations. Despite these exceptions, it was concluded that the results produced support the implementation of standardized gene targets for targeted PGx tests.

Our results demonstrate additional measures that can be taken to further increase the clinical utility of CPGx guided medication therapy. For three out of the five CYP enzyme genes evaluated, peak detection rates were achieved with different CPGx tests depending on ethnic background because of gene selection variations (**Supplementary Table 8**). Additionally, while higher detection rates were often achieved by tests selecting a higher number of variants, tests selecting fewer variants achieved the same detection rate in many instances. This is due to the fact that low frequency variants contribute minimally to the overall detection rates. These results show providers considering ethnic background in CPGx test selection can ensure their patients' have the best chance to have their phenotypes correctly identified. Physicians and pharmacists can better identify situations when more selective tests are sufficient and when broader coverage is needed. Careful consideration of CPGx test selection with respect to ethnicity and variant selection has the potential to improve patient care by better charactering altered metabolizing phenotypes. With the growing emphasis on personalized patient care, our results show meaningful ways to further individualize genetic testing and ultimately improve outcomes.

Our results also provide insight into how the clinical utility of informatics approaches can be enhanced. The findings of this study can be potentially implemented in operationalizing PGx test ordering, data sharing, and cascade testing with an integrative informatics approach (Roosan et al., 2021). We demonstrated altered metabolizers are very prevalent in diverse patient populations, and some ethnic populations have a significantly higher proportion of altered metabolizers. This gene variance implies some ethnic populations may be more likely to benefit from the CPGx test. This is consistent with recent data from PGx gene frequency studies in large diverse populations (McInnes et al., 2021). If the patient ethnic background was included in models predicting patients in need of PGx testing, it could improve the models' performance. Studies have demonstrated ethnicity can be a predictive factor in disease progression and medication efficacy with respect to various cardiovascular diseases and more effective interventions can be initiated when it is considered (Taylor and Wright, 2005). Besides, incorporating ethnic background into the choice of CPGx test can assure more reliable PGx results. Patients in need of PGx tests with expanded gene coverage can be identified, ensuring metabolizing status is assessed correctly. Thus, incorporating factors of ethnicity and ethnicity in informatics tools can improve patient selection for PGx testing and PGx test choice.

This study chose to evaluate CPGx tests due to the emerging evidence showing they are needed to optimize clinical outcomes. Studies comparing CPGx testing against single-gene testing to guide medication decisions for patients with major depressive disorder demonstrated that combinatorial approaches better predicted phenotypes and clinical outcomes than single-gene tests (Winner and Dechairo, 2015). Additionally, CPGx testing approaches provide better opportunities for preemptive PGx practice. In a sample of 10,000 patients preemptively genotyped

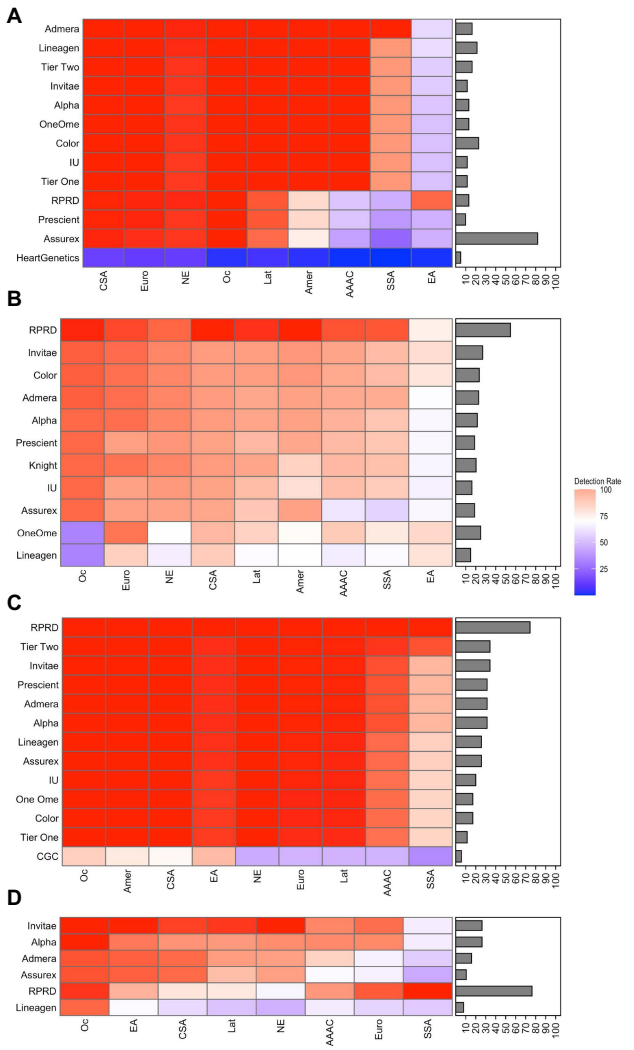

FIGURE 3 | Heatmaps displaying detection rate values and coverage percentage of PGx tests covering **(A)** CYP2C9, **(B)** CYP2D6, **(C)** CYP2C19, and **(D)** CYP2B6. The brighter the red, the higher the detection rate. On the contrary, the darker blue, the lower the detection rate. In addition to the PGx tests, detection rate was calculated for selected Tier 1 and Tier 2 alleles of Association for Molecular Pathology (AMP) recommendations for CYP2C9 and CYP2C19. PGx test coverage percentage is included with each heatmap as a bar chart on the right side of each heatmap. Abbreviations for ethnic groups listed on the bottom of the figure are as follows: Amer, American; CSA, Central/South Asian; EA, East Asian; Eur, European; Lat, Latino; NE, Near Eastern; Oc, Oceanian; and SSA, Sub-Saharan African.

within the PREDICT program with Vanderbilt University, 91% had at least one actionable variant, and 42% of these patients had been exposed to a risk-associated medication in the past (Hockings et al., 2020). Given the growing body of evidence supporting the clinical benefits of CPGx testing, it is necessary to evaluate these assays.

Utilizing reported racial and ethnic backgrounds in healthcare decision-making is challenging for a variety of reasons. A recent study suggests that ethnicity-based PGx decision-making is limited by intrapopulation genetic variation and fluidity (Goodman and Brett, 2021). Additionally, concerns have been expressed regarding medical decision tools incorporating ethnicity having bias that causes sub-optimal therapeutic outcomes for patients of different ethnic backgrounds (Vyas et al., 2020). However, the FDA and current PGx consortiums have adopted ethnicity-based recommendations for PGx screenings (Chang et al., 2020). This practice has been adopted as a common practice with respect to other disease states. For example, ties between familial genetic history and breast cancer risk have been well documented and this information has been included in decision-making guidelines to screen patients as well (Owens et al., 2019). Until universal PGx testing is widespread, using genetic ancestry can prioritize patients most likely to benefit from a more appropriate CPGx test representing known genetic variations.

There are several limitations to this study. With respect to the availability of data, our evaluation only included voluntarily shared commercial clinical laboratory data, which totaled 14 CPGx tests, 24% of total tests found. This is comparable to an evaluation done on direct-to-consumer genetic tests in 2017, which found only 20% of tests reporting gene selection data (Hall et al., 2017). Therefore, our evaluation is biased by only voluntarily shared data. Gene frequency information is only sufficiently studied/reported in PharmGKB to perform our analysis on CYP enzymes, even though, there are several other genes commonly used in PGx practice.

Including all variants within PharmGKB regardless of their function or frequency in our analysis also has its limitations. For instance, CYP2D6 has nearly 150 variants identified with less than half of them having reported gene frequencies or known metabolic function. While a test may report a very low gene coverage percentage, it may include several common clinically relevant genes to achieve high detection rate. Sound gene selection practices would be reflected in higher detection rate for that test in a specific ethnic population. We considered all known variants reported, rare or frequent, in calculating detection rate. However, detection rates were not considerably influenced by rare variants with unknown or very low frequencies. Diplotype combinations occurring at very low frequencies relative to other genes contribute very little to the total altered metabolizer phenotype frequency in a population. Coverage percentages indicate the extent of known variants included in an assay, while detection rates indicate how well an assay captures the overall diversity of the gene variants in a population. Therefore, both gene coverage percentage and detection rate together provide useful insight into gene variant selection practices for CPGx tests.

Our results highlight the prevalence of false-negative test results when common gene variants are not included in CPGx tests. Recent evaluations of the performance of sequencing technologies utilized in CPGx tests demonstrate they have precision and accuracy values greater than 99.9% (Jablonski et al., 2018). While labs are required to meet standards of analytical performance, they are not evaluated based on the genes they select. Thus, high analytical performance measures can lead to a false sense of security for consumers and providers if relevant genes are not included in the assay. Conflict with respect to false negative test results has come forward with respect to direct-to-consumer genetic tests and breast cancer screenings. Many of the home

testing kits incorporate BRCA variants specific to Ashkenazi-Jewish descent, while not including other variants more common in diverse populations leading to false negatives (Landi, 2019). Our data show that this oversight leads to potential false negative results in CPGx tests used in pharmacogenomics practice as well. These false-negatives may significantly impact treatment decisions for patients. Our work supports the continued implementation of institutions like AMP creating recommended genes to include in assays as a standard to ensure reliable results. However, the landscape needs to be continually evaluated as gene frequency and gene functionality information becomes more readily available.

In summary, the proportions of altered metabolizers in a given population can range from 10 to 90% depending on the CYP enzyme sub-class. The majority of assays targeted less than 50% of known gene variants listed in the PharmGKB gene frequency tables. Calculated detection rates of CPGx tests showed high variation across different ethnic groups. Therefore, the patient's ethnicity receiving the PGx test and the variants targeted by the test should be carefully considered to ensure the optimal utility of PGx.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**. All analysis codes are available at https://github.com/sayer108/CPGx_test_evaluation.

AUTHOR CONTRIBUTIONS

MS, ML, KP, JV, BV, and DP performed research, analyzed the data, and wrote the manuscript. AD and TN performed research, analyzed the data, and reviewed the manuscript. RB and DR analyzed the data and gave critical feedback on the manuscript. MR designed the study, reviewed data analysis, gave critical feedback on manuscript writing, and supervised the research. All authors contributed to the article and approved the submitted version.

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Pharmacogenomics Factors Influencing the Effect of Risperidone on Prolactin Levels in Thai Pediatric Patients With Autism Spectrum Disorder

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We investigated the association between genetic variations in pharmacodynamic genes and risperidone-induced increased prolactin levels in children and adolescents with autism spectrum disorder (ASD). In a retrospective study, variants of pharmacodynamic genes were analyzed in 124 ASD patients treated with a risperidone regimen for at least 3 months. To simplify genotype interpretation, we created an algorithm to calculate the dopamine D2 receptor (*DRD2*) gene genetic risk score. There was no relationship between prolactin levels and single SNPs. However, the H1/H3 diplotype (A2/A2-Cin/Cin-A/G) of *DRD2*/ankyrin repeat and kinase domain containing 1 (*ANKK1*) *Taq*1A, *DRD2* -141C indel, and *DRD2* -141A>G, which had a genetic risk score of 5.5, was associated with the highest median prolactin levels (23 ng/ml). As the dose-corrected plasma levels of risperidone, 9-OH-risperidone, and the active moiety increased, prolactin levels in patients carrying the H1/H3 diplotype were significantly higher than those of the other diplotypes. *DRD2* diplotypes showed significantly high prolactin levels as plasma risperidone levels increased. Lower levels of prolactin were detected in patients who responded to risperidone. This is the first system for describing *DRD2* haplotypes using genetic risk scores based on their protein expression. Clinicians should consider using pharmacogenetic-based decision-making in clinical practice to prevent prolactin increase.

Keywords: risperidone, prolactin, autism spectrum disorder, genetic risk score, dopamine D2 receptor (DRD2)

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INTRODUCTION

Risperidone is an atypical antipsychotic used to treat autism spectrum disorder (ASD). Its effect is mediated via dopamine D2 receptor and 5-hydroxytryptamine type 2A receptor antagonism (Corena-McLeod, 2015). The US Food and Drug Administration has approved risperidone for the treatment of irritability in children and adolescents aged 5–16 years with ASD (Goel et al., 2018). Risperidone has a more favorable safety and efficacy profile than typical antipsychotic drugs (Peuskens et al., 2014). Two clinical trials (McCracken et al., 2002; Shea et al., 2004) established the efficacy and tolerability of risperidone in patients with ASD and showed that risperidone significantly attenuated disruptive behaviors compared with the placebo over 8 weeks, as measured by a reduction in the irritability subscale of the aberrant behavior checklist (ABC) scores. The improvement in the risperidone treatment group was higher (56.9–64.0%) compared with that in the placebo group (14.1–31.0%) (McCracken et al., 2002; Shea et al., 2004).

Several studies (Baumann et al., 2004; Riedel et al., 2005) have explored the utility of plasma drug monitoring as a biomarker of treatment response. Therapeutic drug monitoring can improve the efficacy and safety of risperidone. Plasma levels of 20–60 ng/L of the active moiety lead to better clinical outcomes in adults with schizophrenia (Baumann et al., 2004; Nazirizadeh et al., 2010). Plasma levels of the active moiety in non-responders were significantly higher than in responders in a 6 weeks clinical trial of risperidone treatment in schizophrenia patients (Riedel et al., 2005). However, no therapeutic drug monitoring data are available for pediatric patients with ASD treated with risperidone.

There is considerable variation in the response to risperidone that may be explained, at least partly, by genetic variation in drug targets. A polymorphism in the dopamine D2 receptor (*DRD2*)/ankyrin repeat and kinase domain containing 1 (*ANKK1*) gene (often referred to as the *Taq*1A polymorphism; rs1800497) was associated with a clinical response in risperidone-treated ASD patients (Nuntamool et al., 2017). The *DRD2/ANKK1 Taq*1A A2 or C allele (Sukasem et al., 2016) have been associated with high dopamine receptor densities (Jonsson et al., 1999), which may contribute to blocking dopaminergic activity. *DRD2* -241A>G (rs1799978), located in the 5′-promoter region of *DRD2*, may contribute to increased expression levels. (Nyman et al., 2009). *DRD2* -141C insertion/insertion has been associated with high prolactin levels in antipsychotic-treated male schizophrenia (Zhang et al., 2011). Carriers of the dopamine D3 receptor Gly/Gly allele (rs6280) showed significantly better response rates compared with the Ser/Ser genotype in children with ASD (Firouzabadi et al., 2017). Genetic variation in the 5-hydroxytryptamine type 2A receptor (*HTR2A*), 5-hydroxytryptamine type 2C receptor (*HTR2C*), and ATP binding cassette subfamily B member 1 genes also contribute to clinical outcomes and are possible markers for predicting a positive response to risperidone therapy in ASD (Correia et al., 2010). Moreover, the dopamine transporter [*DAT*; also known as solute carrier family 6 member 3 (SLC6A3)] and serotonin transporter [5-hydroxytryptamine transporter-linked promoter region (*5-HTTLPR*) also known as solute carrier family 6 member 4] genes, although not directly targeted by antipsychotic medications, may influence neurotransmitter availability, and thus contribute to the variability in treatment response (Kirchheiner et al., 2007; Porcelli et al., 2012; Outhred et al., 2016).

The anterior pituitary hormone prolactin has essential physiological functions in the brain. Prolactin acts as a neuropeptide, regulating neuroendocrine and emotional stress responses (Torner, 2016). Serum prolactin levels may also reflect the antipsychotic treatment response. Several clinical studies (Zhang et al., 2002; Ates et al., 2015; Stern et al., 2018) revealed that prolactin may mediate effects on the neuropsychiatric response to risperidone. Zhang et al. (2002) observed a significant positive relationship between the reduction rate of positive subscale scores of the Positive and Negative Syndrome Scale (PANSS) and the change in prolactin levels before and after treatment in chronic schizophrenia. Ates et al. (2015) found that in patients with hyperprolactinemia, the PANSS negative symptom scores were significantly higher than in patients without hyperprolactinemia (p = 0.041). Furthermore, several moderators and mediators affecting risperidone response have been described (Stern et al., 2018), and lower baseline levels of prolactin predict responder status in autistic children. Additionally, genetic polymorphisms in the prolactin (*PRL*) (Lee et al., 2007; Ivanova et al., 2017) and prolactin receptor (*PRLR*) (Lee et al., 2007) genes may also contribute to increased prolactin levels. The strongest association was between a single SNP of *PRL* (A>T, rs2244502) and prolactin levels, which showed higher prolactin levels in T carriers than in A carriers. Patients with hyperprolactinemia carried the G/G genotype of −1149 G>T (rs1341239) in the *PRL* gene more frequently than patients without hyperprolactinemia (p = 0.009) (Ivanova et al., 2017). Therefore, prolactin is a promising candidate biomarker for risperidone response.

The impact of pharmacogenetics on increased prolactin levels has not been well investigated. Stern et al. (2018) reported the effect of hyperprolactinemia in pediatric ASD patients with disruptive behaviors undergoing risperidone therapy. Therefore, in this study we investigated the association between genetic variations in pharmacodynamic genes and risperidone-induced increases in prolactin levels in children and adolescents with ASD.

MATERIALS AND METHODS

Participants

ASD children and adolescents aged 3–18 years were recruited at the Yuwaprasart Waithayopathum Child Psychiatric Hospital, Samut Prakan, Thailand, in 2017 and 2018. All participants were ethnic Thai. The clinical neuropsychiatric diagnosis was made according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition criteria. The Ethics Committee of the Faculty of Medicine Ramathibodi Hospital, Bangkok, Thailand (MURA2017/556) and Yuwaprasart Waithayopathum Child Psychiatric Hospital, Samut Prakan, Thailand approved the

study. All participants or parents of the children signed an informed assent or consent after the study objectives and procedures were explained. Sociodemographic data (gender, age at assessment, daily risperidone dosage, duration of risperidone treatment, and concomitant medication) were collected with a questionnaire. Patients were excluded if they were receiving concomitant medication that could affect risperidone metabolism (e.g., haloperidol, fluoxetine, paroxetine, carbamazepine, and phenytoin) or prolactin levels (e.g., haloperidol, sertraline, and fluoxetine).

Study Protocol

The retrospective study included 124 ASD patients treated with a risperidone-based regimen for at least 3 months. Serum prolactin levels, plasma levels of risperidone, 9-OH-risperidone, and the active moiety were measured, and the candidate genes were genotyped. We also included 19 risperidone-naïve patients who underwent a baseline assessment before starting risperidone therapy. They were available for a follow-up assessment 3–20 months after risperidone treatment was started. At the first and follow-up visits, assessments were performed using ABC subscales and serum prolactin and plasma drug levels.

Behavior Assessments

The ABC subscale assessment consisted of 58 items, divided into the following five categories of behavior: irritability, agitation, and crying (15 items); lethargy and social withdrawal (16 items); stereotypic behavior (7 items); hyperactivity and non-compliance (16 items); and inappropriate speech (4 items). The probands were rated by a primary patient's caregiver for the different severity of behavior problems from zero (no problems) to three (severe problems), with higher scores indicating problems that were more severe (Narkpongphun and Charnsil, 2018b). The ABC-irritability subscale score is an accepted gold standard for measuring irritability and aggression in medication trials for ASD (Fung et al., 2016). We used the ABC-C Thai version, which was created with a cross-cultural adaptation method, has been validated, and is highly reliable (Narkpongphun and Charnsil, 2018a). Patients with ASD were divided into responders and non-responders according to the reduction rate of the total ABC scores. Patients classified as responders had a reduction rate of total ABC scores higher or equal to 30%, whereas patients with ABC score reduction rates of less than 30% were classified as non-responders.

Serum Prolactin Measurement

A fasting morning blood sample was analyzed with a chemiluminescent immunoassay system (IMMULITE1000, Siemens Healthcare Diagnostics Products Ltd., Erlangen, Germany) at the Yuwaprasart Waithayopathum Child and Adolescent Psychiatric Hospital, Thailand.

Plasma Drug Assay

Steady-state plasma concentrations of risperidone and 9-OH-risperidone were quantified, between 8:00 and 10:00 AM, approximately 12 h after the bedtime dose, using a validated high-performance liquid chromatography method (Puangpetch et al., 2016; Vanwong et al., 2016). The active moiety levels were calculated by summing the levels of risperidone and 9-OH-risperidone. Plasma drug levels of risperidone, 9-OH-risperidone, and active moiety were corrected by the daily dose to give the dose-corrected risperidone (RIS C/D), dose-corrected 9-OH-risperidone (9-OH-RIS C/D), and dose-corrected active moiety (active moiety C/D).

Pharmacogenetic Testing

Genomic DNA was obtained from EDTA blood using the MagNa Pure automated extraction system according to the manufacturer's instructions (Roche Diagnostics, Basel, Switzerland). The DNA samples were subsequently genotyped for the following sequence variations: *DRD2/ANKK1 Taq*1A A2>A1 (rs1800497), *DRD2* -141C indel (rs1799732), and -241A>G (rs1799978); *HTR2A* -1438G>A (rs6311); *HTR2C* -759C>T (rs3813929); and *PRL* 13096T>A (rs2244502), and *PRLR* 163444A>C (rs37364). SNPs were selected based on functional significance (Kirchheiner et al., 2007; Correia et al., 2010; Porcelli et al., 2012; Outhred et al., 2016; Firouzabadi et al., 2017; Nuntamool et al., 2017) and minor allele frequencies of >0.05 across the Asian population. All SNPs were genotyped using the commercially available TaqMan Drug Metabolism Genotyping assay (Life Technologies, Carlsbad, CA, United States). Genotyping was carried out as recommended by the manufacturer using a real-time PCR system (ViiA7, Applied Biosystems, Life Technologies).

To determine the number of variable tandem repeats (VNTRs) of *DAT*, 60 ng genomic DNA was amplified in 25 µl PCR reactions, containing 2X Green PCR master mix (12.5 µl, BiotechRabbit, Hennigsdorf, Germany), and 1 µl of each 10 µM primer (forward, 5′-TCCTTGCGGTGTAGGGAACG-3′; reverse 5′-CCAGGCAGA GTGTGGTCTG-3′). Denaturation at 95°C for 2 min was followed by 35 cycles of 95°C for 30 s, 65°C for 40 s, and 72°C for 1 min, and a final extension step at 72°C for 10 min. The fragment sizes were 263 bp (5 repeats), 423 bp (9 repeats), 463 bp (10 repeats), and 503 bp (11 repeats), respectively. For *5-HTTLPR*, 8 µl PCR reactions contained 4 µl KAPA 2G^{TM} Fast ReadyMix (KAPA Biosystems, Woburn, MA, United States), 0.6 µl of each 5 µM primer (forward, 5′-CACAAACATGCTCATTTAAGAAGTG-3′; reverse, 5′-AAA GGAAATAGCAGTGACAAGTTTG-3′), and 20 ng genomic DNA. PCR was initiated by a 2 min incubation at 95°C, which was followed by 40 cycles of 95°C for 15 s, 62°C for 40 s, and 72°C for 30 s, and a final extension at 72°C for 1 min. Amplicons representing the short (733 bp) and long (777 bp) alleles were separated by 2% agarose gel electrophoreses.

Genetic Risk Score

We created an algorithm to describe haplotypes with genetic risk scores. For protein expression affecting the prolactin level, the alleles *DRD2 Taq*1A, -141C indel, and -241A>G had values of 1 assigned for a high-expression allele (A2, Cin, G) and 0.5 for a low-expression allele (A1, Cdel, A) (Arinami et al., 1997; Jonsson et al., 1999; Nyman et al., 2009). The score of each haplotype was the sum of the values assigned to each allele. A high risk score indicated a high prolactin level.

TABLE 1 | Patient demographics (n = 124).

Clinical information	Median (IQR)
Age (years)	8.00 (5.00–12.00)
Males, *n* (%)	105 (84.68)
Daily risperidone dosage (mg/day)	0.75 (0.50–1.00)
Risperidone treatment duration (months)	37.94 (11.01–94.05)
Risperidone monotherapy, *n* (%)	74 (59.68)
Prolactin level (ng/ml)	15.70 (8.85–22.85)
RIS level (ng/ml)	0.51 (0.14–1.41)
9-OH-RIS level (ng/ml)	5.28 (2.94–9.29)
Active moiety level (ng/ml)	6.11 (3.44–11.63)
Ratio of risperidone/9-OH-RIS	0.09 (0.03–0.21)
RIS C/D (ng/ml/mg)	0.71 (0.22–2.03)
9-OH-RIS C/D (ng/ml/mg)	7.72 (4.94–12.04)
Active moiety C/D (ng/ml/mg)	9.06 (5.82–13.14)

RIS, risperidone; 9-OH-RIS, 9-hydroxy-risperidone; Active moiety, the sum of RIS plus 9-OH-RIS; C/D, dose-corrected concentration; IQR, interquartile range [quartile 1 (Q1) and quartile 3 (Q3)].

Statistical Analysis

Statistical analyses were carried out using SPSS v24 for Windows (IBM, Armonk, NY, United States). Statistical significance is reported as $p < 0.05$ for a two-tailed distribution. Descriptive analyses were performed for the sociodemographic variables. Data are expressed as mean (standard deviation; SD) or median (interquartile range; IQR) in normal or non-normal distribution data, respectively. Parametric analysis of variance (comparisons between more than two groups) and Student's *t*-test (comparisons between two groups) were used to assess at each time point the association between prolactin or drug levels and the genotypes. The nonparametric Kruskal-Wallis (comparisons among more than two groups) and Mann-Whitney (comparisons between two groups) tests were used to assess the association between prolactin or drug levels and the genotypes at each time point. The nonparametric Spearman rank correlation test was used to measure the correlation between serum prolactin level and plasma drug level. Because nine variants were tested, the *p*-value significance threshold was adjusted for multiple comparisons. Bonferroni's correction was applied to adjust for multiple comparisons. According to Bonferroni's procedure, the corrected *p*-values were calculated by multiplying the *p*-values by 9 for the numbers of variants. The significance threshold of the corrected *p*-values was set as 0.05 (Yi et al., 2014). The Hardy-Weinberg equilibrium, allele, and genotype frequencies of all candidate SNPs were analyzed using Haploview v4.2 (Broad Institute, Cambridge, MA, United States). PHASE v2.1.1 was used to reconstruct haplotype pairs on the same chromosome (Stephens and Scheet, 2005). Fisher's exact test was used to compare the difference in patient characteristics between responders and non-responders in children and adolescents with ASD. Differences in serum prolactin levels between the *DRD2* diplotypes were assessed by analysis of covariance (ANCOVA), controlling for plasma drug level as a covariate. Receiver operating characteristic (ROC) curves were analyzed and plotted. Performance parameters such as sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of the association between serum cut-off value of serum prolactin levels and status of risperidone response were analyzed using MedCalc (https://www.medcalc.org/calc/diagnostic_test.php).

RESULTS

Clinical Characteristics

Our sample cohort consisted of 124 children and adolescents with a mean age of 8.81 years (*SD* = 4.04). All patients were diagnosed with ASD and treated with risperidone. Seventy-four patients (59.68%) received risperidone monotherapy. The remaining patients received concomitant medications that did not affect cytochrome P450 2D6 metabolite and prolactin levels. Demographic data are summarized in **Table 1**.

Association Between Genetic Variations, Serum Prolactin Levels, and Response to Risperidone

Genotype frequencies of the tested polymorphisms are shown in **Supplementary Table S1**. Considering codominant, dominant, and recessive genetic models, there were no differences in serum prolactin levels for *DRD2*/*ANKK1 Taq*1A A2>A1, *DRD2* -141C indel, *DRD2* -241A>G, *HTR2A*-1438G>A, *HTR2C* -759C>T, *PRL* g.13096T>A, *PRLR* g.163444A>C, and the number of variable tandem repeats of *DAT* and *5-HTTLPR*.

Haplotypes were constructed using three SNPs of *DRD2* on chromosome 11 (*DRD2*/*ANKK1 Taq*1A A2/A1, *DRD2* -141C indel, and -241A>G) with PHASE v2.1.1. Six haplotypes with minor allelic frequencies of >1% were identified. The four main haplotypes were A2-Cin-A (H1), A1-Cin-A (H2), A2-Cin-G (H3), and A2-Cdel-A (H4), accounting for 91.4% of the observations (**Table 2**). Fifteen diplotypes accounted for 99.2% of the observations (**Table 3**). We compared the association of each pair of diplotype groups. Participants with the H1/H3 diplotype showed significantly higher prolactin levels than those of other diplotypes (23.00 ng/ml, $p < 0.05$).

We created an algorithm to describe a haplotype with a genetic risk score based on *DRD2* expression of protein affecting the prolactin level. Forty-five (36.29%) patients had a common risk score of 4.5 and had a median prolactin level of 16.60 ng/ml. We compared the common risk score and found that prolactin levels in patients with a common genetic

TABLE 2 | *DRD2* haplotype frequencies predicted by computational phasing using PHASE v2.1.1.

Type	Haplotype	Observation (n = 124)	Frequency (%)
H1	A2-Cin-A	86	34.68
H2	A1-Cin-A	75	30.24
H3	A2-Cin-G	35	14.11
H4	A2-Cdel-A	31	12.50
H5	A1-Cdel-A	11	4.44
H6	A1-Cin-G	10	4.03

Haplotype presented as DRD2/ANKK1 Taq1A, DRD2 -141C indel, and DRD2 -241A>G.

TABLE 3 | Associations between *DRD2* gene diplotypes and serum prolactin levels.

Types	Diplotypes	Observation (*n* = 124)	Percent (%)	Genetic risk score	Prolactin levels (ng/ml)
H2/H6	A1/A1-Cin/Cin-A/G	4	3.23	4.5	29.40 (15.65–67.70)
H3/H3	A2/A2-Cin/Cin-G/G	3	2.42	6	28.20 (16.00–29.75)
H2/H5	A1/A1-Cin/Cdel-A/A	3	2.42	3.5	25.90 (21.80–31.15)
H1/H3	A2/A2-Cin/Cin-A/G	12	9.68	5.5	23.00 (17.50–35.25)[a,b,c,d]
H3/H5 or H4/H6	A2/A1-Cin/Cdel-A/G	4	3.23	4.5	16.90 (13.15–25.30)
H1/H5 or H2/H4	A2/A1-Cin/Cdel-A/A	17	13.71	4	16.80 (12.15–21.80)[a]
H1/H4	A2/A2-Cin/Cdel-A/A	14	11.29	4.5	16.25 (10.10–24.00)
H3/H4	A2/A2-Cin/Cdel-A/G	2	1.61	5	14.40 (8.00–20.80)
H1/H6 or H2/H3	A2/A1-Cin/Cin-A/G	17	13.71	5	13.30 (7.50–21.40)[b]
H1/H2	A2/A1-Cin/Cin-A/A	23	18.55	4.5	13.00 (7.80–19.40)[c]
H1/H1	A2/A2-Cin/Cin-A/A	14	11.29	5	13.00 (9.10–24.00)
H4/H5	A2/A1-Cdel/Cdel-A/A	1	0.81	3.5	12.40
H2/H2	A1/A1-Cin/Cin-A/A	10	8.06	4	10.60 (7.40–21.80)[d]

Diplotype presented as DRD2/ANKK1 Taq1A, DRD2 -141C indel, and DRD2 -241A>G.
[a]*Significant at* p = *0.042 when compared between H1/H3 and H1/H5 or H2/H4.*
[b]*Significant at* p = *0.028 when compared between H1/H3 and H1/H6 or H2/H3.*
[c]*Significant at* p = *0.014 when compared between H1/H3 and H1/H2.*
[d]*Significant at* p = *0.038 when compared between H1/H3 and H2/H2.*

TABLE 4 | Associations between genetic risk scores for *DRD2* gene haplotypes and serum prolactin levels.

Genetic risk score	Diplotypes	Types	N (%) (*n* = 124)	Prolactin levels (ng/ml)	*p*-value
3.5	H2/H5, H4/H5	A1/A1-Cin/Cdel-A/A, A2/A1-Cdel/Cdel-A/A	4 (3.23)	21.80 (15.05–31.15)	0.231
4	H1/H5 or H2/H4, H2/H2	A2/A1-Cin/Cdel-A/A, A1/A1-Cin/Cin-A/A	27 (21.77)	12.70 (8.00–21.60)	0.504
4.5	H1/H2, H1/H4, H2/H6, H3/H5 or H4/H6	A2/A1-Cin/Cin-A/A, A2/A2-Cin/Cdel-A/A, A1/A1-Cin/Cin-A/G, A2/A1-Cin/Cdel-A/G	45 (36.29)	16.60 (9.00–22.70)	Reference
5	H1/H1, H1/H6 or H2/H3, H3/H4	A2/A2-Cin/Cin-A/A, A2/A1-Cin/Cin-A/G, A2/A2-Cin/Cdel-A/G	33 (26.61)	13.00 (8.00–21.40)	0.498
5.5	H1/H3	A2/A2-Cin/Cin-A/G	12 (9.68)	23.00 (17.50–35.25)	0.033[a]
6	H3/H3	A2/A2-Cin/Cin-G/G	3 (2.42)	28.20 (16.00–29.75)	0.413

Diplotype presented as DRD2/ANKK1 Taq1A, DRD2 -141C indel, and DRD2 -241A>G respectively as follow: high expression allele (A2, Cin, G) = 1 and low expression allele (A1, Cdel, A) = 0.5. A high-risk score assumed a high prolactin level.
[a]*Significant at* p < *0.05.*

risk score of 4.5 (n = 45, 36.29%) were significantly lower than those with a genetic risk score of 5.5 (n = 12, 9.68%) (16.60 vs. 23.00 ng/ml, p = 0.033). Thus, higher *DRD2* expression was related to higher prolactin levels (**Table 4**). However, there were high prolactin levels at the lowest and highest risk score (21.80 ng/ml at a risk score of 3.5 and 28.20 ng/ml at a risk score of 6, respectively), but there were no significant differences ($p > 0.05$).

There was no association between any of the interrogated sequence variations in the candidate pharmacodynamic genes and the risperidone response (**Supplementary Table S2**).

Relationships Between Serum Prolactin Levels and Plasma Levels of RIS C/D, 9-OH-RIS C/D, and Active Moiety C/D

Plasma levels of risperidone, 9-OH-risperidone, and the active moiety were corrected with the daily dose. Serum prolactin levels were significantly correlated among RIS C/D (r_s = 0.227, p = 0.012), 9-OH-RIS C/D (r_s = 0.305, p = 0.001), and active moiety C/D (r_s = 0.343, $p < 0.001$).

Relationships Between Serum Prolactin, Plasma RIS C/D, 9-OH-RIS C/D, Active Moiety C/D and Sequence Variations in Candidate Pharmacodynamic Genes

We conducted ANCOVA to compare the effect of *DRD2* diplotypes on prolactin levels, using RIS C/D, 9-OH-RIS C/D, and active moiety C/D as covariates to adjust for possible confounding factors. Serum levels of prolactin were significantly higher in the H1/H3 diplotype group compared with the H1/H2 (**Figure 1A**; F = 5.420, p = 0.026), H1/H5 (**Figure 1B**; F = 4.552, p = 0.042), H1/H6 (**Figure 1C**; F = 4.848, p = 0.037), and H2/H2 (**Figure 1D**; F = 5.761, p = 0.027) groups after controlling for plasma drug levels of active moiety C/D.

Effects of Prolactin in Response to Risperidone Therapy

A total of 124 participants were enrolled in this study; however, only 19 patients were available for naïve and follow-up after being treated for a minimum of 3 months. The mean age at baseline of the 19 patients was 5.21 years (SD 2.82), and most were male (n = 16,

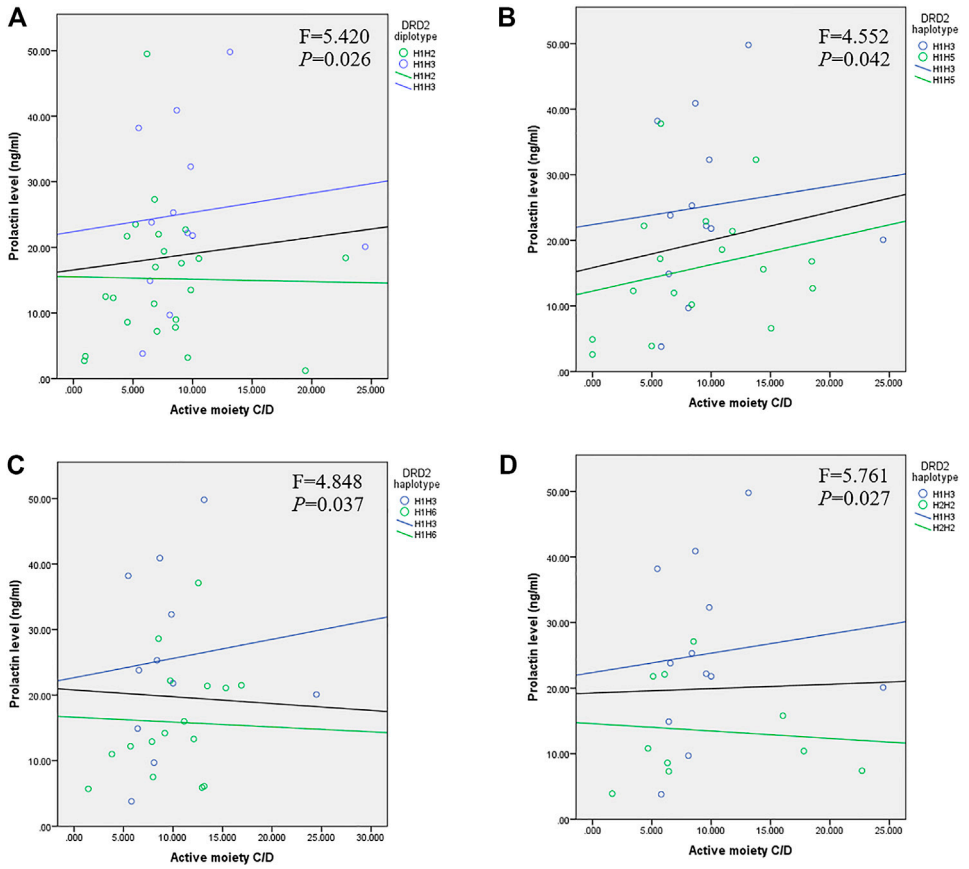

FIGURE 1 | Relationship between prolactin and active moiety C/D plasma levels in children and adolescents with *DRD2* diplotypes of **(A)** H1/H3 and H1/H2 (p = 0.026), **(B)** H1/H3 and H1/H5 (p = 0.042), **(C)** H1/H3 and H1/H6 (p = 0.037), and **(D)** H1/H3 and H2/H2 (p = 0.027). The black line represents the linear relationship between prolactin and drug levels in patients carrying an H1/H3 or other diplotype.

84.21%). **Table 5** summarizes the findings for the 19 patients by responder status according to the percentage decrease in the total ABC score. Of these, 53% (n = 10) responded to risperidone treatment, whereas 47% (n = 9) were non-responders. The mean age of responders was 5.50 ± 2.84 years and that of non-responders was 4.89 ± 2.93 years. Median prolactin levels after treatment in non-responders were higher compared with those in responders (20.10 vs. 10.25 ng/ml, p = 0.013). ABC subscale scores after 3 months were significantly lower than those before treatment (paired t-test; p < 0.05). Plasma levels of RIS C/D, 9-OH-RIS C/D, and active moiety C/D were not significantly different between responders and non-responders. Moreover, there was no significant difference in prolactin levels before and after treatment in responders (7.65 vs. 10.25 ng/ml, p = 0.878). In contrast, the prolactin level in non-responders after risperidone treatment was about twice that in responders (9.40 vs. 20.10 ng/ml, p = 0.028).

ROC Curve of Serum Prolactin for Responders

The ROC curve showed that serum prolactin levels predicted the risperidone response (**Figure 2** and **Table 6**). The area under the ROC curve for prolactin was 0.833 (p = 0.014), which was above the acceptable accuracy level of 0.8. The prolactin cut-off value with sensitivity and specificity of 0.5 or greater was 10.25–18.85 ng/ml, with an optimal prolactin cut-off value of 10.9 ng/ml (sensitivity, 100%; specificity, 60%; PPV, 69.23%; NPV, 100.00%; highest accuracy, 78.95%). The second accuracy for serum prolactin levels of 10.25, 12.20, 15.0, and 16.8 ng/ml was 73.68%.

DISCUSSION

Several studies have reported that genetic variation (Young et al., 2004; Calarge et al., 2009; Yasui-Furukori et al., 2011) or plasma drug levels of risperidone and its metabolite (Knegtering et al., 2005; Melkersson, 2006; Ngamsamut et al., 2016) are related to hyperprolactinemia. However, there are no studies on the relationship between prolactin elevation and genetic variation, or whether controlling plasma drug levels of RIS C/D, 9-OH-RIS C/D, and active moiety C/D can prevent prolactin elevation. The findings of the present study suggest that the patients with the H1/H3 haplotype of *DRD2* were at higher risk than patients with

TABLE 5 | ABC score and serum prolactin levels at baseline and after 3 months of treatment between responders and non-responders (*n* = 19).

Variables	Responders (*n* = 10)	Non-responders (*n* = 9)	*p*-value
Male, *n* (%)	10 (100)	6 (66.67)	0.087
Age (years), median (IQR)	4.50 (4.00–6.00)	5.00 (4.00–7.00)	0.905
Baseline			
Body weight (kg), median (IQR)	17.58 (15.70–25.00)	20.00 (15.30–27.00)	0.968
Risperidone dose (mg/day), median (IQR)	0.20 (0.20–0.50)	0.25 (0.15–0.50)	0.842
Weight-adjusted dose (mg/kg day), median (IQR)	0.01 (0.01–0.01)	0.02 (0.01–0.02)	0.315
ABC total score, mean ± SD	85.70 ± 29.73	91.22 ± 26.29	0.675
ABC-irritability, mean ± SD	20.40 ± 8.17	21.89 ± 11.42	0.746
ABC-social withdrawal, mean ± SD	16.80 ± 9.31	22.78 ± 6.80	0.132
ABC-stereotype, mean ± SD	9.50 ± 6.08	9.56 ± 5.64	0.984
ABC-hyperactivity, mean ± SD	33.10 ± 8.90	32.44 ± 5.88	0.854
ABC-inappropriate speech, mean ± SD	5.90 ± 4.12	4.56 ± 3.32	0.448
Prolactin level (ng/ml), median (IQR)	7.65 (6.00–17.70)	9.40 (7.10–16.60)	1.000
After 3 months			
Body weight (kg), median (IQR)	20.18 (17.00–30.00)	23.60 (16.70–32.00)	0.905
Risperidone dose (mg/day), median (IQR)	0.50 (0.20–0.60)	0.30 (0.20–0.50)	0.315
Weight-adjusted dose (mg/kg day), median (IQR)	0.02 (0.01–0.03)	0.01 (0.01–0.02)	0.356
Risperidone duration (months), median (IQR)	8.64 (3.00–13.77)	4.37 (3.70–7.03)	0.780
ABC total score, mean ± SD	41.50 ± 18.00	84.67 ± 14.63	<0.001
ABC-irritability, mean ± SD	7.90 ± 5.26	20.78 ± 11.55	0.011
ABC-social withdrawal, mean ± SD	8.20 ± 3.94	19.89 ± 6.90	<0.001
ABC-stereotype, mean ± SD	3.40 ± 2.68	7.78 ± 5.14	0.041
ABC-hyperactivity, mean ± SD	18.60 ± 8.21	31.44 ± 4.98	0.001
ABC-inappropriate speech, mean ± SD	3.40 ± 2.91	4.89 ± 3.55	0.330
Prolactin level, median (IQR), ng/ml	10.25 (6.50–16.00)	20.10 (15.80–27.40)	0.013
Risperidone, median (IQR), ng/ml	0.19 (0.02–0.90)	0.33 (0.12–0.58)	0.720
9-OH-RIS, median (IQR), ng/ml	3.04 (1.67–5.26)	4.57 (3.26–7.27)	0.400
Active moiety, median (IQR), ng/ml	3.86 (1.67–5.56)	5.41 (3.28–8.02)	0.447
Risperidone C/D, median (IQR), ng/ml	23.56 (3.17–40.30)	7.19 (3.55–36.12)	0.624
9-OH-RIS C/D, median (IQR), ng/ml	9.68 (7.12–18.64)	8.77 (6.52–15.09)	0.935
Active moiety C/D, median (IQR), ng/ml	13.58 (9.06–19.42)	9.17 (6.77–16.90)	0.638

RIS, risperidone; 9-OH-RIS, 9-hydroxy-risperidone; Active moiety, the sum of RIS plus 9-OH-RIS; C/D, dose-corrected concentration; IQR, interquartile range [quartile 1 (Q1) and quartile 3 (Q3)]; SD, standard deviation.

other diplotypes of presenting with significantly higher prolactin levels with increasing plasma RIS C/D, 9-OH-RIS C/D, and active moiety C/D. Furthermore, low prolactin levels were associated with response to risperidone treatment in Thai ASD children and adolescents.

Hyperprolactinemia is commonly observed in patients treated with antipsychotics. In our study, 44.35% (55 of 124) of patients had high prolactin levels, which is a concern for adverse drug reactions in children and adolescents treated with risperidone. The reported prevalence of hyperprolactinemia in patients on risperidone ranges between 44 and 61% (Hongkaew et al., 2015; An et al., 2016; Bonete Llacer et al., 2019), and abnormal prolactin levels occur in about 27% of patients taking risperidone (Lally et al., 2017). Hypothalamic dopamine inhibits prolactin secretion by acting on dopamine D2 receptors, which inhibits lactotroph cells (Ben-Jonathan and Hnasko, 2001). Hypothalamic dopamine inhibits pituitary prolactin secretion and proliferation of prolactin-producing lactotroph cells by activating lactotroph dopamine D2 receptors (Ben-Jonathan and Hnasko, 2001; Fitzgerald and Dinan, 2008). There are several hypotheses for why prolactin elevation occurs during treatment with certain antipsychotics. Risperidone may cause high prolactin levels because of its slow dissociation from dopamine D2 receptors, which increases central dopamine D2 receptor occupancy (Peuskens et al., 2014), resulting in a prolonged blockade and higher rates of prolactin release. Risperidone is also associated with weaker blood-brain barrier penetration (Peuskens et al., 2014) compared with olanzapine and quetiapine (Arakawa et al., 2010), resulting in greater dopamine D2 receptor occupancy in the pituitary area, which also increases prolactin levels.

