PHARMACOGENETICS RESEARCH AND CLINICAL APPLICATIONS: AN INTERNATIONAL LANDSCAPE OF THE ACCOMPLISHMENTS, CHALLENGES, AND OPPORTUNITIES

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PHARMACOGENETICS RESEARCH AND CLINICAL APPLICATIONS: AN INTERNATIONAL LANDSCAPE OF THE ACCOMPLISHMENTS, CHALLENGES, AND OPPORTUNITIES

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EDITORIAL
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Editorial: Pharmacogenetics Research and Clinical Applications: An International Landscape of the Accomplishments, Challenges, and Opportunities

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Editorial on the Research Topic

Pharmacogenetics Research and Clinical Applications: An International Landscape of the Accomplishments, Challenges, and Opportunities

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Pharmacogenomics (PGx) is a major pillar of personalized medicine. With the effort to tailor therapeutic interventions to patients depending on their genetic profile came a higher demand for further PGx research. As naturally expected, discrepancies have been noted, with some institutions leading the way in this field. The majority of the documented efforts in research and clinical applications are concentrated mostly in the US and Europe, while relatively little information is available from other parts of the world (Abou Diwan et al., 2019; Zgheib et al., 2020). Therefore, the goal of this Research Topic is to shed more light onto worldwide accomplishments in PGx research and clinical applications with a focus on current challenges, lessons learned, and opportunities for further advances in the field towards better clinical uptake of PGx.

This issue includes 13 contributions to the field emanating from 11 countries scattered thorough the globe and spanning five continents. The data presented are quite representative of the status of PGx research worldwide, and relate to the following topics: validation of already established or extensively studied PGx markers in new populations, frequency distribution of actionable pharmacogenes in multiethnic groups, attempts at the discovery of novel PGx markers that are peculiar to certain populations, evaluations of the implementation of PGx guided practice, perspectives of the challenges of PGx applications in the clinic, and economic evaluation of various reimbursement models for PGx testing.

Perhaps one of the most commonly studied drug-gene pairs is that of oral anticoagulants such as acenocoumarol and warfarin with *CYP2C9*, *CYP4F2*, and *VKORC1* candidate polymorphisms (Johnson et al., 2017). Nevertheless, the published guidelines may not necessarily apply to all populations, hence the need for validation studies in different contexts. As such, three manuscripts address the issue from three different angles. Roco et al. provide a pharmacogenetically guided algorithm that explains almost 50% of the variability of acenocoumarol dosing in Chilean patients. Roche-Lima et al. compare and contrast seven machine learning algorithms for the prediction of

warfarin dosing from PGx data and conclude that Random Forest Regression (RFR) outperforms all other models. Finally, Zhang et al. show that gene-based warfarin dosing provides clinical benefits in Chinese patients when compared with clinically fixed dosing.

The PGx of thiopurines is another topic that has been extensively studied, and updated guidelines for preemptive genotyping for *Thiopurine-S-methyltransferase (TPMT)* and *NUDT15* genetic polymorphisms have been published recently (Relling et al., 2019). Nevertheless, the minor allele frequencies of polymorphisms in these genes vary among populations and may be quite rare in, for example, Middle Eastern populations. Accordingly, Moradveisi et al. reveal that two variants in *Inosine triphosphatase (ITPA)* might also be relevant in predicting 6-mercaptopurine toxicity in Arab and Kurdish children treated for Acute Lymphoblastic Leukemia.

The results described above highlight the importance of identifying and characterizing both novel and previously known PGx variants in various populations. As such, Galaviz-Hernandez et al. report on the frequency of *CYP3A5*3* allele in eight different ethnic groups from Northwest Mexico and its association with hypertension. Another study by Gonzalez-Covarrubias et al. characterizes variations in actionable PGx markers in 1,284 Mestizos and 94 natives from Mexico. Of note in the latter study is the multi-institutional collaborative aspect of the endeavor that allowed the investigators to access a large number of samples with deep sequencing data (Gonzales-Covarrubias et al.). A relatively small sample size may be an issue in gene discovery studies, especially if dealing with relatively less common phenotypes or in countries of lower income. For example, an evaluation of 15 variants in *Reelin (RELN)* shows that two novel loci may be associated with response to antipsychotics in 260 Chinese Hans, yet, the statistical significance was lost after multiple corrections (Xu et al.). Similarly, another study, the first of its kind to explore innate immune genetic polymorphisms in 154 patients treated with tacrolimus for kidney transplant in Australia, also shows loss of statistical significance after Bonferroni adjustment (Hu et al.). On the other hand, another evaluation of a much smaller number (N = 76) of kidney transplant patients from Egypt indicates that the common *CYP3A5*3* polymorphism is associated with tacrolimus daily requirements in these patients (Mendrinou et al.).