We also investigated whether *DRD2* haplotypes are more informative than each SNP on its own, and the H1/H3 diplotype had the highest serum prolactin levels. These findings are consistent with increased prolactin levels in patients carrying the *DRD2/ANKK1 Taq*1A A2 or C allele (Sukasem et al., 2016). These alleles are associated with high dopamine receptor densities (Jonsson et al., 1999), which may contribute to blocking dopaminergic activity. *DRD2* -241A>G (rs1799978), which is located in the 5′-promoter region of *DRD2*, may contribute to increased expression levels. Data obtained with lymphoblastoid cell lines suggested that -241G carriers have higher *DRD2* expression levels than non-carriers (Nyman et al., 2009), which is consistent with our conclusion that *DRD2* -241A>G is involved in hyperprolactinemia. Our results are also consistent with previous findings. Calarge et al. (2009) found that the -241G allele had the largest effect on serum

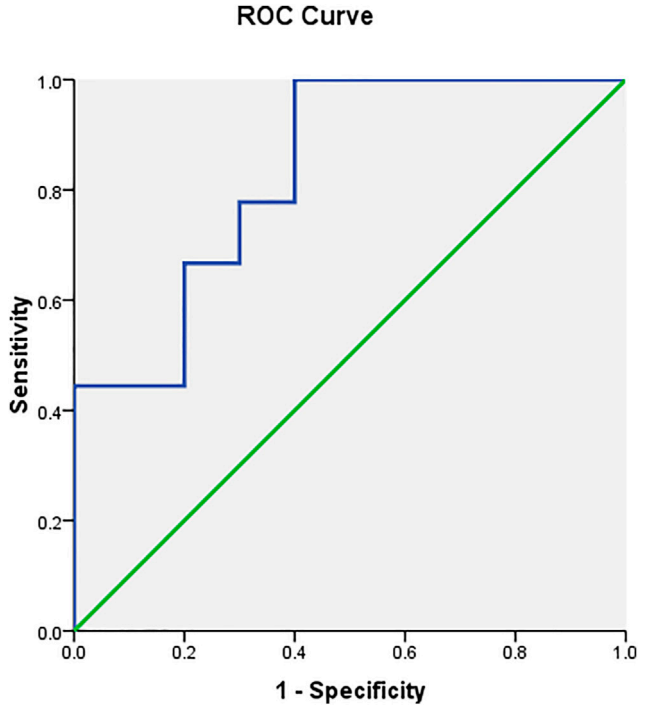

FIGURE 2 | ROC curve analysis showing that serum prolactin level has a high accuracy (68–78%) for identifying responders and non-responders.

prolactin levels when combined with the *Taq*1A A1 allele, whereas Arinami et al. (1997) reported an association between the *DRD2* -141C indel and high *DRD2* expression levels *in vitro*. Stronger binding of risperidone to dopamine D2 receptors in the pituitary area is more likely to inhibit dopaminergic activity in prolactin secretion (Peuskens et al., 2014). Therefore, the findings of these studies support our observation that increased *DRD2* expression levels, mediated by the *DRD2* -141C indel, contribute to the increase in prolactin levels. Our finding is also consistent with a study describing antipsychotic-induced sexual dysfunction in male schizophrenia, which was attributed to high prolactin levels in the presence of the *DRD2* -141C indel (Zhang et al., 2011). Therefore, the synergistic effects of the *DRD2/ANKK1 Taq*1A A2>A1, *DRD2* -141C indel, and -241A>G SNPs may be better captured by the association of haplotypes, rather than SNPs, with serum prolactin levels. Based on our results, we suggest that clinicians should perform DNA analysis of the three SNPs of the *DRD2* haplotype as pre-emptive genetic testing, and prolactin levels should be monitored before and after 3 months to prevent hyperprolactinemia, especially in patients with the H1/H3 diplotype.

We developed an algorithm to categorize *DRD2* haplotypes simply according to their *DRD2* expression (Arinami et al., 1997; Jonsson et al., 1999; Nyman et al., 2009). High-expression alleles (A2, Cin, G) were assigned a score of 1 and low-expression alleles (A1, Cdel, A) were assigned a score of 0.5. The score of each haplotype was the sum of the values of each allele. A high risk score was expected to indicate a high prolactin level. In this study, a higher *DRD2* expression score was significantly related to a higher prolactin level. The increase in prolactin levels with risperidone can be explained by risperidone inhibiting dopamine binding to dopamine D2 receptors (Rosenbloom, 2010). The higher the number of dopamine receptors is, the greater the inhibition by risperidone, and the higher the increase in prolactin. Thus, high prolactin levels occurred at the highest risk score of 6. However, this study also observed the high prolactin level at the lowest risk score of 3.5. This could be explained by the upregulation of dopamine receptors in patients with low *DRD2* expression and who received long-term risperidone treatment. Lidow and Goldman-Rakic (1997) found that 6 months treatment with antipsychotics upregulated prefrontal and temporal cortical dopamine D2 receptor mRNA expression in primates. However, there was no significant difference in expression due to the small sample size. The genetic risk score system is an easy-to-use tool for translating genotype data into *DRD2* expression prediction in a clinical setting. However, further study is needed to validate this tool.

Because variations in the *HTR2A* gene (encoding the 5-hydroxytryptamine type 2A receptor) and *HTR2C* gene (encoding the 5-hydroxytryptamine type 2C receptor) influence the binding affinities of antipsychotic medication, in previous studies, two genetic polymorphisms related to hyperprolactinemia in schizophrenic patients treated with classical and/or atypical antipsychotic treatment have been analyzed. Several studies found contradictory associations between hyperprolactinemia and *HTR2A* (Correia et al., 2010; Ivanova et al., 2017) and *HTR2C* polymorphisms (Alladi et al., 2017; Koller et al., 2020). Our study was consistent with a study of risperidone in 289 Indian schizophrenia patients (Alladi et al., 2017). They reported no association between *HTR2C* (−759 C>T) genetic variants and prolactin levels during risperidone treatment. Genetic polymorphisms in the promoter region of the *HTR2C* gene (−759 C>T) have been investigated in antipsychotic drug-induced weight gain (Vanwong et al., 2021). Moreover, transporter and prolactin-related genes involving the neurotransmitter mechanism of prolactin secretion were studied. Osmanova et al. (2019) found an association between two variants in the *DAT* (*SLC6A3*) gene and hyperprolactinemia in the subgroup of patients taking risperidone/paliperidone in 446 Caucasian schizophrenia patients. Smith et al. (2004) reported that the short allele of the *5-HTTLPR* indel polymorphism was associated with less of an increase in prolactin and cortisol than the long/long genotype in control participants. Lee et al. (2007) found a nominally significant association between *PRL* and *PRLR* tagSNPs and plasma prolactin levels in 95 advanced breast cancer cases. However, the results were inconsistent because of the different underlying disease groups and treatments. Thus, until our results are validated in a prospective study, they should not be applied to patients with other diseases or undergoing other treatments.

There were no significant differences between any genetic variants selected and responses to risperidone in this study. Thus, although the *DRD2* haplotypes relate to prolactin increase, they are likely to be predictive for poor markers for treatment response. Our results agree with Sakumoto et al. (2007), who found that non-responders without the deletion allele of the

TABLE 6 | Sensitivity, specificity, and accuracy of prolactin levels predicting risperidone response.

Prolactin levels (ng/ml)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
1.70	100.00	0.00	47.37	-*	47.37
3.30	100.00	10.00	50.00	100.00	52.63
5.20	100.00	20.00	52.94	100.00	57.89
6.55	100.00	30.00	56.25	100.00	63.16
8.35	100.00	40.00	60.00	100.00	68.42
10.25	100.00	50.00	64.29	100.00	73.68
10.90	100.00	60.00	69.23	100.00	78.95
12.20	88.89	60.00	66.67	85.71	73.68
13.60	77.78	60.00	63.64	75.00	68.42
15.00	77.78	70.00	70.00	77.78	73.68
15.90	66.67	70.00	66.67	70.00	68.42
16.80	66.67	80.00	75.00	72.73	73.68
18.85	55.56	80.00	71.43	66.67	68.42
20.80	44.44	80.00	66.67	61.54	63.16
21.85	44.44	90.00	80.00	64.29	68.42
23.00	44.44	100.00	100.00	66.67	73.68
25.60	33.33	100.00	100.00	62.50	68.42
28.80	22.22	100.00	100.00	58.82	63.16
30.45	11.11	100.00	100.00	55.56	57.89
31.70	0.00	100.00	-*	52.63	52.63

PPV, positive predictive value; NPV, negative predictive value.
Bold values represented cut-off value associated with higher and equal 50% in both sensitivity and specificity.
The italicized value represented the cut-off value associated with higher and equal 50% in both sensitivity and specificity with the highest degree of accuracy.
**PPV or NPV cannot be estimated.*

DRD2 -141C indel showed a higher score for psychiatric, extrapyramidal, and total side-effects than those with the deletion allele after 3 weeks of treatment with dopamine antagonists. The small number of 19 patients in our study cohort may explain why there were no significant differences among the associations between genetic variants and treatment response.

Several studies have examined optimizing risperidone dosing in children and adolescents based on their clinical response (Research Units on Pediatric Psychopharmacology Autism, 2005; Haas et al., 2009; Pozzi et al., 2016). We found no significant association between plasma drug levels and risperidone response due to the small sample size, although non-responders had numerically higher drug plasma levels than responders, which is consistent with other published work (Wang et al., 2007; Lostia et al., 2009). However, our results contradict other studies that show a relationship between therapeutic drug levels and clinical efficacy (Baumann et al., 2004; Riedel et al., 2005). This discrepancy may be a result of the different underlying disease in patients with schizophrenia. In addition, there may be differences in the timing of the clinical outcome assessment and the sociodemographic profile of the patient population among studies. We performed our assessment at least 3 months into risperidone treatment rather than at 1–6 weeks because prolactin levels in children receiving long-term risperidone tend to peak within the first 2 months, and then steadily decline to values within or close to normal after 3–5 months (Findling et al., 2003). Although we did not discover an association between plasma drug concentration and efficacy, the association between plasma drug and serum prolactin levels remained significant. Therefore, monitoring risperidone plasma levels may help to prevent adverse drug reactions, such as hyperprolactinemia.

Although other studies have found that prolactin levels are inconsistently associated with efficacy (Wang et al., 2007; Lostia et al., 2009), our study revealed that prolactin levels may induce changes in neurogenesis, potentially affecting aberrant behaviors in ASD, consistent with several other studies (Zhang et al., 2002; Lee and Kim, 2006; Ates et al., 2015). Prolactin is an anterior pituitary peptide hormone, under inhibitory control by dopamine released from the tuberoinfundibular dopaminergic neurons (Grattan, 2015). Because of dopamine regulation of serum prolactin levels through dopamine D2 receptors in the hypothalamic-pituitary pathway, the serum prolactin levels may be a marker of central dopamine function. Risperidone could induce hyperprolactinemia in excessive dopamine receptor blockade (Oda et al., 2015) due to its high dopamine D2 receptor occupancy (68–70%) (Tsuboi et al., 2013), resulting in hyperprolactinemia in early treatment. Long-term treatment with antipsychotics could lead to increased dopamine D2 receptor density according to the dopamine supersensitivity psychosis hypothesis (Oda et al., 2015), and thus could cause adequate blockade, leading to behavioral improvement. This hypothesis may explain why some ASD patients had increased prolactin levels early in risperidone treatment and subsequently presented with improved symptoms.

The association between high prolactin and worse response could be explained by the brain/plasma concentration ratio of risperidone. Arakawa et al. (2010) reported that risperidone showed a slightly higher brain/plasma concentration ratio. The brain/plasma concentration ratio was calculated from dopamine D2 receptor occupancy in the temporal cortex and pituitary, which

represented the permeability of antipsychotics into the brain. The brain/plasma concentration ratio of risperidone was 1.61 (Arakawa et al., 2010), indicating that the risperidone concentration in the temporal cortex was 1.5-fold greater than that in the pituitary. The permeability of risperidone into the brain resulted in a higher concentration in the brain than in plasma. Dopamine D2 receptor occupancy of risperidone in the temporal cortex was higher than that in the pituitary, causing an improvement in behaviors and reducing the risk of prolactin elevation. Therefore, our results suggest that low prolactin levels are an indicator of response to risperidone, and thus serum prolactin levels could be used as an indirect biomarker for risperidone response.

The present study is the first to report a cut-off value for prolactin-related poor behavioral responses of children treated with risperidone of less than 1 mg/day. The prediction of risperidone response in ASD had an area under the ROC curve of 0.833. The cut-off point of the prolactin level associated with sensitivity and specificity of 0.5 or higher was 10.25–18.85 ng/ml, and the optimal cut-off value for prolactin of 10.9 ng/ml had the highest accuracy of 78.95%. We applied the prolactin measurement as a biomarker for unusual behaviors in patients who did not respond to risperidone treatment. It might be used as a screening test to exclude false negatives. When NPV was 100%, meaning that when the test was negative (low prolactin value), the patient was responding to treatment, when the PPV was 60%, meaning that when the test was positive (high prolactin value) of 100 patients, only 60 patients were not responding to treatment. This information would be beneficial to health practitioners in clinical practice. However, further studies are needed to determine the optimal daily dose of risperidone for prolactin monitoring.

There are several limitations to this study. The small sample size in the cohort study prevented us from detecting significant genotype-phenotype associations, and thus the results should be viewed with caution. No relationship was observed for risperidone dose, weight-adjusted dose, and treatment duration between responders and non-responders. However, the study used cohorts under clinical conditions and did not control for risperidone dose and treatment duration.

CONCLUSION

We created a system for representing the expression of *DRD2* haplotypes using genetic risk scores. Our findings suggest that patients with the H1/H3 diplotype, which had a *DRD2* genetic risk score of 5.5, showed the highest serum prolactin levels, correlated with increasing plasma RIS C/D, 9-OH-risperidone C/D, and active moiety C/D. Lower levels of prolactin were detected in patients who responded to risperidone. These findings further our understanding of hyperprolactinemia, which is a commonly observed adverse effect of risperidone treatment. Preemptive pharmacogenetic testing may allow clinicians to identify patients at risk of developing high prolactin levels at the outset of therapy and use this information to guide therapeutic decision-making.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Faculty of Medicine Ramathibodi Hospital, Bangkok, Thailand (MURA 2017/556) and Yuwaprasart Waithayopathum Child Psychiatric Hospital, Samut Prakan, Thailand. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

YH, AG, BW, and CS conceptualized the study; YH, RG, AP, and RS analyzed the data; WK, NN, and PL collected the samples YH wrote the original draft; YH, AG, BW, and CS reviewed and revised the paper. All authors have read and agreed to the published version of the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.743494/full#supplementary-material

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Human Arylamine *N*-Acetyltransferase 1 (NAT1) Knockout in MDA-MB-231 Breast Cancer Cell Lines Leads to Transcription of NAT2

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Many cancers, including breast cancer, have shown differential expression of human arylamine *N*-acetyltransferase 1 (NAT1). The exact effect this differential expression has on disease risk and progression remains unclear. While NAT1 is classically defined as a xenobiotic metabolizing enzyme, other functions and roles in endogenous metabolism have recently been described providing additional impetus for investigating the effects of varying levels of NAT1 on global gene expression. Our objective is to further evaluate the role of NAT1 in breast cancer by determining the effect of NAT1 overexpression, knockdown, and knockout on global gene expression in MDA-MB-231 cell lines. RNA-seq was utilized to interrogate differential gene expression (genes correlated with NAT1 activity) across three biological replicates of previously constructed and characterized MDA-MB-231 breast cancer cell lines expressing parental (*Scrambled*), increased (*Up*), decreased (*Down, CRISPR 2–12*), or knockout (*CRISPR 2–19, CRISPR 5–50*) levels of NAT1. 3,889 genes were significantly associated with the NAT1 *N*-acetylation activity of the cell lines (*adjusted* $p \leq 0.05$); of those 3,889 genes, 1,756 were positively associated with NAT1 *N*-acetylation activity and 2,133 were negatively associated with NAT1 *N*-acetylation activity. An enrichment of genes involved in cell adhesion was observed. Additionally, human arylamine *N*-acetyltransferase 2 (NAT2) transcripts were observed in the complete NAT1 knockout cell lines (*CRISPR 2–19* and *CRISPR 5–50*). This study provides further evidence that NAT1 functions as more than just a drug metabolizing enzyme given the observation that differences in NAT1 activity have significant impacts on global gene expression. Additionally, our data suggests the knockout of NAT1 results in transcription of its isozyme NAT2.

Keywords: NAT1, arylamine *N*-acetyltransferase 1, RNA-seq, NAT2, arylamine *N*-acetyltransferase 2, cell adhesion, breast cancer

1 INTRODUCTION

It is estimated by the American Cancer Society that breast cancer will account for 30% of new cancer cases and 15% of cancer-related deaths in women in 2021 (Siegel et al., 2021). When breast cancer is detected at the localized stage the 5-year survival rate is 99% however when detected at the distant stage the 5-year survival rate is a disappointing 28% (Siegel et al., 2018). These statistics highlight the need for a better understanding of breast cancer risk and progression so that interventions can be provided at the early stage when breast cancer is the most treatable and to identify novel molecular targets for treatment.

Human arylamine *N*-acetyltransferase 1 (NAT1) has been extensively studied in breast cancer. NAT1 is a classically described phase II xenobiotic metabolizing enzyme, but more recently has been shown to have additional, orthogonal, and diverse roles in metabolism including the hydrolysis of acetyl-Coenzyme A (acetyl-CoA) (Laurieri et al., 2014; Stepp et al., 2015; Stepp et al., 2019). There is a strong association between estrogen receptor positive breast cancer and high NAT1 expression (Carlisle and Hein, 2018; Minchin and Butcher, 2018; Zhang et al., 2018). The role of NAT1 in breast cancer, until recently, was thought to center on NAT1's ability to metabolize/activate carcinogens, however it has been shown that rats with higher Nat2 expression (orthologous to human NAT1) had greater mammary tumor susceptibility, independent of carcinogen metabolism (Stepp et al., 2017).

Additionally, our recent work examining differences in the metabolome between breast cancer cell lines expressing varying levels of NAT1 suggested a role for NAT1 in the metabolism of L-asparagine, putrescine, and L-lysine. We also observed a relationship between NAT1 and the conjugation of carnitine with fatty acyl-CoA moieties (Carlisle et al., 2020). Others have shown deletion of the *NAT1* gene in MDA-MB-231, HT-29 and HeLa cancer cell lines leads to increased collagen adherence, decreased invasion, and morphological changes but no changes to migration ability in all three cell-lines (Li et al., 2020). Despite numerous studies investigating NAT1 in breast cancer, the exact effect of increased NAT1 expression in breast cancer remains unknown. It also remains unknown what additional, diverse roles NAT1 may have in metabolism that are independent of xenobiotic metabolism. In this study our objective was to further investigate the role of NAT1 in pathways commonly dysregulated in cancer.

2 MATERIALS AND METHODS

2.1 Description of Cell Line Samples

Six cell lines constructed via both siRNA and CRISPR/Cas9 technologies from the MDA-MB-231 triple negative breast cancer cell line to have varying levels of human NAT1 *N*-acetylation activity were utilized in this study: *Scrambled* (transfection control with parental MDA-MB-231 activity; 9.41 ± 1.15 nmoles acetylated *p*-aminobenzoic acid/min/mg protein), *Up* (stable integration of plasmid overexpressing NAT1 with increased activity; 63.5 ± 10.3 nmoles acetylated *p*-aminobenzoic acid/min/mg protein), *Down* (stable transfection with siRNA specific to NAT1 with decreased activity; 6.55 ± 0.826 nmoles acetylated *p*-aminobenzoic acid/min/mg protein), *CRISPR 2–12* (single allele deletion with CRISPR/Cas9 guide RNA 2 with decreased activity; 5.33 ± 0.572 nmoles acetylated *p*-aminobenzoic acid/min/mg protein), *CRISPR 2–19* (double allele deletion with CRISPR/Cas9 guide RNA 2 with complete knockout of NAT1; no detectable activity), and *CRISPR 5–50* (double allele deletion with CRISPR/Cas9 guide RNA 5 with complete knockout of NAT1; no detectable activity). Details on the construction and characterization of these cell lines has been described in detail elsewhere (Carlisle et al., 2018; Stepp et al., 2019). The *Scrambled*, *Up*, and *Down* cell lines are non-clonal cell lines while the *CRISPR 2–12*, *CRISPR 2–19*, and *CRISPR 5–50* cell lines were established from a single isolated clone following transfection. Additionally, we acknowledge that the *Scrambled* cell line may have changes in gene expression not present in the other cell lines if the randomly generated scrambled shRNA utilized was complementary to anywhere in the genome. This risk was minimized by the chosen design/sequence of the shRNA.

2.2 Collection of Samples

Figure 1 illustrates the experimental approach and workflow of sample collection described below. Cells were plated in triplicate per biological replicate at a density of 500,000 cells per 150 mm × 25 mm cell plate for both transcriptomics and metabolomics analysis. All cell lines were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM), with 10% fetal bovine serum, 5% glutamine, and 5% penicillin/streptomycin added. Cells were allowed to grow for 3 days at 37°C and 5% CO_2 in an incubator.

Cells were then harvested on ice by adding 5 ml 0.25% trypsin and scraping the cells from the plate. Three plates were combined to form one sample (biological replicate). Three biological replicates for each cell line were collected for transcriptomics analysis. After harvesting the cells, cells were washed 3 times with ice-cold 1 x PBS. All supernatant was removed and 100 µl of cell pellet was reserved for transcriptomics analysis. The remaining cell pellet was reserved for metabolomics analysis. Samples were then flash frozen by placing in a pool of liquid nitrogen for 1 min and stored in an −80°C freezer until RNA isolation.

2.3 Transcriptomics

2.3.1 RNA Isolation

Total RNA was isolated from MDA-MB-231 breast cancer cells expressing parental (*Scrambled*), increased (*Up*), decreased (*Down*, *CRISPR 2–12*), and knockout (*CRISPR 2–19, CRISPR 5–50*) levels of NAT1 using the RNeasy® Mini Kit (Qiagen Sciences, Germantown, Maryland) according to manufacturer's instructions. RNA quality was evaluated and concentrations were measured in each sample using a NanoDrop Bioanalyzer (Thermo Fischer Scientific).

2.3.2 Library Preparation

Libraries were prepared using the TruSeq Stranded mRNA LT Sample Prep Kit- Set A (Illumina, San Diego, California; Cat# RS-122-2101) with poly-A enrichment per manufacturer's instructions. One µg of total RNA (in a volume of 50 µl) from

each sample was used in library preparation. Briefly, the total RNA was fragmented to improve sequence coverage over the transcriptome. Next, the first strand of cDNA was synthesized from the cleaved RNA fragments that were primed with random hexamers using reverse transcriptase and random primers. Then the second strand of cDNA was synthesized, thus giving us double stranded blunt end cDNA. Next, a single 'A' nucleotide was added to the 3′ ends of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction. A corresponding single 'T' nucleotide on the 3′ end of the adapter provides a complementary overhang for ligating the adapter to the fragment. Then, multiple indexing adapters were ligated to the ends of the double stranded cDNA, preparing them for hybridization onto a flow cell. Next the DNA fragments were enriched using PCR to selectively enrich those DNA fragments that have adapter molecules on both ends and to amplify the amount of DNA in the library. The PCR was performed with a PCR Primer Cocktail that anneals to the ends of the adapters. Finally, 30 µl of eluted library was collected and stored at −20°C. To avoid skewing the representation of the library, the number of PCR cycles are minimized. This kit includes steps to validate and normalize constructed libraries and methods to check quality control.

2.3.3 Library Validation

Quality: Size, purity and semi quantitation were performed on an Agilent Bioanalyzer using the Agilent DNA 1000 Kit. The final fragment size for all the samples was approximately 300bp which is expected according to the protocol.

Quantity: Sequencing library quantitation was performed by qPCR using the KAPA Library Quantitation Kit for Illumina Platforms. The standard curve method was used for quantitation using one to five DNA standards that came with the kit.

Normalize and Pool Libraries: Ten µl of sample was transferred from the wells to a new MIDI plate. We then normalized the concentration of the libraries to 10 nM using Tris-HCl 10 mM, pH 8.5 with 0.1% Tween 20. Five µl of each sample was then transferred to be pooled into a new LowBind 1.5 ml micro centrifuge tube for a total volume of 60 µl pooled 10 nM library. Then 4 nM dilution was made from the 10 nM pooled library by diluting with Tris-HCl 10 mM, pH 8.5 with 0.1% Tween 20.

Denaturing and diluting Libraries for the Nextseq 500: A total volume of 1.3 ml of 1.8 pM denatured library is needed for sequencing using the v2 kit. Pooled 4 nM library was denatured by mixing with diluted NaOH and incubated at room temperature for 5 min 200 mM Tris HCl, pH 7.0 was then added. The reaction mixture was diluted to 20 pM using a pre-chilled Hybridization Buffer. 20 pM denatured library was further diluted to 1.8 pM using the same Hybridization Buffer. Before loading onto the reagent cartridge, 1.3 µl of denatured 20 pM Phix control was added to the 1,299 µl of denatured 1.8 pM library to a total volume of 1.3 ml for the first sequencing run (for the 2nd sequencing run 1.9 pM library was used).

2.3.4 Sequencing

Sequencing was performed on the University of Louisville Center for Genetics and Molecular Medicine's (CGeMM) Illumina NextSeq 500 using the NextSeq 500/550 75 cycle High Output Kit v2 (FC-404-2005). A second run was performed to increase the number of reads. For each run, 72 single-end raw sequencing files (.fastq) (Cock et al., 2010) representing six conditions with three biological replicates and four lanes per replicate were generated. Reads were 75 base pair, single-end reads.

2.3.5 RNA-Seq Analysis

Adapter sequences were trimmed from resulting reads using Trim Galore (version 0.6.6). Quality of the sequencing reads (after trimming) were interrogated with FastQC (Andrews, 2014) (version 0.11.9) and found to be excellent. Reads were then aligned to the GENCODE release 31 GRCh38.p12 genome using the GENCODE v31 transcript annotation with the STAR (Dobin et al., 2013) aligner (version 2.7.6) using basic 2-pass mapping, with all 1st pass junctions inserted into the genome indices on the fly. **Supplementary Table S1** indicates the number of reads successfully aligned for each of the samples.

Analysis and visualization of the resulting data were performed using R version 4.0.3 (R Foundation for Statistical Computing, Vienna, Austria). Aligned reads were quantified using the GenomicAlignments (Lawrence et al., 2013) package (Bioconductor) with the "IntersectionStrict" setting. Resulting read counts were log base two transformed and normalized using the rlog function of DESeq2 (Love et al., 2014). Additionally, DESeq2 was utilized to estimate linear regression models relating gene expression and NAT1 *N*-acetylation activity. This method allows us to identify genes whose expression is significantly associated with NAT1 *N*-acetylation activity. Significance of linear regression analysis is reported as adjusted (for multiple comparisons) Wald test p-values. Principal component analysis (PCA) was conducted by singular value decomposition of the centered data matrix on the most 1,000 variable genes in the entire dataset. The scores of the first (*x*-axis) and second (*y*-axis) principal component were plotted.

Log_2 change in gene expression per unit change in NAT1 *N*-acetylation activity and significance (adjusted p-values) from regression analysis were visualized using volcano plots. Unbiased clustering of samples by hierarchal clustering was conducted using the WPGMA method using the most significant 100 differentially expressed genes; resulting data were plotted as heatmaps for visualization. Pathway enrichment analysis was conducted utilizing the linear regression results as input with the absolute value of the Wald test statistic as the variable for ordering. We utilized the normalized enrichment score to determine the relative degree of enrichment. **Figure 1** illustrates the overall experimental approach and data analyses methods.

2.4 Quantitative Measurement of *NAT1* and *NAT2* mRNA

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR) was conducted for *NAT1* and arylamine N-acetyltransferase 2 (*NAT2*) in each of the six constructed cell lines as previously described (Husain et al., 2007a; Husain et al., 2007b; Millner et al., 2012). Briefly, total RNA was isolated from

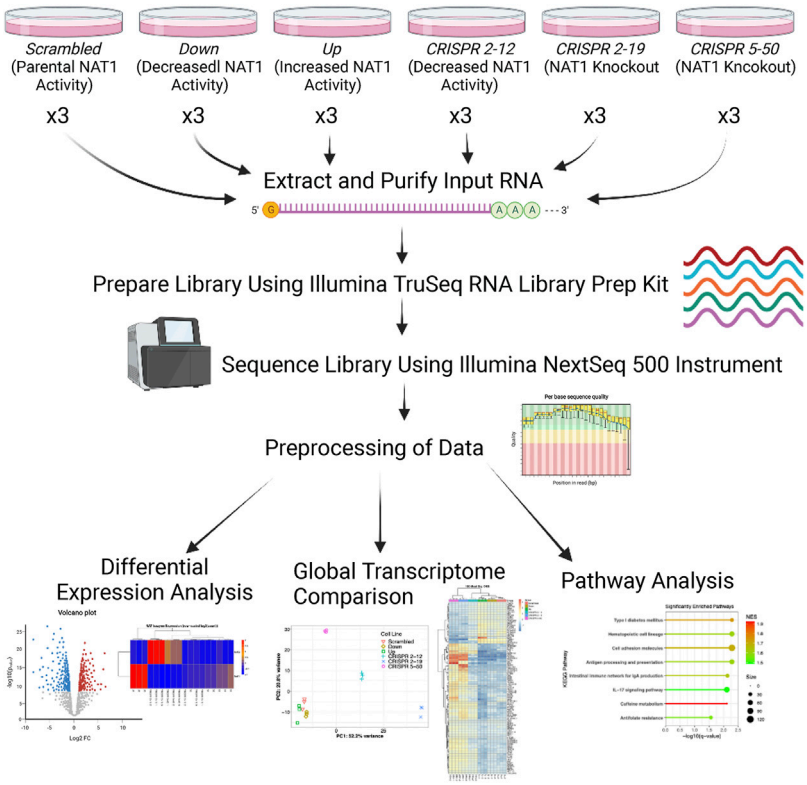

FIGURE 1 | Transcriptomics Experimental Approach. Three biological replicates were collected for each cell line, RNA was extracted and purified, and libraries were prepared using the input RNA. Once library preparation was complete, libraries were sequenced using the Illumina NextSeq 500 instrument. Resulting sequencing data were aligned to the hg38 human reference genome via STAR and read counts were generated for each gene using the Bioconductor R package GenomicAlignments. Next, differential expression analysis, PCA, hierarchical clustering, and pathway analysis was performed.

each cell line using the RNeasy Mini Kit (Qiagen, Germantown, MD). Isolated RNA was used to transcribe cDNA using the High Capacity Reverse Transcriptase kit (Life Technologies, Carlsbad, CA). Resulting cDNA was utilized for quantitative measurement of NAT1 and NAT2 via qRT-PCR. TaqMan analysis was performed using the ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA). The gene probes utilized were designed previously to discriminate between *NAT1* and *NAT2*. Student's t-tests were utilized for testing significance.

2.5 NAT2 *N*-Acetylation Activity Assays

In vitro NAT2 *N*-acetylation activity was determined in each constructed cell line as previously described (Grant et al., 1991; Hein et al., 1993). Briefly, cell lysate from each cell line was incubated with 1 mM acetyl-coenzyme A and 300 μM sulfamethazine (SMZ) at 37°C for 10 min. Reactions were terminated with the addition of 1/10 reaction volume 1 M acetic acid. Reaction products were collected and analyzed using an Agilent Technologies 1,260 Infinity high performance liquid chromatography (HPLC) using a LiChrospher 100 RP-18 (5 μm) column to determine the amount of acetylated product. Our reported limit of detection for *N*-acetylated SMZ is 0.15 nmol/min/mg.

3 RESULTS

PABA *N*-acetylation activity was measured in constructed MDA-MB-231 cell lines and reported previously (Carlisle et al., 2020). Briefly, the *Scrambled* cell line had approximately the same activity as the *Parent* MDA-MB-231 cell line with no manipulation while the *Up* cell line had an approximate 700% increase in activity. Additionally, the *Down* and *CRISPR 2–12* cell lines had approximately 65 and 50% of the activity of the *Parent* and *Scrambled* cell lines, respectively, while the *CRISPR 2–19* and *CRISPR 5–50* cell lines had no detectable activity.

On a per-sample average, 91% of reads aligned to the human genome resulting in approximately 34.6 M uniquely aligned reads. Regression analysis revealed that the expression of 3,889 genes were significantly associated with the NAT1 *N*-acetylation activity of the cell lines (*adjusted* $p \leq 0.05$); of those 3,889 genes, 1,756 were positively associated with NAT1 *N*-acetylation activity and 2,133 were negatively associated with NAT1 *N*-acetylation activity (**Figure 2**).

Principal component analysis of the 1,000 most variable genes in the dataset revealed that all cell lines in this study had distinct transcriptomic profiles as each clustered independently by group membership (**Figure 3**). In our dataset, principal component 1 explains 52.2% of the variance in the data while principal component 2 explains 20.8% of the variance. The *CRISPR*

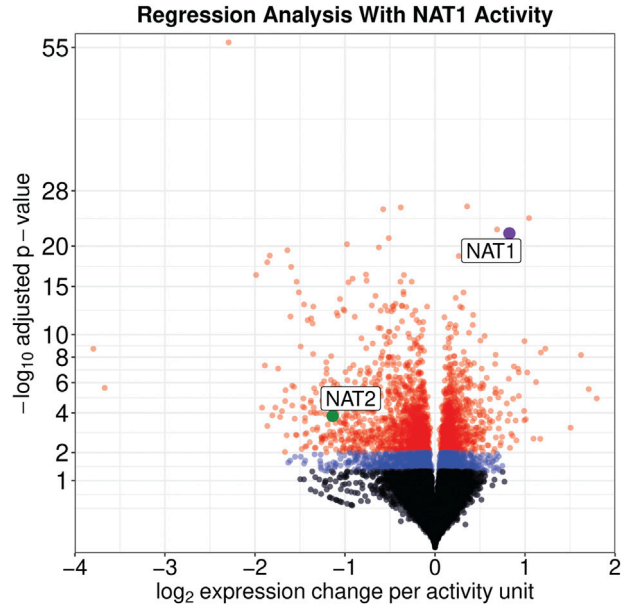

FIGURE 2 | Expression of Many Genes, including NAT1 and NAT2, are significantly associated with NAT1 Activity. Significance and effect size are visualized for all genes in the dataset. Genes with an adjusted significance value greater than 0.05 are shown by black dots, genes with an adjusted significance value between 0.05 and 0.01 are shown by blue dots, and genes with an adjusted significance value less than 0.01 are shown by red dots. NAT1 is shown by a purple dot and NAT2 is shown by a green dot; both are labeled.

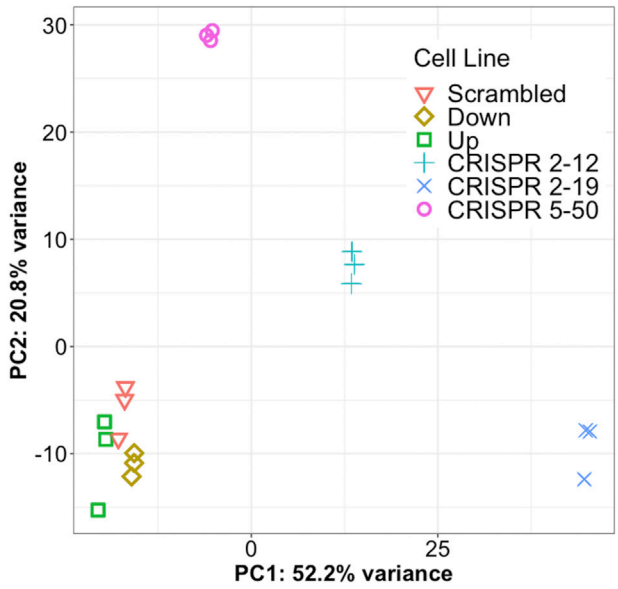

FIGURE 3 | Each cell line has a unique global transcriptome. Principal component analysis, a statistical method for reducing the dimensionality of a dataset to better understand total variance, was conducted for all samples. Each symbol represents a single biological replicate. This analysis shows most of the total variance in the dataset (52.2%) is between the CRISPR-Cas9 generated cell lines from the other three cell lines. This analysis also shows each cell line has a distinct transcriptomic profile but the biological replicates are very similar to each other.

2–12, CRISPR 2–19, and *CRISPR 5–50* groups are separated from the other three groups along principal component 1. The *CRISPR 2–12* and *CRISPR 5–50* cell lines were additionally separated from the remaining 4 cell lines along principal component 2. This revealed that the Scrambled, Up, and Down groups had transcriptomic profiles similar to each other but different from the other three groups suggesting the construction method utilized may have led to unique differences.

Unsupervised hierarchical clustering of the 100 most significant genes associated with NAT1 *N*-acetylation activity was conducted to visualize gene expression patterns within all samples and to identify groups of genes with similar expression patterns (**Figure 4**). We observed one cluster of 24 genes, that included NAT1, to be positively associated with NAT1 *N*-acetylation activity. Gene expression in this cluster was generally low in the *CRISPR 2–19* and *CRISPR 5–50* cell lines, higher in the Down, *CRISPR 2–12*, and Scrambled cell line, and highest in the Up cell line. The remaining 76 genes which grouped as a separate cluster, exhibited the opposite pattern; gene expression in this cluster was generally highest in the *CRISPR 2–19* and *CRISPR 5–50* cell lines, lower in the Down, *CRISPR 2–12*, and Scrambled cell line, and lowest in the Up cell line.

Genes encoding proteins with functions related to acyl/acetyl transfer and binding were significantly associated with NAT1 *N*-acetylation activity. Vesicle amine transport 1 like (*VAT1L*) which is involved in acyl group transfer, was inversely related to NAT1 activity. Acyl-CoA binding domain containing 7 (*ACBD7*), was positively associated with NAT1 *N*-acetylation activity. Glucosaminyl (*N*-acetyl) transferase 4 (*GCNT4*) was negatively associated with NAT1 *N*-acetylation activity. Additionally, a negative association between 5-hydroxytryptamine receptor 1F (*HTR1F*) and NAT1 *N*-acetylation activity was observed.

Many protocadherin and cadherin genes were found to be significantly associated with NAT1 activity in the cell lines (**Supplemental Table S2**). Twenty-nine protocadherin genes were significantly associated with NAT1 activity (with no significant association observed in 33) and all but five of those genes were associated with an increase in expression per unit NAT1 activity. These included alpha, beta, and gamma subfamilies. Five cadherin genes (*CDH2*, *CDH3*, *CDH11*, *CDH13*, *CDH18*) were significantly associated with NAT1 activity and expression of all five were decreased per unit of NAT1 activity. Additionally, three of the four FAT atypical cadherin genes (*FAT1*, *FAT3*, *FAT4*) were significantly associated with NAT1 activity however the fold-changes were very small per NAT1 activity unit and in different directions; *FAT1* and *FAT4* were increased with NAT1 activity while *FAT3* was decreased with NAT1 activity. Transmembrane O-mannosyltransferase targeting cadherins 1 (*TMTC1*) was significantly associated with NAT1 activity and expression was decreased per unit NAT1 activity.

3.1 NAT Isozyme Expression in Transcriptomics Dataset

The expression of *NAT1* and its isozyme, *NAT2*, were compared between all cell lines. As expected, given the *N*-acetylation activities

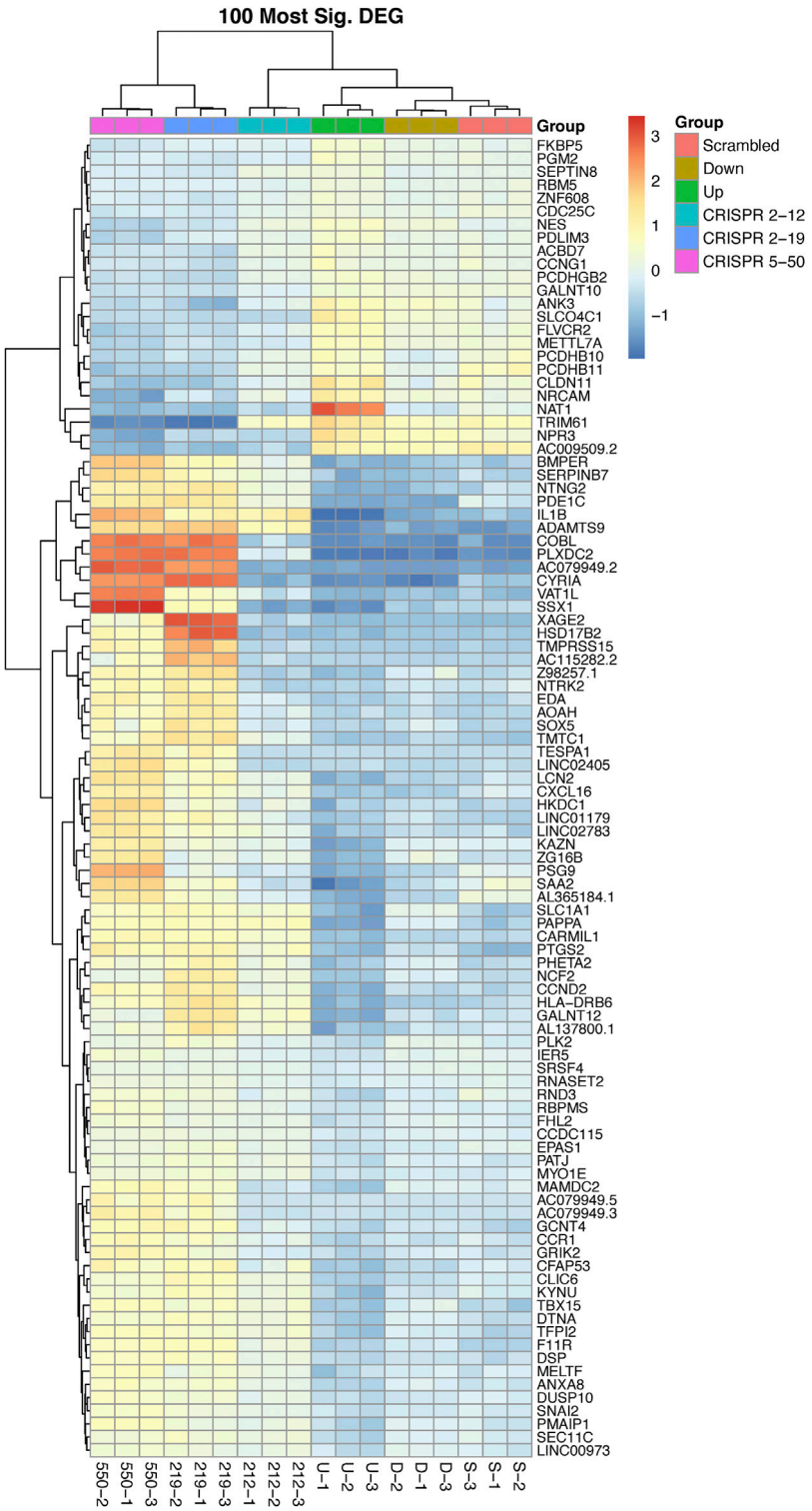

FIGURE 4 | The 100 most differentially expressed genes cluster into two overall groups with distinguishable expression profiles. This analysis shows each cell line has a distinct expression profile for the 100 most significant genes associated with NAT1 activity and that there many genes with similar expression profiles across the samples. There are two overall groups that form in terms of similar patterns of expression, those that are positively associated with NAT1 activity (24—top cluster) and those that are negatively associated with NAT1 activity (76—bottom cluster). Each row of the heatmap shows the expression for a single gene while each column shows gene expression for an individual sample. Expression values have been transformed to Z-scores with red colors representing high expression and blue colors representing low expression.

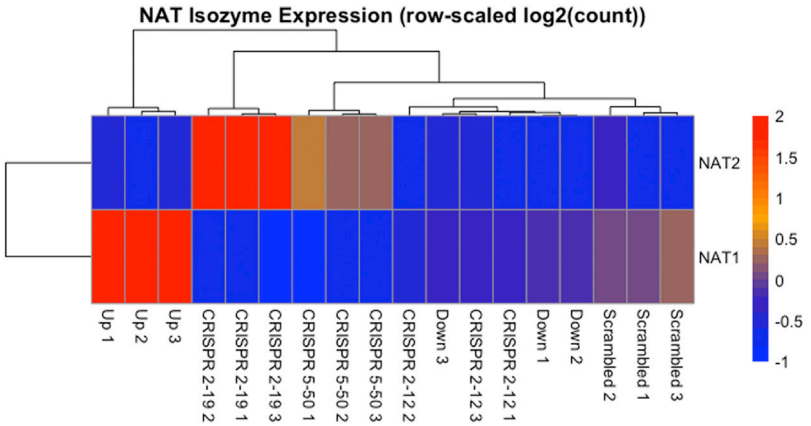

FIGURE 5 | NAT Isozyme Expression is Inversely Related. The expression of NAT1 and its isozyme NAT2 were plotted as a heatmap for comparison. This data shows the inverse relationship between expression of NAT1 and NAT2 in each sample. When NAT1 transcript expression is high, NAT2 transcript expression is low, and conversely, when NAT1 transcript expression is low, NAT2 transcript expression is high. Each row of the heatmap shows the expression for a single gene while each column shows gene expression for an individual sample. Expression values have been transformed to Z-scores with red colors representing high expression and blue colors representing low expression.

measured, NAT1 expression was decreased in the Down, *CRISPR 2–12*, *CRISPR 2–19*, and *CRISPR 5–50* cell lines and increased in the Up cell line compared to the Scrambled cell line. Notably, in the two complete NAT1 knockout cell lines (*CRISPR 2–19* and *CRISPR 5–50*), *NAT2* expression was observed while the expression was much lower in the other cell lines (**Figure 5**). We visualized the RNA-seq reads that were mapping to *NAT2* using the Integrated Genomics Viewer (IGV) to double check that the reads were not NAT1 reads mis-mapping to *NAT2* given the isozymes high degree of sequence homology (**Figure 6**); we confirmed these were not NAT1 misreads as we observed known NAT2 SNPs in the mapped sequence reads. The reads mapping to NAT2 had the following SNPs within them: rs1801280T > C and rs1799929C > T which corresponds with the NAT2*5A haplotype. We also verified the observation of NAT2 reads with qRT-PCR analysis for *NAT1* and *NAT2* in all 6 cell lines (**Figure 7**). Although not significant for all cell lines, we observed a pattern of decreased *NAT1* transcripts and increased *NAT2* transcripts in samples. We conducted NAT2 activity assays with cell lysate from all six constructed cell lines using the prototypic NAT2 substrate sulfamethazine (SMZ) however no NAT2 activity was detected in any cell line (data not shown). Currently, the functionality of the *NAT2* transcripts produced in the NAT1 knockout cell lines is unknown.

3.2 Pathway Analysis

Pathway enrichment analysis was conducted using Gene Set Enrichment and the KEGG pathways to determine what metabolic pathways were enriched for genes associated with NAT1 activity (**Figure 8**). We focused on pathways that had a normalized enrichment score of >1.40. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were used (Ogata et al., 1999; Kanehisa and Goto, 2000). Disease associated pathways were removed from the analysis results. Many pathways were significantly enriched for differences including hematopoietic cell lineage, cell adhesion molecules, antigen processing and presentation, IL-17 signaling, intestinal immune network for IGA production, caffeine metabolism, and antifolate resistance pathways were found to be significantly enriched. This further supports our observation that NAT1 activity is strongly associated with the expression of cell adhesion genes. Additionally, pathways that we would expect to be affected by NAT1 given previous literature and knowledge, such as caffeine metabolism and antifolate resistance, were found to be significantly enriched for differences. Some of the enriched pathways additionally suggest a relationship between the immune system and NAT1.

4 DISCUSSION

As our samples for this study were genetically modified breast cancer cell lines that had been passaged for a few growth cycles, we decided to utilize a regression approach for data analysis rather than pair-wise comparisons to our control cell line. This approach decreases the likelihood that genes identified to be differentially expressed between groups are due to off-target effects of CRISPR/Cas9 editing and/or additional mutations incurred during passaging of cell lines or during clonal selection after CRISPR/Cas9. One limitation of this approach, however, is that it relies on NAT1 *N*-acetyltransferase activity being the functional measure for this enzyme's activity and may limit us identifying genes that are affected if NAT1 has any enzyme functions independent of this activity. Nevertheless, we should still be able to detect some of those possible independent effects because we know our complete NAT1 knockout cell lines have mutations that lead to very truncated NAT1 protein that have lost nearly all protein domains. Additionally, it has been noted by others that CRISPR/Cas9 genetic editing frequently leads to on-target mRNA misregulation which leads to degradation of the mutant mRNAs produced (Tuladhar et al., 2019).

In recent years many roles for NAT1 independent of *N*-acetylation of exogenous xenobiotics have been shown. One challenge of identifying all of the potential roles of NAT1 in

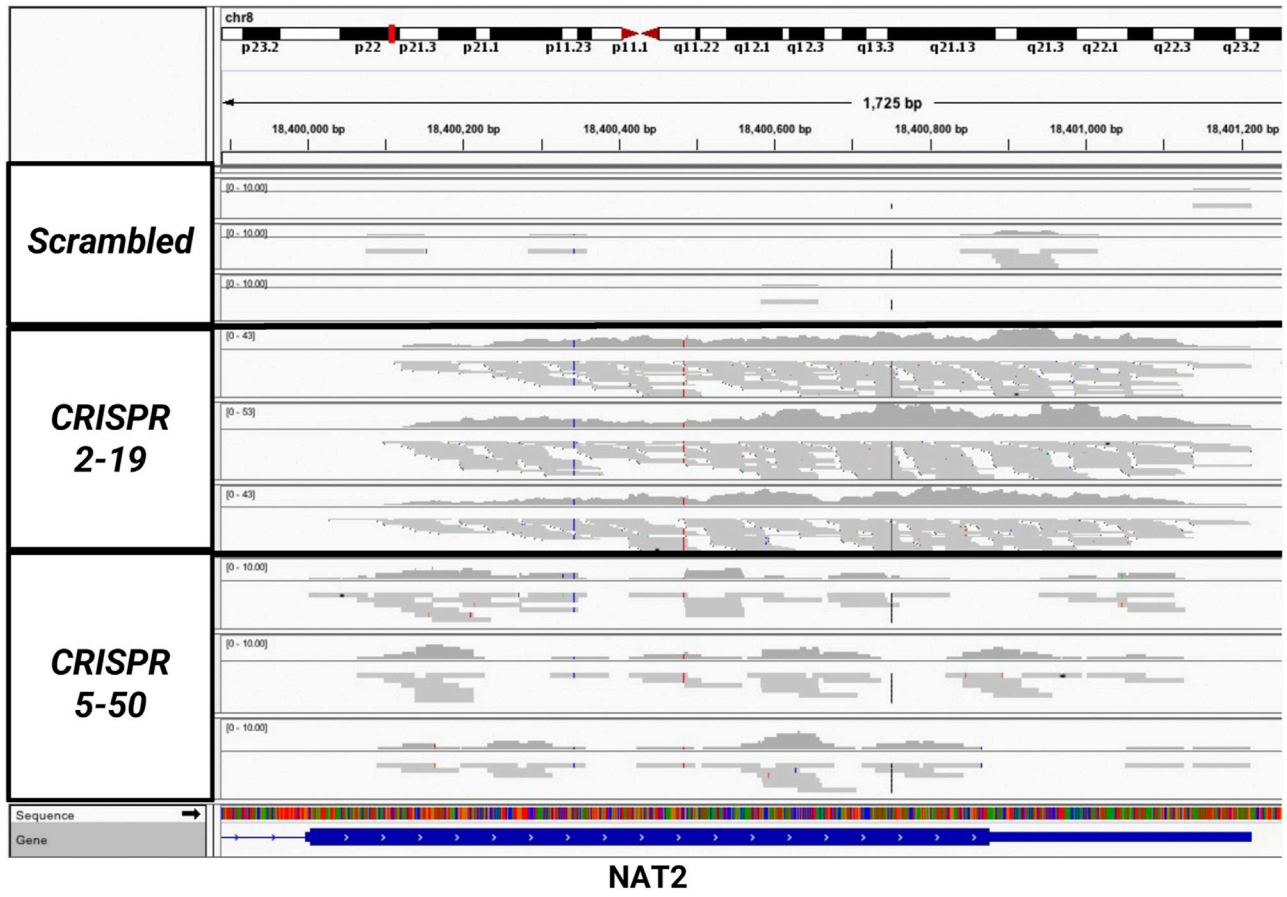

FIGURE 6 | Sequencing Reads Map to the NAT2 Gene But Only in the Two Knockout Cell Lines, *CRISPR 2–19* and *CRISPR 5–50*. The mapping of reads to the NAT2 gene were visualized using the Integrated Genomic Viewer in the *Scrambled*, *CRISPR 2–19*, and *CRISPR 5–50* cell lines. Each linear gray bar represents an individual read. The red and blue lines represent positions in the read where a single nucleotide polymorphism compared to the reference genome is present. Zero to very few reads mapped to NAT2 in the *Scrambled* cell line samples, many reads mapped to NAT2 in the *CRISPR 2–19* cell line samples, and a medium amount of reads mapped to NAT2 in the *CRISPR 5–50* cell line samples.

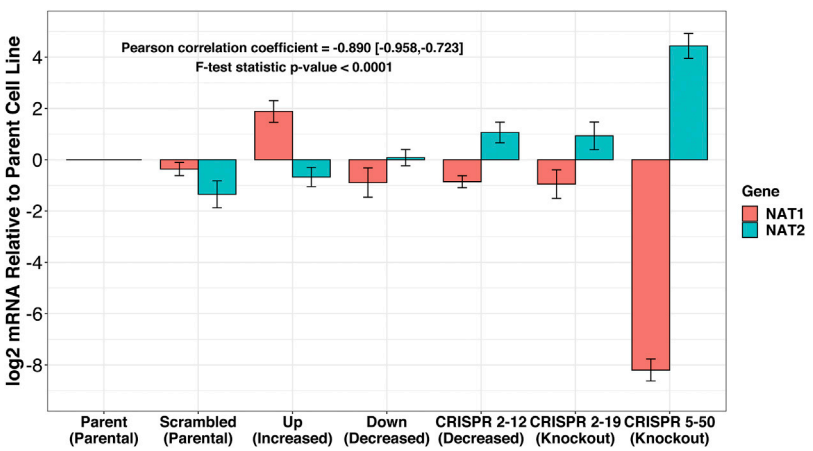

FIGURE 7 | RT-qPCR confirms inverse relationship between NAT1 and NAT2 expression in our cell lines. PCR probes designed to discriminate between NAT1 and NAT2 were utilized for real-time quantitative polymerase chain reactions. mRNA expression was normalized to the expression of the parental MDA-MB-231 cell line. A trend of decreased NAT1 expression and increased NAT2 expression was observed. $^{*}p \leq 0.05$, $^{***}p \leq 0.001$.

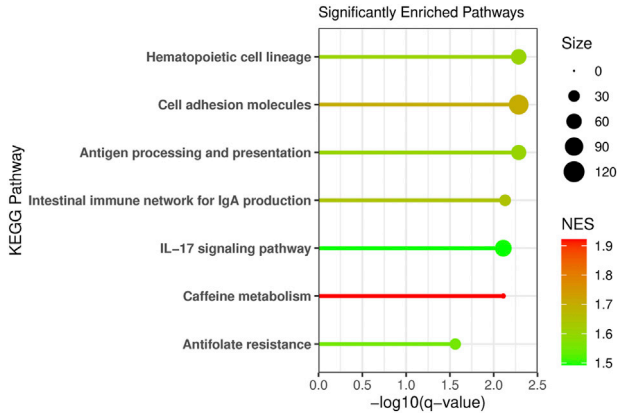

FIGURE 8 | Pathway Analysis Reveals Enrichment of Specific Pathways for Differences Between Cell Lines. Pathway analysis was conducted using Gene Set Enrichment Analysis (GSEA) and the human Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Genes were ranked by the absolute value of the Wald test statistic generated from the linear regression results. The KEGG pathway name is labeled on the *y*-axis, the –log10 of the q-value is plotted on the *x*-axis, the line and dot plotted are colored by the normalized enrichment score (NES) with the highest NES plotted in red and the lowest (from the significant pathways plotted) in green. The size of the dot in the figure represents the number of genes from our input that were in that specific pathway.

metabolism has been the approaches taken thus far. When performing targeted assays probing NAT1's effect on specific pathways/genes we are limited by what is known when deciding where to look. In this study we have taken a global systems biology approach which removes that previous limitation.

In this study we identified additional supportive evidence of an association between NAT1 and cell adhesion molecules. Many have noted that overexpression NAT1, specifically in breast cancer, is associated with increased metastasis (Smid et al., 2006; Savci-Heijink et al., 2016; Zhao et al., 2020) and growth (Adam et al., 2003) and conversely knockdown or deletion of NAT1 leads to increased adherence to collagen (Li et al., 2020) and decreased invasion (Tiang et al., 2010, 2015; Li et al., 2020), E-cadherin up-regulation and cell-cell contact growth inhibition (Tiang et al., 2011), and reduced anchorage independent growth (Stepp et al., 2018; Stepp et al., 2019). Here we have shown the expression of many members of the cadherin family of proteins are associated with NAT1 *N*-acetylation activity. Particularly, the negative association between the genes *CDH2*, *CDH3*, *CDH11*, *CDH13*, *CDH18* and NAT1 *N*-acetylation activity supports observations of high NAT1 expression in breast cancer and increased metastasis.

The cadherin family of genes are subject to numerous regulatory mechanisms (Gumbiner, 2000) complicating the interpretation of the relationship between cadherins and NAT1 activity directly. However, we additionally observed that expression of *TMTC1*, a gene that encodes a protein important for adding O-mannosyl post-translational modifications to members of the cadherin family, was inversely related to NAT1 activity; it has been shown by others that TMTC3, a closely related family member to TMTC1, is important for ensuring E-cadherin operates properly in cell adhesion (Graham et al., 2020); when TMTC1 was knocked out cells showed increased migration ability (Graham et al., 2020). Our pathway analysis indicated the KEGG pathway: cell adhesion molecules, was one of the most significantly enriched pathways for genes associated with NAT1 activity. Although the mechanism behind this relationship remains unclear, this adds additional evidence of a relationship and further impetus to study this phenomenon more closely in order to potentially elucidate NAT1 as an anti-metastasis drug target.

The observation that the complete knockout, but not knockdown, of NAT1 lead to NAT2 transcript production leads us to hypothesize that there may be a compensation mechanism that occurs when NAT1 *N*-acetylation is lost, suggesting NAT1 has an essential role. There have been examples of humans that have no detectable NAT1 *N*-acetylation activity (Bruhn et al., 1999) however NAT2 expression and/or activity has never been investigated specifically in this population. Notably, a retrospective analysis of publicly available NAT1 and NAT2 gene expression data in established breast cancer cell lines, primary breast tumor tissues, and normal breast tissues showed a small positive correlation between the two genes (Carlisle and Hein, 2018). One reason that study did not observe an inverse relationship between NAT1 and NAT2 gene expression may be because complete knockout of NAT1 may be necessary before the compensation mechanism occurs given we only observed NAT2 transcripts in the complete NAT1 KO cell lines but not the cell lines with decreased NAT1. Data reported by the Cancer Cell Line Encyclopedia (CCLE) indicates the MDA-MB-231 cell line is diploid for NAT2 and does not show copy number variation (Ghandi et al., 2019).

The relationship between NAT1 and NAT2 expression has not been studied in-depth although it is known each has tissue specific expression. NAT1 is expressed throughout most tissues while NAT2 is most highly expressed in the liver and digestive tract. Since NAT1 and NAT2 are isozymes with overlapping substrate specificities (although distinct) it has been thought that the isozyme with the highest expression would be most important to understand in a given tissue. While our RNA-seq data showed the highest levels of NAT2 transcripts in the *CRISPR 2–19* cell line, our qRT-PCR showed the highest levels of NAT2 transcripts in the *CRISPR 5–50* cell line. We hypothesize that this is because the samples for each set of experiments were collected at different times; the phase of cell growth as well as growth conditions may affect the mechanism responsible for transcription of *NAT2*. Additionally, others have noted that CRISPR/Cas9 genetic editing can lead to degradation of the mutant mRNAs produced (*NAT1* in our experiments); we hypothesize that this process may also be affected by the growth conditions or growth phase during sample collection.

Additionally, the associations observed between NAT1 *N*-acetylation activity and other genes closely related to transfer of acyl groups such as, *VAT1L* (inversely related to NAT1 activity), *GCNT4* (negatively associated with NAT1 *N*-acetylation activity), and *ACBD7* (positively associated with NAT1 *N*-acetylation activity) suggest there are mechanisms occurring to maintain homeostasis as it relates to acetyl group transfers. *ACBD7* is a member of a family of proteins involved in

numerous intracellular processes including fatty acid-, glycerolipid- and glycerophospholipid biosynthesis, β-oxidation, cellular differentiation and proliferation as well as in the regulation of numerous enzyme activities (Neess et al., 2015). This association may help us better understand the finding in our previous metabolomics study that the β-oxidation of fatty acids seemed to be impaired in NAT1 deficient cell lines (Carlisle et al., 2020).

As NAT1 is known to *N*-acetylate serotonin (Steinberg et al., 1969; Backlund et al., 2017), albeit at very low levels in comparison to aralkylamine N-acetyltransferase (AANAT), we find the negative association between 5-hydroxytryptamine receptor 1F (*HTR1F*) and NAT1 *N*-acetylation activity interesting. The negative association between *HTR1F* and NAT1 *N*-acetylation activity indicates an upregulation of the serotonin receptor when NAT activity is low and presumably less serotonin is N-acetylated and a downregulation of the serotonin receptor when NAT activity is high and presumably more serotonin is N-acetylated. In our previous metabolomics study of the same samples, a positive association was observed between NAT1 *N*-acetylation activity and serotonin metabolite abundance (Carlisle et al., 2020). Taken together, this study suggests there are complex homeostatic/compensatory mechanisms occurring based on NAT1 levels as well as other factors such as AANAT levels.

5 CONCLUSION

This is the first study to measure the global transcriptome profile of breast cancer cells with varying levels of NAT1 *N*-acetylation activity. The expression of many genes, especially genes in the cadherin family, were significantly associated with NAT1 *N*-acetylation activity suggesting either regulation by NAT1, regulation by acetyl-CoA (or products of its hydrolysis), co-regulation mechanisms, or mechanisms compensating for the loss of NAT1. The most notable finding of this study was the observation that *NAT2* gene expression was significantly inversely associated with NAT1 *N*-acetylation activity and this relationship was also observed via RT-PCR methods. It appears only the complete knockout of NAT1 stimulates transcription of *NAT2* suggesting complete loss of NAT1 *N*-acetylation activity is necessary. This observation suggests *NAT2* transcripts are produced as a compensation mechanism for the complete loss of *NAT1* however the mechanism by which the cell senses the loss of *NAT1* and transcribes *NAT2* has not been identified and requires further investigation. As the two NAT1 knockout cell lines (nor any of the other cell lines) did not have any detectable *N*-acetylation activity toward the prototypic NAT2 substrate SMZ (limit of detection = 0.15 nmol/min/mg), the functionality of the *NAT2* transcripts remains unknown and warrants further investigation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA774803.

AUTHOR CONTRIBUTIONS

SC and DH contributed to conception and design of the study. SC and MD prepared samples. SC analyzed the resulting data and prepared figures. PT assisted with statistical analysis of the data. SC wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.803254/full#supplementary-material

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Do Genetic Polymorphisms Affect Fetal Hemoglobin (HbF) Levels in Patients With Sickle Cell Anemia Treated With Hydroxyurea? A Systematic Review and Pathway Analysis

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Hydroxyurea has long been used for the treatment of sickle cell anemia (SCA), and its clinical effectiveness is related to the induction of fetal hemoglobin (HbF), a major modifier of SCA phenotypes. However, there is substantial variability in response to hydroxyurea among patients with SCA. While some patients show an increase in HbF levels and an ameliorated clinical condition under low doses of hydroxyurea, other patients present a poor effect or even develop toxicity. However, the effects of genetic polymorphisms on increasing HbF levels in response to hydroxyurea in patients with SCA (Hb SS) have been less explored. Therefore, we performed a systematic review to assess whether single-nucleotide polymorphisms (SNPs) affect HbF levels in patients with SCA treated with hydroxyurea. Moreover, we performed pathway analysis using the set of genes with SNPs found to be associated with changes in HbF levels in response to hydroxyurea among the included studies. The systematic literature search was conducted on Medline/PubMed, EMBASE, Cochrane Central Register of Controlled Trials, Cumulative Index to Nursing and Allied Health Literature (CINAHL), Scopus, and Web of Science. Seven cohort studies were included following our inclusion and exclusion criteria. From the 728 genetic polymorphisms examined in the included studies, 50 different SNPs of 17 genes were found to be associated with HbF changes in patients with SCA treated with hydroxyurea, which are known to affect baseline HbF but are not restricted to them. Enrichment analysis of this gene set revealed reactome pathways with the lowest adjusted *p*-values and highest combined scores related to VEGF ligand–receptor interactions (R-HSA-194313; R-HSA-195399) and the urea cycle (R-HSA-70635). Pharmacogenetic studies of response to hydroxyurea therapy in patients with SCA are still scarce and markedly heterogeneous regarding candidate genes and SNPs examined for association with HbF changes and outcomes, suggesting that further studies are needed. The reviewed findings highlighted that similar to baseline HbF, changes in HbF levels upon hydroxyurea therapy are likely to

be regulated by multiple loci. There is evidence that SNPs in intron 2 of *BCL11A* affect HbF changes in response to hydroxyurea therapy, a potential application that might improve the clinical management of SCA.

Systematic Review Registration: (https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=208790).

Keywords: *BCL11A* gene, fetal hemoglobin (HbF), genetic polymorphisms, hydroxyurea (HU) therapy, pathway analysis, pharmacogenetics, sickle cell anemia (SCA), systematic review

INTRODUCTION

Sickle cell anemia (SCA) is a global health problem, and approximately 300,000 infants are born with SCA every year (Azar and Wong, 2017). SCA is defined as a monogenic hemoglobin disorder caused by homozygosis for A-to-T transversion at codon 7 (c.20A > T, p.E7V) in the hemoglobin subunit beta (*HBB*) gene (den Dunnen and Antonarakis, 2000; Steinberg, 2008). The pathophysiology of SCA is directly related to polymerization of deoxygenated hemoglobin (HbS; α2βS2), leading to a cascade of pathologic events including erythrocyte sickling, vaso-occlusion, and hemolysis (Kato et al., 2018). It is important to note that higher levels of fetal hemoglobin (HbF; α2γ2) ameliorate clinical outcomes and hematological parameters of SCA, since it reduces HbS concentration and inhibit copolymerization between hemoglobin tetramers (Kato et al., 2018). Notably, higher persistent HbF concentration is often observed in patients with SCA than in subjects without SCA (Lettre and Bauer, 2016).

Hydroxyurea (HU) was approved by the U.S. Food and Drug Administration for the treatment of adults with severe SCA in 1998, and it has been associated with improved survival for both adults and children with SCA, as reviewed elsewhere (McGann and Ware, 2015). The clinical effectiveness of HU is related to the induction of the production of HbF, but it is not restricted to it. HU selectively kills cells in the bone marrow and increases the number of erythroblasts producing HbF, which inhibits the intracellular polymerization of HbS and prevents the sickling process in erythrocytes, thereby decreasing the number of sickled cells (McGann and Ware, 2015). Erythrocytes with high HbF have longer survival, thereby attenuating hemolysis (Steinberg, 1999). Furthermore, HU increases the hemoglobin levels; reduces neutrophils, monocytes, and reticulocytes; and alters the expression of adhesion molecules in the endothelium and the generation of nitric oxide. These hematological changes decrease the risk of vaso-occlusion in patients with SCA (Steinberg, 1999; McGann and Ware, 2015; Rigano et al., 2018).

Because HU has dose-related effects, the laboratory and clinical benefits of HU were shown to be optimized when dimensioned for the maximum tolerated dose (MTD). Almost all patients with SCA show a significant increase in HbF concentration at the MTD (McGann and Ware, 2015). The American Society of Hematology guideline panel suggests HU therapy with at least 20 mg/kg/day at a fixed dose or the MTD (DeBaun et al., 2020). However, there is substantial interpatient variability both in the MTD itself and in the percentage of HbF (% HbF) achieved (Ware et al., 2011; McGann and Ware, 2015). For example, the % HbF achieved with the MTD of HU reaches 10–15% in some patients, but it can reach 40% in other patients (Ware et al., 2011). Moreover, while some patients tolerate high HU doses, such as 30–35 mg/kg/day, others develop severe myelosuppression even at lower doses (Lettre et al., 2008). These findings suggest that important individual differences on pharmacokinetics and pharmacodynamics, and genetic factors contribute to the phenotypic variability in both the dosing and response to HU therapy (McGann and Ware, 2015). However, the effect of genetic polymorphisms on increasing HbF levels in response to HU therapy in patients with SCA has been less explored.

Therefore, the aim of the present study was to perform a systematic review to assess whether genetic polymorphisms affect HbF levels in patients with SCA treated with HU. In addition, we performed pathway analysis using the set of genes which had single-nucleotide polymorphisms (SNPs) that were found to be associated with changes in HbF levels in response to HU therapy among the studies included in the systematic review.

MATERIALS AND METHODS

This study was conducted according to the Cochrane Handbook for Systematic Reviews of Interventions (Higgins et al., 2020), and the results were reported in accordance with the Meta-analysis Of Observational Studies in Epidemiology (MOOSE) checklist (Stroup et al., 2000). The protocol of the current study was registered on the International Prospective Register of Systematic Reviews [PROSPERO (CRD42020208790); https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=208790].

Search Strategy

The search strategy was defined based on the PECO question: Participants (P) = Sickle cell anemia patients (HbSS); Exposition (E) = Minor alleles; Control (C) = Major alleles of genetic polymorphisms and; Outcomes (O) = Fetal hemoglobin levels. A literature review was conducted by searching the electronic databases EMBASE, Medline/PubMed (Medical Literature Analysis and Retrieve System Online), Cochrane Central Register of Controlled Trials (CENTRAL), Cumulative Index to Nursing and Allied Health Literature (CINAHL), Scopus, and Web of Science (WoS) to identify studies published until July 2021. The initial search included the Medical Subject Headings (MeSH) entry terms: “Anemia, Sickle Cell”; and “Hydroxyurea”; and “Polymorphism, Genetic” or “Amplified Fragment Length Polymorphism Analysis” or “Polymorphism,

Single Nucleotide," or "Polymorphism, Restriction Fragment Length"; and "Fetal Hemoglobin," which were then included for a high-sensitivity search strategy in Medline/PubMed (**Supplementary Table S1**).

The same terms were used to search for gray literature and conference proceedings. The reference lists of included articles were also checked to identify additional relevant citations. All potentially eligible studies were considered for review, regardless of the language and publication date.

Inclusion and Exclusion Criteria

The inclusion criteria were restricted to studies that described the pharmacogenetics of response to HU therapy in patients with SCA measured by HbF levels (primary outcome). We included only cohort studies that examined patients with the SS genotype, with a minimum age of three y at the time of HU initiation and with a minimum period of six months of HU therapy.

We excluded studies that did not differentiate patients with SCA from patients with another sickle cell disease (SCD), studies that focused on haplotypes and not on individualized SNPs, and studies that did not examine whether SNPs affect HbF levels in patients with SCA treated with HU. Review articles, conference proceedings, case reports, and commentary studies were also excluded.

Study Selection and Data Extraction

Initially, the studies retrieved from the databases were input into a single electronic library, and duplicates were excluded using EndNote® software. Two reviewers (R.R.S. and B.L.N.) independently analyzed the titles and abstracts of the articles retrieved, reviewed the full text of the published articles, and used a standard data extraction protocol. Any disagreements between the two reviewers were resolved by a third reviewer (J.A.G.T.).

The extracted data from selected studies included study design, country, sample size, follow-up duration, median/mean age of participants, gender of patients, eligibility criteria, median/mean of HU dose, changes in HbF levels after HU therapy, genes, and polymorphisms associated with the primary outcome. The associated genes found in the included studies were used for pathway analysis.

Assessment of Bias Across Studies

The quality assessment of included studies was carried out independently by two reviewers (R.R.S. and B.L.N.), following the approach of the Joanna Briggs Institute for the synthesis of evidence (Moola et al., 2020), and any disparity between the two reviewers was resolved by a third reviewer (J.A.G.T.). The approach indicates the application of critical assessment tools used in systematic reviews, in which the checklist for cohort studies is applied (Moola et al., 2020). The instrument is structured around eleven questions, in which the selected studies were evaluated: 1) the two groups were similar and recruited from the same population; 2) how they were similarly measured to designate exposed and unexposed groups as people; 3) exposure was measured in a valid and reliable manner; 4) confounding factors have been identified; 5) the instrument was created to deal with confounding factors; 6) the groups were free of the outcome at the beginning of the study; 7) the results were measured in a valid and reliable way; 8) the follow-up time was reported and long enough for the results to occur; 9) the follow-up was complete and, if not, whether the reasons for the loss of follow-up were obtained and explored; (10) the instrument was used to deal with incomplete follow-up; and 11) statistical statistics was applied. The answer options for signaling questions are 1) yes, 2) no, 3) unclear, and 4) not applicable (Moola et al., 2020).

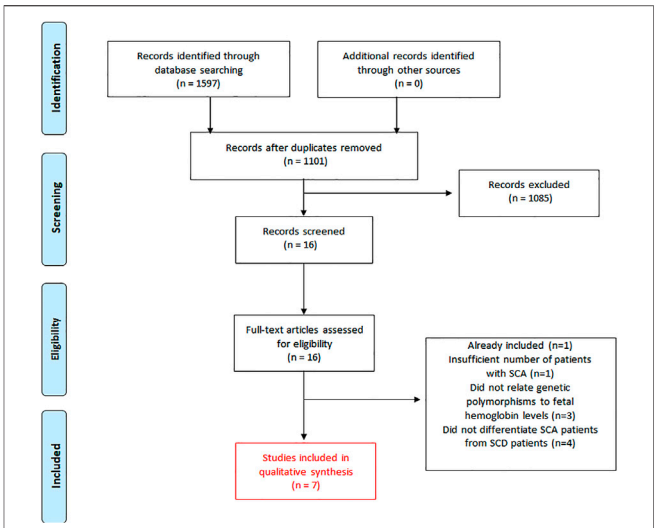

FIGURE 1 | Flow diagram of the literature search and the study selection for this systematic review of published articles related to the effect of genetic polymorphisms on HbF levels in patients with SCA (Hb SS) treated with hydroxyurea.

Gene Set Enrichment Analysis and Pathway Analysis

After the data extraction, we manually curated the genes which had SNPs that were found to be associated with changes in HbF levels in patients with SCA treated with HU among the seven studies included in the systematic review (listed in **Table 2**). Next, we interrogated this gene set for significant well-curated signaling pathways obtained from the Reactome Pathway Knowledgebase (Jassal et al., 2020). The pathways found were sorted both by the adjusted p-values ranking <0.05, which were calculated using a Benjamini–Hochberg method (Benjamini and Hochberg, 1995), and the combined scores according to the gene set enrichment analysis web server Enrichr (Kuleshov et al., 2016; Xie et al., 2021).

RESULTS

Study Selection

We identified 1,597 records in the initial search (**Figure 1**). After the exclusion of duplicates, 1,101 articles were selected for title and abstract analyses. Of these, 1,085 articles were subsequently excluded due to the following reasons (as stated before in the "exclusion criteria"): 1) studies that focused on haplotypes rather than individualized SNPs; 2) studies that did not differentiate patients with SCA from patients with another SCD; or 3) studies

TABLE 1 | Characteristics of the seven cohort studies included in the systematic review, which examined the effects of genetic polymorphisms on fetal hemoglobin (HbF) levels in patients with SCA treated with hydroxyurea (HU).

Author, data; country	Sample (n)	Average age (years)*	Gender (M/F)	Dose of HU (mean ± SD; mg/kg/day)	Time of follow-up on HU therapy (months)	HbF changes	HbF measurement	Number of genes (SNPs) studied	Multiple test correction
Friedrisch et al. (2016); Brazil	111	21 ± 14 (from 4 to 54)	38/62	23 ± 7.6	Minimum of 6	Δ MTD HbF (%)[a]	Capillary electrophoresis	3 (6)	Not applied
Ware et al. (2011); United States	88	9.6 ± 4.8	57/31	23.9 ± 5.1	Minimum of 6	Δ MTD HbF (%)[b]	HPLC	Not informed (331)	Applied
Aleluia et al. (2017); Brazil	42	15.2 ± 11.1	70/71	15 (47.6%) 20 (23.8) 25 (26.2%)**	Mean of 13.4 ± 9.7	Not informed	HPLC	3 (6)	Not applied
Green et al. (2013); United States	38	12.5 ± 4.9	57/60	25.3 ± 3.0	Minimum of 6	Δ HbF (%)[c]	HPLC	9 (20)	Applied
Kumkhaek et al. (2008); United States	32	Not informed	Not informed	Not informed	Minimum of 8	Δ HbF (% and g/dl)[b]	HPLC	1 (20)	Not applied
Sheehan et al. (2014); United States	Discovery cohort (171)	10.4 ± 4.5	Not informed	25.1 ± 4.5	Minimum of 6	Δ MTD HbF (%)[b]; Final HbF	HPLC	Whole exome	Unclear
	Validation cohort (130)	8.1 ± 4.0	Not informed	27.1 ± 4.3	Minimum of 6	Δ MTD HbF (%)[b]; Final HbF	HPLC	24 (25)	Unclear
Ma et al. (2007); United States	137	Not informed	Not informed	Not informed	Minimum of 21	Δ HbF (% and g/dl)[a]	Alkali denaturation	29 (320)	Not applied

Abbreviations: HbF, fetal hemoglobin; M, male; F, female. All selected studies were part of cohort studies.
**age at the time of hydroxyurea initiation.*
***dose (case percentage).*
[a](ΔHbF = MTD HbF—baseline HbF).
[b](ΔHbF = final HbF—baseline HbF).
[c](ΔHbF = maximum HbF—baseline HbF).

that did not focus on SNPs related to HbF levels in patients with SCA treated with HU. Subsequently, 16 full-text articles were thoroughly assessed for inclusion. Following review, nine articles were removed due to the following reasons (**Figure 1**): One cohort study had an insufficient number of patients with SCA (Sclafani et al., 2016). Three studies did not assess whether the SNPs affected HbF levels (Italia et al., 2010; Zhu et al., 2017; Yahouedehou et al., 2020). Four studies did not differentiate patients with SCA from patients with SCD (Borg et al., 2012; Gravia et al., 2016; Chondrou et al., 2017; Elalfy et al., 2017). One study was part of an oral session and their results were later published in an original article already included in this systematic review (Wyszynski et al., 2004). Finally, seven cohort studies were included in this systematic review (Ma et al., 2007; Kumkhaek et al., 2008; Ware et al., 2011; Green et al., 2013; Sheehan et al., 2014; Friedrisch et al., 2016; Aleluia et al., 2017) (**Figure 1**).

Characteristics of the Included Studies

Out of the seven included studies, five studies had data from the United States and two studies had data from Brazil. Sample size in the included studies ranged from 42 to 174 patients with SCA. The publication date ranged from 2007 to 2018, and the sample mean age ranged from 8.1 to 21 y. The mean dose of HU ranged from 19 to 27.1 ± 4.3 mg/kg/day. The mean duration of treatment with HU ranged from 13.4 to 102 months (**Table 1**). Two studies calculated the change in HbF levels for each patient from baseline to the MTD (delta HbF) (Ware et al., 2011; Friedrisch et al., 2016), while other four studies used the increment in HbF after treatment with HU (final HbF) (Ma et al., 2007; Kumkhaek et al., 2008; Green et al., 2013; Sheehan et al., 2014), and one study calculated from the baseline to maximum HbF during treatment with HU (Aleluia et al., 2017).

Overall, 728 genetic polymorphisms were assessed for their association with changes in HbF levels in patients with SCA under treatment with HU, and 11 candidate genes were the most examined in the seven included studies. Four studies examined *BCL11A* and the *HBSIL-MYB* intergenic region (Ware et al., 2011; Green et al., 2013; Sheehan et al., 2014; Friedrisch et al., 2016; Aleluia et al., 2017). Three studies focused on arginase 1 and 2 (*ARG1* and *ARG2*) genes (Ma et al., 2007; Ware et al., 2011; Green et al., 2013). Two studies evaluated the secretion-associated Ras-related GTPase 1A (*SAR1A*) gene (Ma et al., 2007; Green et al., 2013). Two studies examined the *XmnI* gene (Ware et al., 2011; Friedrisch et al., 2016). The Fms-related receptor tyrosine kinase 1 (*FLT1*), hydroxyacid oxidase 2 (*HA O 2*), nitric oxide synthase 1 (*NOS1*), and olfactory receptor family 51 subfamily B member 5 and 6 (*OR51B5/6*), genes were mentioned only once by two studies (Ma et al., 2007; Aleluia et al., 2017).

Regarding the quality assessment according to the Joanna Briggs Institute checklist (**Supplementary Table S2**), one of the seven articles answered affirmative in all the 11 questions. Five studies responded affirmative to ten out of the 11 questions. One

study answered affirmative to six out of the 11 questions, while three of the questions were negative and two were not applicable.

Pharmacogenetics of Response to HU Therapy in Patients With SCA

Among the included studies, a cohort study involving 137 adult African-Americans with SCA from the Multicenter Study of Hydroxyurea in Patients With Sickle Cell Anemia (MSH) examined the association of 320 tagging SNPs from 29 candidate genes with changes in HbF concentrations (measured by %, g/dL and F-cell count) after two years of HU treatment (Ma et al., 2007). Candidate genes were involved in the regulation of DNA transcription, cell proliferation/differentiation, and drug metabolism functions. The daily dose of HU started at 15 mg/kg and increased by 5 mg/kg each week up to the MTD, which means 35 mg/kg, unless toxicity was established. The authors found 17 SNPs to be associated with % HbF change and 20 SNPs to be associated with change of absolute HbF (g/dl), most of them being located in introns or untranslated genomic regions. The SNPs found to be associated with the higher mean of squared were rs2182008 in the *FLT1* gene and rs10483801 in the *ARG2* gene, which is involved in the metabolism of HU. This MSH cohort study performed analysis with age, sex, and *β-globin* haplotypes as covariates and showed several SNPs in other genes as predictors for HbF response. In the random forest algorithm, the SNP rs21822998 in *FLT1* and the SNP rs9376173 in the phosphodiesterase 7B (*PDE7B*) gene had a higher mean of squared residuals as predictors for % HbF and absolute HbF, respectively (Ma et al., 2007).

The hypothesis for a later pharmacogenetic study (Kumkhaek et al., 2008) was supported by an experimental study on the molecular mechanisms underlying the increase in HbF levels induced by HU (Tang et al., 2005). The authors searched for differential gene expression in human adult erythroid cells and identified a small guanosine triphosphate (GTP)–binding protein, whose secretion is associated with Ras-related (SAR) protein, as a specific gene induced by HU. SAR was shown to play a key role in γ-globin (*HBG*) gene induction by promoting cell apoptosis and G1/S-phase arrest by the reduction of PI3K and extracellular signal–regulated kinase phosphorylation and increasing p21 and GATA-binding protein 2 expression (Tang et al., 2005). From these experimental findings, variations of the *SAR1A* gene were hypothesized to explain differences in individual responses to HU treatment (Kumkhaek et al., 2008). The authors tested whether 20 variants in the upstream promoter region, exon 1, and intron 1 of *SAR1A* were associated with HbF changes in response to HU compared to baseline in 32 adults with SCA from the Sickle Cell Pulmonary Hypertension Screening Study, prospectively followed up during two y of HU therapy. The SNP rs231099 was found to be associated with the change in % HbF, and the SNPs rs2310991, rs76901216, rs76901216, and rs4282891 were found to be associated with the change in absolute HbF (g/dl). The intronic SNP rs4282891 showed stronger association, which is phylogenetically conserved in vertebrates (Kumkhaek et al., 2008).

The Hydroxyurea Study of Long-Term Effects (HUSTLE) was a prospective clinical trial for pediatric patients with SCA receiving HU designed to understand the interpatient variability in the responses and toxicities to HU (Ware et al., 2011). A candidate gene study was conducted to carry out pharmacogenetic analyses for the HU end points of % HbF and the MTD. The dose administrated in patients who were included before beginning HU therapy (new cohort; $n = 88$) started with 15 mg/kg/day, and it was escalated every eight weeks to a maximum dose of 30 mg/kg/day or the MTD, with careful monitoring of blood counts every two weeks. If hematologic toxicity occurred twice at the same dose, the MTD was set at 2.5 mg/kg below the toxic dose. Pharmacogenetic analyses included 331 SNPs in candidate genes that were selected based on their presumed pharmacogenetic and pharmacodynamic effects of HU. The *ARG1* rs175999586 and *ARG2* rs2295644 SNPs were associated with the change in % HbF between baseline and MTD. The SNP rs1427407 of the *BCL11A* gene was associated with the MTD, but none was associated with the MTD after adjustment for baseline % HbF (Ware et al., 2011).

The association of several SNPs with HbF levels induced by HU was also examined in a multi-site observational study of 117 pediatric patients (5–21 y), which was mainly composed of SCA patients (93% of HbSS and 7% of Sβ0-thalassemia) (Green et al., 2013). SNPs of *BCL11A*, *HBSIL-MYB*, *HBB*, hemoglobin subunit beta (*HBE*), *OR51B6*, glucagon-like peptide 2 receptor (*GLP2R*), *SAR1A*, *ARG1*, and *ARG2* genes, which were reported as associated with baseline HbF levels, were also examined for their association with HbF under HU therapy ("maximum HbF" and "delta HbF," from baseline to maximum). Clinical indications for HU therapy were comparable across sites (nearly all for repetitive painful crises and/or acute chest episodes) at least for six months. Stable dosing was reached at three months or near maximal dose by absolute neutrophil count criteria, excluding data from subjects on less than 20 mg/kg/day, even for dose-limiting toxicity. The SNPs of *BCL11A* (rs766432, rs11886868, rs4671393, and rs7557939), *HBE* (rs7130110), and *GLP2R* (rs12103880) were associated with maximum HbF under HU. Only the SNP rs7130110 of *HBE* was associated with delta (Δ) HbF (Green et al., 2013).

A cohort composed of 171 patients from the HUSTLE study and 51 patients from the Stroke with Transfusions Changing to Hydroxyurea (SWiTCH) (called "discovery") was examined to identify genetic predictors of HbF response to HU, with focus on protein coding regions (Sheehan et al., 2014). Whole-exome sequencing was performed in two prospective pediatric cohorts with robust HbF phenotype data and a standardized dose escalation regimen to the MTD, which were genotyped for SNPs of *BCL11A* (rs1427407, rs4671393, rs11886868, and rs7599488) and *HBSIL-MYB* (rs9399137 and rs9402686). HbF responses to HU were measured by maximum % HbF at the MTD ("final HbF") or the change in % HbF from baseline to final ("delta HbF"). The patients had baseline HbF measured after three y of age. The HU therapy initiated with 20 mg/kg, and then dose was escalated to mild myelosuppression. The average age of the patients at the time of HU initiation was 10.4 ± 4.5 y. However, they found no associations of *BCL11A* or *HBSIL-MYB* variants with HbF response. Whole-exome sequencing identified 13 and 12 variants associated with final HbF and delta HbF (p-value $< 5 \times 10^{-4}$), respectively. Although these

associations did not achieve the genome-wide significance level (p-value < 1.3×10^{-6}), they did provide suggestive signals (Sheehan et al., 2014). By using functional prediction methods, the authors identified that 13 variants associated with HbF response to HU were predicted to introduce an amino acid change, inducing damage in the protein structure or function (Sheehan et al., 2014). These 13 variants were then genotyped in a validated cohort composed of 130 unrelated children with SCA receiving HU at Texas Children's Hospital Hematology Center for at least six months prior to the MTD. One variant (P840R; rs61743453) in the spalt-like transcription factor 2 (*SALL2*) gene was associated with higher delta HbF and with final HbF in the discovery and the validated cohorts, respectively. A meta-analysis combining the discovery and validation cohorts ($n = 301$) found that the P840R variant was associated with both delta HbF ($p = 8.30 \times 10^{-4}$) and final HbF ($p = 1.48 \times 10^{-4}$) (Sheehan et al., 2014).

A cohort of 121 patients with SCA under regular HU therapy for at least six months at the Sickle Cell Center of the Clinical Hospital from Porto Alegre (Southern Brazil) was examined for the effect of genetic variants at the major loci modifier of baseline HbF on HU-induced HbF levels (Friedrisch et al., 2016). Patients who received any other drugs stimulating HbF (e.g., erythropoietin) or blood transfusion within three months prior to the study were excluded. HbF measurements were obtained before HU (baseline HbF) and at the MTD (MTD HbF), and the change from baseline to the MTD (delta HbF) was calculated for each patient. The association tests were performed by linear regression analyses adjusted for age at start HU, gender, and absolute neutrophil count at MTD. SNPs of hemoglobin subunit gamma 2 (*HBG2*) (rs7482144), *BCL11A* (rs1427407, rs4671393, and rs11886868), and *HBS1L-MYB* (rs9399137 and rs9402686) were assessed, and patients with variations in SNPs of *BCL11A* had a favorable probability of producing more HbFs in response to HU treatment (Friedrisch et al., 2016).

A cross-sectional study of 141 patients with SCA, including 99 patients under HU treatment, followed up at the Sickle cell Disease Reference Center in Itabuna (Northeastern Brazil) was examined for the role of *HBB* haplotypes and SNPs at quantitative trait loci (QTL) associated with baseline HbF in regulating HbF response to HU (Aleluia et al., 2017). HbF measures were not performed in patients with clinical manifestations of vaso-occlusive crisis or transfusions in the last three months. Patients were genotyped for β^S-*globin* gene cluster haplotypes and SNPs of *BCL11A* (rs6732518 and rs766432), *HBS1L-MYB* (rs11759553 and rs3595442), and *OR51B5/6* (rs4910755 and rs7483122). Almost 48% of the patients received 15 mg/kg/day, while 23.8% received 20 mg/kg/day and 26.2% received 25 mg/kg/day. The only patient who received the maximum dose of 35 mg/kg/day was excluded from the analysis. Multiple linear regression analysis adjusted for gender and age were used to investigate the association of SNPs with HbF induction, and the authors concluded that homozygous subjects for the minor allele of rs766432 of *BCL11A* had higher increases in HbF (Aleluia et al., 2017).

In summary, seven studies involving patients with SCA treated with HU identified 50 SNPs of 17 different genes to be associated with HbF changes from baseline to HU (**Table 2**; **Figure 2**). Five out of the seven included studies examined SNPs of *BCL11A*, of which four (80%) found SNPs to be associated with HbF changes (Ware et al., 2011; Green et al., 2013; Friedrisch et al., 2016; Aleluia et al., 2017). These studies confirmed the associations of the *BCL11A* SNPs rs1427407 (Ware et al., 2011; Friedrisch et al., 2016), rs4671393, rs11886868 (Green et al., 2013; Friedrisch et al., 2016), rs766432 (Green et al., 2013; Aleluia et al., 2017), and rs7557939 (Green et al., 2013). In addition, two studies found associations for SNPs of *ARG1* (rs17599586, rs2781667, and rs1799586) and *ARG2* (rs2246012, rs2295644, rs10483801, and rs10483802) (Ma et al., 2007; Ware et al., 2011). Among them, only the SNP rs1799586 of *ARG1* was found to be associated with HbF changes in the two studies (Ma et al., 2007; Ware et al., 2011).

Gene Set Enrichment Analysis and Pathway Analysis

Reactome pathways were obtained from the enrichment analysis using the set of genes that had SNPs found to be associated with changes on HbF levels in patients with SCA under HU therapy (**Figure 3**; **Supplementary Table S3**). The reactome pathways with both lowest adjusted p-values and highest combined scores were related to VEGF binding, namely, "VEGF ligand–receptor interactions" (R-HSA-194313; adjusted p-value = 0.0002847; combined score = 4,826.43) and "VEGF binds to VEGFR leading to receptor dimerization" (R-HSA-195399; adjusted p-value = 0.0002847; and combined score = 4,826.43). Moreover, we obtained the reactome pathway "urea cycle" (R-HSA-70635; adjusted p-value = 0.0003048; combined score = 3,461.84)] (**Figure 3**; **Supplementary Table S3**). The reactome pathway "nitric oxide stimulates guanylate cyclase" (R-HSA-392154; **Figure 3**) ranked fourth but with a lower combined score (200.68; p-value = 0.02105; **Supplementary Table S3**).

DISCUSSION

Genetic variability in response to HU therapy is scarcely explored, despite almost 50 y of HU use and 30 y of the treatment of patients with SCA (Ware et al., 2011). Notably, the literature regarding the effects of genetic polymorphisms on HbF levels in patients with SCA (Hb SS) treated with HU is remarkably scarce. In this systematic review, only seven studies met the inclusion criteria.

Importantly, patient-specific factors, including age, concomitant diseases, diet, and genetic factors, contribute to the interindividual variability in drug efficacy and risk of adverse reactions, and genetic polymorphisms explain around 20–30% of the interindividual variability in drug response (Lauschke et al., 2017). Indeed, the knowledge of how genetic variation contributes to variation in drug response has been expanded (Gong et al., 2021), and guidelines for the clinical implementation of pharmacogenetics have been published (Relling et al., 2020).

Single-Nucleotide Polymorphisms and HbF Modulation

As expected, some genes previously associated with baseline HbF and known for acting in the HbF regulation pathway were found

TABLE 2 | Genes and chromosomes for the 50 different SNPs found to be associated with changes on HbF [described as delta (Δ) % HbF, Δ HbF (g/dl), maximum tolerated dose (MTD) % HbF, or maximum HbF] in response to hydroxyurea therapy in the seven cohort studies included in the systematic review. *The SNP rs17599586 of *ARG1* and three SNPs of *BCL11A* (rs1427407, rs4671393, and rs11886868) were found to be associated by two different cohort studies.