While some countries are still investigating the effects or frequencies of PGx markers in their populations, others with higher income such as The Netherlands and Singapore are at stages of evaluating the implementation of clinical practice guidelines in their settings (Martens et al.; Rigter et al.; Sung et al.). More specifically in The Netherlands, a multi-stakeholder perspective of the implementation of PGx in primary care suggests that, despite the ability to formulate actions to truly integrate PGx, there is no consensus on the prioritization of these actions (Rigter et al.). Furthermore, Martens et al. disclose that the frequency of *DPD* testing before initiation of fluoropyrimidine treatment for patients with colon cancer significantly increased only after the update of a National guideline and local consensus meetings. Along the same lines in Singapore, the incidence of severe cutaneous reactions in association with carbamazepine was significantly decreased after the issuing of National recommendations for *HLA-B*15:02* genotyping (Sung et al.). These results undoubtedly affirm the essential role of practice guidelines for the clinical applications of PGx testing; nevertheless, the cost of these tests may be problematic especially if not or only partially reimbursed. As such, Simeonidis et al., who undertook a very elegant systematic analysis and economic evaluation to assess the feasibility of compensation for PGx testing, conclude that such data are lacking from the literature, hence the need for more cost-utility analyses within various healthcare systems.

In summary, the compilation of manuscripts in this Research Topic gives a taste of the ongoing PGx research and clinical applications worldwide, especially in developing countries. Further studies are needed to cover more diverse populations and ethnicities and to unravel the challenges and solutions to make personalized medicine a global reality (Zgheib et al., 2020).

AUTHOR CONTRIBUTIONS

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

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ORIGINAL RESEARCH
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Application of Economic Evaluation to Assess Feasibility for Reimbursement of Genomic Testing as Part of Personalized Medicine Interventions

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Background: The incorporation of genomic testing into clinical practice constitutes an opportunity to improve patients' lives, as it makes possible the implementation of innovative, individualized clinical interventions that maximize efficacy and/or minimize the risk of adverse drug reactions. In order to ensure equal access to genomic testing for all patients, the costs associated with these tests should be reimbursed by their respective national healthcare systems. Given that funding for the public health sector is decreasing in real terms, it is of paramount importance that the emerging interventions are thoroughly evaluated both in terms of their clinical effectiveness and their full economic cost.

Objective: The aim of this study was to identify those genome-guided interventions that could be adopted and reimbursed by national healthcare systems. Further, we recorded the underlying factors determining the broad adoption of genome-guided interventions in clinical practice, in order to identify potential reimbursement criteria.

Methods: We performed a systematic review of published (PubMed-listed) scientific articles on the economic evaluation of those individualized clinical interventions that include genomic tests. Information on genomic tests reimbursed by the US Medicare program was also included. Subsequently, we correlated the regulatory guidance given for the interventions collated in our systematic review with the corresponding economic evaluation results and policies of the Medicare program. Regulatory guidance information was collected from the PharmGKB online knowledgebase and the Clinical Pharmacogenetics Implementation Consortium (CPIC).

Results: Most of the included studies constitute cost-utility analyses, in which the outcome of the interventions has been measured in quality-adjusted life years (QALYs) whereas an estimate of the total cost has been based upon direct medical cost data. Favorable economic evaluation results, as well as concrete evidence demonstrating the clinical utility of pre-emptive genotyping, are considered as prerequisites for the broad

adoption and reimbursement of the costs incurred during genomic testing. Indicatively, pre-emptive *HLA-B*5701* and *TPMT* testing before administration of abacavir and azathioprine, respectively, is reimbursed by Medicare based on both economic and efficacy evidence. Likewise, the medical necessary screening for *MMR* and *BRCA1/2* genes are reimbursed for high-risk populations.

Conclusions: Our findings further underline the need for further cost-utility analyses within different national healthcare systems, in order to promote the reimbursement of the cost of innovative genome-guided therapeutic interventions.

Keywords: economic evaluation, pricing, reimbursement, genetic and genomic tests, personalized medicine, quality of life, willingness-to-pay

INTRODUCTION

Genomic analysis constitutes the basic tool of personalized medicine, as it allows the identification of specific nucleotide changes in patient genomes, thereby delineating their variomes in relation to predisposition to genetic diseases and/or to the effectiveness or otherwise of specific therapeutic drugs or the likelihood of adverse drug reactions (Phillips et al., 2013; Lee et al., 2014; Phillips et al., 2016). It is thus reasonable to expect that the introduction of genomic testing in clinical practice will contribute to the rationalization of established drug-prescription regimens and lead to the design of new, individualized interventions with maximized efficacy and minimized adverse drug reactions (Phillips et al., 2013; Sun et al., 2013).

Health economics and economic evaluation together aim to allocate the limited resources available in the most effective ways in various healthcare systems (Williams, 1987). Faced with the challenge of achieving optimal benefit for patients, while maintaining the sustainability of national healthcare systems, economic evaluation analyses are deemed to be an essential part of the decision-making process as to whether a new intervention should or should not be adopted (Jonsson, 2009; McFarland, 2014). Further, it is of great importance both in terms of patients' need and equal access that these innovative interventions are reimbursed by national healthcare systems. To this end, there is a need for scientists to provide health policymakers with evidence of clinical validity, utility data associated with the genomic tests, as well as reliable evidence of economic benefit (Snyder et al., 2014). In other words, it is essential to demonstrate i) the clinical utility of all pharmacogenomic biomarkers used and ii) support from reliable economic data demonstrating that reimbursing the cost of such genomic tests will not only improve patient life quality but also reduce the costs of the overall national healthcare expenditure while increasing the efficiency of the public healthcare sector by guiding patients to personalized treatment recommendations (Vozikis et al., 2016). However, the available clinical and financial data are still very limited, and as such, more reliable economic evaluation studies are urgently required (Snyder et al., 2014).

Most economic evaluation studies deal with cohort studies, be they prospective or retrospective, where a group of patients is monitored over time with respect to their progression in relation to a particular disease or after exposure to a given drug or risk factor (dos Santos Silva, 1999; Song and Chung, 2011). In economic evaluation studies, most of the prospective cohort studies involve hypothetical cohorts, based on hypothetical/simulated patients. In such cases, the characteristics of hypothetical patients correspond to the characteristics of real patients taken from the literature or previous clinical trials (*46*)[1]. Moreover, primary data pertaining to treatment efficacy and the clinical progression of patients are computer-simulated. Based on these data, scientists can follow hypothetical cohorts of patients over time (*77*, *79*).

There are four types of economic evaluation study, depending upon the way in which the outcome is measured and evaluated, namely, cost-minimization analysis (CMA), cost-effectiveness analysis (CEA), cost-utility analysis (CUA), and cost-benefit analysis (CBA) (Veenstra et al., 2000; McFarland, 2014), where the outcomes are measured in monetary units, number of life years gained (LYs), or quality-adjusted life years (QALYs), respectively. As a result, different utility values may be measured for a specific health state (Prieto and Sacristán, 2003). CUA studies are considered to be of the utmost importance (Veenstra et al., 2000), although they are subject to significant constraints. Common limitations include difficulties in expressing test utility and in interpreting test results, given the lack of actual clinical utility data and the heterogeneity of patients' overall clinical features. As long as these limitations remain to be overcome, genomic tests are unlikely to be reimbursed from the public purse (Snyder et al., 2014). In spite of this, it is encouraging that the methodology of economic evaluation has improved considerably in recent years; at the same time, several solutions have been proposed in order to overcome the aforementioned constraints, such as conduct of sensitivity analysis and/or value of information analysis (Buchanan et al., 2013).

In economic evaluation analysis, cost is invariably monetized and is classified either as a direct or indirect medical cost, which is the cost associated with providing healthcare in order to deal with an illness or the cost related to the expenditure incurred by the patient and their family as a consequence of healthcare provision, respectively (Riewpaiboon, 2014). The cost of

[1]The in-text references in italics represent the identification number of the article included in the systematic review literature as shown in **Supplementary Table 1**.

genomic testing falls into the category of direct medical costs and is therefore always taken into account by health economists in such economic evaluation studies. The so-called societal cost comprises both the direct non-medical cost and the indirect cost, for which there are no official records, and which may help to explain why societal cost is never reimbursed from public funds. If measurement of the total cost of an intervention is based solely on direct medical cost data, the analysis is from the health-payer perspective. By contrast, if both the direct medical cost and the societal cost are considered, the analysis can be said to be from a societal perspective (Russell et al., 1999).

In most cases, the time period of disease progression exceeds the period of economic evaluation; thus, there is need for measurement of future costs and outcomes resulting from the application of the new intervention, which is accomplished using a variety of models such as decision trees and Markov models for short-term and long-term analysis, relevantly (Naimark et al., 1997).

If an innovative intervention (in this case, genome-guided) outweighs the conventional intervention on the basis of scientific effectiveness data, the decision as whether or not to adopt it depends on its overall cost. If the total estimated cost (including the economic benefits of reducing the incidence of the disease) is lower than that of the conventional intervention, the new intervention may be described as "cost-saving" (or dominant) and its adoption by the national healthcare system is highly recommended. By contrast, the new intervention is said to be "dominated" by the conventional intervention as long as the former is more costly and less effective; in this case, the new intervention should be overruled or put on hold. More frequently, inclusion of genomic testing in clinical practice leads to novel interventions that exceed the cost of their predecessors but also prevail in terms of overall effectiveness. For this reason, it is important that economic evaluation analyses are performed in order for society to decide whether the extra cost is "worth" paying (in order to reap the societal benefits of genomic testing). The decision to adopt a new intervention depends on the amount of money that society is willing to spend on each QALY gained (willingness-to-pay threshold). Whether the incremental cost-utility ratio (ICUR) of an intervention lies below or above this threshold determines the likelihood (or not) of being adopted by the national healthcare system (Bertram et al., 2016).

The willingness-to-pay threshold varies with respect to the country (e.g., $50,000–$100,000/QALY in the United States, £20,000–£30,000/QALY in the UK, etc.). (Stolk et al., 2004; Mccabe et al., 2008). In the absence of officially declared thresholds, health economists suggest a range of thresholds based on the medical literature and other official data, such as the per capita gross domestic product as suggested by the WHO (Marseille et al., 2014).