Gene	Chromosome	SNP	HbF response	Reference
ARG1	6	*rs17599586	Δ % HbF; Δ HbF (g/dL)	Ma et al. (2007)
ARG1	6	rs2781667	Δ % HbF; Δ HbF (g/dL)	Ma et al. (2007)
ARG1	6	*rs17599586	Δ % HbF	Ware et al. (2011)
ARG2	14	rs2246012	Δ HbF (g/dl)	Ma et al. (2007)
ARG2	14	rs2295644	Δ % HbF	Ware et al. (2011)
ARG2	14	rs10483801	Δ % HbF; Δ HbF (g/dl)	Ma et al. (2007)
ARG2	14	rs10483802	Δ % HbF; Δ HbF (g/dl)	Ma et al. (2007)
ASS	9	rs7860909	Δ % HbF; Δ HbF (g/dl)	Ma et al. (2007)
ASS	9	rs10793902	Δ % HbF	Ma et al. (2007)
ASS	9	rs10901080	Δ % HbF	Ma et al. (2007)
ASS	9	rs543048	Δ HbF (g/dl)	Ma et al. (2007)
BCL11A	2	rs766432	Δ % HbF	Aleluia et al. (2017)
BCL11A	2	*rs1427407	MTD % HbF; Δ % HbF	Friedrisch et al. (2016)
BCL11A	2	*rs4671393	MTD % HbF	Friedrisch et al. (2016)
BCL11A	2	*rs11886868	MTD % HbF; Δ % HbF	Friedrisch et al. (2016)
BCL11A	2	rs766432	Maximum HbF	Green et al. (2013)
BCL11A	2	*rs11886868	Maximum HbF	Green et al. (2013)
BCL11A	2	*rs4671393	Maximum HbF	Green et al. (2013)
BCL11A	2	rs7557939	Maximum HbF	Green et al. (2013)
BCL11A	2	*rs1427407	MTD, mg/kg	Ware et al. (2011)
FIGF	X	rs6632521	Δ % HbF	Ma et al. (2007)
FLT1	13	rs9319428	Δ % HbF; Δ HbF (g/dl)	Ma et al. (2007)
FLT1	13	rs2182008	Δ % HbF; Δ HbF (g/dl)	Ma et al. (2007)
FLT1	13	rs3751395	Δ HbF (g/dl)	Ma et al. (2007)
FLT1	13	rs8002446	Δ % HbF; Δ HbF (g/dl)	Ma et al. (2007)
FLT1	13	rs2387634	Δ HbF (g/dl)	Ma et al. (2007)
GLP2R	17	rs12103880	Maximum HbF	Green et al. (2013)
HA O 2	1	rs10494225	Δ % HbF	Ma et al. (2007)
HBE	11	rs7130110	Maximum HbF; Δ % HbF	Green et al. (2013)
MAP3K5	6	rs9376230	Δ % HbF	Ma et al. (2007)
MAP3K5	6	rs9483947	Δ % HbF	Ma et al. (2007)
NOS1	12	rs7309163	Δ HbF (g/dl)	Ma et al. (2007)
NOS1	12	rs816361	Δ % HbF	Ma et al. (2007)
NOS1	12	rs7977109	Δ % HbF; Δ HbF (g/dL)	Ma et al. (2007)
NOS2A	17	rs1137933	Δ % HbF	Ma et al. (2007)
NOS2A	17	rs944725	Δ % HbF	Ma et al. (2007)
PDE7B	6	rs2327669	Δ HbF (g/dl)	Ma et al. (2007)
PDE7B	6	rs11154849	Δ HbF (g/dl)	Ma et al. (2007)
PDE7B	6	rs9376173	Δ HbF (g/dl)	Ma et al. (2007)
PDE7B	6	rs1480642	Δ HbF (g/dl)	Ma et al. (2007)
PDE7B	6	rs487278	Δ HbF (g/dl)	Ma et al. (2007)
PIR	X	rs2071182	Δ HbF (g/dl)	Ma et al. (2007)
SALL2	14	rs61743453	Δ % HbF	Sheehan et al. (2014)
SAR1	10	rs2310991	Δ % HbF; Δ HbF (g/l)	Kumkhaek et al. (2008)
SAR1	10	rs76901216	Δ HbF (g/dl)	Kumkhaek et al. (2008)
SAR1	10	rs76901220	Δ HbF (g/dl)	Kumkhaek et al. (2008)
SAR1	10	rs4282891	Δ HbF (g/dl)	Kumkhaek et al. (2008)
TOX	8	rs2693430	Δ HbF (g/dl)	Ma et al. (2007)
TOX	8	rs826729	Δ % HbF	Ma et al. (2007)
TOX	8	rs765587	Δ % HbF; Δ HbF (g/dl)	Ma et al. (2007)
TOX	8	rs9693712	Δ % HbF; Δ HbF (g/dl)	Ma et al. (2007)
TOX	8	rs172652	Δ % HbF	Ma et al. (2007)
TOX	8	rs380620	Δ % HbF; Δ HbF (g/dl)	Ma et al. (2007)
TOX	8	rs12155519	Δ HbF (g/dl)	Ma et al. (2007)

to be associated with HbF changes in response to HU therapy in the included studies. There is evidence that SNPs of intron 2 of *BCL11A* affect HbF changes in patients with SCA treated with HU. Five out of the seven included studies examined the role of the three main QTLs associated with baseline HbF levels: *BCL11A*, *XmnI*, and *HBS1L-MYB* intergenic region (Ware et al., 2011; Green et al., 2013; Sheehan et al., 2014; Friedrisch et al., 2016; Aleluia et al., 2017). Noteworthy, BCL11A is a negative regulator of HbF expression. Subjects with variation in any of the established SNPs of *BCL11A* are known to have

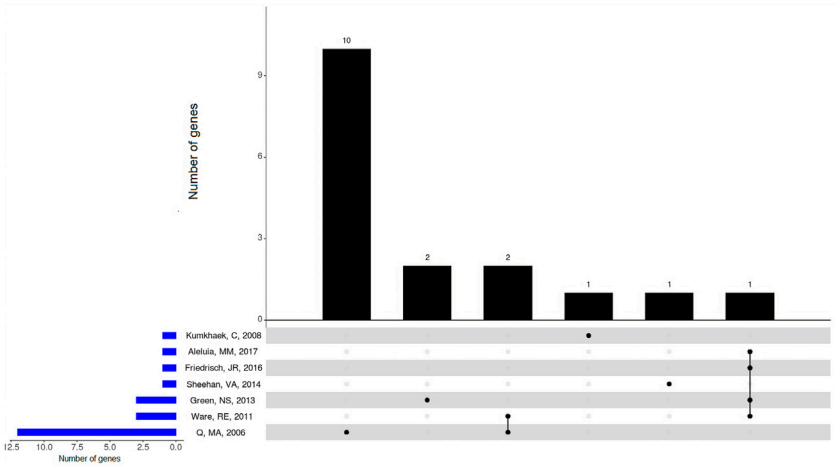

FIGURE 2 | Overlapping genes with SNPs associated with changes on HbF levels in the included studies involving patients with SCA (Hb SS) under HU therapy. *BCL11A*, *ARG1*, and *ARG2* genes showed overlap between included studies. Upset plot showing the total number of genes with SNPs found to be associated with changes on HbF levels identified in each study (horizontal bars), and the number of genes exclusive to one study or shared by different studies (vertical bars). Black dots below vertical bars indicate genes quantified.

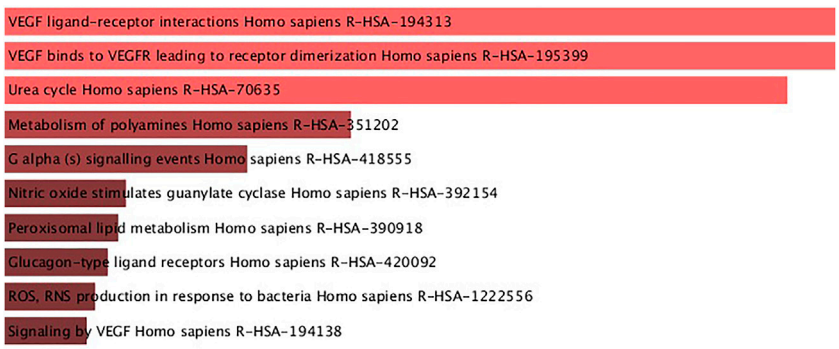

FIGURE 3 | Reactome pathways obtained from enrichment analysis of the set of genes with SNPs found to be associated with HbF changes in patients with SCA treated with hydroxyurea. Pathways are ordered from top to bottom according to the lowest adjusted *p*-values, and light red bars indicate adjusted *p*-values < 0.001.

decreased *BCL11A* expression, which results in increased HbF production (Lettre et al., 2008; Bauer et al., 2013). *HBS1L-MYB* genes are expressed in the erythroid precursor cells (Lettre et al., 2008; Bauer et al., 2013). *HBS1L* encodes a protein with apparent GTP-binding activity and is involved in a variety of cellular processes, while *MYB* encodes a transcription factor for erythroid differentiation in hematopoiesis (Thein et al., 2007). The *HBS1L-MYB* intergenic region is known to contain several common QTLs associated with HbF levels and a long-range erythroid enhancer that regulates *MYB* expression by chromatin looping (Stadhouders et al., 2014). Finally, the *XmnI* restriction site at -158 position of the *HBG* gene is associated with an increased expression of γ-globin and higher HbF production (Sripichai and Fucharoen, 2016). Together, they account for approximately 20–50% of the variation in HbF levels in patients with SCA and β-thalassemia, and even in healthy adults (Galarneau et al., 2010).

Four out of five studies that examined SNPs of *BCL11A* found associations with HbF response (Ware et al., 2011; Green et al., 2013; Sheehan et al., 2014; Friedrisch et al., 2016). These SNPs (rs1427407, rs4671393, rs11886868, rs766432, and rs7557939) are located in intron 2 of *BCLL1A*, which is a region marked by functional elements. These SNPs are located nearby several DNase hypersensitive sites, which indicate a genomic region of open chromatin. Noteworthy, the critical SNP rs1427407 (G > T) falls within a +62 DNase I hypersensitive site, an erythroid enhancer of *BCL11A*, and overlaps a peak of GATA1 and TAL1 transcription factor–binding sites. Notably, the minor T-allele for the SNP rs1427407 disrupts the G nucleotide of a consensus sequence [CTG (n9)] enriched for GATA1 and TAL1 transcription factors. GATA1- and TAL1-binding sites are more frequent in the G-allele than T-allele in the primary erythroblast samples (Bauer et al., 2013). In agreement with our findings regarding the effect of SNPs of *BCL11A* on HbF response, a recent

functional *in vitro* study based on gene editing comparative analysis showed that *BCL11A* is the most clinically relevant approach focused on HbF resurgence (Lamsfus-Calle et al., 2020). This functional information supports the effect of *BCL11A* SNPs on baseline HbF, but the way it affects the response to HU remains to be elucidated. Biological network analysis integrating effects of HU on gene expression in erythroid precursors could highlight pathways involved in this process.

The *Xmnl* variant in the *HBBP1* gene was also examined. One study found the SNP rs7482144 to be associated only with an increase in baseline HbF (Ware et al., 2011) but not with HbF changes to HU, while other studies excluded this SNP because it had a very low allele frequency (0.45%, only one heterozygous subject) in a Brazilian cohort (Friedrisch et al., 2016).

The SNPs rs9399137 and rs9402686 are located in the *HBS1L-MYB* intergenic region but were not found to be associated with the increase in HbF in two included studies (Sheehan et al., 2014; Friedrisch et al., 2016). *SAR1A*, a gene belonging to the small GTPase superfamily that encodes a GTP-binding protein called SAR1A, has been reported to be associated with *HBG* expression. The SNP rs2310991 of *SAR1A* was associated with the change in absolute HbF concentration (Kumkhaek et al., 2008). Conversely, other studies found no association of rs2310991 with posttreatment HbF levels (Kumkhaek et al., 2008; Green et al., 2013).

Our findings may potentially guide the selection of candidate gene regulatory sequences within these genomic regions to be validated by *in vitro* functional assays in cells treated with HU, such as luciferase reporter assays. Further studies may examine whether the variation in these SNPs would affect the activity of the gene regulatory element, such as an enhancer or a silencer. Therefore, the present findings can contribute to guide further functional studies, which may advance the research focused on genomics of HbF changes in response to HU therapy.

Signaling Pathways Underlying HbF Changes in Response to HU Therapy

Pathway analysis using genes with SNPs found to be associated with HbF changes in patients with SCA treated with HU in the included studies revealed pathways underlying HbF changes in response to HU. For example, we found enrichment of the reactome pathway "urea cycle" (R-HAS-70635; **Figure 3**), which is directly related to arginine (Friebe and Koesling, 2003). Indeed, cytosolic arginase 1 is a canonical enzyme of the urea cycle. Arginase 2 was described to play a role in the regulation of the urea cycle arginine metabolism and in downregulation of nitric oxide synthesis (Mori, 2007). Arginase isoforms encoded by *ARG1* and *ARG2* genes were also related to the increase in HbF levels induced by HU (Ware et al., 2011). The SNPs rs175999586 and rs2295644 of *ARG1* and *ARG2* were associated with the changes in HbF, respectively. Notably, rs2295644 has been implicated in kidney disease, so it could affect the renal clearance of HU and possibly the dose of the MTD (Ware et al., 2011). Another *ARG2* SNP (rs10483801) was also associated with the absolute HbF change (Ma et al., 2007).

We also found the enrichment of the reactome pathway "nitric oxide stimulates guanylate cyclase" (R-HSA-392154; **Figure 3**). Noteworthy, HU was suggested to act as a nitric oxide donor in patients with SCA (King, 2004). Nitric oxide is synthetized from L-arginine, stimulates vasodilatation of the endothelium and disaggregation of platelet aggregates, and inhibits platelet activation, an important modulator of SCA pathophysiology (Radomski et al., 1987). Moreover, HU was shown to modulate the nitric oxide signaling pathway in erythrocytes, rheology of erythrocytes, and oxidative stress through its effects on HbF and possibly on nitric oxide bioavailability (Nader et al., 2018).

A complex regulatory environment determines the HbF concentration in the blood, as well as chromosome remodeling, transcription factors, erythropoiesis modulation, gene regulatory elements linked to the β-globin gene cluster, and the kinetics of erythroid cell differentiation and differential red cell survival (Ma et al., 2007). Therefore, there is a large opportunity for the genetic modulation of HbF production. Consistent with this complex regulation apparatus, even with the restricted number of studies, our systematic review suggests that there is huge heterogeneity in genetic elements modulating the HbF levels in response to HU treatment. Unfortunately, some genetic associations with HbF response have not been reproduced by other studies, and further investigations are needed to conclude their use in predicting HbF response to HU.

Dosing and monitoring regimens of HU have yet to be determined (Ware, 2010). The best results from treatment with HU are found when the dose is escalated to the MTD, improving laboratory variables and reducing clinical complications. The dose escalation of HU is a labored process that requires risk monitoring of cytopenias, mainly neutropenia, and the clinical response to treatment with HU may take up to six months after reaching the MTD (NIH US, 2014). Therefore, severe patients with clinical recommendation for HU might have to experience a long exposure time until deducing that the treatment with HU is ineffective. Therefore, the prediction of HbF induction in response to treatment with HU by using SNPs in the intron 2 of *BCL11A* gene may have potential clinical applicability in the management of SCA.

The induction of HbF is a powerful mechanism of action of HU. However, since several other mechanisms of actions are known, further research is needed to conclude whether such SNPs are able to predict a subgroup of patients as "responders" to HU. Noteworthy, these SNPs were previously associated with increased baseline HbF levels, milder hematological parameters, and lower risk of clinical complications (Sales et al., 2020). Interestingly, not all these associations were dependent on HbF. Therefore, future studies should evaluate if the SNPs located in intron 2 of the *BCL11A* gene are also able to distinguish patients who show a reduced rate of clinical complications when treated with HU from those patients who do not show this reduction. This evidence is of huge importance to assess the cost-effectiveness of the use of pharmacogenetic tests for these SNPs in the SCA management.

Some studies do not meet the inclusion criteria of this review due to the different genotypes of the study subjects (Borg et al., 2012; Chondrou et al., 2017; Elalfy et al., 2017). They involve other β-type hemoglobinopathies, and known differences in their hematological parameter could bias the review. However, these studies highlight specific points regarding pathways related to the HbF regulation. Two studies suggested that *KFL1* expression and the SNP rs3191333 of *KFL1* play a role in the HbF regulation and are biomarkers of HU response in β-type hemoglobinopathies. It makes biological sense, since KLF1 regulates *BCL11A* expression and the γ- to β-globin gene switching (Zhou et al., 2010). Further studies can confirm their influence in HU therapy in patients with SCA (Hb SS).

Another study suggests that the vascular endothelial growth factor (*VEGFA*) gene is a biomarker in β-type hemoglobinopathies severity and efficacy of HU therapy (Chondrou et al., 2017). These findings are in agreement with a study included in this systematic review that found SNPs in the *FLT1* gene, encoding VEGF receptor 1, associated with HbF changes by HU therapy in Hb SS patients (Ma et al., 2007). Interestingly, we found enrichment of two reactome pathways related to VEGF ligand–receptor interactions (R-HSA-194313 and R-HSA-195399; **Figure 3**). The binding of VEGF ligands to VEGFR receptors in the cell membrane triggers intracellular signaling cascades, which results in proliferation, survival, migration, and increased permeability of vascular endothelial cells (Matsumoto and Mugishima, 2006). It is important to SCA pathophysiology, since endothelial dysfunction plays a key role in sickle cell vasculopathy, as reviewed elsewhere (Wood et al., 2008).

Our systematic review highlighted the role of SNPs on HbF induction upon HU therapy. However, it is important to note that this is one of the several mechanisms underlying response to HU. Indeed, a previous systematic review reported on the molecular mechanisms of HbF induction by HU in SCD (Pule et al., 2015). The reviewed findings pointed out three main pathways: epigenetic modifications, signaling pathways involving HU-mediated response, and posttranscriptional pathways, focusing on regulation by small non-coding RNAs (miRNAs). In this context, several miRNAs were identified as differentially expressed in patients with SCD under HU treatment, most of them being functionally related to genes known to regulate HbF, including *BCL11A* (Mnika et al., 2019). Notably, an experimental study showed that downregulation of *BCL11A*, *MYB*, and *KLF1* induces *γ-globin* expression by miRNA-mediated mechanisms, and miR-26b directly interacted with the 3′-untranslated region of *MYB* (Pule et al., 2016). Since miRNAs have been associated with a multitude of regulatory mechanisms, their functions may add to the complex mechanisms underlying response to HU.

In summary, the regulation of HbF involves both *cis*- and *trans*-regulatory elements, which interact in a complex network. HU promotes the induction of HbF, and the mechanisms by which it interacts with genetic modifiers of HbF affecting drug response are not fully understood. In this context, SNPs located within gene regulatory elements can have a major effect on differences in drug response (Luizon and Ahituv, 2015). A proposed schematic diagram to HbF regulation in response to HU is shown in **Figure 4**, including the functional findings of genes found in this systematic review as candidates to modify the HbF response to HU in patients with SCA.

Confounding Factors

The first clinical manifestations of SCA appear along with the replacement of HbS by HbF (Rumaney et al., 2014). After 10 y, age is no longer an indicator of red cell deformability in patients with SCA; instead, this hemorheological parameter is mainly affected by the level of HbF, sex, and HU treatment (Thein et al., 2007).

The level of HbF is the best predictor of clinical severity of SCA (Wonkam et al., 2014). However, there is no threshold or value that characterizes the high and low baseline HbF levels for response to HU treatment. It was established that subjects who start with baseline HbF values between 5 and 10% can have a 2- to 3-fold HbF increase, whereas subjects with very low baseline HbF can have a 10-fold increase after treatment with HU (Wonkam et al., 2014). In the MSH cohort study, the baseline HbF was not predictive to HbF response to HU. On the other hand, baseline HbF was found as a predictor of the direction of association to % HbF at MTD (Ware et al., 2011). However, the change in HbF outcome in patients with SCA treated with HU was largely heterogeneous among the seven included studies, which examined different HbF outcomes in response to HU. Changes in HbF upon HU therapy was calculated as absolute HbF (g/dl), % HbF, and F-cell count from baseline until the MTD or a defined time of therapy (about 2 y). Although the % HbF and the amount of F cells are highly correlated, some patients with high levels of HbF develop severe complications of SCA probably due to the heterogeneous distribution of HbF among erythrocytes (Khandros et al., 2020). The number of F cells with polymer-inhibiting concentrations of HbF is likely to be a more accurate predictor of clinical benefits of HU therapy than HbF levels. However, the distribution of HbF among F cells is often unavailable, mainly in health centers of least developed countries. Using HbF under the MTD to calculate delta HbF probably provides the maximum level that the patient can achieve.

Patients with SCA experience several acute clinical events involving pronounced changes in hematological parameters (Novelli and Gladwin, 2016). Moreover, they commonly receive blood transfusion for treating and avoid a range of complications, which introduces biases on evaluating the association with hematological variables, including HbF. However, three of the seven included studies did not describe whether strategies were used to deal with these established confounding factors, which introduces bias in our analyses and constitute a limitation of this systematic review.

Although clinical experience of HU therapy for patients with SCA has been related for more than 25 y, there is still much questioning about the pharmacokinetics, pharmacodynamics,

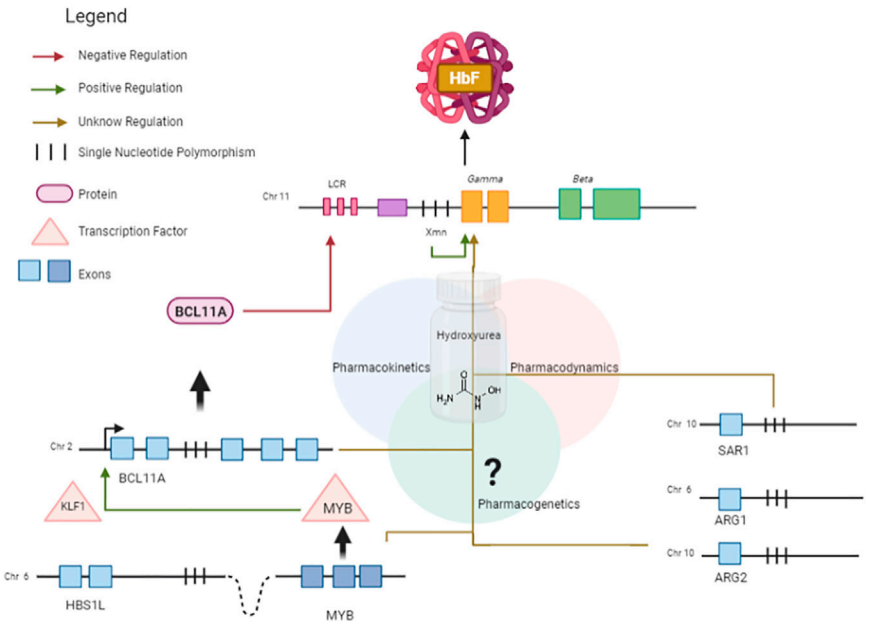

FIGURE 4 | Schematic diagram showing HbF regulation in response to HU, an HbF-inducing drug. *BCL11A* is a master repressor of gamma genes. The erythroid transcription factor KLF1 activates the transcription of *BCL11A* by binding to its promoter region. The expression of *KLF1* is possibly regulated by MYB. The *HBS1L-MYB* intergenic region contains several trait-associated common genetic variations and a distal enhancer of *MYB*, promoting long-range transcription regulation by chromatin looping. *SAR1A* encodes a GTP-binding protein which has been reported to be associated with γ-globin expression. Variations in the candidate genes *ARG1* and *ARG2* are associated with HbF changes. Pharmacokinetic genes can affect absorption, distribution, and metabolism of HU.

and pharmacogenetics of HU (Ware et al., 2011). To better understand the interpatient variability, polymorphisms in genes encoding drug-metabolizing enzymes and solute transporters were recently examined to learn their role in HU bioavailability and metabolism (Yahouedehou et al., 2020). The authors found evidence for the involvement of enzymes of the CYP450 family and catalases in the metabolism of HU, and the association between urea transporter-B (UTB) and response to HU in erythroid cells. SNPs in the *CYP2D6* (rs3892097), *CAT* (rs7943316 and rs1001179), and *SLC14A1* (rs2298720) genes were found to be linked to reduced metabolism or the elimination of HU, which may increase its therapeutic effects in patients with SCA (Yahouedehou et al., 2020). Unfortunately, this study did not examine the association between the SNPs with HbF response to HU, and thus, it was not included in the systematic review.

There was great heterogeneity in the patients' age in the included studies. For example, one study examined patients aged 4–54 y (mean 21 ± 14 y) (Friedrisch et al., 2016). The average age of initiation of HU was 9.6 ± 4.8 y in another study (Ware et al., 2011).

The present study has layers of complexity linked to the multifactorial characteristic of the disease. The heterogeneity of HU dose, patient age, HbF outcomes in response to HU, and candidate genes brought limitations to the search and contributed to the result being only seven included studies. These findings highlight that the pharmacogenetics of response to HU in patients with SCA is a fertile field for further investigations.

CONCLUSION

The literature about the pharmacogenetics of response to HU therapy in patients with SCA is highly heterogeneous regarding the chosen candidate genes and SNPs examined for the possible association with changes in HbF levels, and regarding the HbF outcomes measured during HU therapy. Nevertheless, the findings of the studies included in this systematic review point out two main conclusions. First, as well as the baseline HbF, changes in HbF levels in response to HU therapy are likely to be regulated by genetic variations on multiple loci. Second, there is evidence that SNPs located in intron 2 of the *BCL11A* gene affect HbF changes in patients with SCA treated with HU. However, further studies are needed to test whether such SNPs may also predict the success of the treatment in ameliorating other hematological parameters and reducing the incidence of clinical complications.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**; further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

RS, BN, JT, KG, and ML made substantial contributions to the conception or design of the work; RS, BN, and JT acquired the data,

and all authors analyzed and interpreted the data for the work; RS, BN, and ML drafted the manuscript, and all authors revised it critically for important intellectual content; and all authors read and approved the final version of the manuscript for submission.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Elucidation of Cellular Contributions to Heparin-Induced Thrombocytopenia Using Omic Approaches

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Heparin-induced thrombocytopenia (HIT) is an unpredictable, complex, immune-mediated adverse drug reaction associated with a high mortality. Despite decades of research into HIT, fundamental knowledge gaps persist regarding HIT likely due to the complex and unusual nature of the HIT immune response. Such knowledge gaps include the identity of a HIT immunogen, the intrinsic roles of various cell types and their interactions, and the molecular basis that distinguishes pathogenic and non-pathogenic PF4/heparin antibodies. While a key feature of HIT, thrombocytopenia, implicates platelets as a seminal cell fragment in HIT pathogenesis, strong evidence exists for critical roles of multiple cell types. The rise in omic technologies over the last decade has resulted in a number of agnostic, whole system approaches for biological research that may be especially informative for complex phenotypes. Applying multi-omics techniques to HIT has the potential to bring new insights into HIT pathophysiology and identify biomarkers with clinical utility. In this review, we review the clinical, immunological, and molecular features of HIT with emphasis on key cell types and their roles. We then address the applicability of several omic techniques underutilized in HIT, which have the potential to fill knowledge gaps related to HIT biology.

Keywords: heparin-induced thrombocytopenia, adverse drug reaction, t-cell, neutrophil, genomics, transcriptomics, metagenomic, proteomic

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INTRODUCTION

Heparin is a widely used anticoagulant indicated for a broad range of diseases and procedures. Approximately one third of all hospitalized patients in the United States receive heparin therapy (Campbell et al., 2000). This frequency of administration is due in part to multiple pharmacologic benefits of heparin, including immediate onset of action, rapid reversibility, and relative ease of titration (Bauer et al., 1997). However, these advantages are potentially offset by the immune-mediated complication, heparin-induced thrombocytopenia (HIT). HIT occurs in 0.2–2.7% of patients exposed to heparin anticoagulants and this risk increases in patients undergoing cardiac surgery (Warkentin et al., 1995; Warkentin et al., 2000; Girolami et al., 2003; Martel et al., 2005; Smythe et al., 2007). Despite the high prevalence and potentially life-threatening prognosis of this immune-mediated adverse drug reaction (ADR), the pathophysiology of HIT is still poorly understood making it difficult to predict and prevent.

HIT pathogenesis is initiated when heparin molecules, bound to circulating Platelet Factor 4 (PF4) proteins, are recognized by immunoglobulin G (IgG) antibodies (Kelton et al., 1994). These PF4/heparin antibodies then engage with platelets, leading to platelet activation and ultimately

thrombocytopenia. Despite extensive research efforts towards understanding the immunopathology of HIT, fundamental knowledge gaps persist regarding HIT etiology (Cuker 2011; Greinacher et al., 2011; Karnes 2018). The HIT immunogen, the roles of antigen presenting cells and T-cells, and the B cell subtypes that produce the antibody remain unknown (Arepally 2017). The contributing role of non-platelet cell types in HIT, the interactions between cell types (platelets, monocytes, neutrophils, T-cells, etc.), and the heterogeneity of cell types intrinsic to HIT have yet to be fully resolved. Furthermore, the clinical significance of non-pathogenic PF4/heparin antibodies and the molecular basis that distinguishes them from pathogenic PF4/heparin antibodies remain unclear (Khandelwal and Arepally 2016). Transgenic mouse models (Zheng et al., 2015; Tutwiler et al., 2016; Perdomo et al., 2019), microfluidics devices (Tutwiler et al., 2016), and *in vitro* work on isolated cells (Kasthuri et al., 2012; Zhou et al., 2016) including platelets, peripheral blood mononuclear cells (PBMCs) (Kasthuri et al., 2012), and neutrophils (Duarte et al., 2019; Lelliott et al., 2020) have previously been employed to understand the pathophysiology of HIT. Although informative, prior targeted molecular approaches have not fully identified the mechanisms of HIT, likely due to the complicated and unusual nature of the HIT immune response. The rise in omics and "Big Data" over the last decade has resulted in a number of agnostic, whole system approaches for biological research that are especially informative for complex phenotypes. The common omics disciplines including genomics, transcriptomics, proteomics, metabolomics, and metagenomics, all have made great strides to answer questions in a wide range of biological topics. In the HIT field, however, these techniques have been under-utilized with studies employing only genome-wide association (Karnes et al., 2015; Karnes et al., 2017a; Witten et al., 2018) and array-based transcriptomic (Haile et al., 2017) approaches. This review will outline the current understanding of HIT pathogenesis within the context of specific cell types. This review will then evaluate omics techniques which might answer many unresolved questions related to HIT pathogenesis.

Clinical Features of Heparin-Induced Thrombocytopenia

In contrast to most immune-mediated ADRs, the immune response in HIT is atypical and transient (Karnes et al., 2019). HIT is characterized by a fall in platelets (thrombocytes), 5–14 days after exposure to heparin (Prince and Wenham 2018). Mortality associated with HIT can reach 30% (Franchini 2005; Martel et al., 2005). PF4/heparin antibodies are necessary but not sufficient for HIT to occur, and these antibodies are typically produced 5–10 days after heparin exposure (Reilly et al., 2001; Staibano et al., 2017). IgG antibodies bind to PF4/heparin complexes to form ultra-large complexes (ULCs). These PF4/heparin antibodies are rarely detected in healthy individuals, with one study identifying ~3% (Khandelwal and Arepally 2016) of the general population had detectable antibodies, using an optical density (OD) threshold of 0.4. A second study corroborated these findings and observed that 4.4% of healthy patients had antibodies against PF4/heparin (OD threshold = 0.5) (Krauel et al., 2011). In patients taking heparin, PF4/heparin antibodies are seen in 8–50% of patients (Arepally, 2017).

Up to half of patients with confirmed HIT experience thromboembolic complication including limb-threatening and life-threatening venous or arterial thrombosis (Prince and Wenham 2018). When thromboembolic events occur, the condition is sometimes referred to as HIT-associated thrombosis or HIT with thrombosis (HITT). In many patients, thromboembolic complications occur before a decrease in platelet count is observed (Prince and Wenham 2018). Less common presentations can also occur, such as skin necrosis and venous limb gangrene (Arepally 2017). Female sex, intravenous route of administration, and major surgery increase the risk of HIT (Arepally and Ortel 2010; Linkins et al., 2012). Patients receiving heparin within the last 90 days may experience rapid onset HIT within 24 h (Greinacher 2015). Patients receiving unfractionated heparin (UFH) are at higher risk of HIT compared to those receiving low molecular-weight heparin (LMWH) (Stein et al., 2009). One meta-analysis showed an absolute HIT risk of 0.2% with LMWH and 2.6% with UFH (Martel et al., 2005). Fondaparinux, a synthetic pentasaccharide fragment of heparin, shows almost no cross-reactivity with PF4/heparin antibodies and HIT is rare during fondaparinux treatment (Greinacher et al., 2017). Fondaparinux-associated HIT cases may be due to autoimmune HIT rather than fondaparinux. Autoimmune HIT occurs even in the absence of heparin but exhibits many clinical features of HIT (Greinacher et al., 2017). Proposed mechanisms of autoimmune HIT include endogenous polyanions, such as non-heparin glycosaminoglycans (GAGs), binding to PF4 complexes and exposing a neoepitope similar to heparin, which is subsequently recognized by IgG antibodies.

Confirmation of HIT requires both clinical and experimental confirmation. Clinically, a key indicator of HIT is an absolute drop in platelet count of 30–50% or a drop in platelet count of 40,000 to 80,000 per cubic mL (Greinacher 2015; Warkentin and Greinacher 2016). Platelet count along with other clinical indicators such as timing of onset of thrombocytopenia, presence of thrombosis or other sequelae, and alternative causes of thrombocytopenia collectively comprise the 4T scoring system (Warkentin and Greinacher 2016). Laboratory testing is also frequently used to confirm diagnosis of HIT. These laboratory tests include enzyme-linked immunosorbent assay (ELISA), which is used to test for PF4/heparin antibodies, and washed platelet activation assays such as the serotonin-release assay (SRA) and the heparin-induced platelet activation assay (HIPA) (Warkentin and Greinacher 2016), which quantify platelet activation in suspected patients' serum. The SRA and HIPA assays are the "gold-standard" for detecting HIT but vary slightly in their assay endpoints. The SRA assay measures the release of radiolabel C^{14}–serotonin from activated platelets while the HIPA test measures formation of platelet aggregation (Bakchoul et al., 2014). These functional assays are time-intensive and technically challenging, resulting in few

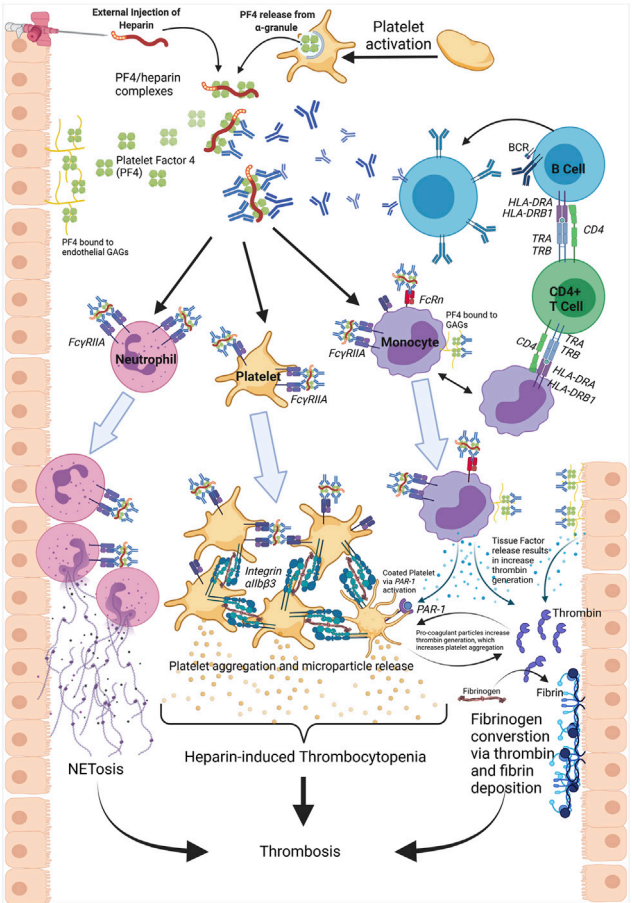

FIGURE 1 | Heparin-induced thrombocytopenia (HIT) is initiated by the binding of heparin to platelet factor 4 (PF4), released from platelet α-granules. This binding results in a conformational change and exposure of a neo-epitope. PF4/heparin IgG antibodies generated from B cells bind to the neo-epitope, forming PF4/heparin/IgG ultra-large complexes (ULCs). The Fc region of the antibodies engage with FcγRIIa receptors on platelets, neutrophils, and monocytes. In platelets, this ULC binding results in intracellular activation, release of pro-coagulant microparticles, and subsequent platelet-platelet aggregation via Integrin αIIb/β3 engagement. The consumption of platelets into thrombi and platelet disintegration via microvesiculation causes thrombocytopenia. Neutrophil activation via ULC binding results in neutrophil recruitment to the endothelium and release of Neutrophil extracellular traps (NETs). The process of NETosis is a driver of thrombus formation and thrombosis. Monocyte activation leads to tissue factor (TF) expression and an increase in thrombin generation. Conversion of fibrinogen to fibrin via thrombin also contributes to an increased risk of thrombosis. TF released from monocytes additionally transactivates platelets via PAR-1 receptors, leaded to highly thrombotic, coated platelets. Furthermore, GAGs expressed on the endothelium act as binding partners for PF4 and subsequent antibody recognition. This antibody deposition results in increased TF release, thrombosis risk and serves as a site for activation of cells containing FcγRIIa receptors.

laboratories having the regulatory and safety measures in place to perform these assays (Bakchoul et al., 2014). This limits their timeliness in clinical decision making and in confirming HIT cases.

Immunological Features of Heparin-Induced Thrombocytopenia Pathogenesis

HIT is initiated by ULCs cross-linking with the Fc domain of PF4/heparin antibodies via the low affinity IgG Fc region receptor II-a (FcγRIIa) on the surface of platelets. This initiates platelet activation and release of procoagulant microparticles (Warkentin et al., 1994), culminating in thrombocytopenia and/or thrombotic complications (Rollin et al., 2015). ULCs have been shown to interact with monocytes (Arepally and Mayer 2001; Pouplard 2001; Kasthuri et al., 2012; Tutwiler et al., 2016), endothelial cells (Arepally and Mayer 2001; Arepally 2002; Arepally 2017), neutrophils (Perdomo et al., 2019), and platelets (**Figure 1**).

The immunopathogenesis of HIT is atypical and has characteristics of both innate and adaptive immunity (Greinacher 2015; Arepally 2017). Rapid production of PF4/heparin IgG antibodies, typical of a secondary adaptive immune or anamnestic response, is seen as early as 5 days after an initial heparin exposure. This response may be due to prior expose of PF4 bound to heparin-like GAGs or polyanions (Krauel et al., 2011; Staibano et al., 2017). However, PF4/heparin antibodies are transient, disappearing after approximately 90 days, characteristic of a non-anamnestic response (Warkentin and Kelton 2001). Heparin re-exposure after this 90 day period also does not reliably induce HIT, and patients with a previous HIT episode may be rechallenged with heparin (Warkentin and Sheppard 2014). PF4/heparin complexes activate complement, characteristic of an innate immune response. Binding of ULCs to B cells is mediated through the interaction between C3/C4 complement and complement receptor 2 (CR2 [CD21]). (Khandelwal et al., 2016). Polyreactive immunoglobulin M (IgM) antibodies initiate complement activation by binding to PF4/heparin complexes. IgM binding generates ULCs, facilitates the deposition of complement components, and promotes ULC binding to B cells (Khandelwal et al., 2018).

Molecular Features of Heparin-Induced Thrombocytopenia Pathogenesis

Heparin is a naturally occurring, endogenous GAG, and it has been extensively studied in both the context of HIT and in its pharmacologic action. In HIT, heparin molecules bind to complexes of circulating PF4, a chemokine (chemokine C-X-C motif 4 [CXCL4]) that is stored in α-granules of platelets (Delcea and Greinacher 2016). Polyanionic heparin binds to positively charged PF4, but this must occur in specific molar ratios or charge imbalances and complex instability will prevent ULC formation (Suvarna et al., 2007). Studies have identified structural (Cai et al., 2016) and thermodynamic (Brandt et al., 2014; Kreimann et al., 2014) features necessary for formation of ULCs. While PF4 exists in monomer, dimer, and tetramer states, PF4 must exist in a tetrameric configuration for ULCs to form and HIT to occur (Mayo and Chen 1989; Cai et al., 2015). The tetrameric structure of PF4 displays a pseudosymmetry exhibiting an "open" and

"closed" end characterized by distances between two salt bridge forming amino acids, glutamic acid 28 and lysine 50. In PF4 monomers A and C, amino acids E28 and K50 are ~3 angstroms (Å) apart and form stable salt bridges. The same amino acids in monomers B and D are too far apart to form a salt bridge (~8 Å) without a bond mediator (Cai et al., 2015). Heparin binds in the "closed" end of the tetramer and stabilizes the self-association of the PF4 tetramer (Cai et al., 2016). Binding of heparin results in a conformational change in the PF4 tetramer complex and stabilizes the "open" end of the tetramer, exposing the epitope recognized by PF4/heparin IgG antibodies (Cai et al., 2016).

Circulating PF4 interacts with other polyanions, including nucleic acids, liposaccharides on bacteria (Greinacher 2015), and cellular GAGs, but PF4 has a greater affinity for heparin than for other GAGs (Arepally 2017). The atomic weight and structure of GAGs dictates the binding strength to PF4. UFH is more likely to stabilize the intramolecular interactions within the PF4 tetramer compared to LMWHs (Cai et al., 2016). Fondaparinux, shows an even greater loss of stabilizing potential for the PF4 tetramer.

CELLULAR CONTRIBUTIONS TO HEPARIN-INDUCED THROMBOCYTOPENIA PATHOGENESIS

Platelets are a primary driver of HIT and platelet activation contributes to HIT's thromboembolic consequences. While the role of platelets and megakaryocytes have been extensively studied, other cells play critical roles in HIT pathogenesis, including T-cells, monocytes, endothelial cells, and neutrophils (**Table 1**) (Arepally and Mayer 2001; Pouplard 2001; Xia and Kao 2002; Tutwiler et al., 2016) (Cines et al., 1987; Arepally 2002) (Zheng et al., 2015) (Perdomo et al., 2019).

Platelets (Megakaryocytes)

Platelets, the circulating anuclear fragments derived from megakaryocytes, express a single class of Fcγ receptors, FcγRIIa (Gratacap et al., 1998). Binding of ULCs to the FcγRIIa receptor triggers intracellular signaling involving the spleen tyrosine kinase 50 (SYK50) (Arepally 2017). SYK is phosphorylated when bound to FcγRIIa, activating SYK and triggering downstream signaling through calcium mobilization and PI3-kinase activation (Zhou et al., 2016). This leads to integrin αIIb/β3 activation and release of procoagulant microparticles from platelet granules. Two types of granules are released from activated platelets: dense granules, containing serotonin, calcium, adenosine triphosphate, and adenosine diphosphate and α-granules containing PF4, P-selectin, and fibrinogen (Fitch-Tewfik and Flaumenhaft 2013; Arepally and Padmanabhan 2020; Tardy et al., 2020). As shown in **Figure 1**, this signaling cascade leads to platelet activation and aggregation upon fibrinogen binding to the integrin receptor (Gratacap et al., 1998), resulting in a positive feedback loop facilitating a hypercoagulant state.

While the mechanisms of platelet activation via the FcγRIIa receptor are well understood, the mechanism of thrombocytopenia in HIT is not fully resolved. Clearance of antibody-coated platelets in the spleen (Prince and Wenham 2018), the consumption of platelets by thrombi, and platelet disintegration through platelet-derived microvesicles are proposed mechanisms for thrombocytopenia in HIT (Liu et al., 2013; Liu et al., 2016). A recent study showed that platelet activation was accompanied by cell death through complex apoptotic and non-apoptotic pathways, which may contribute to thrombocytopenia (Mordakhanova et al., 2020). Platelet activation and aggregation into thrombi reduce platelet count (Greinacher 2015). More recently, von Willebrand factor (VWF) and complexes of PF4-VWF-IgG antibodies were shown to bind extensively to platelets (Chen and Chung 2018) (Johnston et al., 2020).

Mutations in the FcγRIIa receptor have been shown to be associated with both HIT and HITT (Karnes 2018). The polymorphism H131R in the IgG binding region of FcγRIIa receptor has been associated with HIT in several populations, but a meta-analysis showed no difference in HIT between the wild-type and 131R variant (Trikalinos et al., 2001). However, individuals homozygous for the 131-RR genotype were at higher risk of HITT, likely due to the inability of endogenous IgG2 antibodies to bind to the RR genotype receptor. IgG2 antibodies compete with PF4/heparin antibodies (IgG1) for binding to FcγRIIa (Rollin et al., 2015). Decreased IgG2 affinity allows increased engagement of the IgG1 antibody, further stimulating the intracellular pathway involving FcγRIIa, SYK50 and platelet activation.

T-Cells

After maturation, selection, and transport out of the thymus, subsets of T-cells including regulatory T-cells (Treg), naïve T-cells, and memory T-cells are critically important for the maintenance of immune response and immunological memory. T-cells, particularly those subsets involved in antigen presentation, have been implicated in the immune response seen in HIT patients. HIT exhibits T-cell dependent characteristics (Xia and Kao 2002, Xia and Kao 2003; Chudasama et al., 2010; Joglekar et al., 2015), such as the requirement of CD4 and CD40 ligands on T-cells for PF4/heparin IgG production (Zheng et al., 2015). Observed associations between Human Leukocyte Antigen (HLA-DR) alleles and HIT suggest T-cell dependent mechanisms (Karnes et al., 2015; Karnes et al., 2017b; Zhang et al., 2019). However, HIT also presents T-cell independent characteristics. PF4/heparin complexes have similar characteristics to T-cell independent antigens, including similar molecular weight, repetitive epitopes, and spacing between elements (Vos et al., 2000; Staibano et al., 2017). The lack of memory B cells after heparin exposure (Selleng et al., 2010), and the role for marginal B cells in PF4/heparin IgG production in mouse models (Jouni et al., 2016) point to T-cell independent mechanisms.

Neutrophils

Neutrophils are phagocytes and the most abundant type of white blood cell in the body (Van Rees et al., 2016). They migrate through blood vessels and perform a variety of function such as recruitment to sites of inflammation, phagocytosis of pathogens, degranulation, and generation of neutrophil extracellular traps

(NETs). Activation of neutrophils via FcγRIIa receptor promotes phagocytosis, degranulation, and production of reactive oxygen species (ROS) (Gollomp et al., 2018). Neutrophils exposed to PF4 and PF4/heparin antibodies show enhanced adhesion to venous endothelium. In a microfluidics model, enhanced neutrophil involvement within venular thrombi was observed after induction of PF4 and PF4/heparin antibodies (Gollomp et al., 2018). Changes in absolute neutrophil counts have also been observed in HIT patients (Hui et al., 2019).

Neutrophils may play a role in HIT through generation of neutrophil extracellular traps (NETs). NETs are complexes of intracellular components including histones, myeloperoxidase, and elastase. NETs, when released from neutrophils, bind to pathogens and have anti-microbial activity (Van Rees et al., 2016). NETs have also been shown to contain prothrombotic components, such as tissue factor (TF), factor XII, VWF, and fibrinogen (Perdomo et al., 2019). Increased levels of cell-free DNA (cf-DNA), myeloperoxidase (MPO), elastase, VWF, and citrullinated histone H3 (CitH3), which are all markers of NETosis, have been reported in HIT patient plasma (Perdomo et al., 2019). In one study, whole blood from HIT patients included a second population of neutrophils that were not present in healthy controls, classified as activated neutrophils or low-density granulocytes (LDGs) (Perdomo et al., 2019). Using microfluidics models, complexes of NET and PF4 were shown to be recognized by KKO antibodies, which are synthetic antibodies that mimic PF4/heparin antibodies, and IgG from the plasma of HIT patients (Gollomp et al., 2018). Recently, murine HIT models (FcγRIIa+/hPF4+) recapitulated *ex vivo* results, in which NETosis was required for thrombus formation. Inhibition of NET formation by the NET inhibitor, GSK484, caused a marked reduction in thrombus deposition and neutralization of FcγRIIa completely abolished thrombi. However, blocking of platelet-neutrophil interactions via anti-CD62p did not inhibit thrombus formation (Perdomo et al., 2019).

More recent studies show neutrophils and the release of NETs do not initiate thrombosis in HIT mouse models, but play a role in thrombus growth and stabilization. Loss of peptidyl arginine deiminase type IV (PAD4) abolishes neutrophils' ability to release NETs. Pad4 −/− HIT mice following KKO injection had thrombocytopenia comparable to Pad4 +/+ HIT mice. However, loss of PAD4 led to smaller venular thrombi following injury but did not abrogate thrombosis all together (Gollomp et al., 2018). Other studies have shown that heparin-induced NETs display two markedly different phenotypes. The first NETs (described above) are from neutrophils where cell nuclear membranes are disrupted, and the second subset are from neutrophils where small amounts of extracellular DNA were released but those neutrophils maintained their structural integrity (Lelliott et al., 2020). One study showed neutrophil activation induced by ULCs varied among individuals. In fact, an individual's neutrophil response to ULCs remained fixed over a longitudinal 1-year period (Duarte et al., 2019), suggesting that susceptibility to neutrophil activation by ULCs is specific to the host and may be genetic in nature. These studies indicate that NETosis is a driver of thrombosis in HIT, but inter-individual variability in neutrophil count and heterogeneity, including polymorphism in cell surface receptors, are potential modifiers of HITT risk.