The Medicare program is the largest third-party payer that provides reimbursement for medical services on behalf of US citizens (De Lew, 2000). Eligible for Medicare coverage are US citizens aged 65 or older who have worked in the US and have paid payroll taxes, employees in government agencies, as well as people under the age of 65 with certified disability. Insurance coverage can also be provided through the spouse's work. The federal agency responsible for managing the Medicare program is the Centers for Medicare and Medicaid Services (CMS) and is responsible for concluding contracts with private insurance companies to serve as fiscal agents between healthcare service providers and the US government (De Lew, 2000).

Although genomic tests constitute a useful tool for the diagnosis and personalized treatment of genetic diseases, concerns about their validity and cost hinder their extensive use in clinical practice and, subsequently, reimbursement. Here, we have conducted a systematic review of scientific articles describing the economic evaluation of individualized interventions, with the aim of recording the important characteristics of economic evaluation analysis, as well as evaluating the different genomic tests in terms of both their cost and effectiveness. The main objective of this approach is to identify those interventions that are more likely to be reimbursed by national healthcare systems. Furthermore, we triangulated the regulatory guidance of the genome-guided interventions recorded in our study with the corresponding economic evaluation results and reimbursement policies of major programs, such as Medicare. Such a correlation should reveal the criteria that need to be met in order for a new therapeutic intervention to be broadly implemented in the clinic and then reimbursed.

METHODS

A systematic literature search was conducted using the PubMed database using the following search terms: "pricing and genomics," "pricing and personalized medicine," and "reimbursement and genomic tests." Using the aforementioned terms, a total of 2,843 publications were collected, from which 2,686 publications were not processed further, based on their title and/or their abstract. From the remaining 157 publications, the full text was obtained in order to assess their suitability in accordance with the main purpose of this review, based on the following criteria. Firstly, only publications that were published since 2006 were included. Secondly, every enquiry had to be an economic evaluation of a therapeutic strategy involving one or more genomic tests assessed in comparison to another more or less applied therapeutic strategy not involving genomic tests. Another essential inclusion criterion was the nature of the economic evaluation study, which had to have been appropriately conducted. Moreover, clear reference must have been made either to the trade name of the test or the gene(s) examined, as well as to the disease(s) for which the therapeutic recommendations were designed. Based on the above criteria, 64 publications were rejected, leaving 96 publications to be further evaluated in this systematic review (**Supplementary Table 1**). The overall search methodology is depicted in **Figure 1**.

The 96 scientific articles were examined in detail in order to gather all the available information relevant to the scope of the present study. More specifically, we collected information pertaining to the nature of the economic evaluation studies (**Supplementary Table 2**) and the corresponding individualized interventions (**Supplementary Table 3**), while collating

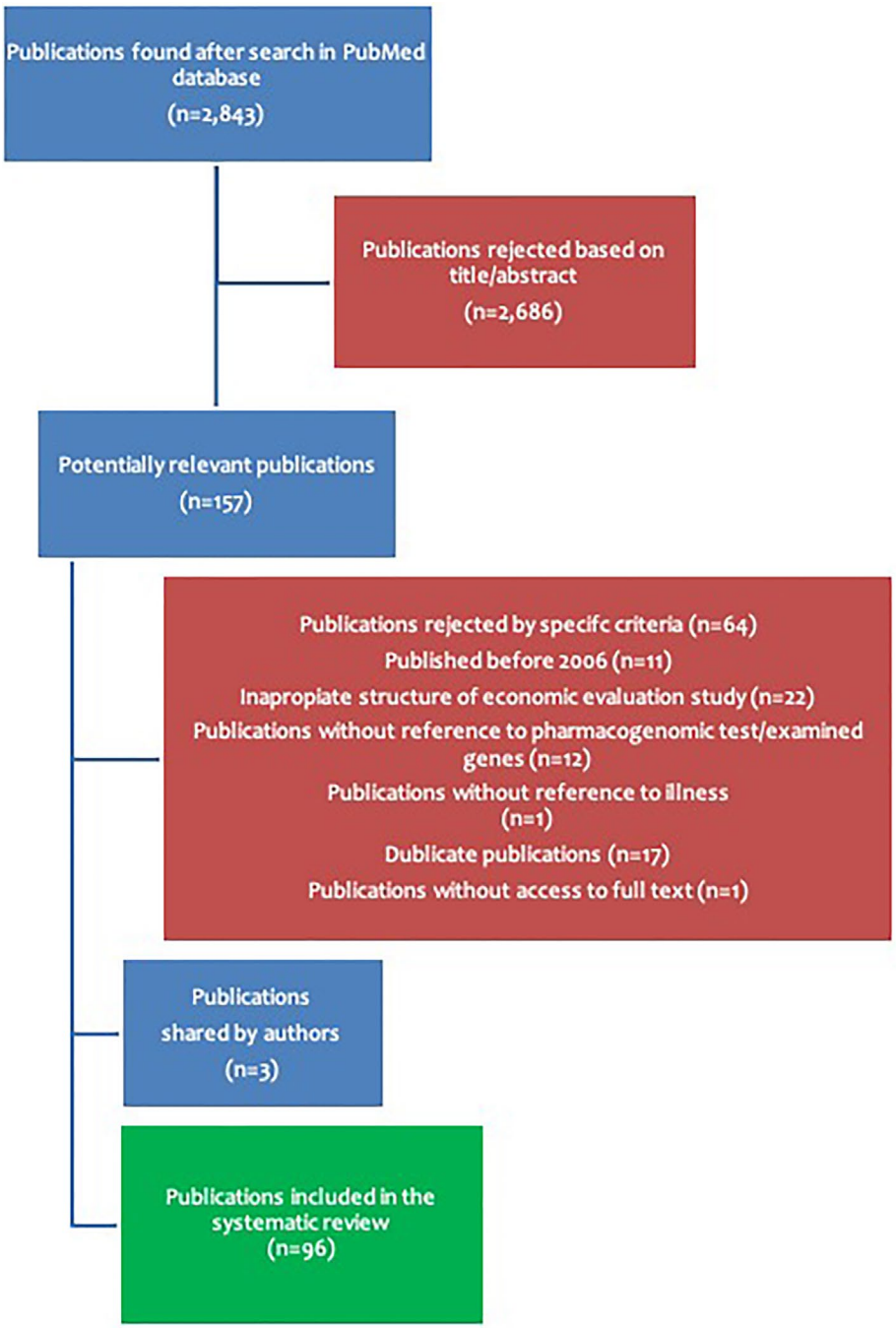

FIGURE 1 | Overview of the article querying methodology in the PubMed literature database.

quantitative data, such as the cost of the genomic tests, the overall cost of the new interventions and their corresponding cost-utility, and/or cost-effectiveness ratios (**Supplementary Table 4**). Our literature mining effort was enriched with further data on those genomic tests that are reimbursed by the Medicare program. Emphasis was placed on the Medicare program, as its reimbursement policies constitute basic principles for many private insurance payers (Ball, 1995).

We subsequently cross-correlated the genome-guided therapeutic interventions reported in our literature review with the corresponding guidance from the American regulatory body, the Food and Drug Administration (FDA), as well as the online Clinical Pharmacogenetics Implementation Consortium (CPIC, 2019) resource (https://cpicpgx.org). Of note, we collected the FDA-approved drug labels *via* the PharmGKB knowledgebase (PharmGKB, 2019). "PharmGKB" is an online knowledge

resource that integrates the pharmacogenomic information which is documented in the drug labels of major regulatory bodies, such as the FDA. In terms of the requirement to conduct a pharmacogenomic test before the administration of a given drug, pharmacogenomic testing may be classified as "required," "recommended," "actionable PGx," or "informative PGx" in order of decreasing necessity (https://www.pharmgkb.org/). As far as the CPIC® is concerned, it is an international consortium that provides freely available, evidence-based, and detailed gene/drug clinical practice guidelines in an effort to overcome the barrier of the implementation of pharmacogenomic testing in the routine clinical setting. CPIC has already published 21 guidelines covering 44 gene-drug pairs that have been meticulously curated and systematically updated. For each gene-drug pair, CPIC assigns a corresponding level ranging from A–D (in order of decreasing necessity). As far as the gene-drug correlations classified as CPIC level A are concerned, it is highly recommended that prescription of the affected drug should be changed with regard to the available genetic information, given that the strength of such a recommendation is of high or moderate importance. CPIC level B supports similar action in terms of clinical context while bearing in mind that alternative therapies/dosing are likely to be as effective and as safe as non-genetically based dosing (https://cpicpgx.org/).

RESULTS

In the present systematic review, we examined only economic evaluation studies published since 2006, the majority of which were published after 2010. More specifically, only 23 publications were published between 2006 and 2010, while 73 publications were published between 2010 and the present. The number of studies published per year is presented in **Figure 2A**. Moreover, the majority of the studies were performed either in a European country (34 studies) or in the USA (41 studies). The majority of the economic evaluation studies were conducted in two countries, namely, the USA (39 studies) and the United Kingdom (10 studies). The number of publications per country is presented in **Figure 2B**.

Qualitative Characteristics of Economic Evaluation Studies

Cohort Study and Economic Evaluation Model

In this systematic review, 83 prospective cohort studies and 13 retrospective cohort studies were identified. It should be noted that 80 of the 83 prospective studies were based on hypothetical cohorts of patients, while only three were based on real patients. The models used with regard to the measurement of future costs and outcomes are presented in **Figure 2C**.

Measurement of Outcome and Cost

In our systematic review, 27 studies employed CUA, 17 CEA, while only two adopted CMA. 40 publications employed a combination of CEA and CUA, whereas one was a combination of CEA and CMA. The remaining nine publications were simple economic analyses, as the interventions were evaluated taking into consideration only the total cost of each intervention.