Monocytes

Monocytes, the immature precursor to macrophages and dendritic cells, serve as vehicles for antigen presentation, cytokine production, tissue remodeling, and phagocytosis (Guilliams et al., 2018). Monocytes express GAGs that react with PF4 to generate structural changes in PF4, which allows binding of PF4/heparin antibodies (Rauova et al., 2009). Monocytes primarily express two GAGs, heparin sulfate (HS) and dermatan sulfate (DS) (Madeeva et al., 2016). These GAGs may be the preferred target for PF4 binding and formation of ULCs, compared to platelets, as platelets express a low affinity GAG, chondroitin sulfate (Rauova et al., 2010). The binding of PF4/heparin antibodies to these PF4/GAG complexes on monocytes results in activation of Fc-receptors on monocytes.

Binding of PF4/heparin antibodies to FcγRIIa receptors trigger monocyte TF expression and release of TF particles (Kasthuri et al., 2012). TF binds to Factor VII (FVII)/FVIIa complexes and initiates coagulation by activating FX and FIX. The conversion of FX to FXa in complex with FVa activates prothrombin to thrombin (Mackman, 2004) (Tutwiler et al., 2016). The removal of monocytes from whole blood decreases platelet accretion and fibrin deposition. When levels of monocytes are brought back to normal physiological levels, the ability of KKO antibodies to stimulate platelet accretion and fibrin deposition is restored (Madeeva et al., 2016). Recent work has shown that in HIT patients, absolute monocyte levels decrease over a 7-days span post-HIT (Hui et al., 2019). However, overall counts of monocytes did not vary between patients with HIT and HITT (Hui et al., 2019).

The activation of monocytes and release of TF has recently been shown to involve the neonatal Fc receptor (FcRn) (Cines et al., 2020). Dual receptor engagement of ULCs (FcγRIIa and FcRn) on monocytes may explain why KKO antibodies have higher binding affinity to monocytes compared to platelets (Rauova et al., 2010). Thrombin generated by stimulated monocytes can *trans*-activate platelets via protease-activated receptor 1 (PAR-1), a subfamily of G protein-coupled receptors (Tutwiler et al., 2016). Coactivation of platelets via PAR and FcγRIIa leads to the formation of highly prothrombotic, coated platelets (**Figure 1**) (Tutwiler et al., 2016). Following whole blood exposure to KKO and PF4, a population of platelets exist with increase expression of P-selectin and other markers of coated platelets (Tutwiler et al., 2016). Expression of coated platelet markers was considerably reduced in the absence of monocytes in mouse platelet-rich plasma and in monocyte-depleted whole blood (Tutwiler et al., 2016). The amount of PF4 deposition onto monocytes is likely correlated with the extent of platelet transactivation (Madeeva et al., 2016). This amplification loop where activated platelets release PF4, this PF4 binds to monocytes, resulting in release of TF particles, which then further activates platelets and generates coated platelets, illustrates the complex interplay of cells in HIT.

Endothelial Cells

Endothelial cells line blood vessels and play a critical role in regulating blood flow, vascular homeostasis, vascular tone, and platelet function (Jain et al., 2016; Madeeva et al., 2016; Sturtzel 2017). Signaling between vascular endothelial cells and platelets is vital for regulation of thrombosis (Jain et al., 2016). The basal state of endothelium is anticoagulant in nature, but at the onset of tissue trauma the endothelium facilitates coagulation (Sturtzel 2017). The endothelium expresses a mosaic sheath of glycoproteins, proteoglycans, and GAGs, known as the glycocalyx, under normal physiological conditions (Madeeva et al., 2016). Endothelial cells may bind PF4 with a greater affinity than platelets or monocytes given the predominant GAG on endothelial cells is heparin sulfate (Madeeva et al., 2016).

As shown in **Figure 1**, platelet activation at the site of injured endothelium releases PF4, which is deposited onto the endothelium due to a high affinity for GAGs. The PF4/GAG complex then becomes a target for PF4/heparin antibody binding. This binding contributes to increased endothelial activation and recruitment of platelets in a feed forward loop resulting in fibrin deposition and thrombosis (Madeeva et al., 2016). PF4 release was associated with binding of ULCs on thrombi following laser injury of endothelial cells (Hayes et al., 2017). Complexes of PF4 and VWF, released from endothelium following injury, may also be an antigenic site for PF4/heparin antibodies (Johnston et al., 2020). Microfluidics injury models also support that endothelial cells are the main initial harbor for PF4 released from activated platelets and a target for PF4/heparin antibodies (Hayes et al., 2017). The higher affinity of endothelial GAGs for PF4 may help to explain why antiplatelet therapy is not efficacious in HIT (Madeeva et al., 2016) and why thrombotic conditions may persist after heparin is no longer present. As approaches to date have yet to resolve these questions, applying multi-omics techniques to HIT has the potential to bring new insights and fundamentally improve our understanding.

APPLICATION OF OMIC APPROACHES TO HEPARIN-INDUCED THROMBOCYTOPENIA

Application and integration of omics disciplines, such as genomics, transcriptomics, metagenomics, proteomics, and metabolomics, has the potential to probe cellular roles discussed above and reveal previously unknown aspects of HIT biology. The complex, immune-mediated nature of HIT makes it well-suited for multi-omics approaches to solve questions surrounding the pathogenesis of HIT.

Genomics

The majority of genetic studies related to HIT have been candidate gene studies. (Arepally et al., 1997; Pouplard et al., 2012; Rollin et al., 2012; Rollin et al., 2013; Rollin et al., 2015; Karnes et al., 2017b; Zhang et al., 2019). These studies focus on genes or single nucleotide polymorphisms (SNPs) with suspected biological relevance to the disease (Tabor et al., 2002). Genomic association studies for HIT and HITT are summarized in **Table 2**. Such studies have identified multiple associations with HIT and HITT (Brandt et al., 1995; Arepally et al., 1997; Rollin et al., 2015). Candidate gene/SNP studies can be cost effective, but these approaches have multiple limitations. They include the need for *a priori* information on gene function and a historical inability to replicate observed associations (Tabor et al., 2002; Patnala et al., 2013). Genome-wide association studies (GWAS) are an "agnostic" approach, in contrast to the hypothesis-driven candidate gene studies, and have become a powerful tool in discovering the genetic influence of complex disease. GWAS investigates the association between millions of genetic variants and a phenotype of interest. Despite clear successes in GWAS in identifying genetic polymorphisms associated with other phenotypes/diseases, very few GWAS studies have been performed for HIT (Karnes et al., 2015; Witten et al., 2018). One study identified a SNP in chromosome 5 in AC106799.2 as a risk allele for HIT (Witten et al., 2018). This GWAS only contained 96 suspected HIT cases and a replication cohort of 86 suspected HIT cases. Another GWAS observed a significant association with HIT near TDAG8 (or GPR65) (Karnes et al., 2015). Low sample sizes, particularly in HIT cases, were a limitation in both studies and reduce the impact of these findings. Furthermore, many of the HIT cases in both studies were determined through antibody testing and 4Ts scores and HIT was not confirmed with functional assays. Additionally, discovery cohorts in both studies did not include differentiation between antibody positive patients who develop HIT and antibody positive patients who did not develop HIT. This creates ambiguity in these results as the associations seen may be with PF4/heparin antibody levels and not HIT itself.

Within the HIT field, there is a need for a well-powered GWAS study which can overcome the limitations of the previous literature. Applying GWAS strategies to HIT research could have its largest impact in 1) identifying novel variants associated with HIT and 2) corroborating previous findings of variants which alter HIT risk. Large GWAS could provide valuable information on additional polymorphisms that alter thrombosis risk in HIT. GWAS could be used to identify genetic variants associated with antibody production independent of HIT and bring new insights into why only a subset of antibody positive patients develop HIT. However, the acquisition of enough HIT cases to create a sufficiently powered GWAS for HIT is a challenge. Whole-genome sequencing (WGS) might also be used to discover new polymorphisms with an influence on HIT. Declining sequencing costs will permit large scale interrogation of entire genomes, including rare variants, in the near future (Tam et al., 2019). However, WGS approaches will likely require even greater sample sizes for statistical power and additional resources for data storage, processing, and analysis.

Metagenomics

Microorganisms, present throughout the human body, including bacteria, viruses, and fungi, collectively defined as the human microbiota, may have more biological influence that previously expected. Metagenomics, or the surveying of all ("Meta-") microbial genomes (genomics), is typically performed via shotgun or 16s rRNA sequencing. Briefly, 16s rRNA

TABLE 1 | Mechanisms of PF4/heparin antibody binding, involvement in pathogenesis of heparin-induced thrombocytopenia, and involvement in HIT-associated thrombosis of various cell types.

Cell Type	PF4/heparin antibody binding	Involvement in HIT Pathophysiology	Involvement in HIT-Associated Thrombosis
Megakaryocytes (Platelets)	Direct: via FcγRIIa receptor	• PF4/heparin antibodies bind to platelets • Activation leads to pro-coagulant particle release Warkentin et al. (1994) • Apoptotic and non-Apoptotic depletion of platelets occur; leads to thrombocytopenia Mordakhanova et al. (2020) • TULA-2 deficiency increases thrombocytopenia Zhou et al. (2016)	• Activated platelets release microparticles with procoagulant activity Warkentin et al. (1994) • platelet-NETs lead to thrombus formation Martinod and Wagner (2014) • PF4-VWF-IgG on ECs leads to platelet deposition and thrombi enlargement Johnston et al. (2020) • FcγRIIa 131 R R allele leads to increased thrombosis Rollin et al. (2015)
T-cells	Precursor: No direct evidence for PF4/heparin antibody mediated activation	• CD4 T-cells are necessary for antibody response Zheng et al. (2015) • Antigen presenting cells associated with PF4/heparin antibodies Karnes et al. (2017b), Zhang et al. (2019) • Lack of memory B cells and PF4/heparin complex similarities to T-cell independent antigen confounds T-cells role Warkentin and Kelton (2001)	• Lack of evidence for T-cells influence on thrombosis
Neutrophils	Direct: via FcγRIIa receptor	• Activation leads phagocytosis, degranulation, and NETs generation, which contain prothrombotic components Duarte et al. (2019)	• NETs are critical for thrombus formation exhibiting engagement with platelets, red blood cells and procoagulant proteins Perdomo et al. (2019) • L-selectin and CD11b/CD18 upregulation promote adhesion of neutrophil-platelet aggregation Xiao et al. (2008)
Monocytes	Direct: via FcγRIIa receptor and neonatal Fc receptor	• Activation of monocytes triggers TF expression and release of TF particles activates platelets Kasthuri et al. (2012)	• Binding of ULCs increase procoagulant activity indicated by release of TF containing particles • TF containing microparticles promote thrombin activation • PF4 bound to cell-surface GAGs serve as reservoirs for PF4/heparin antibody binding Rauova et al. (2010), Tutwiler et al. (2016)
Endothelial	Indirect: EC bound ULCs mediate activation of other cell types	• Source for TF release and site for PF4 deposition onto GAGs, subsequent binding of antibodies and engagement to FC-receptor on platelets Madeeva et al. (2016)	• Platelet adhesion to EC exacerbates thrombus formation and promotes thrombosis • EC activation releases VWF strings that bind PF4 and serve as new antigenic sites Hayes et al. (2017) • ECs, in the presence of platelets upregulate adhesion markers–E-selectin, ICAM-1 and VCAM Herbert et al. (1998)

EC indicates endothelial cells; FcγRIIa, low affinity immunoglobulin gamma Fc region receptor II-a; GAG, glycosaminoglycans; ICAM-1, intracellular adhesion molecule 1; NET, neutrophil extracellular traps; PF4-VWF-IgG, platelet factor 4–von Willebrand factor–immunoglobulin G complexes; TULA-2, T-Cell Ubiquitin Ligand-2; TF, tissue factor; ULC, ultra-large complexes; VCAM, vascular cell adhesion molecule; VWF, von Willebrand factor

sequencing is a cost-effective solution to surveying the communities of a bacterial microbiome via a single gene, the 16S ribosomal gene. Whereas shotgun sequencing surveys the entire genome of all organism present in a sample, including human, viral and fungal DNA (Ranjan et al., 2016). Recent evidence suggests that HIT is a misguided immune response, involving a prior bacterial exposure serving as a "priming" event. PF4/polyanion antibodies are produced and later recognize heparin as a "foreign" entity. PF4 has been shown to interact with both gram-positive and gram-negative bacteria and antibodies obtained from HIT patients recognize PF4 bound to bacteria (Krauel et al., 2011). Additional studies show PF4/heparin antibodies present in a cohort of subjects with periodontal disease (Greinacher et al., 2011) as well as inpatients with bacteremia. Patients with gram-negative bacteria showed higher levels of PF4/heparin antibodies relative to healthy controls (Pongas et al., 2013). Mice with polymicrobial sepsis produced PF4/heparin antibodies which followed the expected seroconversion from IgM to IgG (Jouni et al., 2016). The rapid antibody class switching observed in HIT, may in fact be due to a prior exposure of PF4 bound to polyanions expressed on bacteria or those with ongoing infections (Pongas et al., 2013). This evidence suggests the response against PF4/heparin complexes may be a misguided immune reaction, in that the epitope generated by PF4/heparin "mimics" the epitope of PF4 when bound to bacteria cell wall components. As this theory of a misguided immune response in HIT has yet to identify specific classes or even phyla of bacteria, sequencing the microbiome may provide powerful insights into HIT pathogenesis.

TABLE 2 | Genomic studies identifying associations with Heparin-Induced Thrombocytopenia.

Locus	Variant(s)	Phenotype [Assay for Confirmation]	Study Design	Study Limitations–Pathogenesis Evidence	Citation
TDAG8 (or GPR65)	*rs1887289 rs10782473*	*HIT, Antibody production [ELISA]*	*GWAS*	Small cohort (*n* = 67 HIT, 884 controls); no functional assay; replication of signal in antibody production but not HIT–Signal near TDAG8 gene (T-cell death-associate gene 8) may be involved in antibody response not HIT itself; additional studies/ larger cohorts needed for confirmation of gene influence	Karnes et al. (2015)
Chr 5 near AC106799.2	*rs1433265*	*HIT [ELISA or HIPA]*	*GWAS*	Small discovery (*n* = 96 HIT, 96 controls) and replication (*n* = 86 HIT, 86 controls) cohort; signal did not reach GWAS significance threshold–SNP identified is located in non-coding gene region; influence on HIT requires further (*in vitro/in vivo*) validation	Witten et al. (2018)
IL-10	*IL10G G20 microsatellite*	*PF4/heparin antibody production [ELISA and SRA]*	*Candidate Gene*	No replication: small cohort (82 HIT 84 Ab^{pos}, 85 Ab^{neg})–IL-10 plays critical role in peripheral inflammation; marker of immune response, may not be specific to antibody production in HIT	Pouplard et al. (2012)
FCGR2A	*rs1801274 [H131R]*	*HITT [ELISA and SRA]*	*Candidate Gene*	Small cohort (*n* = 35 HITT, 54 HIT, 160 Ab^{pos}, 174 Ab^{neg}, 206 healthy controls)–Strong evidence for direct influence of polymorphism on phenotype (thrombosis); Amino acid substitution located in extracellular domain of FcγRIIa; interacts with Fc fragment of IgG antibody; multiple other studies have observed same association Burgess et al. (1995), Carlsson et al. (1998)	Rollin et al. (2015)
HLA-DR	*DRB3*01:01*	*HIT [ELISA]*	*Candidate Gene*	Small cohort (*n* = 65 HIT, 350 controls); no functional assay for HIT confirmation; no replication–Lack of evidence for presentation of HIT antigen via HLA using traditional (*in vitro/in vivo*) techniques; lessens study impact of HLA allele as risk factor for HIT	Karnes et al. (2017b)
HLA-DR	*DRB1*03:01 and DQB1*02:01*	*Antibody production [ELISA]*	*Candidate Gene*	No replication: no functional assay–Haplotype has been associated with other autoimmune disorders; lack of evidence for presentation of HIT antigen via HLA using traditional (*in vitro/ in vivo*) techniques lessens impact of findings	Zhang et al. (2019)
ACP1	*rs11553742/ rs11553746 haplotypes*	*Antibody production [ELISA and SRA]*	*Candidate Gene*	No replication: no difference in genotype frequency between Ab^{pos} and HIT patients, solely Ab^{neg} vs Ab^{pos}–Evidence does exist for influence in antibody response as ACP1 regulates ZAP-70, which plays critical role in T-cell development; functional studies necessary to support findings	Rollin et al. (2013)
PTPRJ	*rs1566734 rs1503185*	*HIT [ELISA and SRA]*	*Candidate Gene*	No replication: All patients (Ab^{neg}, Ab^{pos}, HIT) were cardiopulmonary bypass patients; potential enrichment of population–Influence on phenotype supported by amino acid changing variants identified; previous murine studies strengthen support Senis et al. (2009), Ellison et al. (2010)	Rollin et al. (2012)

Ab^{neg}: negative PF4/antibody assay; Ab^{pos}: positive PF4/antibody assay with negative functional assay result; GWAS: genome-wide association study; HIPA: heparin-induced platelet aggregation assay; SRA: serotonin release assay, HIT: heparin-induced thrombocytopenia; HITT: heparin-induced thrombocytopenia associated thrombosis; Chr, chromosome.

Sequencing of microbiomes from antibody positive patients compared with that of antibody negative controls could elucidate if the presence of particular microorganisms is associated with detectable PF4/heparin antibodies. Patients with specific bacterial exposure, identified through presence of bacteria or relevant antibodies, could alert clinicians to the increased risk of HIT prior to heparin exposure. Studies have already observed that gut microbiota alter the risk of cardiovascular disease (Witkowski et al., 2020; Jin et al., 2021). Gut microbiota-derived metabolites may increase the risk of venous thromboembolisms (Zhu et al., 2016; Hasan et al., 2020; Lichota et al., 2020). However, the influence of gut microbiota-derived metabolites has not been evaluated in the context of HIT or HITT. Given the number of patients prescribe heparin relative to the few who develop HIT, there are methodological difficulties in the assessment of all heparin-exposed patients prior heparin administration.

Transcriptomics

Transcriptomics, the comprehensive study of all RNA transcripts within an organism, is a powerful approach to explore cellular functions. The dominant techniques employed today are microarrays and RNA-sequencing (RNA-seq). Microarrays measure the abundance of a predefined set of transcripts and can allow the assay of thousands of transcripts simultaneously. Conversely, RNA-seq quantifies the entire transcriptome of cells via the sequencing of cDNA transcripts. Numerous HIT studies

have looked at targeted mRNA levels using quantitative real-time PCR. Often these studies focus on a specific gene of interest (Lhermusier et al., 2011; Kasthuri et al., 2012; Rollin et al., 2015) as opposed to the broader scope that microarrays provide. Microarray studies could identify new roles of mRNA transcripts and their genes involved in HIT. This information could be used to delineate individuals who may produce higher antibody quantities and increased monitoring of those patients could be implemented to help minimize the progression to thromboembolic complications of HIT. Microarrays require a priori information on specific transcripts, but RNA-Seq provides a more agnostic approach for surveying the transcriptome. RNA-seq, most often utilized for differential gene expression (DGE), allows users to determine quantitative changes in expression levels between experiments, cases/controls studies, different disease states, etc.

Transcriptomics work could help identify specific transcriptional activity present in HIT cases compared to non-HIT controls in cross sectional studies. Also informative may be longitudinal studies on patients during HIT and post-HIT after antibodies have waned to interrogate the transcriptional differences where patients can serve as their own controls. Despite the clear applicability of RNA-seq in investigating HIT cases/controls, or even antibody cases/controls, RNA-seq has not yet been applied to HIT research. The most common RNA-sequencing technology, short read cDNA sequencing for DGE, is a robust and highly reproducible across and between platforms (Stark et al., 2019). Applying RNA-seq to HIT research might have a large impact in determining novel biological pathways involved in HIT pathogenesis. This application might also identify differentially expressed genes responsible for the subset of PF4/heparin antibody positive patients who go on to develop full-blown HIT. RNA-seq has the potential to discovery if difference in transcript levels amplify, or suppress, the antibody signally cascade initiated by IgG-platelet binding.

RNA-seq from bulk heterogeneous cells, as described in the previous paragraph, fails to resolve the role of specific cell types in the biological system being studied. As HIT pathogenesis is complex and involves multiple cell types, single-cell RNA sequencing (scRNA-seq) may reveal specific roles individual cells play in HIT, and potentially identify cell types that were previously not known to be involved in HIT. ScRNA-seq has been successful in discovering previously unknown cells types and mechanisms involved in phenotypes such as cystic fibrosis and myocardial infarction (Montoro et al., 2018) (Li et al., 2019) (Paik et al., 2020). ScRNA-seq could help answer questions surrounding the individual pathogenic roles of distinct cell types, in the case of monocytes and neutrophils, and the role of cell subtypes, in the case of T-cells and B-cells.

Proteomics

Proteomic analysis characterizes the proteome, including expression, structure, function, interaction, and modification of proteins (Aslam et al., 2017). While proteomic analysis has brought new insights into HIT, these studies have been limited in scope thus far (Khandelwal et al., 2018). Probing the proteome has the potential to identify a broader range of proteins and post-translational modifications of proteins involved in HIT. Proteomic approaches might be used to determine how glycosylation or post-translational modifications of proteins alters binding affinities and results in differential antibody response. A recent GWAS identified five novel loci associated with IgG antibody glycosylation (Shen et al., 2017).

Although not specific to HIT, proteomic approaches have been used successfully in cohorts of patients treated with heparin and other anticoagulants. One study identified 25 proteins displaying changes in concentration following heparin administration, with 14 proteins replicating in a validation cohort (Beck et al., 2018). In another study of heparin-treated pregnant women, multiplex protein screening identified increased levels of chemokines CXCL10, CLCL11 and CCL20 when compared to untreated pregnant controls (Rasmark Roepke et al., 2019). Another recent study showed substantive changes to expression of focal adhesion and cytoskeletal proteins in podocytes following chronic heparin exposure (Delézay et al., 2021). In a cohort of 4,200 oral anticoagulant patients with atrial fibrillation, one recent study identified seven novel proteins associated with increased risk of bleeding (Siegbahn et al., 2021). A number of other targeted proteomic studies have reported additional biomarkers associated with risk of bleeding in patients with acute coronary syndromes (Hagström et al., 2016; Hijazi et al., 2016). In a study comparing treatment with warfarin and rivaroxaban, nine metaproteins were shown to be differentially expressed (Mueck et al., 2014; Harter et al., 2015; Kaye et al., 2017; Reçber et al., 2020).

Proteomic profiling still suffers from shortcomings, such as the large samples required to achieve an accurate measurement and difficulty accounting for heterogeneity within a sample. Single-cell proteomics is an emerging technique that is applicable to HIT research. Still in its infancy, initial reports of profiling hundreds of proteins from a single, or small number of mammalian cells were first published in 2018 (Budnik et al., 2018; Zhu et al., 2018). As the transcriptome is not a direct reflection of the proteome, and that post-translational modifications of proteins are unattainable from RNA transcripts, the use of proteomics should be strongly considered to probe unanswered questions within the field. Furthermore, proteomics can be a powerful complement to genomic and transcriptomic studies, by offering systems-level insights into HIT pathogenesis.

Metabolomics

The comprehensive set of small molecules within the body, the metabolome, is another avenue of research for further understanding of HIT pathogenesis. The metabolome can affect biological pathways through the modulation of the genome, transcriptome, and proteome (Rinschen et al., 2019). Metabolites serve as enzyme cofactors and substrates in biochemical processes. Activity of transmembrane receptors and transcription factors can be modulated by metabolites via allosteric regulation and metabolites can influence RNA metabolism by acting on riboswitches (Rinschen et al., 2019).

Metabolites such as physiologically relevant metal cations induce conformational changes in heparin (Seo et al., 2011;

Zhang 2014). Neutralization of heparin via metal ion binding could influence the binding of PF4 to heparin since the electrostatic interaction between heparin and PF4 is critical for complex formation. Calcium (Ca^{2+}) also induces substantive conformational changes in heparin (Seo et al., 2011) and is a critical cofactor in the binding of heparin to particular proteins such as Annexin A2 (Shao et al., 2006). Changes in concentrations of Ca^{2+} and Zinc (Zn^{2+}), alters the binding affinity of heparin to other proteins including fibroblast growth factor-1 and interleukin 7 (Zhang 2014). Zinc (Zn^{2+}) is present in high concentrations in α-granules, is a critical cofactor in non-HIT thrombosis (Vu et al., 2013), and is known to mediate binding of heparin to proteins including fibrinogen, high molecular weight kininogen, and histidine-rich glycoprotein (Fredenburgh et al., 2013; Sobczak et al., 2018). Substantive changes in the concentrations of these physiologically relevant metal cations could be a risk factor for HITT. Profiling of metal cation concentrations in the serum of HIT cases and heparin-treated controls could identify novel biomarkers in HITT. Metabolites, including acetate, citric acid, 3-hydroxybutarate, glucose, and some mono- and polyunsaturated fatty acids have been shown to be altered in arterial and deep venous thrombosis (Quintero et al., 2020). Metabolomics work could be applied to determine differences in metabolite levels affecting differential antibody responses and differences in HIT risk. The identification of metabolites that are risk factors for HIT could be invaluable within the clinic. Patients with metabolic alterations could alert clinicians to prescribe alternative therapies or increase monitoring of these at-risk patients.

CONCLUSION

Heparin-induced thrombocytopenia is a complex, unpredictable, immune-mediated adverse drug reaction involving multiple cell types. While platelets appear to be primarily involved in HIT, strong evidence exists for a critical role of several cell types in HIT pathogenesis. Neutrophils and monocytes have strong evidence for their role in HIT, particularly in HITT. HIT exhibits both T-cell dependent and independent features and genomic studies have observed associations between variation in antigen presentation pathways and HIT. Endothelial cells serve as a deposition site for PF4, likely playing a role in PF4/heparin antibody binding and signal transduction. Despite our understanding of these cell types and their roles in HIT, multiple gaps in our knowledge of HIT pathogenesis remain. Applying multi-omics techniques to HIT has the potential to bring new insights. Larger and functional assay-confirmed HIT cohorts are necessary to overcome the limitations of published HIT genomic studies. Other omics techniques, such as transcriptomics and proteomics, have been underutilized in HIT research. As technologies become more established and less cost prohibitive, single cell transcriptomics and proteomics are particularly well-suited to address for the gaps in our knowledge of HIT pathogenesis. Such omics approaches have great promise to elucidate biological pathways, profile patient samples and discover valuable clinical biomarkers, which could reduce the risk of HIT and improve patient outcomes in the clinic.

AUTHOR CONTRIBUTIONS

JG and JK conceived of project; JG and JK drafted the manuscript; JG, EM, HS, and JK performed literature review and critically revised manuscript. All authors reviewed and approved the final version of the manuscript.

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Genetic Susceptibility Toward Nausea and Vomiting in Surgical Patients

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Postoperative nausea and vomiting (PONV) are frequently occurring adverse effects following surgical procedures. Despite predictive risk scores and a pallet of prophylactic antiemetic treatments, it is still estimated to affect around 30% of the patients, reducing their well-being and increasing the burden of post-operative care. The aim of the current study was to characterize selected genetic risk factors of PONV to improve the identification of at risk patients. We genotyped 601 patients followed during the first 24 h after surgery for PONV symptoms in the absence of any antiemetic prophylaxis. These patients were recruited in the frame of a randomized, placebo controlled clinical study aiming to test the efficacy of dexamethasone as a treatment of established PONV. We examined the impact of selected single nucleotide polymorphisms (SNPs) located around 13 different genes and the predicted activity of 6 liver drug metabolizing enzymes from the cytochromes P450 family (CYP) on the occurrence and recurrence of PONV. Our genetic study confirms the importance of genetic variations in the type 3B serotonin receptor in the occurrence of PONV. Our modelling shows that integration of *rs3782025* genotype in preoperative risk assessments may help improve the targeting of antiemetic prophylaxis towards patients at risk of PONV.

Keywords: PONV, genetic risk factors, *HTR3B* polymorphisms, risk score, serotonin receptor

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INTRODUCTION

Postoperative nausea and vomiting (PONV) are frequently occurring adverse effects following surgical procedures concerning about one third of patients, with a prevalence that can reach up to 70–80% in high risk populations (Amirshahi et al., 2020; Elvir-Lazo et al., 2020; Ziemann-Gimmel et al., 2020). PONV considerably affects patient well-being, increases recovery time and cost through enhanced care and potential secondary effects. The most effective and commonly used anti-emetic treatments consist of 5-HT_3 and D_2-and more recently NK-1 receptor antagonists [reviewed in (Elvir-Lazo et al., 2020; Gan et al., 2020)]. Corticosteroids, especially dexamethasone, complete the spectrum of clinically potent preventive anti-emetic medications (Chu et al., 2014). As systematic administration of antiemetic prophylaxis before surgery is neither safe nor cost-effective (Tramèr et al., 1999), it is important to identify patients with a high-risk profile. A number of patient characteristics, as well as anesthetic or surgical procedures have been identified as risk factors of PONV including, but not limited to: female gender, young age, non-smoking status, previous history

of PONV, use of volatile anesthetics, visceral and gynecological procedures, and perioperative opioid consumption (Palazzo and Evans, 1993; Heyland et al., 1997; Koivuranta et al., 1997; Apfel et al., 1999; Sinclair et al., 1999; Junger et al., 2001; Murphy et al., 2006; Leslie et al., 2008).

The contribution of individual genetic susceptibility to PONV was first supported by the notion of family history of PONV, found to be a significant risk factor in pediatric patients (Eberhart et al., 2004) as well as a potential role of ethnicity that remains however controversial [reviewed in (Gan, 2006)]. Identification of genetic determinants playing a role in nausea and vomiting has been attempted using both candidate gene approaches and genome-wide association (GWAS) techniques in the context of postoperative as well as chemotherapy-induced (CINV) or pregnancy-related (NVP) nausea and vomiting. Candidate genes were mainly selected from signaling pathways known to be involved in emesis such as serotonin (5-HT), dopamine, acetylcholine, and neurokinin-1 (substance P) [reviewed in (Hornby, 2001; Horn et al., 2014; Janicki and Sugino, 2014)]. Research focusing on opioid-induced nausea and vomiting (OINV), uncovered additional polymorphism of interest as opioids are often used to manage perioperative analgesia and belong themselves to the recognized risk factors of PONV (Smith et al., 2012).

The pro-emetic role of serotonin and the efficiency of antagonists of the type 3 serotonin receptors (5-HTR_3), such as ondansetron, as anti-emetic treatment, has prompted a number of studies on the effect of polymorphisms affecting serotonin receptor genes on nausea and vomiting. Several studies uncovered significant associations between *HTR3A* and *HTR3B* related genetic polymorphisms and PONV (Rueffert et al., 2009; Laugsand et al., 2011; Lehmann et al., 2013; Ma et al., 2013; Yan et al., 2021). Genetic variations affecting other serotonin receptors such as 5-HTR_{2A}, might also participate in PONV susceptibility (Kato et al., 2006). Other mutations significantly associated with the occurrence of nausea and vomiting have been found in the type 2 dopamine receptor (*DRD2*) (Nakagawa et al., 2008; Frey et al., 2016), the neurokinin-1 receptor (*TACR1*) (Hayase et al., 2015), the type 3 muscarinic acetylcholine receptor (*CHRM3*) (Janicki et al., 2011; Laugsand et al., 2011; Klenke et al., 2018) or the cathechol O-methyltransferase (*COMT*) gene responsible for degradation of catecholamines (Kolesnikov et al., 2011; Laugsand et al., 2011; Wesmiller et al., 2017).

Besides differences in pro- and anti-emetic signal transduction, variations in the mu type opioid receptor (*OPRM1*) (Kolesnikov et al., 2011; Kong et al., 2018; Aroke and Hicks, 2019) and the efflux transporter encoded by *ABCB1* acting at the blood brain barrier on a broad range of substrate (Coulbault et al., 2006; Zwisler et al., 2010) could contribute to individual susceptibility toward PONV, either directly by their modulation of endogenous signals or through their role in the processing of exogenous opioids and, for *ABCB1*, surgeries or anesthetic agents. Polymorphisms in the fatty acid amide hydrolase (*FAAH*) which degrades endocannabinoids responsible for the mediation of the anti-nociceptive effects of morphine have, also been associated with PONV (Sadhasivam et al., 2015).

In addition, drug metabolizing enzyme (DME) might also participate to patient-related PONV risk factors. This contribution was first suggested by Sweeney (Tornio and Backman, 2018) in the context of the recognized protective effect of smoking on PONV (Sweeney, 2002). Indeed, although a direct link between metabolic activity and PONV has not yet been demonstrated, DMEs and in particular cytochromes from the P450 family (CYP) show a large inter-individual variability in activity resulting both from genetic and environmental origin including smoking [reviewed in (Zanger and Schwab, 2013; Tornio and Backman, 2018)]. The role of CYP activity variability in the context of PONV has most commonly focused on differences in anti-emetic drug metabolism (Candiotti et al., 2005; Nielsen and Olsen, 2008; Wesmiller et al., 2013; Aroke and Hicks, 2019). One example is the metabolism of the 5-HTR_3 receptor antagonist ondansetron by CYP1A2 and CYP3A (Huddart et al., 2019). However, in addition, to a direct detoxification effect of surgery related products, cytochromes may be directly involved in the regulation of pro-or anti-emetic signals, for instance through the regeneration of serotonin by CYP2D6 (Yu et al., 2003; Kirchheiner et al., 2005).

Despite the development of a number of risk scores that aim to identify patients at risk of PONV and the availability of efficient preventive anti-emetic medication that can be targeted toward patients at risk, the overall incidence of PONV remains significant in clinical settings. Characterization of additional risk factors allowing an even better identification of patients at risk of PONV is therefore still highly clinically relevant. The aim of the current study is to validate the association between selected genetic polymorphisms and PONV in a cohort of surgical patients without antiemetic premedication.

MATERIAL AND METHODS

Clinical Trial

The study population was recruited in the frame of a multi-centric clinical study evaluating the efficacy of postoperative intra-venous administration of dexamethasone for the treatment of established PONV symptoms. Patients (>18 years old) undergoing elective surgery were recruited at Geneva University Hospitals (HUG), Lausanne University Hospital (CHUV), Réseau Hospitalier Neuchâtelois, Neuchâtel (RHNe) and Etablissements Hospitaliers du Nord Vaudois, Yverdon les Bains and Saint Loup (eHnv).

The clinical trial, described in detail elsewhere (Czarnetzki et al., in press), was approved by the Ethics Committee of Geneva (CER 11-213/NAC 11-076) and Swissmedic, the Swiss agency of medical products (SM2012DR2118) and registered on clinicaltrials.gov (NCT01975727). The study was conducted according to Swiss national law on clinical trials, following international ICH guidelines and the ethical principles of the Helsinki declaration. All patients were informed, agreed to participate and signed an informed consent form including questions regarding their choices for the handling of their genetic material.

Briefly, patients were included before undergoing surgery and those presenting an initial episode of nausea or vomiting in the

first 24 h following waking after surgery, received the study treatment and were followed for another 24 h for recurrence of PONV symptoms, which consisted the primary end-point of the study. A rescue anti-emetic treatment was offered to patients showing persistent symptoms. Administration of study treatment was double-blinded and randomized between 4 groups receiving either 0 (placebo), 3, 6 or 12 mg dexamethasone.

Inclusion/Exclusion Criteria

All patients had to have an American Society of Anesthesiology (ASA) status below IV.

Patient taking drugs with antiemetic properties (butyrophenones, 5-HT_3 receptor antagonists, dexamethasone) or known emetogenic potency (for instance, L-Dopa, COMT inhibitors) were excluded. Similarly, patients with overt psychosis or taking antipsychotic treatment (such as, antidopaminergic drugs), or patients taking drugs interfering with platelet aggregation (such as aspirin or clopidogrel) in the week preceding the operation were excluded. Specific types of surgery increasing the risk of postoperative bleeding such tonsillectomy, as well as interventions or patients requiring strict prevention of postoperative vomiting were not taken in considerations. Other exclusion criteria included renal of hepatic dysfunction, gastrointestinal ulcer, systemic or local infections as well as the need for prolonged postoperative intubation or use of a gastric tube. For female participants of child-bearing age a negative pregnancy test was mandatory.

Beside prophylactic antiemetic and the exceptions mentioned above, there was no restriction on premedication, and compounds for anesthesia compound and postoperative analgesia, including opioid administration.

Clinical Data Collection and Dichotomization

The data collected during the study included: age (years), gender (male/female), active tobacco or cannabis smoking habits (yes/no), self-reported previous history of PONV (yes/no), type of surgery (gynecologic, orthopedic, visceral, ENT, neurologic or plastic), use of volatile anesthetics during the operation (yes/no) as well as intra and postoperative use of opioids (type, amount and route of administration). Opioid posology was divided into three categories: intraoperative, postoperative until time of first PONV episode or, for patients without PONV, until the end of the 24 h observation period. Perioperative opioid consumption was expressed as mg of oralmorphine equivalents using standard conversion tables (HUG, 2009; Toulouse, 2016).

In case of patients undergoing gender re-assignment surgery (5 over 601 participants), final sex was considered as most relevant due to the potential effect of the hormonal status on PONV occurrence, considering that all genetic determinants explored were located on autosomal chromosomes.

All risk predictors were dichotomized according to their predicted influence on PONV (1 = for increased risk, 0 = no increase of risk). Age <50 years old, female sex, previous PONV history, visceral or gynecological surgery and use of volatile anesthesia, were considered risk factors, while tobacco and cannabis smoking were expected to be protective. Dichotomization for opioid consumption was based on median consumption with a median [IQR] consumption of 19 mg [6–46] mg morphine equivalent and thus 0 for values ≤19 mg and 1 for values above 19 mg morphine equivalent.

DNA Extraction

The DNA purification and concentration at 50 ng/μL was performed in the laboratory for clinical pharmacology of Geneva University Hospitals using the MagJET Whole Blood Genomic DNA kit (ThermoFisher Scientific). DNA quantification was performed with the Qubit dsDNA BR Assay (ThermoFischer Scientific).

Genotyping

Polymorphisms were tested on a QuantStudio 12K instrument using a custom OpenArray® panel (ThermoFisher Scientific) operated by GenePredictis® SA. All selected SNPs were chosen based on literature data suggesting direct correlation with nausea and vomiting in different contexts (PONV, CINV, pregnancy) or functional implication in metabolic pathway potentially related to the process. The full list is found in **Table 3** for isolated SNPs and **Supplementary Data S1** for polymorphisms used for cytochrome activity prediction.

The cytochrome *CYP2D6* copy number was determined in triplicate on a 7900HT Fast Real-Time PCR System (Applied Biosystems™) at the iGE3 Genomics Platform of the University of Geneva using TaqMan® probes located within the exon 9 (Hs00010001_cn from ThermoFischer Scientific) and RNase P as reference. The study population was assumed to be mainly of European descend.

Quality Control and Genotype Assignment

Raw data from the OpenArray® and Copy Number Assay were treated using the TaqMan® Genotyper™ and CopyCaller™ software (ThermoFischer Scientific) respectively. The Hardy-Weinberg equilibrium is respected for all SNP except *rs1065852* and *rs3755468* (cases, $p < 0.001$).

Validated calls were further processed using the Alleletyper™ software (ThermoFisher Scientific) to assign individual genotypes. When available, the star allele nomenclature from the Pharmacogene Variation Consortium (PharmVar, 2021) was used, otherwise the genotypes were reported as single nucleotide polymorphisms. CYP2D6 activity score was calculated following Gaedigk et al. (2008). CYP1A2, CYP2B6 and CYP2C19 activities were calculated on the same model as CYP2D6 with: *1A/*1L = 1, *1F = 2 and *1C = 0 for CYP1A2; *1/*9 = 1, *4/*22 = 2 and *5/*6 = 0 for CYP2B6 and *1 = 1, *17 = 2 and *2/*3 = 0 for CYP2C19. CYP3A activities were predicted following the most recent studies on tacrolimus dose adjustment (Elens et al., 2011) with one category increment for CYP3A7 activation in adults (*1C allele) (Burk et al., 2002). For CYP3A, extensive metabolizers (EM) were given an activity score of 2, intermediate metabolizers (IM) a score of 1 and poor metabolizers (PM) a score of 0. Finally, CYP2C9 activity was expressed as a sum of the number of alleles with reduced activities (Van Booven et al., 2010). When mentionned, CYP1A2 induction by smoke was taken into account by

multiplying the activity score by 1.5× for smokers as previously described (Lesche et al., 2020).

Statistical Analysis

Descriptive statistics were carried out with R v3.5.1 and Microsoft® Office Excel version 2007. Logistic regression analysis according to **Eq. 1** were performed using PLINK v1.07 (Purcell et al., 2007) for single nucleotide polymorphisms or R v3.5.1 for predicted cytochrome activities. Dichotomized values were used for co-variables. Cytochrome activities were considered as continuous values while single nucleotide polymorphisms were considered as ordinal variable. An additive genetic model was applied.

$$Log\left(\frac{p}{1-p}\right) = \beta_0 + \beta_1 \text{Gender} + \beta_2 \text{Age} + \beta_4 \text{Smoking} + \beta_5 \text{Cannabis} + \beta_3 \text{PONVhistory} + \beta_8 \text{SurgeryType} + \beta_6 \text{VolatileAnesthetics} + \beta_7 \text{HighOpioid} + \beta_9 \text{GeneticMarker} \quad (1)$$

Where p = probability of PONV occurence or recurrence.

Haplotypes blocks and corresponding correlations were determined in PLINK v1.07 based on a linkage disequilibrium analysis (Purcell et al., 2007) and were illustrated with Haploview (Barrett et al., 2005).

Statistic tests were considered significant if the p-value < 0.05 and correction for multiple testing was applied unless otherwise specified.

For modeling, PONV risk scores were calculated according to the simplified Apfel score (Apfel et al., 1999) and model parameters together with NNG according to Tonk and al. (2017). Receiver operating characteristic (ROC) curve analyses were performed online using the EasyROC tool (Goksuluk et al., 2016), available at http://www.biosoft.hacettepe.edu.tr/easyROC/.

RESULTS

Study Population Characteristics and Genetic Integrity

Of the 803 study participants, 632 agreed to the genetic blood analysis, Nineteen of them were excluded due to incompleteness of their data record, leaving a preliminary sample pool of 613 patients.

The OpenArray® technology was used to assess the genotype of 60 different single nucleotide polymorphisms (SNPs). Following quality control of the genotyping experiment, a total of 601 samples and 59 SNPs were cleared for final analyses. Both the mean sample genotyping call rate (CR) and the mean SNP call rate for the OpenArray® experiment were >99% (99.35 vs. 99.41% CR respectively).

The characteristics of the final population is presented in **Table 1**. From the 601 participants, 264 (43.9%) suffered from PONV in the 24 h following their awakening from surgery. Of those, 229 patients (86.7%) were followed, and 157 (68.6%) of them presented a second episode of PONV. Administration of study treatment did not show any benefit on recurrence at neither dose (p > 0.1 whatever the model) compared with placebo (Czarnetzki et al., in press), leading to an early termination of the clinical trial for futility purposes.

The minor allele frequencies (MAF) observed in the cohort are consistent with those of a standard population of European descent (according to the Ensembl and GnomAD repositories (Karczewski et al., 2020; Howe et al., 2021)) and similar between case and controls (**Table 2**, **Supplementary Data S1, S2**). Cytochrome P450 (CYP) enzymatic activities are often affected by combinations of genetic polymorphisms. Use of prediction tools reflecting the overall genetic impact of a set of mutations on protein activity show a resulting metabolizer profile for the study population that is consistent with the expected distribution in the general European population (**Supplementary Data S3**).

Classical Risk Factor Evaluation

Recognized risk factors for PONV include: female gender, non-smoking status, a history of previous PONV, use of volatile anesthesia and perioperative opioid consumption. The role of patients' age and the type of surgery itself remains less clear. The impact of each of those factors on the occurrence and recurrence of PONV our cohort are presented in **Table 3**.

Using dichotomized values to describe the cohort, we found that, female gender, history of previous PONV and use of volatile anesthetics were, as expected, significant risk factors for PONV occurrence. Advanced age showed a significant protective effect while tobacco smoking and cannabis consumption tended to be protective although the values did not reach statistical significance. It should however be taken in consideration that only 6.3% of the whole study population reported smoking cannabis. Although not statistically significant for the model, the trend observed for the type of surgery or level of opioid consumption did not follow the expected behavior. Interestingly, none of the factors known to influence the occurrence of PONV seemed to predict its reoccurrence, with even an apparent protective effect of high opioid consumption which seemed to reduce the risk with an odds ratio (OR) of 0.35 [95% confidence interval (CI): 0.21–0.71, p-value = 6.99E-03].

Using linear parameters for age (year) and opioid consumption (expressed as grams of morphine equivalent) did improve the overall model parameter for the prediction of PONV occurrence as well as the statistical significance of age and opioid consumption, however with an OR very close to 1.0 for both parameters (**Supplementary Data S4**). The lack of detailed timing of administration of opioids following surgery precluded further dissection of their specific role in the occurrence of PONV in this study.

Co-variable independence is one of the fundamental prerequisites of mathematical regression models. It is, however, clear in the current case that all variables in the model are not fully independent for each other. For instance, 71% of the participants with previous PONV

TABLE 1 | Population characteristics.

	All	With initial PONV	Without PONV	PONV patients with follow-up	With recurrence	Without recurrence
Total population (Nbr.)	601	264	337	229	157	72
Female (%)	52.6	70.8	38.3	73.8	73.2	75.0
<50 years (%)	57.7	60.2	55.8	62.0	63.7	58.3
Nonsmoking (%)	68.1	71.2	65.6	72.1	75.2	65.3
No cannabis (%)	93.7	94.7	92.9	95.2	95.5	94.4
With PONV history (%)	23.8	33.7	16.0	34.9	38.2	27.8
Visceral or gynecological surgery (%)	40.8	37.5	43.3	38.0	35.7	43.1
With volatile anesthesia (%)	87.5	90.2	85.5	91.7	91.7	91.7
With high opioid (%)	48.4	47.0	49.6	48.0	40.1	65.3

TABLE 2 | Correlation between PONV and selected single nucleotide polymorphisms.