Further, regarding the measurement of the outcome, in 44 publications, the outcome was measured in QALYs and in 12 publications in LYs, whereas in 23 publications, a combination of QALYs and LYs was preferred. In the vast majority of the publications included in this study (76 out of 96 studies), the researchers took into consideration only the direct medical cost when determining the total cost of individualized interventions. The applied methods of measuring the outcome and cost in all studies are presented in **Figure 3**.

Estimation of Cost-Effectiveness and Cost-Utility of Individualized Interventions

In our systematic review, we attempted to establish which interventions were likely to be adopted by each national healthcare system in their corresponding countries, based on the available willingness to pay threshold. The "cost-saving" interventions suggested to be adopted and reimbursed are shown in **Table 1**. Moreover, the interventions that were found to be either cost-effective or not, according to ICUR data, and the willingness to pay thresholds (suggested by authors) are presented in **Supplementary Tables 5A** and **Supplementary 5B,** respectively.

Regulatory Instructions and Economic Evaluation in Reimbursement Decisions

Subsequently, we aimed to cluster the pharmacogenomic correlations for which we have collected evidence from the Medicare program regarding the corresponding guidance and economic studies conducted (**Tables 2** and **3**). As may be readily assumed, those gene-drug correlations that have the strongest evidence to support the necessity of genetic testing, and that are accompanied by reliable cost-utility studies which prove the cost-effectiveness of pre-emptive genotyping, are those that are reimbursed. Abacavir-*HLA-B*5701* probably constitutes the best example of the concordance between scientific and economic data. Indeed, both the FDA and CPIC highlight the contraindication of this medication to carriers of the *HLA-B*5701* allele due to the high risk of hypersensitivity reactions (HSR) (Centers for Medicare & Medicaid Services, 2018; BlueCross Blueshield of Western New York, 2018; Quest Diagnostics, 2015; PGX Tests Determined to be Medicially Necessary for Medicare Coverage.; Local Coverage Determination (LCD)), while economic evaluation results with an ICUR of $36,700/QALY (lower than the $50,000/QALY threshold) justify its cost-effectiveness (*60*). Another noteworthy gene-drug correlation is the *TPMT*-azathioprine; pre-emptive genotyping (for the administration of this drug) is classified as level A by CPIC and "recommended" by the FDA, indicating that changes in dosing should be done with regard to the pharmacogenomic results. Apart from the aforementioned strong clinical evidence, adoption and reimbursement of *TPMT* testing (Local Coverage Determination (LCD); Blue Regence, 2019) are also supported by the results of economic evaluation, given that *TPMT* testing

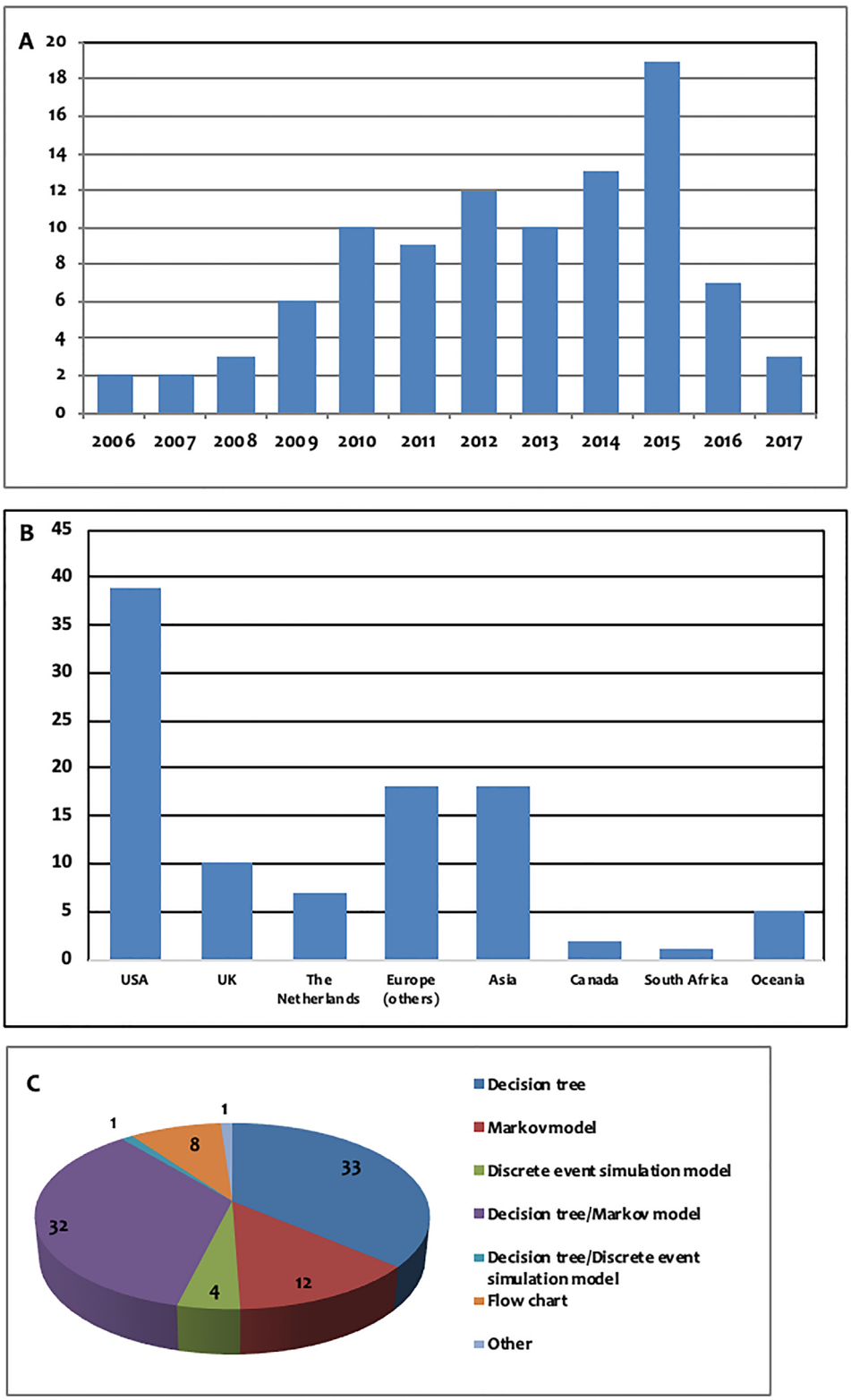

FIGURE 2 | (A) Number of publications per year, **(B)**: number of publications per continent/country: Europe (others): Austria (*n* = 1), France (*n* = 2), Germany (*n* = 2), Denmark (*n* = 1), Switzerland (*n* = 3), Sweden (*n* = 1), Spain (*n* = 5), Italy (*n* = 1), Croatia (*n* = 1), Serbia (*n* = 1), Asia: China (*n* = 5), Japan (*n* = 3), South Korea (*n* = 1), Singapore (*n* = 4), Thailand (*n* = 3), Oceania: Australia (*n* = 3), New Zealand (*n* = 2), **(C)**: models used for measurement of future costs and outcomes.