Gene	SNP ID	Chr	Major allele	Minor allele	MAF EUR[a]	MAF study	PONV Occurrence			PONV recurrence		
							OR	95% CI	P-value[b]	OR	95% CI	P-value[b]
COMT	*rs4680*	22	G (VAL)	A (MET)	0.50	0.47	1.09	0.85–1.41	0.493	0.92	0.60–1.41	0.693
	rs4633	22	C	T	0.50	0.47	1.06	0.83–1.37	0.629	0.93	0.61–1.43	0.745
	rs165722	22	C	T	0.50	0.48	1.09	0.85–1.41	0.492	0.95	0.62–1.45	0.800
	rs6269	22	A	G	0.41	0.43	0.91	0.71–1.18	0.484	0.95	0.62–1.45	0.801
	rs4818	22	C	G	0.40	0.41	0.90	0.70–1.17	0.434	0.89	0.58–1.37	0.594
CHRM3	*rs2165870*	1	G	A	0.34	0.34	0.97	0.75–1.26	0.845	0.97	0.63–1.48	0.878
	rs10802789	1	C	T	0.44	0.43	0.93	0.72–1.19	0.539	1.05	0.71–1.57	0.797
	rs685550	1	A	G	0.24	0.26	1.14	0.85–1.52	0.375	1.25	0.76–2.05	0.371
HTR1A	*rs6295*	5	C	G	0.46	0.49	0.89	0.69–1.14	0.360	0.88	0.59–1.32	0.550
HTR2A	*rs6313*	13	G	A	0.44	0.45	0.82	0.64–1.05	0.115	0.62	0.41–0.93	**0.022***
HTR3A	*rs10160548*	11	T	G	0.33	0.39	0.97	0.76–1.24	0.809	1.24	0.81–1.81	0.321
	rs1985242	11	T	A	0.32	0.34	0.80	0.61–1.04	0.098	0.94	0.60–1.46	0.772
	rs1176713	11	A	G	0.21	0.25	0.79	0.59–1.04	0.097	0.93	0.58–1.51	0.782
HTR3B	*rs1176744*	11	A (TYR)	C (SER)	0.31	0.30	0.76	0.58–1.00	0.051	1.10	0.68–1.78	0.700
	rs3758987	11	T	C	0.29	0.27	0.73	0.55–0.97	**0.033***	1.05	0.64–1.73	0.838
	rs1672717	11	A	G	0.42	0.35	1.45	1.12–1.89	**0.005****	0.63	0.40–0.98	**0.043***
	rs3782025	11	A	G	0.50	0.44	1.40	1.09–1.79	**0.009****	0.66	0.43–1.00	0.052
	rs76124337	11	CA	—	0.40	0.33	1.47	1.12–1.91	**0.005****	0.67	0.43–1.05	0.079
	rs45460698	11	AAG	—	0.07	0.14	0.84	0.58–1.21	0.352	0.92	0.51–1.66	0.772
HTR3D	*rs6443930*	3	G (GLY)	C (ALA)	0.47	0.49	1.01	0.79–1.30	0.916	1.26	0.83–1.92	0.275
OPRM1	*rs1799971*	6	A (ASN)	G (ASP)	0.16	0.15	0.88	0.62–1.25	0.474	0.98	0.55–1.76	0.949
DRD2	*rs1800497*	11	G	A	0.19	0.18	0.99	0.71–1.38	0.935	1.31	0.74–2.33	0.350
TACR1	*rs3755468*	2	C	T	0.45	0.44	0.77	0.60–0.98	**0.032***	1.01	0.67–1.50	0.978
FAAH	*rs324420*	1	C (PRO)	A (THR)	0.21	0.20	1.15	0.85–1.56	0.364	0.63	0.38–1.05	0.076
ABCB1	*rs1128503*	7	G	A	0.42	0.40	0.96	0.74–1.24	0.750	0.91	0.60–1.40	0.679
	rs1045642	7	G	A	0.52	0.47	0.80	0.62–1.03	0.078	0.89	0.58–1.35	0.578
	rs2032582[c]	7	C	A/T	0.41/0.02	0.38/0.02	0.91	0.70–1.18	0.472	1.06	0.70–1.61	0.782

[a] *[59, 60].*

[b] *Signif. codes: 0 "***" 0.001 "**" 0.01 "*" 0.05.*

[c] *Triallelic SNP: P-value calculated in PLINK, in A vs C (MAF, for T allele = 0.016).*

history were women and 67% of the smokers were below 50 years of age. Although statistical independence test of each variable pair evaluated using Fisher exact test showed there were significant associations (*p*-values in **Supplementary Data S5**), a multicollinearity analysis showed that all variance inflation factors (vif) were <1.2 (see **Supplementary Data S5**), indicating that the observed parameter interdependence did not affect the overall model integrity. As patients were randomized and study treatment was administrated only after the first episode of PONV, dexamethasone administration had no influence on the occurrence of the initial PONV symptoms. However, while both administration and dosage of dexamethasone were thought to interfere with PONV recurrence, neither did show the intended effect (Czarnetzki et al., in press). Using the current regression model, we confirm that dexamethasone administration does not prevent reoccurrence of PONV in patients suffering from PONV symptoms nor does it influence the weight and the significance of the other model parameters of the logistic regression (**Supplementary Data S6**). Moreover, administration of dexamethasone, within the tested dose range, does not impact the weight or the significance of any of the genetic determinants tested (**Supplementary Data S7**).

TABLE 3 | Logistic regression model based on dichotomous clinical factors.

	PONV occurrence			PONV recurrence		
	OR	**95% CI**	**P-value[a]**	**OR**	**95% CI**	**P-value[a]**
Intercept	—	—	5.01E-06***	—	—	0.483
Gender (0 = male, 1 = female)	4.11	2.85–5.96	**6.02E-14*** **	0.92	0.50–1.96	0.800
Age group (0 ≥ 50, 1 < 50 years)	1.52	1.05–2.20	**0.026***	1.35	0.68–2.31	0.332
Smoking (0 = yes, 1 = no)	1.35	0.91–2.01	0.140	1.56	0.85–3.21	0.193
Cannabis (0 = yes, 1 = no)	1.08	0.50–2.39	0.849	1.27	0.34–5.68	0.735
History of PONV (0 = no, 1 = yes)	2.38	1.56–3.65	**6.87E-05*** **	1.86	0.95–3.56	0.067
Surgery (0 = other, 1 = visc, gyneco)	0.72	0.50–1.04	0.082	0.70	0.41–1.37	0.245
Volatile anesthetics (0 = no, 1 = yes)	2.88	1.66–5.12	**2.30E-04*** **	0.92	0.29–2.67	0.886
High opioid (0 = no, 1 = yes)	0.85	0.59–1.22	0.378	0.35	0.21–0.71	**6.99E-03****

[a]*Signif. codes: 0 "***" 0.001 "**" 0.01 "*" 0.05.*

TABLE 4 | Predicted P450 cytochrome activities correlation with PONV.

Gene	Determinant	Chr	PONV Occurrence			PONV recurrence		
			OR	**95% CI**	**P-value[1]**	**OR**	**95% CI**	**P-value[1]**
CYP2D6	Activity score	22	1.02	0.79–1.32	0.862	0.88	0.56–1.39	0.594
CYP3A	Activity group	7	1.17	0.85–1.63	0.332	0.80	0.46–1.38	0.418
CYP2C9	Nbr. of reduced allele	10	0.78	0.56–1.09	0.147	1.42	0.80–2.63	0.249
CYP2C19	Activity score	10	1.09	0.88–1.35	0.415	1.00	0.70–1.42	0.987
CYP1A2	Activity score	15	1.17	0.90–1.52	0.239	**0.55**	**0.34–0.87**	**0.012***
CYP2B6	Activity score	19	0.93	0.74–1.17	0.536	1.12	0.75–1.66	0.578

[a]*Signif. codes: 0 "***" 0.001 "**" 0.01 "*" 0.05.*

PONV Association With Single Nucleotide Polymorphism or Metabolic Enzyme Activities

The impact of each SNP or DME activity on occurrence or recurrence of PONV was assessed using logistic regression including the following covariates: age, gender, tobacco smoking, cannabis consumption, previous PONV history, use of volatile anesthetic and high perioperative opioid consumption. An additive genetic model, where each mutated allele contributes to the signal (i.e., AA = 0, AB = 1, BB = 2) was chosen for all polymorphisms tested. For metabolic enzymes, activity scores were considered as ordinal values. The association *p*-values and corresponding OR for first PONV occurrence and recurrence are presented in **Table 2** for individual SNPs and **Table 4** for cytochrome activities respectively. None of the associations reached statistical significance if a strict Bonferroni correction was applied (ie if $p = 0.05/27$—> corrected *p*-value = 0.002 or 2E-03).

Four of the five polymorphisms tested showing an association with the occurrence of PONV map to the serotonin receptor 3B encoding gene (*HTR3B*). In addition, we found an association between the *rs3755468* mutation in *TARC1* and PONV occurrence, as well as between the *HTR2A rs6313* mutation and PONV recurrence (**Table 2**). Regarding predicted cytochrome activities, our results suggest an involvement of the highly inducible CYP1A2 enzyme in the recurrence of PONV [OR 0.55 (95% CI 0.34–0.87), *p*-value = 0.012]. None of the other predicted CYP activities seemed to significantly influence the occurrence or recurrence of PONV in our cohort (**Table 4**).

There was no effect of dexamethasone treatment at either dose nor globally on the results presented in **Tables 2**, **4** (**Supplementary Data S7**). As different genetic factors are likely to act on PONV through various mechanisms, we considered the influence of each genetic factor on the impact of classical risk determinants of PONV by looking for confounding. A systematic analysis of the influence of each polymorphism on the estimates of significant co-variable revealed only 4 cases of weak confounding effect (**Supplementary Data S8**).

Interestingly, two of the *HTR3B* polymorphism had an effect on the age association with PONV occurrence. Indeed, age segregation for heterozygous carriers of the *rs3782025* or *rs76124337* mutations reveals a clear risk difference. This effect, while already visible in the whole cohort for *rs76124337*, was highly predominant in women (**Supplementary Data S9**). Interestingly, both serotonin signaling and PONV risk are believed to be sex and age dependent (Yonezawa et al., 1989; Meltzer et al., 1998; Laugsand et al., 2011), further underlying the involvment of variations in brain serotonin homeostasis in PONV.

The *HTR3B* Haplotype Associated With the Most Severe PONV Risk is Present in One Third of the Patients

Serotonin signaling through type 3 receptors is known to play an important role in emesis. The strong association observed between three of the *HTR3B* SNPs and PONV confirms previous reports (Rueffert et al., 2009; Ma et al., 2013; Yan et al., 2021). Moreover, the weaker associations found between

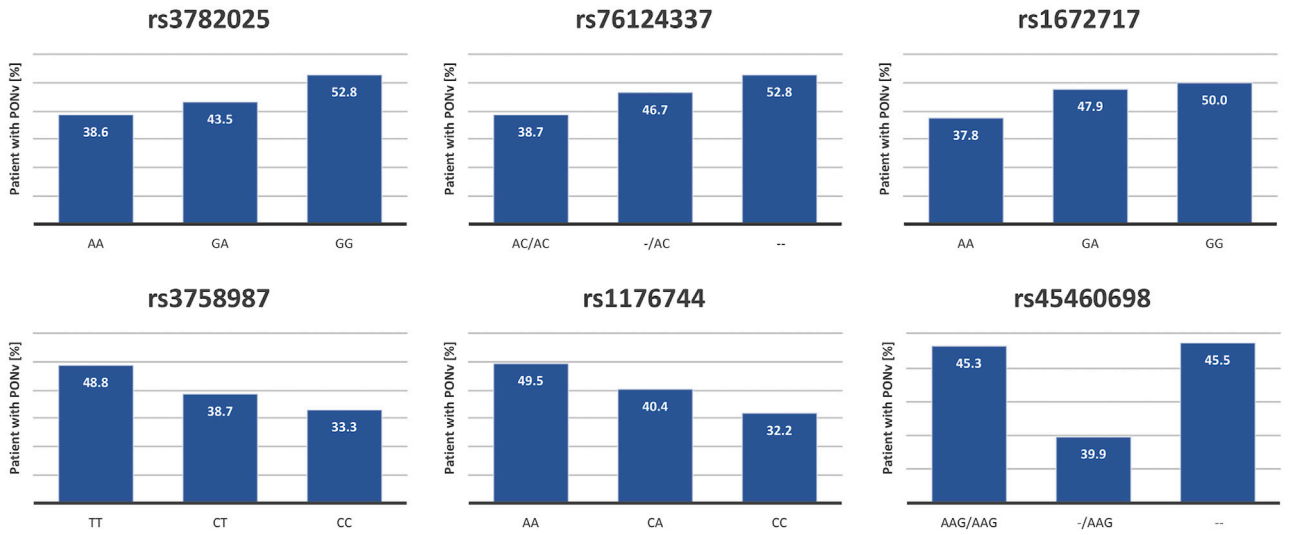

FIGURE 1 | Correlation between *HTR*$_{3B}$ polymorphisms and PONV occurrence. Five of the six *HTR*$_{3B}$ polymorphisms tested in the screen were significantly correlated with the risk of PONV occurrence. The figure shows, for each of those SNPs, the proportion (%) of patients suffering from PONV in each genotypic category. The homozygous genotype corresponding to the major allele is always depicted on the left.

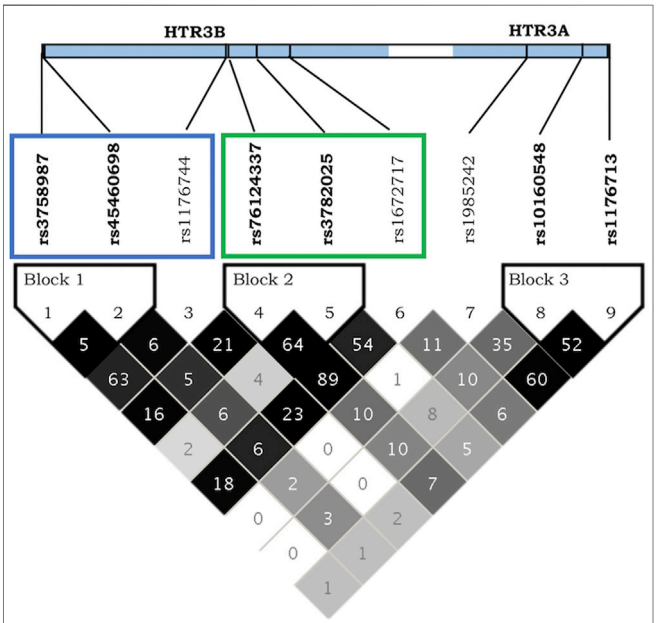

FIGURE 2 | LD plot for *HTR3A* and *HTR3B* receptors on Chromosome 11. The shading of the haplotype boxes indicates the magnitude of LD coefficient D', with darker color indicating stronger LD. 100-fold pairwise r^2 squared numbers are displayed. Blue rectangle highlights *HTR3B* mutations with positive influences on PONV prediction while the green box indicates *HTR3B* mutation with increased risks of PONV. Produced with Haploview (Barrett et al., 2005).

the remaining *HTR3B* and *HTR3A* related SNPs and PONV occurrence, and to a lesser extend recurrence, further supports the observed link between nausea and vomiting and type 3 serotonin receptors.

The logistic regression analysis was based on an additive model where the presence of each additional allele contributes independently to the phenotype. Testing of the significant associations between *HTR3B*-related SNPs and PONV occurrence using dominant or recessive genetic models for the minor alleles, shows that the best description of the association for *rs3782025* is additive. Additive and dominant models are nearly equivalent for *rs76124337* and *rs1672717*, while a dominant model is more favorable for *rs3758987*. It is noteworthy that the association between *rs3782025* remained significant whatever the model used (**Supplementary Data S10**).

Interestingly, not all *HTR3B* mutations showed the same trend of association with PONV: some alleles had a protective effect while others were clear risk factors suggesting the presence of two blocks of mutations (**Figure 1**). A linkage disequilibrium analysis detected three haplotype blocks on chromosome 11, two within *HTR3B* (*rs3758987* and *rs45460698*; *rs76124337* and *rs3782025*) and one within *HTR3A* (*rs10160548* and *rs1176713*) (**Figure 2**). As highlighted in **Figure 2**, the two *HTR3B* variant blocks identified by the haplotype analysis corresponded to the positive and negative PONV prediction trends observed for *HTR3B* mutations.

Association between PONV and the haplotypes blocks on chromosome 11 confirms the predominance of the second block composed of *rs76124337* and *rs3782025* for the prediction of PONV (**Table 5**) with the–G genotype increasing the risk of PONV by a factor of 1.49. Similarly, the combination of all six mutations of *HTR3B* together also highlights the importance of the same SNPs for prediction of PONV occurrence. Interestingly the predicted "wild-type" or reference haplotype, corresponding to the major alleles of each independent SNPs was present in only 19% of the population, while the worst combination in terms of PONV occurrence, with an odd ratio of 1.5, was found in 32% of the study population.

TABLE 5 | Correlations between serotonin receptor haplotypes and PONV occurrence.

Gene	Haplotype Block[a]	Nbr SNP	Population coverage [%]	Haplotype	Frequency	PONV Occurrence		
						OR	STAT	P-value[b]
HTR3B	1	2	100	T-	0.14	0.84	0.86	0.355
				CAAG	0.26	0.74	4.27	**0.039***
				TAAG (wt)	0.60	1.39	6.33	**0.012***
HTR3B	2	2	100	-G	0.33	1.47	7.93	**0.005****
				ACG	0.11	1.01	1.52E-3	0.969
				ACA (wt)	0.56	0.71	7.12	**0.008****
HTR3A	3	2	100	GG	0.25	0.78	3.00	0.083
				GA	0.14	1.41	3.57	0.059
				TA (wt)	0.62	1.03	0.04	0.840
HTR3B	All	6	92	TAAGAG-G	0.32	1.49	8.23	**0.004****
				TAAGAAACG	0.02	0.95	1.26E-2	0.911
				CAAGCGACA	0.07	0.79	0.76	0.382
				TAAGCGACA	0.01	1.27	0.20	0.656
				CAAGCAACA	0.17	0.70	3.72	0.054
				TAAGCAACA	0.05	0.83	0.35	0.555
				T-AAACA	0.13	0.79	1.42	0.233
				TAAGAAACA (wt)	0.19	0.99	8.36E-3	0.927

[a]*Block 1: HTR3B rs375987 + rs45460698.*
Block 2 HTR3B rs76124337 + rs3782025.
Block 3 HTR3A rs10160548 + rs1176713.
All rs375987/rs45460698/rs1176744/rs3782025/rs76124337/rs1672717.
[b]*Signif. codes: 0 "***" 0.001 "**" 0.01 "*" 0.05.*

Taken together, our genetic analysis has identified two single nucleotide polymorphisms located in the open reading frame of type 3B serotonin receptor on chromosome 11 as the most important genetic predictor of PONV in our cohort.

Serotonin Type 3B Receptor Mutation Versus Pharmacological Modulation of Serotonin

As serotonin is known to play an important role in emesis and as we were able to involve several SNPs related to type 3B serotonin receptors in the occurrence of PONV, we examined the effect of *HTR3B* genotype in patients having received tramadol as part of their postoperative analgesic treatment. Tramadol is an opioid with additional serotonin and norepinephrine reuptake inhibitor properties, thus promoting an increase in the synaptic cleft serotonin concentrations.

In the current study, 50 patients (8.3% of the study population) received tramadol as part of their postoperative analgesia. Despite known emetic properties of this opioid, only 10 (20%) of the patients under tramadol experienced PONV, versus 43.9% in the total cohort suggesting a selection bias. However, when comparing the effect of the *HTR3B* variant on PONV occurrence in patients under tramadol with patients without, two different tendencies became apparent (**Figure 3**). First, administration of tramadol seems to limit the negative impact of *rs3782025*, *rs76124337* and *rs1672717* variants. Second, the risk of PONV in patients with double mutation at position *rs3758987* or *rs1176744* receiving tramadol seems to be considerably diminished. Actually, the protective effect of those two last mutations relied to some extend on patients receiving tramadol (**Supplementary Data S11**). The current clinical study was not designed to address the impact of tramadol on PONV and the available data clearly lack the appropriate power for formal statistical analysis on this point.

Incorporation of *HTR3B* Genotyping Information can Improve Prediction Capacity of the Classical Risk Scores

The simplified Apfel score is a tool for PONV prediction (Gan et al., 2020). In this score, the risk category of each patient is obtained by adding one unit for each of the following risk factors: female gender, non-smoking status, history of previous PONV and opioid consumption, and each risk category is associated with an increasing probability of suffering from PONV (Apfel et al., 1999).

Application of the Apfel score to our study population followed the expected risk trend with a near linear increase of PONV risk from one category to the next as shown in **Figure 4A**. According to standard nomenclature, category 0 and 1 are generally considered at low-risk of PONV, category 2 patients are at medium risk while categories 3 and 4 regroup high-risk patients (Gan et al., 2020). While in our study population the *HTR3B* SNP *rs3782025* was the most relevant polymorphism for the prediction of PONV, the strength of the association of this parameter did not support a cost-effective genotyping in the whole cohort. However, considering that the classification of low and high-risk patients is unlikely to change due to genotype, medium-risk patients are the ones that are most likely to benefit from genotyping. In addition, the confounder analysis described above and shown in **Supplementary Data S9**, highlighted the predominance of the impact of *rs3782025* in younger women. Thus, we developed two models focalizing on medium risk (Apfel

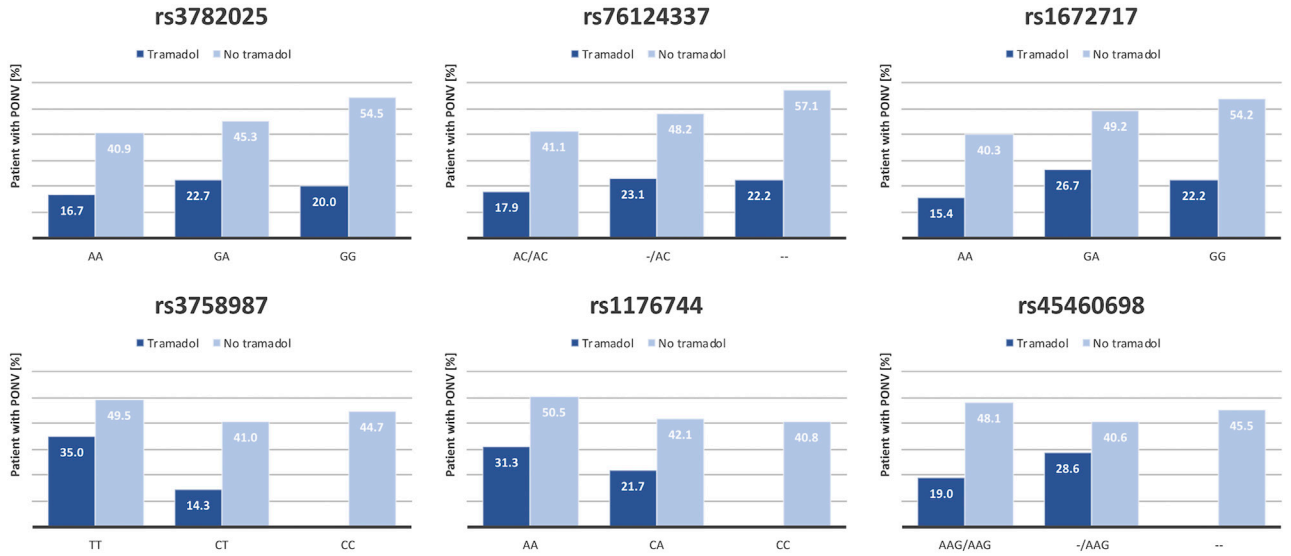

FIGURE 3 | Effect of tramadol on association between *HTR3B*-related polymorphisms and PONV occurrence. Tramadol is a selective serotonin reuptake inhibitor that could influence *5-HTR*$_3$ receptor function and thus modulate the effect of *HTR3B* polymorphisms on PONV occurrence. The figure shows the proportion (%) of patients suffering from PONV in function of tramadol administration in each genotypic category for each of the six *HTR3B* SNP tested.

category 2) female patients to evaluate the clinical impact of genotyping. In Model 1, only category 2 female patients below 50 years old were genotyped while in Model 2 the genotype of all category 2 female patients was taken into consideration. In all cases, *rs3782025* genotypic risk was assigned according to the following rules: A/A = 0 and G/G = 1 in all cases, while A/G = 0 if patient is > 50 years old and = 1 if younger.

As summarized in **Table 6**, the Model 1 group covered 80 of our 601 patients, with an average PONV risk of 64% before adjustment. Adding the genetic information at locus rs3782025 for this group resulted in accurate PONV prediction for 68% of the patients with an average PONV occurrence of 75% in the higher risk group and 44% in the lower risk group. Clinical relevance can be estimated by calculating the number needed to genotype (NNG), which is obtained by dividing the number needed to treat (NNT) by the frequency of the risk allele occurrence (Tonk et al., 2017). In our case, this represents the number of patients that needs to be genotyped for one to be classified into the proper risk group and thus to be likely to benefit from PONV prophylaxis. Selection of patients according to Model 1 results thus in an NNG of 5 if considering identification of patients which might benefit of a reinforced PONV prophylaxis versus an NNG of 9 to avoid administration of unnecessary anti-emetic treatment. **Figure 4B** shows the actual incidence of PONV in the genotype-adjusted Apfel risk score categories when Model 1 is applied to our study population. The results for Model 2 are relatively similar to Model 1, with 67% accurate prediction for 124 patients responding to the selection criteria and an improvement from 9 to 7 of the NNG for detection of lower risk patients. Graphical representation of the PONV risk for each category calculated according to Model 2 is shown in **Figure 4C**.

Receiver operating characteristic (ROC) curve analysis presented in Supplementary data S12 shows a significant although modest increase in the area under the curve (AUC) for both models with respect to the classic score with an AUC_{Model1} = 0.648 [95% CI 0.538–0.759], p = 8.27E-03 and AUC_{Model2} = 0.668 [95% CI 0.584–0.753], p = 9.62E-05 for the subgroups of patients where the genotype is taken into account. If considering the whole cohort, the calculated AUC for the classical Apfel score of 0.633 is slightly improved to 0.660 [95% CI 0.617–0.702] using Model 1 and further to 0.665 [95% CI 0.623–0.708] using Model 2.

Effect of Phase 1 CYP450 Predicted Metabolism on PONV Recurrence—A Role for CYP1A2?

CYP1A2 is a highly inducible cytochrome from the P450 family, with a large inter-individual variability. It is an abundant liver phase I enzyme as it represents >10% of all liver cytochromes P450, metabolizing a large number of substrates including drugs (clozapine), endogenous substrates (melatonin, steroids) and dietary products (caffeine). Both genetic and environmental factors are strongly influencing CYP1A2 activity, underscoring the difficulty in predicting its activity (Thorn et al., 2012). CYP1A2 is strongly induced by smoking. Our initial correlation between PONV recurrence and CYP1A2 activity did only take into account the genetics resulting in an OR of 0.55 (95% CI 0.34–0.87, p-value = 0.012). Adjusting the predicted CYP1A2 activity for smoking using the method developed by Lesche et al. (2020) resulted in a slightly improved correlation with an OR of 0.57 (95% CI 0.38–0.85, p-value = 0.006) (**Figure 5**).

CYP1A2 parallels the effect of smoking on the reduction of the risk of PONV and confounder analysis revealed that the two parameters are indeed interconnected. It has already been

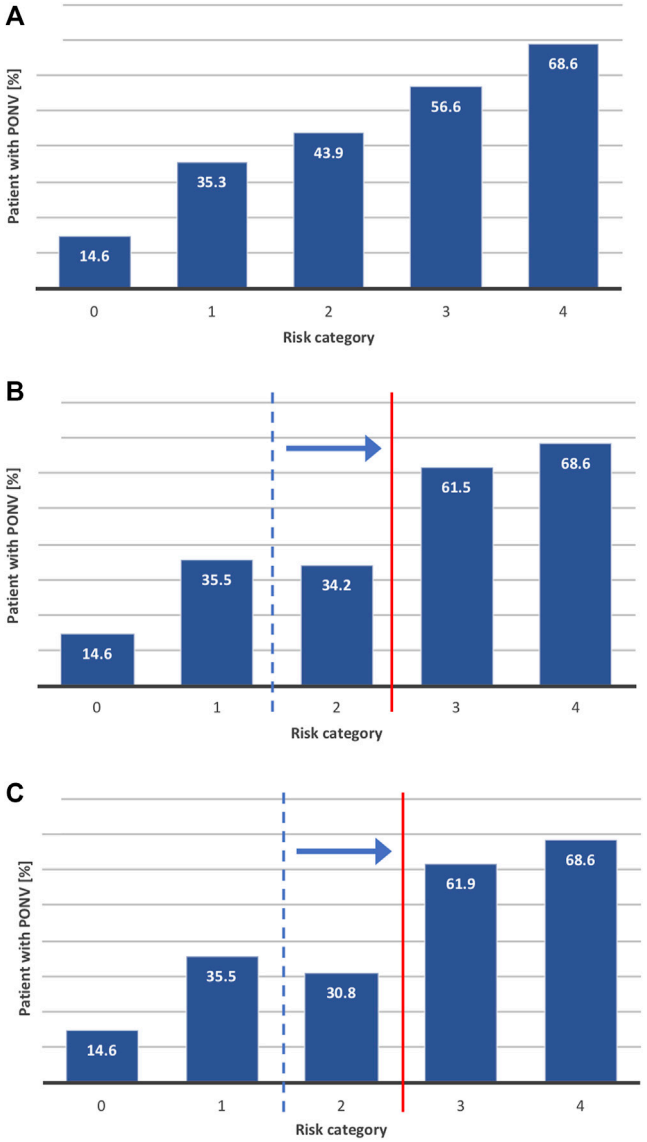

FIGURE 4 | Integration of HTR_{3B} *rs3782025* genetic information in prediction score modeling. We tested three different models to evaluate the potential impact of integrating HTR_{3B} *rs3782025* genotype in the prediction score. The figures shows the proportion (%) of patients predicted to suffer from PONV in each risk category of **(A)** classic risk score (Apfel score) without genetic information **(B)** Apfel score + *rs3782025* genotyping for women below 50 years of age in Apfel score category 2 **(C)** Apfel score + *rs3782025* genotyping for all women in Apfel score category 2.

proposed that part of the protective effect of smoking toward PONV could be mediated by enhanced detoxification resulting from CYP1A2 and CYP2E1 activation (Sweeney, 2002).

DISCUSSION

The current genetic study first examined the impact of 28 individual single nucleotide polymorphisms (SNPs) located around 13 genes as well as the activity of six liver cytochromes (CYP) on the occurrence of PONV in a cohort of 601 patients without anti-emetic prophylaxis followed during the first 24 h after surgery. The overall PONV incidence in the cohort was 44%. We detected five polymorphisms that were significantly associated with PONV occurrence, one located in the promoter of the neurokinin receptor *TACR1* and the four others around the type 3B serotonin receptor gene (*HTR3B*). Further statistically significant risk factors in our cohort were female gender, history of PONV, and the use of volatile anesthetics.

5-HTR_3 receptors are the only ligand-gated ion channel receptors activated by serotonin (Walstab et al., 2010). They are expressed throughout the brain as well as on gastrointestinal tract vagal afferents (Walstab et al., 2010). In the central nervous system, 5-HT_3 receptors mediate fast excitatory synaptic transmission in response to serotonin, promoting neurotransmitter release (Walstab et al., 2010; Rees et al., 2017). HTR3B functions as a penta-heteromeric complex together with HTR3A (GeneCards, 2021). Polymorphisms in both *HTR3A* and *HTR3B* have previously been associated with early onset PONV (<6 h postoperatively) (Rueffert et al., 2009) as well as a response to antiemetic treatment in pregnant women (Lehmann et al., 2013) and CINV (Tremblay et al., 2003; Kaiser et al., 2004). As shown in **Figure 1**, we identified two blocks of mutations with opposite effects on PONV prediction. Additional analyses confirmed the linkage disequilibrium within each of the two mutation blocks and revealed that the haplotype corresponding to the highest risk group in terms of PONV occurrence was present in 32% of the study population and was best represented by *rs3782025*.

From the 6 *HTR3B* SNPs included in this screen, only *rs1176744* is located within an exon and results in a missense mutation (Y129S) known to result in a global increase receptor activity, with longer channel opening time, slower desensitization and deactivation kinetics (Krzywkowski et al., 2008; Walstab et al., 2008). This increase in activity tends to have a protective effect toward PONV occurrence which is in concordance with previous observations (Laugsand et al., 2011). The impact of the other identified mutations on 5-HTR_3 receptor activity has, to our knowledge, not been described, but it is tempting to speculate on a reduced activity for the other block of mutations. The last *HTR3B* polymorphism tested (*rs45460698*), whose impact on CINV is controversial (Tremblay et al., 2003; Perwitasari et al., 2011), did not show any significant trend in the present study.

The confounder analysis suggesting a relationship between *HTR3B rs3782025* and *rs76124337* and patient age and gender is noteworthy. For both mutations, our results suggest a shift from a recessive to a dominant genetic model with age in female patients, while the mutated alleles have little impact in men of either age group. Several studies have suggested differences in serotonin signaling with age and gender, possibly linked, at least in part, to sex hormone signaling in women of child-bearing potential (Hernández-Hernández et al., 2019; Hudon Thibeault et al., 2019; Pottoo et al., 2019). This observation is in line with previous reports showing no gender difference in PONV occurrence in children up to puberty, after which a higher

TABLE 6 | Prediction model parameters.

	Model 1[a]	Model 2[b]
Nbr patients genotyped	80	124
% PONV in genotyped patents	64	58
Nbr of patients with genetic risk factor[c]	51	66
% PONV in patients with genetic risk factor	75	72
% PONV in patients without genetic risk factor	44	40
Sensitivity	0.75	0.67
Specificity	0.56	0.65
NNG for identification of patients with increased PONV risk	5	6
NNG for identification of patients with decreased PONV risk	9	7

[a]*Model 1: rs3782025 genotype for category 2 women <50 years.*
[b]*Model 2: rs3782025 genotype for all category 2 women.*
[c]*rs3782025 G/G = 1; rs3782025 A/G = 1 for women <50 years, rs3782025 A/G = 0 for women >50 years.*

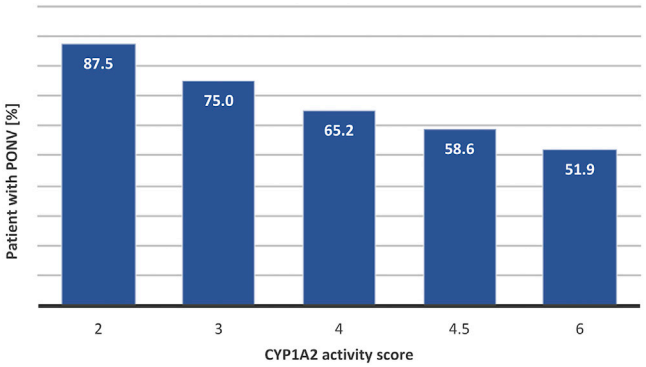

FIGURE 5 | Correlation between CYP1A2 activity and levels of PONV. Predicted CYP1A2 activity scores have been calculated based on genotypic data taking into account activation due to smoking by multiplying the genotypically predicted activity score by 1.5× in presence of smoking (Lesche et al., 2020). The figures showy the proportion of patients experiencing PONV in each CYP1A2 activity category. The R^2 of the linear regression between score activity and PONV risk reaches 0.957.

incidence is observed in girls than boys (Heyland et al., 1997; Eberhart et al., 2004; Ames and Machovec, 2020). However, although an age below 50–55 years is a recurring PONV risk factor, none of the studies reporting age as a significant factor (Sinclair et al., 1999; Junger et al., 2001; Leslie et al., 2008), performed sex specific analysis for different age groups in an adult population. Our data showed no modification in the gender related odds ratios for PONV in function of age. However, a number of factors other than hormones could play a role in the gender susceptibility toward PONV in older adults, including a global decrease in serotonin neurotransmission (Meltzer et al., 1998).

Current guidelines recommend the administration of 1–2 antiemetic agents for patients presenting up to two risk factors versus 3–4 agents for patients with more than two risk factors (Gan et al., 2020). The difficulty is to balance the need for antiemetic prophylaxis with the risk of adverse effects and increased cost, resulting from poly medication. Indeed, none of the available anti-emetic treatments is devoid of potential adverse effects and the concomitant administration of multiple drugs increases the risk of interaction and occurrence of adverse reactions (Alomar, 2014; Bennett and Sofat, 2020). Although the cost of genetic testing is decreasing rapidly, currently, genotyping all surgery patients is unlikely to be cost-effective. By focusing on the group of medium risk patients that would benefit most from it, our model shows that genotyping might improve the management of PONV in this population. Adding the genetic factor to prediction scores could allow evidence-based decision making regarding antiemetic prophylaxis in those patients, both offering an increased protection to patients at risk, and decreasing unnecessary exposure to potentially harmful treatments. Moreover, knowledge about 5-HTR_3 receptor polymorphism might also be used to help predict the efficacy of 5-HTR_3 antagonists, further enhancing the benefit of the genetic testing.

TACR1 encodes a G-protein coupled receptor activated by neurokinin-1 (aka substance P). Neurokinin-1 is a neuropeptide involved in pain signaling and inflammation as well as emesis (Vilisaar and Arsenescu, 2016; Schank and Heilig, 2017). TACR1 receptor antagonists have been successfully used as antiemetic medications in CINV and PONV (Aziz, 2012; Razvi et al., 2019; Gan et al., 2020). The *rs3755468* C > T polymorphism in the *TACR1* promoter has previously been associated with reduced PONV occurrence and gender susceptibility in a Japanese population (Hayase et al., 2015). As the mutation is found in a predicted ERE (estrogen response element), it was hypothesized that this SNP could participate in the gender susceptibility toward PONV (Hayase et al., 2015). While we saw a similar protective effect of the *rs3755468* mutation in our cohort, this effect was approaching significance in men (OR 0.69, 95% CI 0.47–1.01, p-value = 0.06) but not in women (OR = 0.83, 95% CI 0.61–1.14, p-value = 0.24) in the opposite of the previous study. It should be noted that there is no considerable MAF difference between European and Japanese populations at this position. Thus, while the protective effect of the *TACR1 rs3755468* T allele seems reproducible, the differences observed with the results from the Japanese study suggests that more work is needed to unravel the role of *TACR1 rs3755468* polymorphism in PONV.

While both serotonin and substance P are known to play an important role in the transduction of pro-emetic signals, a number of other neurotransmitters involved in PONV have been identified, either through the blocking action of anti-emetic treatments or genetic screening (Gan, 2007; Horn et al.,

2014; Janicki and Sugino, 2014). The current study failed to replicate a number of previously described associations between PONV occurrence and clinically relevant polymorphisms affecting recognized nausea and vomiting signaling pathways, such as dopamine, acetylcholine, opioid or even serotonin signaling through other types of 5-HT receptors (Hornby, 2001; Aziz, 2012; Janicki and Sugino, 2014; Belkacemi and Darmani, 2020). A number of technical reasons might explain differences between our and other studies starting by different ethnic background, the absence of anti-emetic prophylaxis in the present study as well as a potential overlap sometimes observed between PONV and opioid-induced nausea and vomiting. Indeed, as opioid administration did not appear to be a significant risk factor in our cohort, genetic factors affecting pathways induced by opioids are unlikely to show significant associations. In addition, the important cross-talk between the different neurotransmitter signaling pathways in the central nervous system has to be taken into consideration. Indeed, both HTR3 and TACR1 are key modulators of synaptic signal transduction through release of neurotransmitter from pre- and post-synaptic neurons and are in turn regulated by other neurotransmitters (Adell et al., 2010; Rees et al., 2017; Schank and Heilig, 2017; Peters et al., 2021). Thus it is difficult to distinguish whether the action of any anti-emetic drug is direct or through modulation of other neurotransmitter pathways. Similarly, genetic variants resulting in physiological differences in neurotransmitter signal transmission are likely to have a broad impact on central nervous system organization and neuronal interconnection.

The second part of this work examined the influence of the selected genetic markers on the recurrence of PONV following administration of dexamethasone. Although the anti-emetic mechanism of action of dexamethasone remains unclear, it is, in the PONV setting, an effective prophylactic treatment when used alone or in combination with other anti-emetic medication (Chu et al., 2014; Gan et al., 2020). According to the study protocol, patient suffering from PONV during the first 24 h after surgery randomly received an intravenous dose of 0 (placebo control), 3, 6 or 12 mg of dexamethasone (Czarnetzki et al., in press). As the study treatment was administrated only after apparition of the first PONV symptoms, it was of no concern for the search for genetic determinants of PONV occurrence. PONV recurrence after treatment administration on the other hand, would have been expected to depend on both the pharmacokinetic and pharmacodynamic properties of dexamethasone and the inter-individual variability affecting these parameters. Interestingly, administration of dexamethasone to patients suffering from PONV did not show any benefit, resulting in premature termination of the study for futility purpose (Czarnetzki et al., in press). The reanalysis of the study data taking into account the genetic information, in addition to the previously assessed risk factors, failed to reveal any potential genetic bias that might have occluded a positive effect of dexamethasone for the treatment of established PONV. The lack of association between the study treatment and the primary outcome as well as the absence of confounding effects with any of the polymorphisms or DME activity level tested, suggest that the observed associations between PONV recurrence and the *HTR2A rs6313* polymorphism and CYP1A2 activity is a direct result from the surgery and anesthesia rather than dexamethasone administration.

There is little previous information on the involvement of *HTR2A rs6313* in PONV, although *rs6311*, which is in strong LD with *rs6313* (LD r^2 = 1.000, D' = 1.000) has been involved in severe nausea resulting from the adverse effect of paroxetine treatment in Japanese patients suffering from depression (Kato et al., 2006). The protective effect of the A allele, seen here, mirrors the results of Kato et al. (Kato et al., 2006). The rs6313 polymorphism is located in exon 1 and results in a silent mutation at the Ser34 position of the *HTR2A* gene, a neuronal G-coupled serotonin receptor involved in signals transmission from the synaptic cleft via PLC and PLA_2 activation (Yevtushenko and Reynolds, 2010; Masson et al., 2012; Iglesias et al., 2017). Although the *rs6313* SNP has been linked to a number of diseases including schizophrenia, depression and eating disorders, the associations remain weak and the impact of the mutation on receptor activity remains uncertain, with some studies suggesting a decreased expression and protein level for the G allele, while other failed to measure a difference (Polesskaya and Sokolov, 2002; Bray et al., 2004; Parsons et al., 2004; Norton and Owen, 2005). However, 5-HTR_{2A} receptors are intimately involved in the neurotransmitter crosstalk in the brain (Norton and Owen, 2005; Peters et al., 2021) and could be involved in transmission or modulation of pro-emetic signals.

It is important to note that CYP1A2 activity was not correlated with the risk of PONV occurrence but recurrence, decreasing the likelihood that the effect is due to a direct effect of faster turnover of anesthetic drugs. Indeed, a faster metabolism of propofol, which is an intravenous general anesthetic drug shown to be protective of PONV (Dinis-Oliveira, 2018), by CYP1A2 (Murayama et al., 2007), is not coherent with the observed protective effect of this cytochrome. Lidocaine, another known CYP1A2 substrate (Orlando et al., 2004), is a local anesthetic drug that was shown, when administrated intravenously, to decrease the need of postoperative analgesia and thus believed to protect against PONV by decreasing opioid consumption (Wang et al., 2019). While perioperative administration of opioids belongs to the classical risk factors of PONV, this effect was not observed in the current study. Moreover, we could not detect any confounding effect between CYP1A2 activity and opioid dosage (Chang and Kam, 1999; Faber et al., 2005). Although not demonstrated, an interplay between CYP1A2 activity and the serotoninergic system is possible. Indeed, CYP1A2 substrate specificity is quite broad and the enzyme is known to metabolize both ondansetron (Huddart et al., 2019) and melatonin (Skene et al., 2001). Ondansetron is a serotonin receptor antagonist sharing a similar structure with serotonin; melatonin a downstream product of the serotonin pathway. Thus, it is possible that a high basal level of CYP1A2 activity, either due to genetic polymorphism or a habit of smoking, could set a higher degree of tolerance against increased serotonin signaling.

There are several limitations to the current analyses. First, by selecting a limited number of polymorphisms from the literature, we might have missed undescribed associations as well as introduced a bias towards the most often studied genes in the context of PONV. Indeed, four of the five significant associations uncovered for PONV occurrence belong to the *HTR3B* gene, and are in linkage disequilibrium, meaning those associations are not independent from each other. Genome-wide studies offer the advantage of an unbiased selection and the possibility to uncover novel associations, however, the power of such association study relies heavily on a larger sample size (Tam et al., 2019). Secondly, the initial SNP selection included a number of assumptions such as the pro-emetic effect of opioids that we did not see in this cohort, thus decreasing the interest in polymorphisms affecting genes directly related to opioid signaling such as *COMT*, *OPRM1*, *ABCB1* or *FAAH* (Coulbault et al., 2006; Janicki and Sugino, 2014; Sadhasivam et al., 2015). Next, although inter-individual variability, drug metabolism and elimination of volatile anesthetics as well as other intraoperatively administrated drugs might have been expected to play a role, none of the cytochrome activities tested was significantly associated with the occurrence of PONV. This might, on one hand, be due to treatment heterogeneity occulting significant effects, and on the other hand be a consequence of predicting cytochrome phenotypic activity based solely on genetic information. Indeed, enzymatic activity is a result of a combination of genetic and environmental factors. Thus, while genetically-derived predictive activity of most cytochromes tested in the current study is widely used, discrepancy might be observed especially for highly inducible enzymes (Shah and Smith, 2015). Also, regarding the risk score model parameters it should be noted that the non-inclusion of patients having received anti-emetic prophylaxis might have biased our cohort toward lower risk patients and thus enhance the AUC value of our models. Finally, in absence of a more powerful tool, we used the Apfel score to predict the baseline risk of PONV in individual patients. This score is pragmatic and also widely used in clinical practice. However, its discrimination (ability to distinguish between patients with and without PONV) and calibration (agreement between observed and expected outcome frequencies) are limited (van den Bosch et al., 2005).

Future perspectives include the validation of the clinical impact of the risk model including genetic parameters in a prospective study. Moreover, investigations focusing on the effect of tramadol in combination with *HTR3B* mutations could provide valuable information on the molecular mechanism of the 5-HTR_3 receptors and enhance our understanding of its role in PONV. It is indeed difficult to draw conclusions from the effect of tramadol on the *HTR3B* SNPs seen in the current study, both due to the limited number of patients having received the drug and the apparent lack of an emetogenic effect of tramadol in those who did. However, our preliminary observations suggest that administration of tramadol, which, like other selective serotonin receptor inhibitors, tend to increase serotonin levels in the extracellular space, might limit the negative impact of *rs3782025*, *rs76124337* and *rs1672717* variants and further diminish the risk of PONV in patients with a double mutation at position *rs3758987* or *rs1176744*. In a broader view, the next challenge facing genetic studies in the context of PONV will be to unravel the contribution of different SNP to the occurrence of PONV versus response to antiemetic treatments. Although large cohorts including administration of standardized antiemetic treatment will be necessary to reach statistical significance, understanding the interplay between different NT systems and emesis would contribute to the identification of personalized risk factors as well as help selecting the most efficient individualized antiemetic treatment for each patient, further enhancing the benefit and cost-effectiveness of genotyping.

DATA AVAILABILITY STATEMENT

The datasets generated and analyzed during the current study are available in the Yareta repository, DOI: 10.26037/yareta:33pqmyb4kreulpvugeyh4q56vm.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Commission cantonale d'éthique de la recherche (CCCR), République et Canton de Genève, Geneva, Switzerland. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YG, CC, FC, JAD, and MRT designed the study. YG performed the experiments, YG and BG-W processed the clinical data, YG performed the bioinformatics analysis, wrote the manuscript's draft, and prepared the figures and tables. MRT, BG-W and JAD reviewed the draft. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.816908/full#supplementary-material

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Comparative Assessment of Outcomes in Drug Treatment for Smoking Cessation and Role of Genetic Polymorphisms of Human Nicotinic Acetylcholine Receptor Subunits

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Objective: To investigate the effects of genetic polymorphisms of human nicotinic acetylcholine receptor subunits α3, α4 and α5, which are encoded by *CHRNA3*, *CHRNA4 CHRNA5* genes, respectively, on nicotine addiction and outcomes of pharmacological treatments for smoking cessation.

Methods: A total of 143 smokers and 130 non-smokers were included. Genotyping for *CHRNA3 rs578776, CHRNA4 rs1044396-rs1044397, CNRNA5 rs16969968* polymorphisms was performed by PCR, flowed by RFLP. Clinical outcomes and success rates of pharmacological treatments for smoking cessation with nicotine replacement therapy (NRT), bupropion or varenicline were determined at the 12th week of the treatment.

Results: Overall, 52 out of 143 (36.4%) smokers who received pharmacotherapy were able to quit smoking. Success rates for smoking cessation were similar for female (30.3%) and male (41.6%) subjects ($p = 0.16$). The success rate for smoking cessation treatment with varenicline (58.5%) was significantly higher as compared to other treatments with NRT (20.0%), bupropion (32.3%) or bupropion + NRT (40.0%) (chi-square test, $p = 0.001$). Smoker *vs*. non-smoker status and the clinical outcomes of drugs used for smoking cessation were found similar in subjects carrying wild-type and variant alleles of human nicotinic acetylcholine receptor α subunits.

Conclusion: In this study, smoking cessation treatment with varenicline was significantly more effective than treatments with nicotine replacement or bupropion in a cohort of Turkish subjects. Smoker/non-smoker status and the clinical outcomes of treatment with pharmacological agents were similar in subjects with wild-type or variant alleles for human nicotinic acetylcholine receptor subunits α3 (*CHRNA3*), α4 (*CHRNA4*) and α5 (*CHRNA5*).

Keywords: smoking, nicotine addiction, smoking cessation, CHRNA, nicotinic acetylcholine receptor alpha subunit

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INTRODUCTION

Nicotine dependence is a very serious health problem and a leading cause of preventable death in many countries. There is an increase in cigarette consumption rates both in the world (WHO, 2021) and in Turkey. Overall, 26.5% of the total population of Turkey were reported as smokers in 2016 (TSI, 2016). In the same year, 58,631 smokers and, 7,941 non-smokers died because of smoking-related causes (Institute of Health Metrics and Evaluation (IHME), 2016).

Nicotine exerts its abusive effects by stimulating neuronal nicotinic acetylcholine receptors (nAChR) and by participating in cholinergic system functions, which regulate emotion, cognition and rewarding effects. nAChRs are ligand-gated ion channels consisting of five subunits that modulate the release of neurotransmitters (Wen et al., 2016). Upregulation of nAChRs, particularly $\alpha_4\beta_2$ sub-type is important in the development of nicotine addiction (Miwa et al., 2011). Also, α_3 and α_5 auxiliary subunits play a role in the modulation of addiction (Fowler et al., 2011).

Fagerström Test for Nicotine Dependence (FTND) has been used for determining the severity of nicotine dependence (Fagerstrom and Schneider, 1989). According to ENSP Guidelines for Treating Tobacco Dependence, FTND scores between 0-3 are classified as low level of dependence, scores between 4-6 as medium and scores 7 or higher as high level of dependence. Pharmacotherapy is available and recommended for high and medium level nicotine-dependent smokers (ENSP, 2020). Among strategies for the treatment of nicotine addiction, pharmacotherapy and psychological counselling are the main ones and pharmacotherapy has been shown to be more effective than other interventions (Kalkhoran et al., 2018). Nicotine replacement therapy (NRT), bupropion (a serotonin-dopamine re-uptake inhibitor) and varenicline (an $\alpha_4\beta_2$ nAChR partial agonist) are the first-line therapeutics used for smoking cessation. Bupropion and NRT can be used together as a combination treatment (Cahill et al., 2013). Although there has been some advancement in the treatments, clinical success rates remain modest. Absolute smoking cessation rates have been reported to be between 5 and 35% depending on the strategy used (Benowitz, 2009).

It has been reported that success rates of cessation treatments may be altered by pharmacogenetic factors (Lerman et al., 2006; Benowitz, 2008; Bergen et al., 2013; Chenoweth and Tyndale, 2017). Therefore, pharmacogenetic optimization of cessation treatments may potentially improve smoking cessation rates (Chenoweth and Tyndale, 2017). Twin studies indicated that the heritability estimate of smoking cessation is around 50% (Xian et al., 2003; Lessov et al., 2004). Genetic factors, which are indicated in affecting nicotine dependence, include genetic variants of α_3, α_4 and α_5 subunits of nicotinic receptors, which are encoded by *CHRNA3, CHRNA4* and *CHRNA5* genes, respectively (Saccone et al., 2007; Chenoweth and Tyndale, 2017).

CHRNA3 rs578776 polymorphism was reported to be associated with change in nAChR functioning (Wang et al., 2009; Wen et al., 2016), and with nicotine dependence levels (Saccone et al., 2009a), while such associations could not be shown by some other studies (Hubacek et al., 2014; Tyndale et al., 2015). A study in the Chinese population indicated that *CHRNA4* rs1044396 and rs1044397 were associated with nicotine dependence, and *CHRNA4* rs1044396 with smoking initiation (Chu et al., 2011). Moreover, a Brazilian study found an association between *CHRNA4* rs1044396 variant and smoking cessation rates in subjects with varenicline therapy (Rocha Santos et al., 2015). Most of these results have not been replicated by other studies (Chenoweth and Tyndale, 2017). It was reported that *CHRNA5 rs16969968* polymorphism causes disruption of α5 nAChR signaling that resulted in the diminishing of stimulatory effects of nicotine (Fowler et al., 2011). Some clinical studies have identified polymorphic A allele of *CHRNA5 rs16969968* as a risk factor for high nicotine dependence in Caucasians (Tobacco and Genetics, 2010; Ware et al., 2011; Chenoweth and Tyndale, 2017). However, other clinical studies found no association between *CHRNA5* rs16969968 genetic polymorphism and success of smoking cessation treatments (Chen et al., 2012; Bergen et al., 2013; Tyndale et al., 2015).

We previously examined the effects of genetic polymorphisms of a few pharmacokinetic targets, namely metabolizing enzymes of CYP2A6, CYP2B6, and the drug transporter ABCB1 (MDR1) on smoking status and success of smoking cessation therapies in a similar but smaller cohort of Turkish subjects (Muderrisoglu et al., 2020). In the current study, within a population of Turkish subjects with an extended number of patients we aimed to investigate the effects of polymorphic variants of a few pharmacodynamics targets; namely human nicotinic acetylcholine receptor subunits α3, α4 and α5 on smoking status and the clinical outcomes of smoking cessation with pharmacotherapies.

MATERIALS AND METHODS

Subjects

This study was reviewed and approved by Hacettepe University Ethics Committee (GO-14/416-03). The patients and participants provided their written informed consent to participate in this study.

Participants were divided into two groups as smokers and non-smokers. Smokers (n = 143; 66 females, 77 males) were recruited from subjects who applied to the Smoking Cessation Clinic, Department of Chest Diseases, Hacettepe University for smoking cessation between August 2016 and November 2020. The control group of 130 volunteers (73 females, 57 males) were non-smokers. All participants were aged between 18 and 71. Exclusion criteria for the study were as follows: having a serious heart, liver or kidney disease, using or having an addiction history for products other than nicotine, having a severe anxiety disorder and being pregnant. Fagerström Test for Nicotine Dependence (FTND) was used to determine the severity of nicotine dependence in smokers. Exhaled CO levels were measured by using piCO Smokerlyzer (Bedfont Scientific Ltd., Kent, United Kingdom) to verify smoking status. Subjects with CO values higher than 4 ppm were interpreted as active smokers. Routine drug treatments according to the standard procedures

TABLE 1 | Genotyping methods for the *CHRNA3* rs578776, *CHRNA4* rs1044396- rs1044397 and *CHRNA5* rs16969968 polymorphisms.

Gene	Genetic polymorphism	PCR primers	PCR product size	Restriction enzyme	Restriction fragments (bp)	Allele
CHRNA3	rs578776	5′-TTCTTTACTGGGTCTAAAGGGCTATGCC-3′	146 bp	*NlaIII*	82 + 54+10	C
		5′-ATCCACCCAGTTTATGGTGTACTAAG-3′			92 + 54	T
CHRNA4	rs1044396	5′-CTTTGGTGCTGCGGGTCTT-3′	84 bp	*HinP1I*	57 + 27	G
		5′-AGCCCTCTCCGTGCAAATG-3′			84	A
CHRNA4	rs1044397	5′-GTCTGCAATGTACTGGACGC-3′	97 bp	*HinP1I*	69 + 28	C
		5′-CACGGTCAAGACCCGCAG-3′			97	T
CHRNA5	rs16969968	5′ -ATGAAGAAGTCATGTAGACAGGTACTTC-3	165 bp	*Tag$^{\alpha}$I*	97 + 68	G
		5′ -TACACATCACAGACCTCACGGACATC-3			165	A

bp, base pairs.

indicated by the ENSP Guidelines for Treating Tobacco Dependence were administered to smokers (ENSP, 2020). Non-smokers were comprised of individuals who never smoked in their lifetime.

Smokers were divided into 4 groups according to the drug treatment and they were prescribed according to the guidelines published by ENSP: NRT ($n = 40$), bupropion ($n = 47$), bupropion + NRT ($n = 15$), and varenicline users ($n = 41$). NRT group was comprised of both patch and gum users. On the 12th week of the drug treatment, smokers were contacted by phone and inquired whether they were able to quit smoking or not.

Genotyping

Whole Blood DNA Purification Kit (Thermo Fischer Scientific, Waltham, Massachusetts, USA) was used to extract DNA from venous blood. Genotyping was performed by a PCR-RFLP method for the *CHRNA3* rs578776, *CHRNA4* rs1044396-rs1044397 and *CHRNA5* rs16969968 polymorphisms. Previously described methods were modified for genotyping *CHRNA3* rs578776 and *CHRNA5* rs16969968 polymorphisms (Hubacek et al., 2014). New primers were designed for the *CHRNA4* polymorphisms. 200 μM of each dATP, dCTP, dGTP, dTTP, 2.5 mM $MgCl_2$, BSA, 12.5 pmol of each primer, 1 unit of Taq DNA Polymerase and 100 ng of genomic DNA with a volume of 25 μl used as PCR mixture (Solis BioDyne, Tartu, Estonia). Hot start PCR conditions were as follows; 95°C for 15 min following 30 cycles of 95°C for 20 s, 60°C for 30 s, 72°C for 1 min, and 72°C for 10 min. A thermal cycler (Bio-Rad T100 Thermal Cycler, Bio-Rad Laboratories, Taipei, Taiwan) was used to perform PCR cycles. PCR products were cut by restriction enzymes (New England Biolabs, Ipswich, Massachusetts, USA). Restriction products were separated by 2–3% agarose gel electrophoresis and UV light was used to visualize fragments (Kodak, Rochester, New York, USA). Details of genotyping methods are provided in **Table 1**.

Sample Size Calculation and Data Analysis

To predict approximate sample sizes for study groups, we used the Power and Sample Size Program software (Dupont and Plummer, 1990). We applied previously reported frequencies for the variant alleles of interest in Caucasians as 28.1% for the *CHRNA3* rs578776, 52.9% for the *CHRNA4* rs1044396-1044397 and 36.6% for the *CHRNA5* rs16969968 polymorphisms (Genomes Project et al., 2015). The estimated sample sizes were calculated while the type I error probability and the power were set to 0.05 and %80, respectively. With a proposed relative risk of 1.5 for allelic distributions between smokers *vs.* non-smokers and for a study of independent cases and controls with at least one control per case, calculated numbers for cases were 180 subjects for the rs578776 polymorphism, 49 subjects for the rs1044396 and the rs1044397, and 115 subjects for the rs16969968 polymorphisms. Sample sizes needed for each group appeared to be adequate with the estimated numbers for all, but one of the genetic variants examined.

Genotype, haplotype frequencies and gender groups were analyzed by using Chi-Square and Fischer's exact tests. For multiple comparisons Bonferroni's correction was applied. For estimation of *CHRNA* haplotype frequencies and their effects on smoking status and cessation rates, SNPStats internet resource was used as an *in silico* statistical tool (SNPStats, 2016). $p < 0.05$ was accepted as a statistical significance level. Statistical analyses were performed by using GraphPad Prism version 6.01 for Windows (GraphPad Software, La Jolla, California, USA).

TABLE 2 | Demographics and number of individuals in each group according to smoking status.

	Smokers n, (%) (Total $n = 143$)	Non-smokers n, (%) (Total $n = 130$)	*p*-values
Gender			
Male	77, (53.8)	57, (43.8)	0.10
Female	66, (46.2)	73, (56.2)	
Age	39.2 ± 1.1 (37-41.4)	41.6 ± 1.1 (39.4-43.8)	0.14
CO (ppm)	14.2 ± 0.8 (12.7-15.7)	N/A	N/A

Ages are shown as mean ± standard errors of means, S.E.M., and (95% Confidence Intervals).
N/A, not applicable.

RESULTS

Demographics, Smoking Status and Cessation Rates

Demographic variables are shown in **Table 2**. There were no statistically significant differences between smokers and non-smokers regarding the genders and ages. Thirty-two (41.6%) of male smokers and 20 (30.3%) of female smokers, and

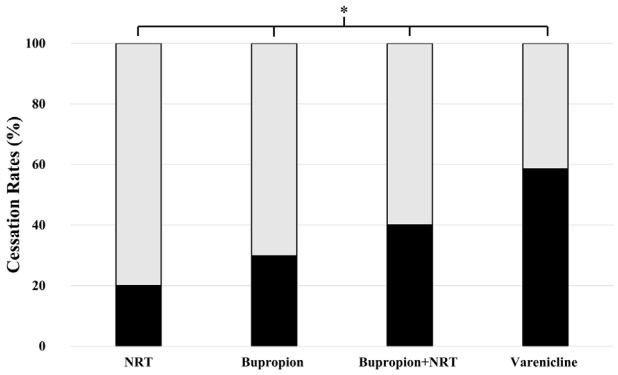

FIGURE 1 | Smoking cessation rates among drug-treatment groups. Cessation rates were as follows: 20.0% (95% CI= 7.6-35.2) for NRT users ($n = 40$), 29.8% (16.7-47.6) for bupropion users ($n = 47$), 40% (15.2-77.9) for bupropion + NRT users ($n = 15$) and 58.5% (43.5-88) for varenicline users ($n = 41$) (Chi-square test, $p = 0.001$; $\chi 2$, df = 16.2, 3) (■: Quitters, □: Non-Quitters).

overall 52 (36.4%) smokers were able to quit smoking after 12 weeks from the beginning of the drug treatment.

Smoking cessation rates were significantly different among four drug treatment groups. The success rates were 20.0% ($CI_{95\%}$ = 7.6-35.2) for NRT, 29.8% ($CI_{95\%}$ = 16.7-47.6) for bupropion, 40% ($CI_{95\%}$ = 15.2-77.9) for bupropion + NRT and 58.5% ($CI_{95\%}$ = 43.5-88) for varenicline users (**Figure 1**, $p = 0.001$). Post-hoc analysis with Bonferroni's correction revealed that varenicline treatment was significantly more successful than NRT ($p = 0.0002$) and bupropion treatments ($p = 0.003$).

Smoking Status and Variants of *CHRNA*

Overall, the frequencies for the examined variant alleles in our study population were 37.2% for the *CHRNA3* rs578776, 48.4% for the *CHRNA4* rs1044396, 46.7% for the *CHRNA4* rs1044397 and 40.8% for the *CHRNA5* rs16969968 polymorphisms. The distribution of all genetic variants examined was consistent with the Hardy–Weinberg equilibrium ($p > 0.05$). The genotype and allele distributions of the polymorphic alleles are summarized in **Table 3**. As shown, the distribution of the variant alleles and genotypes were similar for all polymorphisms between smokers and non-smokers (**Table 3**). Therefore, *CHRNA3, CHRNA4* and *CHRNA5* polymorphisms were not associated with smoking status.

Cessation Rates and Variants of *CHRNA*

The distribution of the wild-type and the variant alleles for the *CHRNA3* rs578776, *CHRNA4* rs1044396, *CHRNA4* rs1044397, *CHRNA5* rs16969968 genetic polymorphisms are presented in **Table 4**. The polymorphic variants were not found to be associated with the success of smoking cessation treatments. Likewise, *CHRNA* haplotypes were not associated with smoking cessation rates (**Table 5**).

DISCUSSION

In this study, we sought to investigate whether smoking status and clinical outcomes of pharmacological treatments with nicotine, bupropion or varenicline for smoking cessation were affected by genetic polymorphisms of several pharmacodynamic targets, namely, human nicotinic acetylcholine receptor subunits α3 (*CHRNA3*), α4 (*CHRNA4*) and α5 (*CHRNA5*) in a sample of subjects in Turkish population. For this aim, a total of 143 smokers and 130 non-smokers were examined.

A routine drug treatment according to the standard procedures as indicated by the ENSP Guidelines for Treating

TABLE 3 | Genotype and allele frequencies for the *CHRNA3* rs578776, *CHRNA4* rs1044396, *CHRNA4* rs1044397 and *CHRNA5* rs16969968 polymorphisms in smokers *vs*. non-smokers.

Gene	Genetic polymorphism	Genotypes and alleles	Smokers: n, (%)	Non-smokers: n, (%)	*p*-value ($\chi 2$, df)
CHRNA3	rs578776	CC	58, (40.6)	51, (39.2)	0.97 (0.0563, 2)
		CT	65, (45.5)	60, (46.2)	
		TT	20, (14)	19, (14.6)	
		C	181, (63.3)	162, (62.3)	0.81 (0.0559, 1)
		T	105, (36.7)	98, (37.7)	
CHRNA4	rs1044396	GG	38, (26.6)	46, (35.4)	0.07 (5.33, 2)
		GA	69, (48.3)	45, (34.6)	
		AA	36, (25.2)	39, (30)	
		G	145, (50.7)	137, (52.7)	0.64 (0.217, 1)
		A	141, (49.3)	123, (47.3)	
CHRNA4	rs1044397	CC	39, (27.3)	46, (35.4)	0.23 (2.96, 2)
		CT	70, (49)	51, (39.2)	
		TT	34, (23.8)	33, (25.4)	
		C	148, (51.7)	143, (55)	0.45 (0.579, 1)
		T	138, (48.3)	117, (45)	
CHRNA5	rs16969968	GG	47, (32.9)	44, (33.8)	0.87 (0.268, 2)
		GA	73, (51)	68, (52.3)	
		AA	23, (16.1)	18, (13.8)	
		G	167, (58.4)	156, (60)	0.70 (0.146, 1)
		A	119, (41.6)	104, (40)	

TABLE 4 | Frequencies of genetic variants between quitters and non-quitters.

Gene	Genetic polymorphism	Allele	Quitters; n, (%)	Non-quitters; n, (%)	*p*-value (χ2, df)
CHRNA3	rs578776	C	68, (65.4)	113, (62.1)	0.58 (0.31,1)
		T	36, (34.6)	69, (37.9)	
CHRNA4	rs1044396	G	50, (48.1)	95, (52.2)	0.50 (0.45, 1)
		A	54, (51.9)	87, (47.8)	
CHRNA4	rs1044397	C	52, (50.0)	96, (52.7)	0.65 (0.2, 1)
		T	52, (50.0)	86, (47.3)	
CHRNA5	rs16969968	G	62, (59.6)	105, (57.7)	0.75 (0.101, 1)
		A	42, (40.4)	77, (42.3)	

TABLE 5 | Estimated *CHRNA* haplotype frequencies among quitters and non-quitters and their association for quitting smoking.

CHRNA3 rs578776	CHRNA4 rs1044396	CHRNA4 rs1044397	CHRNA5 rs16969968	Frequency in quitters	Frequency in non-quitters	Odds ratio (95% CI)	*p*-value
C	A	T	A	0.1869	0.2054	1	—
C	G	C	A	0.1623	0.1884	1.33 (0.43–4.12)	0.62
T	G	C	G	0.1832	0.1827	1.2 (0.47–3.06)	0.71
T	A	T	G	0.0877	0.1616	1.57 (0.42–5.9)	0.51
C	G	C	G	0.1147	0.1419	1.15 (0.41–3.19)	0.79
C	A	T	G	0.1605	0.0853	0.64 (0.21–1.91)	0.42
T	A	T	A	0.0443	0.0203	1.17 (0.06–22.3)	0.92
Rare Haplotypes				0.0604	0.0144	0.19 (0.03–1.19)	0.08

Overall Haplotype Association p *Value: 0.28.*

Tobacco Dependence and according to subjects' FTND scores was administered for each individual (ENSP, 2020). We found out that varenicline treatment was significantly more effective than NRT, bupropion treatment and bupropion + NRT (**Figure 1**). Historically, the success rate for smoking cessation has been found significantly higher in subjects who were taking any kind of pharmacotherapies, i.e., with nicotine, bupropion or varenicline, as compared to patients with no drug treatment. However, even with pharmacotherapy applications, cessation rates remain modest because of the tenacious nature of nicotine addiction. Frequent relapse of nicotine addiction has also been attributed to the strong character of nicotine dependence (Benowitz, 2009; Kalkhoran et al., 2018). Absolute abstinence should be the goal since it has been reported that even smoking one single cigarette per day increases the risk of cardiovascular diseases and stroke (Bonnie RJ and Kwan, 2015; Hackshaw et al., 2018).

Our findings for drug efficacy were in agreement with previous studies, as bupropion and varenicline were found more effective than NRT alone (Stead et al., 2012; Cahill et al., 2013). Particularly, the findings in the EAGLES trial, which reported that a 6-months abstinence rate was highest among varenicline users, support our finding of superior efficacy with varenicline (Anthenelli et al., 2016). A meta-analysis study reported that varenicline treatment has been shown to improve the chance of quitting more than other therapies (Cahill et al., 2013). Our findings support the view that bupropion or varenicline treatments should be preferred over treatment with NRT-alone in smoking cessation.

Few previous studies reported associations between genetically polymorphic variants of pharmacokinetic and pharmacodynamic targets. Such studies imply that pharmacogenomic data may facilitate the optimization of drug treatments for smoking cessation (Chenoweth and Tyndale, 2017). We previously studied the effects of genetic polymorphisms of a few pharmacokinetic targets, mainly metabolizing enzymes of CYP2A6, CYP2B6, and the drug transporter ABCB1 (MDR1) on smoking status and success of smoking cessation therapies. In that study, we reported that genetic variants of the drug transporter ABCB1 and a particular haplotype (*1236TT-2677TT-3435TT*) were significantly associated with non-smoking status, while no other associations with genetic variants of ABCB1 or CYP2A6, CYP2B6 with nicotine addiction was found (Muderrisoglu et al., 2020). Therefore, in the current study we aimed to investigate whether the smoking status or the clinical outcomes of pharmacological treatments for smoking cessation might be affected by genetic polymorphisms of pharmacodynamic targets of human nicotinic acetylcholine receptor subunits α3 (*CHRNA3*), α4 (*CHRNA4*) and α5 (*CHRNA5*) subunits.

The SNPs examined were selected on the basis of their functional effects on receptor expression and functioning (as explained in **Table 6**) and their frequent occurrence among Caucasians. In previous studies, frequencies for the variant alleles in Caucasians were found to be 28.1% for the *CHRNA3* rs578776, 52.9% for the *CHRNA4* rs1044396, 52.9% for the *CHRNA4* rs1044397 and 36.6% for the *CHRNA5* rs16969968 polymorphisms (Genomes Project et al., 2015). We did not find any association of *CHRNA* polymorphisms with either smoking status or clinical success of pharmacotherapies for smoking cessation (**Tables 3**, **4** and **5**).

TABLE 6 | Summary of the polymorphic variants examined and their association with previously reported functional consequences.

Gene	SNP	Location	Variant Type	Functional Consequences
CHRNA3	rs578776	chr15:78596058, intron 1 of *CHRNA3*	3′ Prime UTR Variant	No amino acid substitution; a tag SNP associated with the change in mRNA expression of *CHRNA5* (Wang et al., 2009; Wen et al., 2016)
CHRNA4	rs1044396	chr20:63349782, exon 5 of *CHRNA4*	Missense Variant	Amino acid substitution from serine to arginine at position 543 of the α4 subunit of nAChR; alteration of α4β2 nAChR sensitivity (Li et al., 2005; Winterer, 2011)
CHRNA4	rs1044397	chr20:63349752, exon 5 of *CHRNA4*	Synonymous Variant	No amino acid substitution; change of electrophysiological properties of the nicotinic receptor subtype α4β2 (Winterer, 2011; Mobascher et al., 2016)
CHRNA5	rs16969968	chr15:78590583, exon 5 of *CHRNA5*	Missense Variant	Amino acid substitution from aspartate to asparagine at position 398 of the α5 subunit of nAChR; a greater influx of calcium ions and disruption of α5 nAChR signaling (Fowler et al., 2011)

CHRNA3 is responsible for coding the α3 auxiliary subunit of nAChRs. This subunit regulates the function of nAChRs (Miwa et al., 2011). rs578776 is located 3′-UTR region of the *CHRNA3* gene and found to be associated with mRNA expression of *CHRNA5* (Wen et al., 2016). Some of the previous similar studies reported associations between *CHRNA3* rs578776 polymorphism and nicotine dependence in Caucasians (Saccone et al., 2009b; Tyndale et al., 2015; Hubacek et al., 2017). These studies also reported that the treatment outcomes for smoking cessation were not affected by *CHRNA3* rs578776 polymorphism. In contrast, a Chinese study reported the C allele of *CHRNA3* rs578776 polymorphism was significantly related to smoking cessation (Wang et al., 2016). Another study by Chaity et al. (2021) reported that *CHRNA3* rs578776 polymorphism was associated with smoking status in a Bengali population (Chaity et al., 2021). However, unlike the Caucasians, no association between *CHRNA3* rs578776 polymorphism and nicotine dependence was detected in Africans (Saccone et al., 2009b). Our results of the current study in a Caucasian Turkish population yielded a lack of association between *CHRNA3* rs578776 polymorphism and smoking status or success of smoking cessation.

It was reported that the majority of the high-affinity nicotine-binding sites were present on the $\alpha_4\beta_2$ sub-type of nAChRs in the brain (Flores et al., 1992; Lindstrom, 1997). A previous study found that activation of the α_4 subunit of nAChRs is sufficient for the development of reward, tolerance and sensitization mediated by nicotine (Tapper et al., 2004). It has been suggested that genetic polymorphisms of *CHRNA4* exert functional consequences by altering receptor function or mRNA expression (Li et al., 2005). rs1044396 causes an amino acid substitution from serine to arginine at position 543 of the α4 subunit of nAChR (Li et al., 2005). rs1044397 is a synonymous variant and causes no amino acid substitution; however, it has been linked with a change of electrophysiological properties of the nicotinic receptor subtype α4β2 (Mobascher et al., 2016). A previous study measured electrophysiological properties of the nicotinic receptor subtype $\alpha_4\beta_2$ in animals carrying the combined haplotype for both of the C*HRNA4* rs1044396 and rs1044397 variants and reported that this haplotype altered $\alpha_4\beta_2$ nAChR sensitivity (Winterer et al., 2011). An association between the variant (A) allele of *CHRNA4* rs1044396 and psychiatric illnesses such as depression, anxiety disorders and certain other addictions was reported (Markett et al., 2011; Tsai et al., 2012; Jeong et al., 2017). As widely known, most smokers report alleviation of their anxiety with smoking (Brandt, 2007) and nicotine has cognitive enhancer activity (Davis and Gould, 2009). Therefore, it may be conceivable that *CHRNA4* polymorphisms may alter smoking behavior *via* altering cholinergic system functions related to mood change and cognition. Recently, Gu et al. (2020) found that homozygous AA genotype carriers of *CHRNA4* rs1044396 polymorphism were more likely to quit smoking after the diagnosis of lung cancer in their cohort of Chinese patients (Gu et al., 2020). Also, there are studies in the literature that suggested associations of *CHRNA4* rs1044396 with outcomes of bupropion and varenicline treatments (Feng et al., 2004; King et al., 2012; Rocha Santos et al., 2015). In contrast, the associations could not be found by some other studies (Chenoweth and Tyndale, 2017). Our results confirm the lack of association between *CHRNA4* rs1044396 or *CHRNA4* rs1044397 polymorphisms with smoking status or quitting rates in cessation interventions in Caucasians.

CHRNA5 rs16969968 polymorphism causes amino acid substitution from aspartate to asparagine at position 398 of the α5 subunit of nAChR protein sequence and a previous study on knockout mice reported that the amino acid change resulted in a greater influx of calcium ions and disruption of α5 nAChR signaling (Fowler et al., 2011). Their findings suggest that as a result of α5 nAChR signaling disruption, stimulatory effects of nicotine diminishes on the medial habenulo-interpeduncular nucleus pathway. Thereby, greater quantities of nicotine are needed to gain stimulatory effects. (Fowler et al., 2011). In line with this finding, clinical studies identified polymorphic A allele of *CHRNA5* rs16969968 as a risk factor for higher nicotine dependence in Caucasians (Tobacco and Genetics, 2010; Ware et al., 2011; Chenoweth and Tyndale, 2017). Moreover, a meta-analysis study by Chen et al. (2015) examined associations between rs16969968 and age of quitting smoking, age of lung cancer diagnosis in 24 studies of European ancestry. They reported that the rs16969968 variant allele (A) was associated with a lower likelihood of smoking cessation and the AA genotype was associated with a 4-year delay in the median age of quitting compared with the GG genotype, and homozygous variant AA genotype carriers for the *CHRNA5* rs16969968 polymorphism had a 4-years earlier age of diagnosis of lung cancer due to smoking (Chen et al., 2015). A recent study by Hopkins et al. (2021) demonstrated that *CHRNA5* rs16969968 AA genotype independently associated with smoking exposure

and its main complications i.e., chronic obstructive pulmonary disease and lung cancer (Hopkins et al., 2021). In contrast, in African-Americans, an association between *CHRNA5* rs16969968 polymorphism and smoking behavior was not detected (Zhu et al., 2014). Our results in a different Caucasian population of Turkish subjects yielded no association between *CHRNA5* rs16969968 and smoking or cessation status. This discrepancy may be explained by relatively small sizes of samples in the studies, as well as differences in ethnicities. Indeed, Tyndale et al. (2015) and Chenoweth and Tyndale (2017), suggested that *CHRNA5* rs16969968 polymorphism did not affect the success of pharmacotherapies with smoking cessation (Tyndale et al., 2015; Chenoweth and Tyndale, 2017). Also, Breitling et al. (2009) indicated that neither *CHRNA3* rs578776 nor *CHRNA5* rs16969968 was associated with smoking cessation (Breitling et al., 2009). Also, a recent study by Hubacek et al. (2021) demonstrated no association of polymorphisms of *CHRNA3* rs578776 and *CHRNA5* rs16969968 between nicotine dependence, treatment success and nicotine metabolite concentrations (Hubacek et al., 2021). Their findings in Caucasian populations are in agreement with our results in our cohort of Turkish population that suggested lack of association of *CHRNA5* rs16969968 with smoking cessation treatments outcomes.

A limitation of our study is that our study was conducted in a relatively small size of population sample with 143 smokers and 130 non-smoker control subjects. While our finding of superior clinical efficacy of varenicline treatment in smoking cessation has consistently been reported also by other studies in literature (see Cahill et al., 2013; Anthenelli et al., 2016), larger sample sizes in further studies would probably yield clearer results for comparison of effectiveness of drug molecules used for this purpose. Also, studies with larger sample sizes may provide more conclusive results for addressing influence of *CHRNA* variants on smoking status and success of cessation. Another limitation is that we could follow up smokers only by phone-calls at the 12th week, but not afterwards and with a way of communication that could have provided a face-to-face interaction with subjects. It is well-known that smoking cessation rates would decrease over time.

In conclusion, in the present study we report that varenicline treatment was significantly more effective when compared to bupropion or nicotine replacement treatments in smoking cessation. Our results yielded informative, yet inconclusive results for association between genetic polymorphisms of α3, α4 and α5 subunits of nicotinic acetylcholine receptors with either smoking status or clinical outcomes of pharmacotherapies for smoking cessation. Nevertheless, we believe that the present study may be helpful in understanding the nature of smoking habit and the association of smoking cessation with pharmacogenomics of nicotinic acetylcholine receptor subunits.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the Mendeley data repository, accession number: https://doi.org/10.17632/pjf5rdcdf9.1.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hacettepe University Ethics Committee for Human Studies (GO-14/416-03). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AM, EB, and MOB contributed to the conception and design of the study; EB, AM, SE, and ETK recruited the subjects; AM, EB, MOB, SK, and ETK contributed to the acquisition of the data; AM, EB, MOB, SE, and EK contributed to the analysis of data. All authors contributed to the manuscript preparation.

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BRIEF RESEARCH REPORT
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560G>A (rs4986782) (R187Q) Single Nucleotide Polymorphism in Arylamine N-Acetyltransferase 1 Increases Affinity for the Aromatic Amine Carcinogens 4-Aminobiphenyl and N-Hydroxy-4-Aminobiphenyl: Implications for Cancer Risk Assessment

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Human arylamine N-acetyltransferase 1 (NAT1) catalyzes the N-acetylation of arylamine carcinogens such as 4-aminobiphenyl (ABP), and following N-hydroxylation, the O-acetylation of N-hydroxy-arylamine carcinogens such as N-hydroxy-ABP (N-OH-ABP). Genetic polymorphisms in NAT1 are linked to cancer susceptibility following exposures. The effects of individual single nucleotide polymorphisms (SNPs) in the NAT1 coding exon on Michaelis-Menten kinetic constants was assessed for ABP N-acetyltransferase and N-OH-ABP O-acetyltransferase activity following transfection of human NAT1 into COS-1 cells (SV40-transformed African green monkey kidney cells). NAT1 coding region SNPs 97C > T (rs56318881) (R33stop), 190C > T (rs56379106) (R64W), 559C > T (rs5030839) (R187stop) and 752A > T (rs56172717) (D251V) reduced ABP N- acetyltransferase and N-OH-ABP O-acetyltransferase activity below detection. 21T > G (rs4986992) (synonymous), 402T > C (rs146727732) (synonymous), 445G > A (rs4987076) (V149I), 613A > G (rs72554609) (M205V) and 640T > G (rs4986783) (S241A) did not significantly affect Vmax for ABP N-acetyltransferase or N-OH-ABP O-acetyltransferase. 781G > A (rs72554610) (E261K), and 787A > G (rs72554611) (I263V) slightly reduced ABP N-acetyltransferase and N-OH-ABP O-acetyltransferase activities whereas 560G > A (rs4986782) (R187Q) substantially and significantly reduced them. 560G > A (rs4986782) (R187Q) significantly reduced the apparent Km for ABP and N-OH-ABP a finding that was not observed with any of the other NAT1 SNPs tested. These findings suggest that the role of the 560G > A (rs4986782) (R187Q) SNP cancer risk assessment may be modified by exposure level to aromatic amine carcinogens such as ABP.

Keywords: arylamine N-acetyltransferase 1, single nucelotide polymorphisms, 4-aminobiphenyl, N-hydroxy-4-aminobiphenyl, N-acetylation, O-acetylation

INTRODUCTION

Human occupational exposures to 4-aminobiphenyl (ABP) led to excess incidence of urinary bladder cancer with sufficient evidence for listing as a Group 1 carcinogen (IARC, 1987). ABP also is carcinogenic in animal models including liver, intestine, mammary gland, and angiosarcoma (NTP, 2021). Subsequent human epidemiological investigations have focused on the elevated cancer incidence in cigarette smokers, particularly in the urinary bladder (NTP, 2021). In addition to both mainstream and sidestream cigarette smoke, ABP is a byproduct in the synthesis of numerous chemicals and color additives (NTP, 2021). DNA adducts following exposure to ABP serve as a biomarker of internal exposure and possibly carcinogenesis in human tissues (NTP, 2021).

Arylamine carcinogens such as ABP are *N*-hydroxylated by cytochrome P450 to N-hydroxy-ABP (N-OH-ABP) followed by O-acetylation catalyzed by arylamine N-acetyltransferase 1 (NAT1) and 2 (NAT2) to N-acetoxy-ABP which is highly unstable resulting in DNA adducts. DNA adducts not repaired can result in mutations leading to cancer (Wang et al., 2019; NTP, 2021). NAT1 catalyzes ABP N-acetyltransferase and N-OH-ABP O-acetyltransferase activities (Hein et al., 1993; Millner et al., 2012; Zhou et al., 2013; Leggett et al., 2021; Leggett et al., 2022; Hein et al., 2022).

Many arylamines and alkylanilines undergo N- and O-acetylation catalyzed by NAT1 (Minchin et al., 1992; Hein et al., 1993; Zenser et al., 1996; Liu et al., 2007; Leggett et al., 2021). R127 and Y129 amino acids in NAT1 reduce the volume of the NAT1 binding pocket by ~40% compared to NAT2 (Wu et al., 2007; Zhou et al., 2013). NAT1 is selective for acetylation of 4-alkylanilines due to binding to V216, which is replaced by S216 in NAT2 (Liu et al., 2007). NAT1 exhibits higher affinity than NAT2 for the O-acetylation of N-hydroxy-arylamine carcinogens such as N-OH-ABP (Hein et al., 2022).

In addition to its role in the metabolism of exogenous drugs and xenobiotics, NAT1 has additional roles in metabolism of endogenous compounds including the hydrolysis of AcCoA (Laurieri et al., 2014; Stepp et al., 2015; Stepp et al., 2019). Further studies suggest a role for NAT1 in cellular metabolism of endogenous amines (Carlisle et al., 2020).

Human NAT1 has been measured in virtually all human organs and exhibits genetic polymorphism in human populations. Associations between NAT1 genetic polymorphism with cancer risk has been reviewed (Hein et al., 2000; Agundez 2008; Butcher and Minchin, 2012; Zhang et al., 2015). Most of these focus on associations between *NAT1*10* and/or *NAT1*11* and cancer risk, two variant alleles that possess SNPs in the 3′-UTR region outside of the coding exon. Urinary bladder cancer is most frequently associated with NAT1 genetic polymorphism (Cascorbi et al., 2001; Yassine et al., 2012; Dhaini et al., 2018; El-Kawak et al., 2020).

*NAT1*4* is the reference allele. Numerous single nucleotide polymorphisms (SNPs) are present in the NAT1 coding exon. As previously reviewed (Hein, 2009), the identity of these SNPs and their functional effects have been investigated following recombinant expression of *NAT1*4* and NAT1 variant alleles in bacteria, yeast, Chinese hamster ovary, and COS-1 cells and have focused primarily on the N-acetylation of a prototype NAT1 substrates *p*-aminosalicyclic acid and *p*-aminobenzoic acid. The investigations have identified several SNPs in the NAT1 coding exon which reduce expression of NAT1 catalytic activity and protein, and one SNP that reduced substrate affinity. The objective of the current study is to investigate functional actions of the NAT1 coding exon SNPs for ABP N-acetyltransferase and the N-OH-ABP O-acetyltransferase and to interpret these results in the context of cancer risk following ABP exposures.

METHODS

Expression of NAT1 Reference and NAT1 Alleles Possessing Individual SNPs

The human NAT1 coding exon was amplified by polymerase chain reaction (PCR) using plasmid pESP-3 containing *NAT1*4* and other NAT1 SNPs as previously described (Zhu and Hein, 2008). COS-1 cells (SV40-transformed African green monkey kidney cells) were purchased from American Type Culture Collection (Manassas, VA United States) and cultured as previously described (Zhu and Hein, 2008). As described more fully previously (Zhu and Hein, 2008), constructed plasmids were transiently transfected using Lipofectamine and Plus reagent, 10 μg of either *NAT1*4* (no SNP), a mock empty vector, or NAT1 possessing a single SNP containing vector pCR3.1 and 80 ng pCMV SPORT-βgal (Invitrogen) plasmid. Transfected cells were harvested after 48 h and then removed, washed, and pelleted by centrifugation. Cell pellets were disrupted in lysis buffer (20 mM sodium phosphate, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 10 μM phenylmethanesulfonyl fluoride, 10 μM leupetin, 1 μM pepstatin A, 4 μM aprotinin) by sonication on ice. Cell debris was precipitated at 15,000 X g for 15 min at 4°C. No NAT1 activity was detectable in mock-transfected cells. To account for possible differences in transfection efficiency, COS-1 cell lysates were assayed for β-galactosidase activity with O-nitrophenyl β-D-galactopyranoside (Zhu and Hein, 2008).

ABP N-Acetyltransferase Assays

ABP N-acetyltransferase reactions were conducted as previously described (Hein et al., 2006). Briefly, 50 μL cell lysate (buffer composition described above), ABP (0–1 mM) and acetyl coenzyme A (1 mM) were incubated at 37°C for 10 min. HPLC separation of ABP and N-acetyl-ABP was achieved using a gradient of 85:15 sodium perchlorate pH 2.5: acetonitrile to 35:65 sodium perchlorate pH 2.5: acetonitrile over 10 min and was quantitated by absorbance at 260 nm. Mock transfected COS-1 cells were used as a negative control. Transiently transfected cells were normalized to total protein and β-galactosidase activity was used to normalize for transfection efficiency as previously described (Zhu and Hein, 2008). Protein concentrations were determined with the Bio-Rad protein assay (Bradford, 1976).

N-OH-ABP O-Acetyltransferase Assays

N-OH-ABP O-acetyltransferase assays were conducted as previously described (Hein et al., 2006). Briefly assays containing 100 μg cell lysate in buffer described above), 1 mM acetyl coenzyme A, 1 mg/ml deoxyguanosine (dG), and 0–1 mM N-OH-ABP were incubated at 37°C for 10 min. HPLC separation was achieved using a gradient of 80:20 sodium perchlorate pH 2.5: acetonitrile to 50:50 sodium perchlorate pH 2.5: acetonitrile over 3 min and dG-C8-ABP adduct was detected at 300 nm. Transiently transfected cells were normalized to total protein and β-galactosidase activity was used to normalize for transfection efficiency as previously described (Zhu and Hein, 2008). Protein concentrations were determined with the Bio-Rad protein assay (Bradford, 1976).

Data Analysis

ABP N-acetyltransferase and N-OH-ABP O-acetyltransferases activities carried out at various substrate concentrations were subjected to Michaelis-Menten kinetic analysis and the apparent Km and Vmax were calculated using the Michaelis-Menten program in Graphpad Prism (San Diego, CA). Differences in apparent Km and Vmax between reference allele *NAT1*4* and SNP variants were tested for significance using one-way ANOVA followed the Dunnett multiple comparison tests.

RESULTS

The effects of individual single nucleotide polymorphisms (SNPs) in the NAT1 coding exon on Michaelis-Menten kinetic constants was assessed for ABP N-acetyltransferase and N-OH-ABP O-acetyltransferase catalyzed by recombinant human NAT1 expressed in COS-1 cells. As shown in **Figure 1**, NAT1 coding region SNPs 97C > T (rs56318881) (R33stop), 190C > T (rs56379106) (R64W), 559C > T (rs5030839) (R187stop) and 752A > T (rs56172717) (D251V) reduced ABP N-acetyltransferase and N-OH-ABP O-acetyltransferase activity below detection. 21T > G (rs4986992) (synonymous), 402T > C (rs146727732) (synonymous), 445G > A (rs4987076) (V149I), 613A > G (rs72554609) (M205V) and 640T > G (rs4986783) (S241A) did not significantly affect Vmax for ABP N-acetyltransferase or N-OH-ABP O-acetyltransferase. 781G > A (rs72554610) (E261K), and 787A > G (rs72554611) (I263V) slightly decreased ABP N-acetyltransferase and N-OH-ABP O-acetyltransferase whereas 560G > A (rs4986782) (R187Q) substantially and significantly reduced them.

As shown in **Figure 2**, 560G > A (rs4986782) (R187Q) significantly reduced the apparent Km for ABP and N-OH-ABP a finding that was not observed with any of the other NAT1 SNPs tested. None of the other NAT1 SNPs significantly ($p > 0.05$) affected apparent Km towards ABP or N-OH-ABP.

DISCUSSION

The frequency of the various NAT1 SNPs varies with ethnicity (https://www.ncbi.nlm.nih.gov/snp/rs4986782?vertical_tab=true#frequency_tab). 560G > A (rs4986782) (R187Q) is the most frequent SNP in the NAT1 coding exon with a global frequency of 1.75% but a 13-fold higher frequency (23.8%) has been reported in Lebanese resulting in nearly 50% of a Lebanese population heterozygous for the allele (Dhaini and Levy, 2000).

The effects of SNPs in the human NAT1 coding exon on N-acetyltransferase and O-acetyltransferase activities have previously been reported following recombinant expression in bacteria (Doll et al., 1997; Butcher et al., 1998; Hughes et al., 1998; Payton and Sim, 1998), yeast (Fretland et al., 2001; Fretland et al., 2002) COS-1 cells (Zhu and Hein, 2008; Zhu et al., 2011) and/or CHO cells (Millner et al., 2012). Our findings that NAT1 coding region SNPs 97C > T (rs56318881) (R33stop), 190C > T (rs56379106) (R64W), 559C > T (rs5030839) (R187stop) and 752A > T (rs56172717) (D251V) reduced ABP N- acetyltransferase and N-OH-ABP O-acetyltransferase activities below detection is consistent with previous reports on *p*-aminobenzoic acid N-acetyltransferase activity, NAT1-selective substrate, in the expression systems described above. 781G > A (rs72554610) (E261K), and 787A > G (rs72554611) (I263V) caused slight but significant reduction in ABP N-acetyltransferase and of N-OH-ABP O-acetyltransferase activities that likely is relatively unimportant in risk assessment. Previous studies following recombinant expression of NAT1 in yeast reported that 781G > A (rs72554610) (E261K) caused slight reductions in N-acetylation of the arylamine carcinogens 2-aminofluorene (Fretland et al., 2001) and ABP (Fretland et al., 2002) and the O-acetylation of N-hydroxy-2-aminofluorene (Fretland et al., 2002).

560G > A (rs4986782) (R187Q) was identified as a reduced N-acetylation phenotype based upon its effects on the N-acetylation of *p*-aminosalicylic acid in human subjects (Hughes et al., 1998). In the present study 560G > A (rs4986782) (R187Q) substantially reduced both ABP N-acetyltransferase and N-OH-ABP O-acetyltransferase, consistent with the identification of *NAT1*14A* and *NAT1*14B* as reduced function alleles. As previously reviewed (Hein, 2009), the R187 is partially exposed to the protein surface and the active site and forms hydrogen bonds with E182, K188, and T289. These interactions help shape the active site pocket and R187Q may cause partial loss of these interactions leading to destabilization of NAT1 consistent with reduced expression of human NAT1 protein following recombinant expression in yeast (Fretland et al., 2001) and COS-1 cells (Zhu and Hein, 2008). R187Q also may cause changes in the conformation and/or dynamics of the active site or protein structure and thus alter substrate selectivity and catalytic activity.

Previous studies reported that the G560A(R187Q) SNP caused a 6–10-fold *decrease* in the affinity of human recombinant NAT1 for *p*-aminobenzoic acid (Zhu and Hein, 2008; Millner et al., 2012). Following administration of *p*-aminosalicylic acid, individuals with the *NAT1*14A* allele possessing 560G > A (rs4986782) (R187Q) exhibit higher maximum concentrations and overall area under the plasma level versus time curve drug

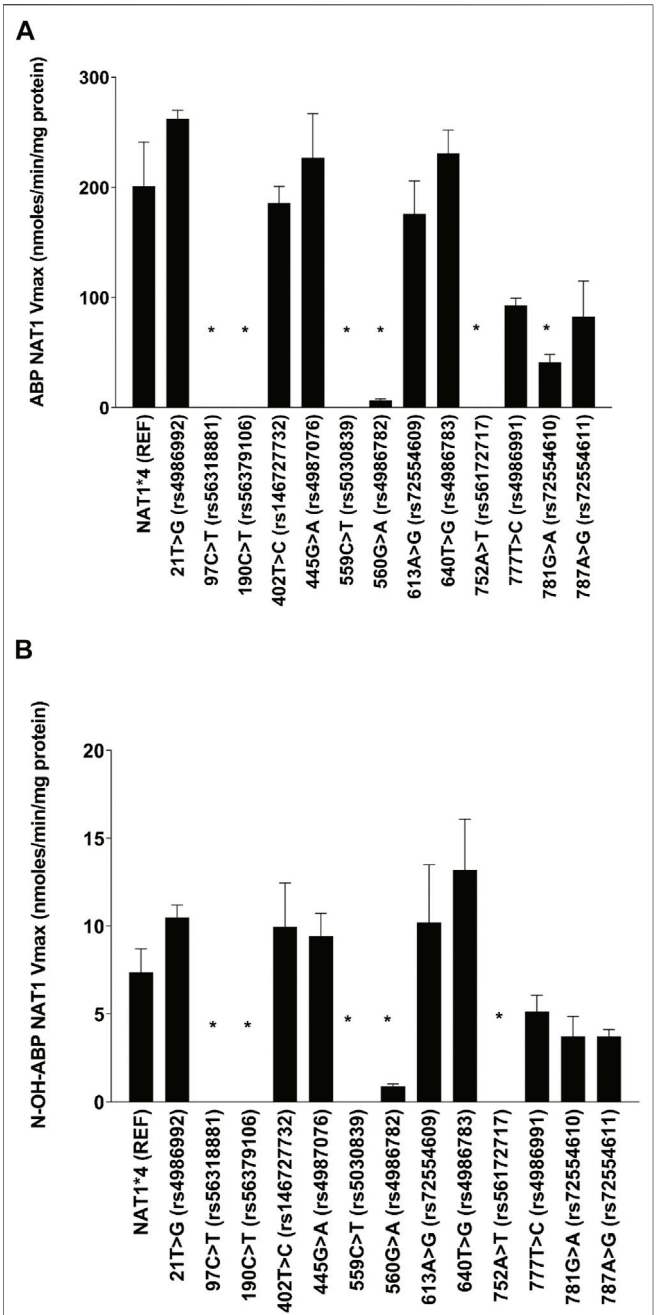

FIGURE 1 | Apparent Vmax for **(A)** ABP N-acetylation and **(B)** N-OH-ABP O-acetylation for NAT1*4 (reference) and *NAT1* variants possessing the SNP labeled on the abscissa. Each bar represents Mean ± SEM from three transfections in duplicate. *Significantly less than *NAT1*4* for ABP-N-acetylation ($p \leq 0.001$) and N-hydroxy-ABP O-acetylation ($p < 0.05$).

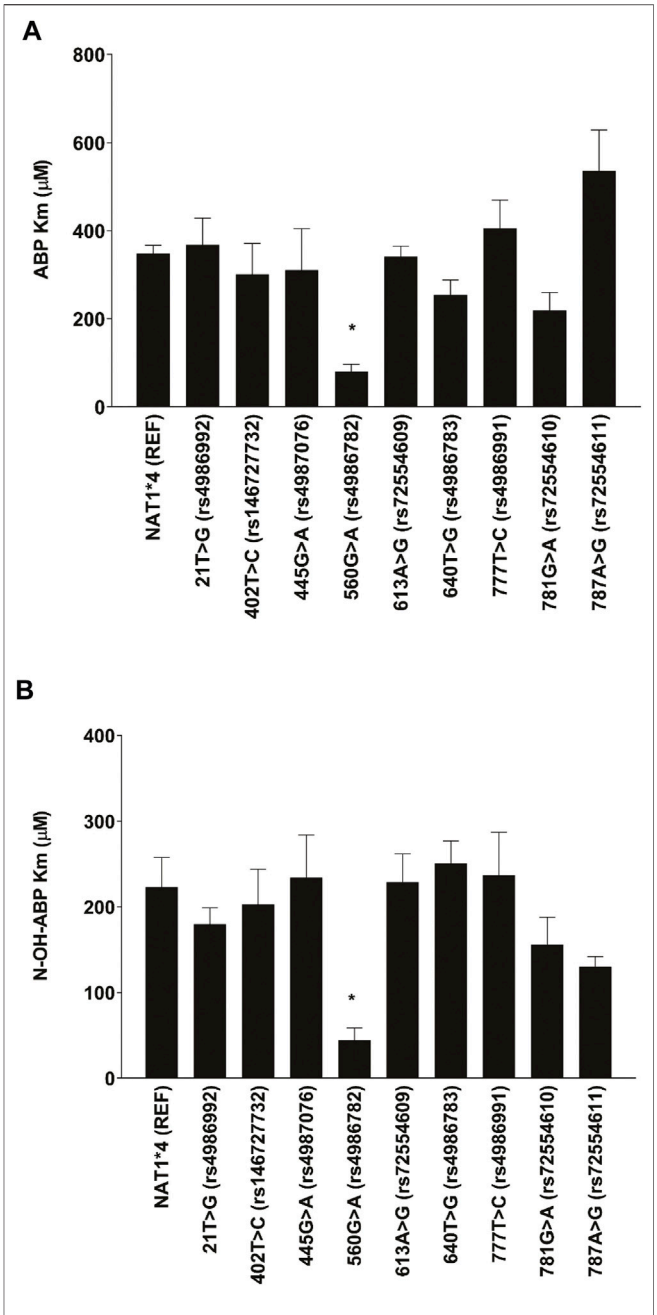

FIGURE 2 | Apparent Km for **(A)** ABP and **(B)** N-OH-ABP for *NAT1*4* (reference) and *NAT1* variants possessing the SNP labeled on the abscissa. Each bar represents Mean ± SEM from three transfections in duplicate. *Significantly less than *NAT1*4* for ABP ($p < 0.05$) and N-OH-ABP ($p < 0.01$).

levels irrespective of dosing regimen (Sy et al., 2015), consistent with *NAT1*14*s designation as a reduced function allele (Hughes et al., 1998; Fretland et al., 2001; Fretland et al., 2002; Zhu and Hein, 2008; Hein et al., 2018).

NAT1 is a major pathway in the urinary bladder mucosa for the bioactivation of urinary N-hydroxy arylamines to reactive N-acetoxy esters that form DNA adducts (Badawi et al., 1995). The frequency of 560G > A (rs4986782) (R187Q) varies with ethnic origin but it was identified in almost half of a Lebanese population (Dhaini and Levy, 2000). Notably, the incidence of urinary bladder cancer has increased markedly in Lebanon and is currently the second most incident cancer among males (Shamseddine et al., 2004). The frequency of 560G > A (rs4986782) (R187Q) is 7-fold higher among urinary bladder cases than controls in Lebanon (Yassine et al., 2012) and is

significantly associated with higher muscle-invasiveness and higher tumor grade of urinary bladder tumors (El Kawak et al., 2020). In the current study 560G > A (rs4986782) (R187Q) caused a 4 to 5-fold *increase* ($p < 0.05$) in affinity for both ABP and N-OH-ABP. None of the SNPs in the NAT1 coding exon affect V93, K100, I106, F125, L209, S215, V216 or F217 residues in the active site that may be important for ABP access (Zhou et al., 2013). The increase in affinity for ABP and N-OH-ABP is consistent with an increased risk for urinary bladder cancer following lower levels of exposure. In contrast, higher activity NAT1 alleles such as *NAT1*10* (Hein et al., 2018) are associated with lower prevalence of urinary bladder cancer (Cascorbi et al., 2001).

560G > A (rs4986782) (R187Q) also has been associated with higher prevalence of lung cancer in cigarette smokers (Bouchardy et al., 1998). In a subsequent study, the association of *NAT1*14* alleles possessing 560G > A (rs4986782) (R187Q) with higher incidence of lung cancer nearly reached statistical significance while an inverse association with the *NAT1*10* allele achieved statistical significance (McKay et al., 2008). Thus the results with lung cancer incidence in smokers are similar to those for urinary bladder cancer incidence described above.

In conclusion, the current study confirms and expands upon previous studies that have characterized the effects of SNPs in the NAT1 coding exon on N-acetylation of ABP and O-acetylation of N-OH-ABP. Our findings confirm previous studies reporting that *NAT1*14* alleles are reduced function alleles, but in contrast to *p*-aminobenzoic acid, the current study showed that the 560G > A (rs4986782) (R187Q) caused a 4 to 5-fold *increase* ($p < 0.05$) in affinity for both ABP and N-OH-ABP. The increase in affinity for ABP and N-OH-ABP shows that in altering the site for substrate binding, 560G > A (rs4986782) (R187Q) can cause opposite effects on substrate affinity. The findings are consistent with an increased risk for urinary bladder cancer and perhaps also lung cancer in individuals possessing *NAT1*14* alleles following lower levels of exposure to ABP as would be expected in active and particularly passive exposure following use of cigarette and other tobacco products.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MD performed experiments, data analysis, assisted with writing of the manuscript. Approved final draft. DH performed data analysis, wrote the manuscript; Approved final draft.

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Use of a Baculovirus-Mammalian Cell Expression-System for Expression of Drug-Metabolizing Enzymes: Optimization of Infection With a Focus on Cytochrome P450 3A4

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Heterologous expression systems are important for analyzing the effects of genetic factors including single nucleotide polymorphisms on the functions of drug-metabolizing enzymes. In this study, we focused on a baculovirus-mammalian cell (Bac-Mam) expression system as a safer and more efficient approach for this purpose. The baculovirus-insect cell expression system is widely utilized in large-scale protein expression. Baculovirus has been shown to also infect certain mammalian cells, although the virus only replicates in insect cells. With this knowledge, baculovirus is now being applied in a mammalian expression system called the Bac-Mam system wherein a gene-modified baculovirus is used whose promotor is replaced with one that can function in mammalian cells. We subcloned open-reading frames of cytochrome P450 3A4 (CYP3A4), UDP-glucuronosyltransferase (UGT) 1A1, and UGT2B7 into a transfer plasmid for the Bac-Mam system, and prepared recombinant Bac-Mam virus. The obtained virus was amplified in insect Sf9 cells and used to infect mammalian COS-1 cells. Expression of CYP3A4, UGT1A1, and UGT2B7 in COS-1 cell homogenates were confirmed by immunoblotting. Optimum infection conditions including the amount of Bac-Mam virus, culture days before collection, and concentration of sodium butyrate, an enhancer of viral-transduction were determined by monitoring CYP3A4 expression. Expressed CYP3A4 showed appropriate activity without supplying hemin/5-aminolevulinic acid or co-expressing with NADPH-cytochrome P450 reductase. Further, we compared gene transfer efficiency between the Bac-Mam system and an established method using recombinant plasmid and transfection reagent. Our results indicate that the Bac-Mam system can be applied to introduce drug-metabolizing enzyme genes into mammalian cells that are widely used in drug metabolism research. The expressed enzymes are expected to undergo appropriate post-translational modification as they are in mammalian bodies. The Bac-Mam system may thus accelerate pharmacogenetics and pharmacogenomics research.

Keywords: drug-metabolizing enzyme, heterologous expression, baculovirus, mammalian cell, bac-mam system, cytochrome P450 3A4, UDP-glucuronosyltransferase

INTRODUCTION

Hydrophobic xenobiotics are difficult to be eliminated and induce adverse effects in the body for a long period. Drug-metabolizing enzymes constitute a defensive mechanism against such chemicals by converting them to less toxic and soluble metabolites, although pharmacologically active or toxic reactive metabolites of drugs are occasionally formed. Hence drug metabolizing enzymes are important for both the detoxication and intoxication of drugs (Guengerich, 2006). Large inter-individual differences in drug metabolism capacity are largely due to genetic and environmental factors. Single nucleotide polymorphisms play a major role among genetic factors (Evans and Relling, 1999). Cytochrome P450 (P450, CYP) and UDP-glucuronosyltransferase (UGT) play crucial roles in respective phase I and II reactions of drug metabolism. Huge numbers of genetic polymorphisms were revealed which can affect functions of P450 and UGT, with some causing adverse effects to drugs (Court, 2010; Sugatani, 2013; Zanger and Schwab, 2013). Hence, it is important to elucidate whether newly observed genetic polymorphisms affect activities of drug-metabolizing enzymes using a simple approach.

An heterologous expression system is an essential approach to address this issue. Actually, many systems have been applied to research of drug metabolism (Guengerich et al., 1997; Crespi and Miller, 1999). Among them, expression systems using mammalian cells are the most physiological since expressed protein is expected to undergo post-translational modification, locate to the correct target organelle, and show proper activity. There are several ways to introduce a target gene into mammalian cells, including transfection of recombinant plasmid with calcium phosphate, chemical reagent, or electroporation and use of recombinant adeno- or other viruses (Khan, 2013). These methods have both advantages and disadvantages. In this study, we focused on a baculovirus-mammalian cell (Bac-Mam) expression system as a safe and efficient method to express introduced genes. Baculovirus was originally utilized for transient expression of protein in insect cells (Luckow, 1993). Because the baculovirus host is restricted to insect cells, the baculovirus-insect cell expression system is considered as a safe expression system without negative impacts on animals (Hu, 2005). Of course, other viral vectors also lack inherent ability to replicate freely in mammalian cells but experimental restrictions in facility and equipment were less in the case of baculovirus. Despite the restricted host, it was reported that baculovirus can infect human cells (Volkman and Goldsmith, 1983). Focusing on this newly observed infection ability, a Bac-Mam system was established in which a promoter-substituted baculovirus, Bac-Mam virus, is generated for expression in mammalian cells (Hu, 2005; Morales-Perez et al., 2016). The Bac-Mam system is considered safer than other virus-used expression systems because the Bac-Mam virus can infect many kinds of mammalian cells but never replicates in these cells. The virus is now widely utilized for gene/siRNA delivery *in vitro* and *in vivo* (Hu, 2005; Makkonen et al., 2015).

In the present study, we applied the Bac-Mam system to transient expressions of major human isoforms of P450 and UGT, namely CYP3A4, UGT1A1, and UGT2B7, in COS-1 cells. We determined infection conditions by monitoring CYP3A4 expression. Further, expression levels were compared: 1) between the Bac-Mam system and transfection of plasmid with a chemical reagent and 2) among several kinds of cells.

MATERIALS AND METHODS

Materials

Synthetic oligonucleotides were purchased from Fasmac (Kanagawa, Japan). Restriction enzymes and shrimp alkaline phosphatase were from Takara Bio (Shiga, Japan). D-luciferin potassium salt was from Nacalai Tesque (Kyoto, Japan) and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) was from Oriental Yeast (Tokyo, Japan). Pooled human liver microsomes (HLM) prepared 50 donors, and Supersomes™ expressing CYP3A4 and NADPH-P450 reductase (CPR) were from BD Gentest (Franklin Lakes, NJ, United States). All other reagents were of the highest quality commercially available.

Subcloning of Drug-Metabolizing Enzyme Genes Into a Bac-Mam and Mammalian Cell Expression Vector

pEZT-BM, a transfer plasmid for the Bac-Mam system, was a gift from Ryan Hibbs (Addgene plasmid # 74099; http://n2t.net/addgene:74099; RRID: Addgene_74099). The plasmid was based on pFastBac Dual (Thermo Fisher Scientific, Waltham, MA, United States), and the original insect polyhedrin promotor was replaced by a cytomegalovirus (CMV) promotor for expression of target gene in mammalian cells (Morales-Perez et al., 2016). The open reading frames (ORFs) of CYP3A4, UGT1A1, and UGT2B7 were amplified with each pair of primers as listed in **Supplemental Table S1**, and each pFastBac1-based construct as template (Ishii et al., 2014; Miyauchi et al., 2015). KOD-Plus-Neo DNA Polymerase (Toyobo Life Science, Osaka, Japan) was used for the polymerase chain reaction, and thermal cycling condition was as follows: initial denaturation, 94°C, 2 min; 40×amplification step, 98°C, 10 s; 52°C, 30 s; 68°C, 1 min; hold, 4°C. The PCR products were digested with DpnI, purified with a FastGene Gel/PCR Extraction Kit (NIPPON Genetics, Tokyo, Japan), and introduced into KpnI/XhoI digested pEZT-BM using an In-Fusion HD Cloning Kit (Takara Bio). The ORF of CYP3A4 was also subcloned into pcDNA3.1/Hygro (–), a mammalian cell expression vector, with KpnI/XhoI sites using Ligation high Ver.2 (Toyobo Life Science). Primers for this CYP3A4 subcloning are also listed in **Supplemental Table S1**. The nucleotide sequences of the constructs were confirmed by an Applied Biosystems 3130xl Genetic Analyzer (Thermo Fisher Scientific), using a BigDye Terminator version 3.1 Cycle Sequencing Kit (Thermo Fisher Scientific).

Preparation of Recombinant Bac-Mam Virus and Culture of Insect Cells

Recombinant Bac-Mam viruses were prepared with the Bac-to-Bac Baculovirus Expression system (Thermo Fisher Scientific). The pEZT-BM constructs were transfected into competent *Escherichia coli* (*E. coli*) DH10Bac. A positive single clone was selected after blue/white colony selection, and recombinant bacmid, a part of the baculoviral DNA, was prepared according to the user manual. Insect cells Sf9 were cultured in a 100 or 500 mL plastic Erlenmeyer flask (Corning, Lowell, MA, United States) containing Sf-900 III medium (Thermo Fisher Scientific) supplemented with 5% fetal bovine serum (FBS). To prepare recombinant Bac-Mam virus, the constructed bacmids were transfected into Sf9 cells as described previously (Miyauchi et al., 2015). The control bacmid, obtained from transfection of mock pEZT-BM, was also transfected to obtain control virus. pEZT-BM contains the p10 promotor followed by the ORF of green fluorescent protein (GFP), which enabled us to confirm viral generation in Sf9 cells by detecting GFP (**Supplemental Figure S1**). Culture media were collected as primary viruses 1 week after transfection. Baculoviral DNA were purified from portions of these media (200 μL) by NucleoSpin Blood (Macherey-Nagel, Düren, Germany), and their titer was determined using a BacPAK qPCR Titration Kit (Clontech, Mountain View, CA, United States). The Bac-Mam viruses were amplified 2–3 times until their titer reached over 5.0×10^7 plaque-forming unit (PFU)/mL.

Culture of Mammalian Cells and Infection of Bac-Mam Virus

In this study, COS-1 cells were mainly utilized for expression of drug-metabolizing genes. COS cells (COS-1 cells, COS-3 cells, and COS-7 cells) were established by transformation of African monkey kidney cells (CV-1 cells) with a mutant of simian virus 40 (SV_{40}) (Gluzman, 1981) and have been widely used in research including the field of drug metabolism (Mackenzie, 1986; Clark and Waterman, 1991; Guengerich et al., 1997; Thomae et al., 2002). In case of P450, it is especially advantageous that COS cells have endogenous CPR, which is sufficient for catalytic activity of transiently expressed P450 (Clark and Waterman, 1991). COS-1, HEK293, and HepG2 cells were grown in Dulbecco's modified Eagle medium (FUJIFILM Wako Pure Chemical, Osaka, Japan, Cat#043-30085) supplemented with 10% FBS. Cells were routinely maintained in a 10 cm dish and seeded to a 35 mm dish at 1.0×10^6 cells/dish the day before infection. After removing the culture medium, recombinant Bac-Mam viruses were added to the dish. Viral amount, multiplicity of infection (MOI), ranged from 25 to 150 and finally fixed at 100. The infection was performed in a CO_2 incubator for 1 hour. Then virus was removed, and new culture medium containing sodium butyrate ranged from 0 to 4.5 mM (finally fixed at 3 mM) was added. Sodium butyrate is used as an inhibitor of histone deacetylase, enhancing expression of recombinant protein in mammalian cells including the Bac-Mam system (Gorman et al., 1983; Condreay et al., 1999; Morales-Perez et al., 2016). The infected cells were further cultured for 24–96 h. The culture time was finally fixed at 48 h.

Plasmid Transfection to COS-1 Cells

COS-1 cells were seeded in a 35 mm dish the day before transfection. Transfection of pcDNA3.1/Hygro (−)_CYP3A4 was conducted as described previously (Miyauchi et al., 2020b). In short, 2 μg plasmid DNA was mixed with 8 μg of Polyethylenimine HCl MAX (PEI; Polysciences, Warrington, PA, United States) in Opti-MEM I Reduced Serum Media (Thermo Fisher Scientific) and incubated at room temperature for 10 min. The DNA-PEI complex was added to COS-1 cells, and the cells were cultured for 48 h.

Preparation of Cell Homogenates

Cells were collected in homogenate buffer containing 10 mM Tris-HCl (pH7.4), 250 mM sucrose, and 10% glycerol, and then sonicated with an AS38A ultrasonic cleaner (AS ONE, Osaka, Japan). Protein concentration was determined using Quick Start Bradford 1×Dye Reagent (Bio-Rad Laboratories, Hercules, CA, United States) with bovine serum albumin as a standard.

Immunoblotting

Cell homogenates (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto a polyvinylidene difluoride membrane (FUJIFILM Wako Pure Chemical, Cat#034-25663). The blots were washed with Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and incubated in TBS-T containing 5% skim milk at room temperature for 30 min. After blocking, the blots were incubated with primary antibody diluted 2,000-fold with TBS-T containing 5% skim milk at 4°C overnight. The following primary antibodies were utilized: mouse anti-CYP3A4 antibody, rabbit anti-UGT1A antibody (respective Cat# sc-53850 and sc-25847; Santa Cruz Biotechnology, Dallas, TX, United States), rabbit anti-UGT2B7 antibody, and rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (respective Cat# 16661-1-AP and 10494-1-AP, Proteintech, Rosemont, IL, United States). The blots were extensively washed with TBS-T and further incubated with secondary antibody diluted 10,000-fold with TBS-T containing 5% skim milk at room temperature for 1 hour. The following secondary antibodies labeled with horseradish peroxidase (HRP) were utilized: HRP-goat anti-mouse IgG (H + L) antibody and HRP-goat anti-rabbit IgG (H + L) antibody (respective Cat# SA00001-1 and SA00001-2, Proteintech). After extensive washing with TBS-T, EzWestLumi plus (ATTO, Tokyo, Japan) was used as a substrate of HRP, and the signal was visualized and analyzed using iBright Imaging System (Thermo Fisher Scientific). To quantify expressed CYP3A4 in 20 μg homogenates, Supersomes™ (BD Gentest) containing 1 pmol CYP3A4 were applied as a standard.

CYP3A4 Assay

CYP3A4 activity was measured with P450-Glo CYP3A4 Assay and Screening System (Luciferin-IPA; Promega, Madison, WI, United States). We used 100 μM NADPH (final concentration). Substrate luciferin-IPA concentration varied from 0.3 to 15 μM

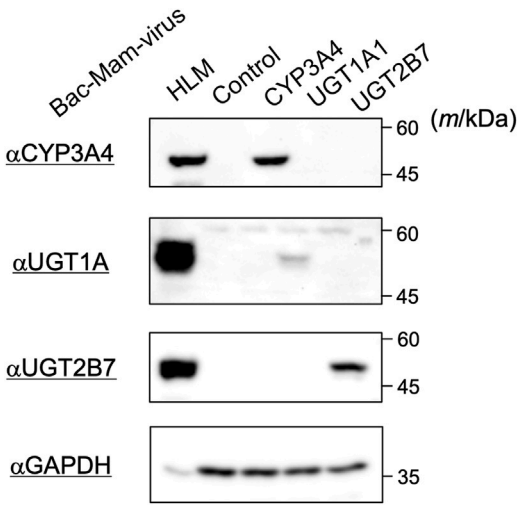

FIGURE 1 | Detection of drug-metabolizing enzymes expressed by the Bac-Mam system. COS-1 cells were infected with the Bac-Mam viruses encoding CYP3A4, UGT1A1, or UGT2B7. Infection conditions were: virus amount, MOI = 100; culture time after infection, 24 h; sodium butyrate, 3 mM. Cell homogenates (20 μg) were analyzed by immunoblotting with HLM (10 μg) and homogenates from cells infected with control virus as positive and negative controls, respectively. HLM, human liver microsomes.

(nine points of concentration). Chemiluminescence was measured using a microplate reader, Infinite M200 Pro (Tecan Group, Männedorf, Switzerland). As enzyme sources, 20 μg HLM, Supersomes™, or COS-1 homogenates were utilized. In kinetic experiments, data were fitted to the Michaelis-Menten model defined by the equation below:

$$V = \frac{Vmax \times S}{Km + S}$$

Where V is the reaction rate, S is the substrate concentration, Vmax is the maximum enzyme velocity, and Km is the Michaelis constant, which is equal to the concentration of substrate for half-maximal velocity. The kinetic parameters were determined with GraphPad Prism 5.04 software (GraphPad software, La Jolla, CA, United States).

RESULTS

Detection of CYP3A4, UGT1A1, and UGT2B7 Expressed With the Bac-Mam System

The drug-metabolizing enzymes were transiently expressed using the Bac-Mam system in COS-1 cells and their expressions were confirmed by immunoblotting (**Figure 1**). Infection conditions were fixed as below: MOI = 100; culture time, 24 h; sodium butyrate, 3 mM. With every enzyme, specific bands were observed that were not detectable in control virus-infected cells. In addition, the enzymes showed the same mobility as those in the positive control, HLM. The antibody that we used in detection of UGT1A1 can react with other UGT1A isoforms in HLM, which resulted in a slight difference of observed bands between HLM and COS-1 homogenates. These results indicated that the Bac-Mac system is suitable for transient expression of the major drug-metabolizing enzymes. In this experiment, we loaded 20 μg homogenates and 10 μg HLM. CYP3A4 showed the closest expression to that in HLM among the three targets, so we optimized infection conditions by monitoring CYP3A4 expression level in subsequent experiments.

Optimization of Viral Amount

We evaluated infection conditions to obtain maximum CYP3A4 expression using the recombinant Bac-Mam virus in COS-1 cells. P450 can be quantified by measuring its CO difference spectrum (Omura and Sato, 1964), but a previous study suggested that the amount of P450 that can be overexpressed in COS-1 cells was below the amount required for detection using the CO difference spectrum assay (Clark and Waterman, 1991). Therefore, we quantified expressed CYP3A4 by immunoblotting in the current study. First, we tested varying the amount of virus. In our previous study, CYP3A4, CPR, and some UGT isoforms were expressed in insect Sf9 cells using an original baculovirus expression system. The viral amounts in the experiments were set at MOI = 0.01–1, which was enough to express the enzymes in Sf9 cells (Ishii et al., 2014; Miyauchi et al., 2015; Miyauchi et al., 2020a). In contrast, more than 10-fold larger amounts of virus seem to be necessary in the Bac-Mam system since the Bac-Mam virus cannot amplify in mammalian cells, and so secondary infection is not expected (Condreay et al., 1999). Hence, COS-1 cells were infected with the prepared Bac-Mam virus at MOI = 0 (not infected), 25, 50, 75, 100, and 150. Culture time and concentration of sodium butyrate were fixed at 24 h and 3 mM, respectively. Immunoblotting and its analyzed result are shown in **Figures 2A,B**, respectively. CYP3A4 expression tended to increase in a MOI-dependent manner and reach a peak around MOI = 75–100. Hence, MOI was fixed at 100 in the later experiments.

Optimization of Culture Time After Infection

Next, we tested varying culture time after viral infection. Viral amount and concentration of sodium butyrate were fixed at MOI = 100 and 3 mM, respectively. Bac-Mam virus-infected cells began to float from dishes 48 h after infection, and almost all the cells were dead at 96 h. Thus, we prepared cell homogenates between 24 and 72 h post infection and quantified CYP3A4 expressions (**Figures 2C,D**). Expression levels increased in a time-dependent manner and reached a maximum at 48–72 h after infection. Given that the cells began to die, we concluded that 48 h is the best culture time after infection.

Optimization of Concentration of Sodium Butyrate

As cell death was also observed in the absence of viral infection, we predicted that sodium butyrate was the cause of this toxicity. We next varied the concentration of sodium butyrate from 0 to 4.5 mM under the fixed condition: viral amount, MOI = 100; culture time, 48 h. As explained, sodium butyrate enhances target gene expression in the Bac-Mam system. Hence,

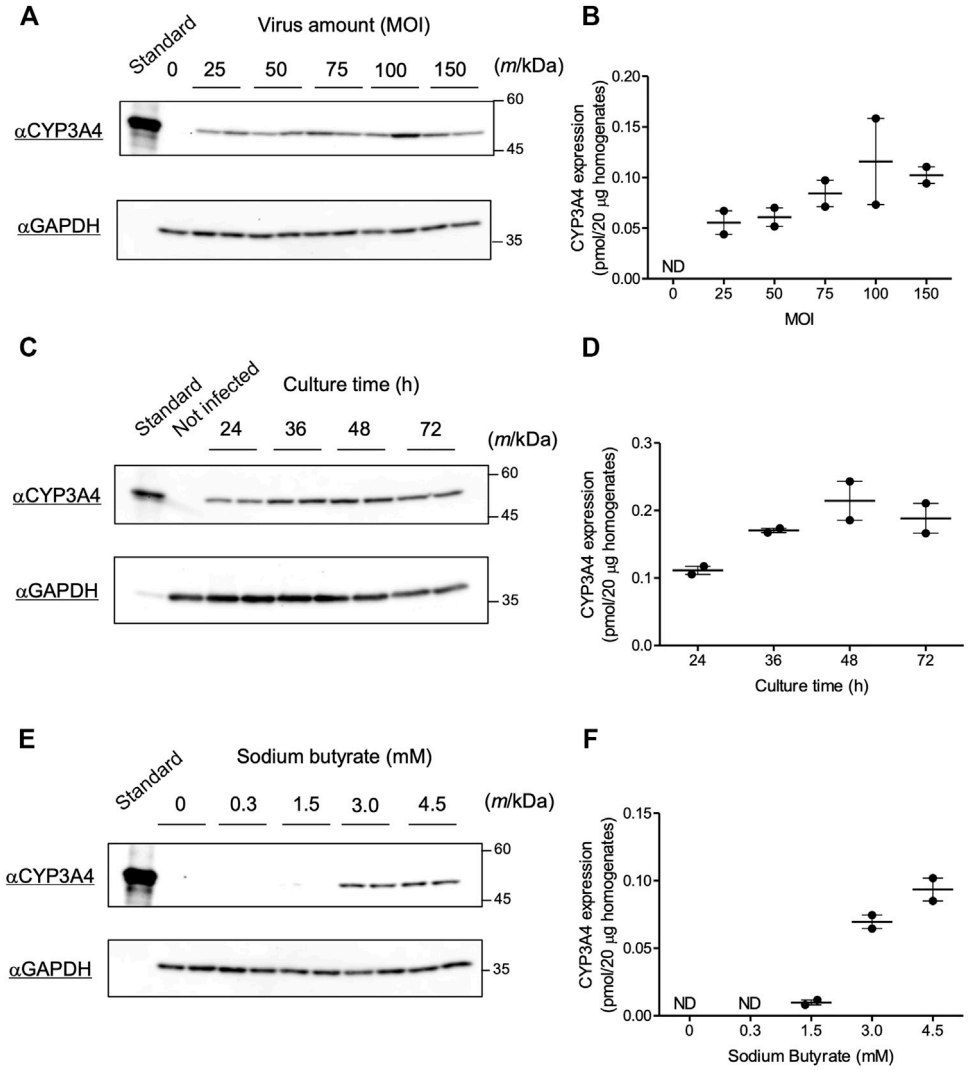

FIGURE 2 | Optimization of infection conditions for the Bac-Mam virus. Conditions for the Bac-Mam virus to infect COS-1 cells were optimized by monitoring CYP3A4 expression. CYP3A4 expression in COS-1 homogenates (20 μg) was determined by immunoblotting with Supersomes™ contained 1 pmol CYP3A4 (BD Gentest) as a standard. Viral infection was independently conducted twice in each experiment. **(A)** Optimization of viral amount (MOI) ranging from 0 to 150. Culture time after infection and concentration of sodium butyrate were fixed at 24 h and 3 mM, respectively. **(B)** The result of quantification of CYP3A4 in the optimization of viral amount. Each determined CYP3A4 level (N = 2), and the mean ± S.E.M. are presented. **(C)** Optimization of culture time after infection ranging from 24 to 72 h. Viral amount and concentration of sodium butyrate were fixed at MOI = 100 and 3 mM, respectively. **(D)** The result of quantification of CYP3A4 in the optimization of culture time. Each determined CYP3A4 level (N = 2), and the mean ± S.E.M. are presented. **(E)** Optimization of the concentration of sodium butyrate ranging from 0 to 4.5 mM. Viral amount and culture time after infection were fixed at MOI = 100 and 48 h, respectively. **(F)** The result of quantification of CYP3A4 in the optimization of concentration of sodium butyrate. Each determined CYP3A4 level (N = 2), and the mean ± S.E.M. are presented. ND, not detected.

CYP3A4 was not detectable under 1.5 mM while significant expression was observed at three and 4.5 mM (**Figures 2E,F**). In accordance with our hypothesis, however, not only significant CYP3A4 expression but also cell toxicity was observed, and COS-1 cells seemed to be more sensitive to sodium butyrate than HEK293 and HepG2 cells. We determined that 3 mM is the best concentration of sodium butyrate for use. Taken together, we concluded that the optimized infection condition was: viral amount, MOI = 100; culture time after infection, 48 h; concentration of sodium butyrate, 3 mM.

Comparison of CYP3A4 Activity With HLM and Supersomes™

According to the optimized infection condition, COS-1 homogenates were prepared, and the activity of expressed CYP3A4 was measured with luciferin-IPA as a substrate,

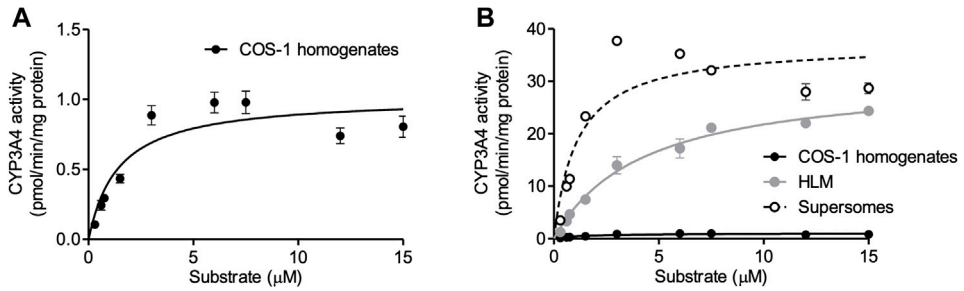

FIGURE 3 | Kinetic analysis of expressed CYP3A4 with the Bac-Mam system. Kinetic analysis of CYP3A4 was conducted with luciferin-IPA (Promega) as a substrate. **(A)** COS-1 homogenates (20 μg) were used as enzyme source, and the data were fitted to the Michaelis-Menten equation. Results are shown as the mean ± S.E.M. of three independent transductions. **(B)** Comparison of CYP3A4 activity between prepared COS-1 homogenates and HLM/Supersomes™. The result of kinetics with HLM/Supersomes™ (20 μg) as enzyme source were merged. In the result of HLM and Supersomes™, each plot represents the mean ± S.E.M. of triplicate assays. Calculated parameters were listed and compared in the Table.

TABLE 1 | Comparison of kinetic parameters of CYP3A4 activity among COS-1 cell homogenates prepared by the Bac-Mam system, HLM, and Supersomes™.

	Vmax (pmol/min/mg protein)	Km (μM)
COS-1 homogenates	1.0 ± 0.1	1.5 ± 0.4
HLM	31.0 ± 1.6	4.3 ± 0.6
Supersomes™	37.2 ± 2.8	1.1 ± 0.3

*Data were fitted to the Michaelis-Menten equation (**Figures 3A,B**). Each parameter is shown as the calculated value ±S.E.M. HLM, human liver microsomes.*

which is converted to luciferin by CYP3A4 but shows minimal cross-reactivity with CYP3A5 and CYP3A7. Kinetic analysis was conducted with the COS-1 homogenates, HLM, and Supersomes™ as enzyme sources, and obtained parameters were compared (**Figure 3** and **Table 1**). CYP3A4 activity was confirmed in the prepared homogenates, which supported our view that the Bac-Mam system can be applied for expression of drug-metabolizing enzymes. The highest CYP3A4 activity was observed when Supersomes™ was used as the enzyme source, followed by HLM and homogenates. In comparison with HLM and Supersomes™, the Vmax value of the Bac-Mam expressed CYP was one 30th of those of HLM/Supersomes™ although the Km value was comparable.

Comparison of Bac-Mam Expressed CYP3A4 Levels With Plasmid Expressed Levels

Transfection of recombinant plasmid with a transfection reagent is one of the major methods to introduce a target gene into mammalian cells and is also utilized in research of drug-metabolizing enzymes. We compared the expressed CYP3A4 levels between the Bac-Mam system and the widely used transfection method (**Figures 4A,B**). With the Bac-Mam system, 2–3 times higher expression of CYP3A4 was observed compared to that expressed using the transfection method.

Application of the Bac-Mam System to Other Kinds of Mammalian Cells

We have applied the Bac-Mam system to introduce genes of CYP3A4 and some UGTs into COS-1 cells and confirmed their expression and CYP3A4 function. Further, we utilized the Bac-Mam system to introducing CYP3A4 into other kinds of cells (HEK293 and HepG2 cells) under the optimized condition determined with COS-1 cells. Although these cells are widely used in research of drug-metabolizing enzymes, there was a large difference in the expressed CYP3A4 levels (**Figures 4C,D**). COS-1 cells showed the highest CYP3A4 level, almost 4- and 20-times higher than that in HepG2 and HEK293 cells, respectively.

DISCUSSION

In this study, we have applied a Bac-Mam system to introduce genes of drug-metabolizing enzymes into COS-1 cells and optimized infection conditions focusing on CYP3A4. The optimized viral amount was determined at MOI = 100 (**Figures 2A,B**), which is over 100 times higher compared to that of the baculovirus-insect cell expression system (Miyauchi et al., 2015). This is because the Bac-Mam virus never replicates in mammalian cells, so there is no secondary infection. However, the viral amount is comparable to the amount of adenovirus needed for maximum CYP3A4 expression in HepG2 cells (Aoyama et al., 2009) and those of Bac-Mam virus in other studies (Condreay et al., 1999; Ramos et al., 2002). Since it is easy to culture insect cells in large scale, such a requirement for a high amount of virus does not matter. Culture time after infection was optimized at 48 h (**Figures 2C,D**), but caution is required to minimize sodium butyrate induced cellular toxicity. As shown in **Figures 2E,F**, sodium butyrate was essential for expression of CYP3A4 but COS-1 cells seemed to be sensitive to this chemical. Sodium butyrate has many cellular effects including inhibition of histone deacetylase, e.g. arresting cell proliferation, affecting the cytoskeleton, and sometimes triggering apoptosis (Kruh, 1982; Shin et al., 2012). The main cause of COS-1 death

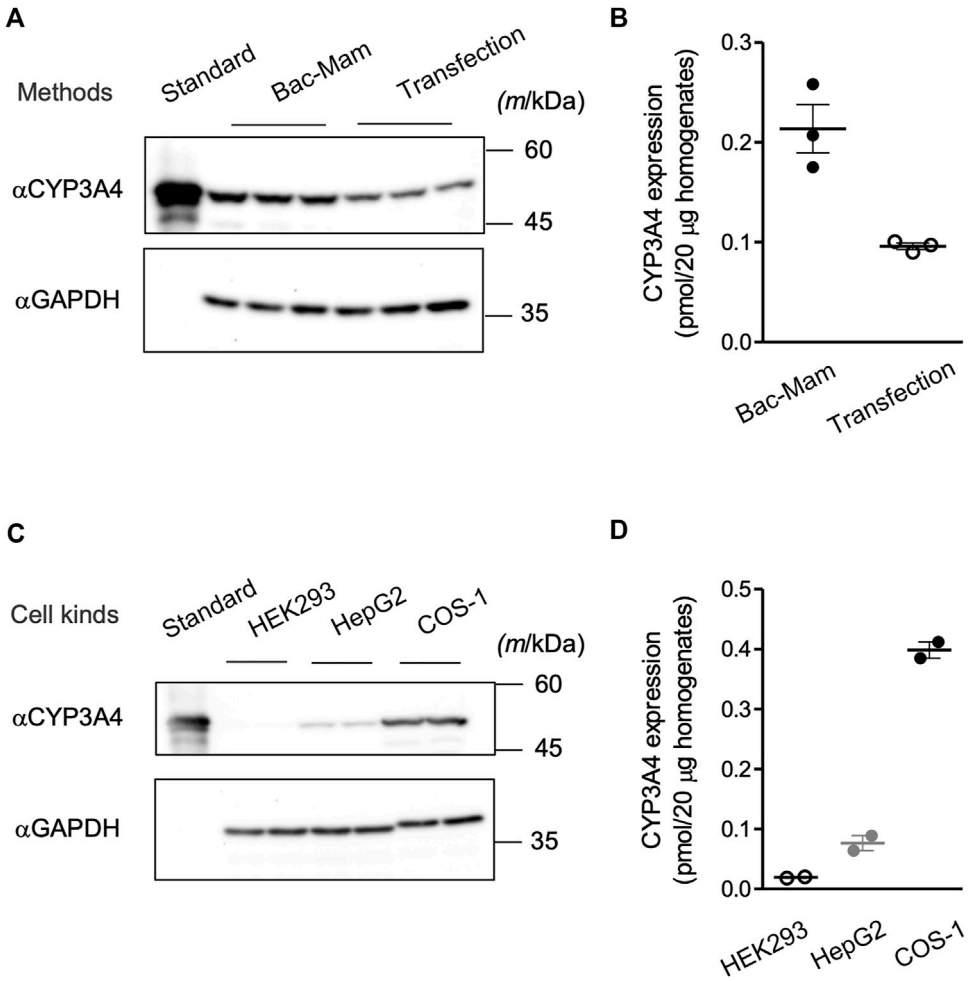

FIGURE 4 | Advantages of the Bac-Mam system. **(A)** CYP3A4 expression levels were compared between the Bac-Mam system and plasmid transfection with polyethylenimine (PEI). Viral infection and transfection were independently conducted three times, and COS-1 cell homogenates (20 μg) were analyzed by immunoblotting with Supersomes™ (BD Gentest) containing 1 pmol CYP3A4 as a standard. **(B)** The result of quantification of expressed CYP3A4 in the comparison between the two expression systems. Each determined CYP3A4 level (N = 3), and the mean ± S.E.M. are presented. **(C)** Transduction efficiency of Bac-Mam virus was compared among three kinds of mammalian cells. Viral infection conditions were: viral amount, MOI = 100; culture time after infection, 48 h; concentration of sodium butyrate, 3 mM. Cell homogenates (20 μg) were applied to determine the expressed CYP3A4 level. Infection was independently conducted twice. **(D)** The result of quantification of CYP3A4 expressed in the three kinds of cells. Each determined CYP3A4 level (N = 2), and the mean ± S.E.M. are presented.

remains unclear but further medium replacement should be carried out to reduce toxicity when experiments on Bac-Mam virus-infected cells require several days of culture.

Since mammalian cells were utilized in this study, it was not necessary to supply hemin and 5-aminolevulinic acid which are essential for holo-CYP3A4 enzyme formation in insect cells and *E. coli* (Asseffa et al., 1989; Kusano et al., 1999). Neither was coexpression of CPR necessary to detect CYP3A4 activity (**Figure 3A**). The Vmax value of CYP3A4 activity in the cell homogenates was 30-fold less than that in HLM and Supersomes™ (**Figure 3B** and **Table 1**). This is not surprising given that P450s are concentrated in the microsomal fraction and that there may be more CPR and cytochrome b5, which localize to the endoplasmic reticulum (ER) membrane and function as electron suppliers for P450, in HLM and Supersomes™ than in COS-1 cells (Crespi and Miller, 1999). In contrast, the Km value was comparable between the cell homogenates and the other microsomal samples. Expressed CYP3A4 levels were 2–3 times higher with the Bac-Mam system than with the plasmid transfection method, one of the main techniques for heterologous expression (**Figure 4A**). Hence, the Bac-Mam system is useful for experiments on drug-metabolizing enzymes such as determination of substrate/isoform specificities, and estimation of effects of genetic factors on enzyme activity.

It has been reported that the Bac-Mam system can deliver genes into a wide range of vertebrate cells, some of which show low transfection efficiency when the plasmid transfection with chemical reagent is applied. For example, primary cells including hepatocytes, induced pluripotent stem cells, and neural cells can be transduced by the Bac-Mam virus (Hu, 2005; Makkonen et al., 2015). HepG2 cells are also difficult to be transduced by the

plasmid transfection methods. However, the Bac-Mam virus could introduce the CYP3A4 gene into HepG2 cells (**Figure 4C**), which is consistent with previous studies (Hofmann et al., 1995; Boyce and Bucher, 1996). There was a large difference in the expression of CYP3A4 among the 3 cell lines (**Figures 4C,D**). As it is known that the CMV promotor is a strong promoter in many cell types, we think that the difference in CYP3A4 expression levels among the 3 cell lines is a result of their sensitivity to infection by baculoviruses. A baculovirus enters mammalian cells via the envelope glycoprotein gp64, and cell surface heparan sulfate proteoglycans and phospholipids have been identified as factors on the mammalian cell side that interact with gp64 (Duisit et al., 1999; Tani et al., 2001). We speculate that quantitative and qualitative differences in these factors among the cells examined in this study led to the differences in CYP3A4 expression levels. In addition, the Bac-Mam system can be applied to large scale expression in mammalian cells which can be grown in suspension culture, including HEK293S GnTI- and CHO cells (Ramos et al., 2002; Morales-Perez et al., 2016). Although it is necessary to optimize the expression conditions in each cell, these findings indicate that the Bac-Mam system is a safe, simple, and efficient approach to analyzing drug-metabolizing enzymes in mammalian cells.

In mammalian cells, it is expected that expressed proteins undergo post-translational modification as they do in the mammalian body, which is a large advantage of a mammalian cell expression system compared to other expression systems with *E. coli*, insect cells, etc. Most UGTs have several *N*-glycosylation sites, Asn-X-Ser/Thr, in their sequence, and glycosylation can affect UGT function (Mackenzie, 1990; Barbier et al., 2000; Nakajima et al., 2010; Nagaoka et al., 2012; Nakamura et al., 2016). Although proteins which have such glycosylation sites undergo modification in insect cells, there is a difference in the glycosylation pattern between mammalian and insect cells (Marchal et al., 2001; Jarvis, 2003). Further, it was reported that UGT activity could be reduced when the enzyme was expressed at high levels with a baculovirus-inset cell system (Oda et al., 2012; Zhang et al., 2012). Taken together, as a next step, we need to analyze UGT using the Bac-mam expression system to confirm if such post-translational modifications are properly performed in this system.

Similar to post-translational modification, protein-protein interactions among drug-metabolizing enzymes can regulate their activities (Backes and Kelley, 2003; Finel and Kurkela, 2008; Ishii et al., 2010). Our team revealed that P450 and UGT also form a complex on the ER membrane and regulate each others function, even though they catalyze far different reactions, and their membrane topologies are opposite (Ishii et al., 2010; Miyauchi et al., 2021). Our previous study indicated that coexpression of P450 and UGT in the same membrane is necessary to see the effects of P450-UGT interactions (Miyauchi et al., 2019). In general, it is difficult to control the expression levels of several target proteins when they were coexpressed using a single expression system. The Bac-Mam system can be easily combined with other methods such as plasmid-transfection, which may aid in the controlled expression of several proteins and permit a more rigorous investigation of protein-protein interactions including those between P450 and UGT.

In conclusion, this study highlights the Bac-Mam system as a convenient approach to analyze drug-metabolizing enzymes in mammalian cells. The Bac-Mam system is safe, simple, and efficient compared to other widely used methods. Hiratsuka's group recently reported their application of a HEK293FT expression system for heterologous expression of several P450 isoforms, which is also a simple and useful approach for estimating P450 function (Kumondai et al., 2020). These methodological advances are an immense aid in analyzing how genetic- and post-translational-factors affect functions of drug-metabolizing enzymes that result in large inter-individual differences in drug metabolism.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

Participate in research design: YM, AK, MS, KF, YH, YT, ST, PM, and YI. Conducted experiments: YM, AK, and MS. Performed data analysis: YM, AK, MS, and YI. Wrote or contributed the writing of the manuscript: YM, YT, ST, PM, and YI.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.832931/full#supplementary-material

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