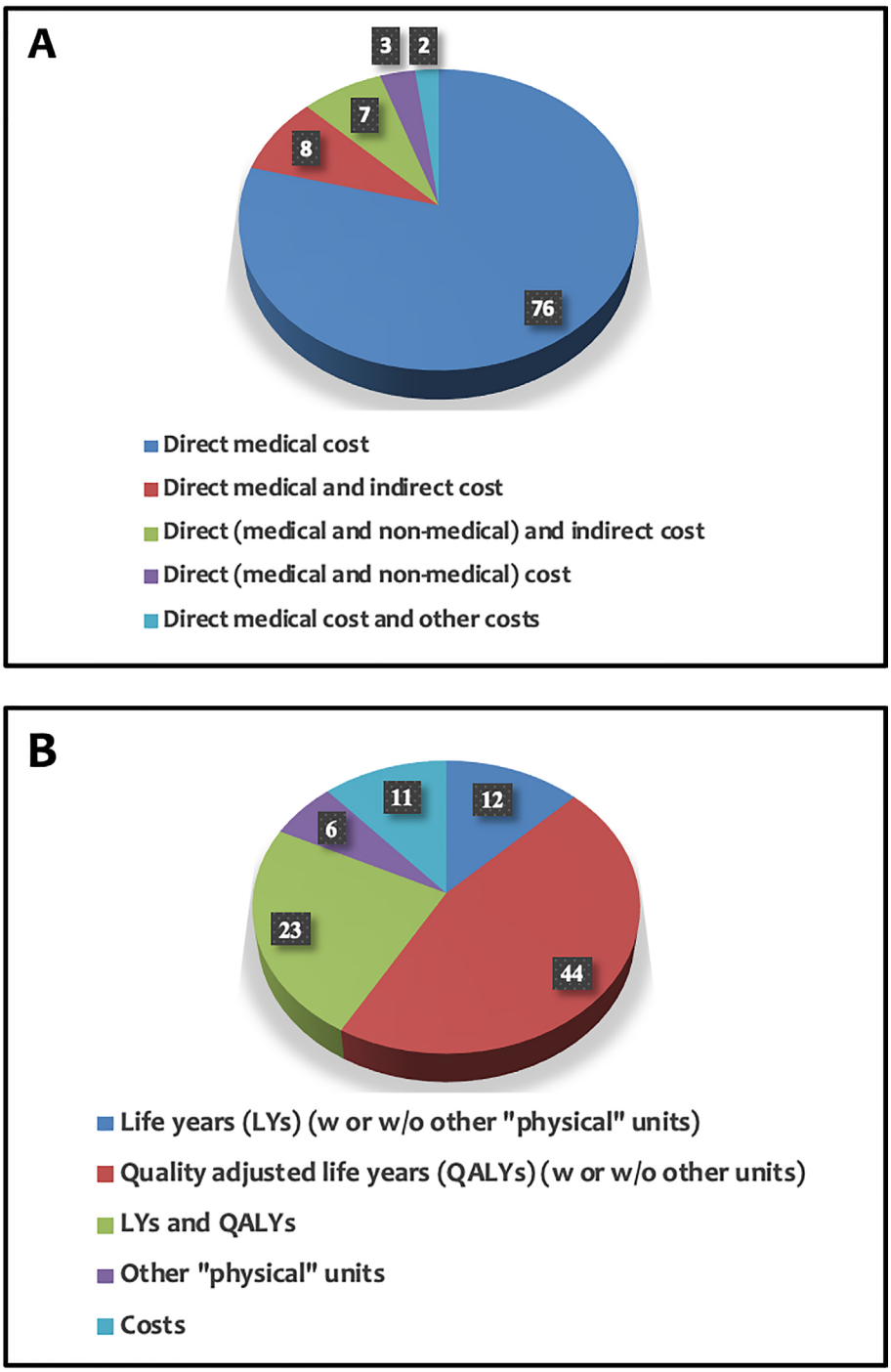

FIGURE 3 | Measurement of cost **(A)** and outcome **(B)** in the articles analyzed within this study.

in idiopathic pulmonary fibrosis prior to azathioprine treatment has an ICUR of $49,156/QALY, below the US $50,000/QALY threshold (*27*).

In our literature mining effort, we also identified pharmacogenomic correlations with variable economic results. Regarding the cost-effectiveness of *HLA-B*1502* genotyping in relation to carbamazepine or phenytoin treatment, the results differ; some of them demonstrate that pharmacogenomic testing is cost-effective, whereas others do not. However, it is noteworthy that carbamazepine has a boxed warning on its FDA drug label stating that screening for *HLA-B*1502* allele is required to be carried out prior to treatment in patients

TABLE 1 | Cost and number of quality-adjusted life years (QALYs) of "dominant" individualized interventions.

Disease	Gene	Cost of intervention		Number of QALYS		Reference++
		W/o PGx test/test 1	With PGx test/test 2	W/o PGx test/ test 1	With PGx test/test 2	
Advanced adenocarcinoma of the lung	*EGFR*	SG$47,100	SG$44,700	0.87	0.91	(Lee et al., 2014)
Colorectal cancer	*KRAS*	¥3,160,000($35,000)	¥2,600,000 ($29,000)	0.48	0.49	(Sun et al., 2013)
	UGT1A1	$13,058	$12,786	1.6347	1.6349	(*54*)
Acute coronary syndrome	*CYP2C19*	$15,800	$14,900	0.966	0.9665	(Jonsson, 2009)
		NZ$85,342	NZ$84,646	8.544	8.650	(Hess et al., 2015)
		$76,906* (CLO) $78,296 (P2Y12)	$76,450	7.4381	7.5301	(*47*)
Neonatal diabetes	*KCNJ11, ABCC8*	$71,784	$59,256	16.29	16.99	(Song and Chung, 2011)
Atrial fibrillation	*4q25*	Cost saving of $250,689		Net gain of 8.8		(Riewpaiboon, 2014)
Familial adenomatous polyposis	*APC*	€13,928.82 (Spain)	€8,038.93	19.92	19.93	(*65*)
Neovascular macular degeneration	*CFH, ARMS2/ HTRA1, C3, C2, CFB*	Cost saving of $493		Gain of 0.0392		(*78*)
Venous thromboembolism	Thrombo inCode®	€1,366.30–€2,795.61 (Spain)	€832.58–€848.38	8.2586–8.4784	8.5871–8.5874	(*61*)
Breast cancer	MammaPrint®	€17,869+ (Spain)	€16,989	18.131	18.357	(Snyder et al., 2014)
		$27,882+	$21,598	7.364	7.461	(*53*)

Currency used is different from the currency of the country in which the corresponding economic evaluation study was carried out. Researchers chose to express costs in US $ ($). +Cost of individualized intervention including Oncotype DX®. PRA, prasugrel; CLO, clopidogrel; P2Y12, other P2Y12 inhibitor (individualized interventions including prasugrel, clopidogrel and other P2Y12 inhibitors, respectively). ++Identification number of the article (Supplementary Table 1**).*

TABLE 2 | Pharmacogenomic tests covered by Medicare.

Drug	Allele	FDA	CPIC	ICUR	Willingness-to-pay threshold	Type	Reference++
Abacavir	*HLA-B*5701*	Required	A	$36,700/QALY	$50,000–$100,000/QALY	CE	(*60*)
				–	–	DT	(*64*)
Azathioprine	*TPMT*	Recommended	A	$49,156/QALY	$50,000/QALY	CE	(BlueCross Blueshield of Western New York, 2018)
Carbamazepine	*HLA-B*1502*[1]	Required	A	$85,697/QALY	$50,000/QALY	Not CE	(Stolk et al., 2004)
				$29,750/QALY	$50,000/QALY	CE	(De Lew, 2000)
Cetuximab	*KRAS*	Required	–	–	–	Cost saving, same effectiveness	(*44*)
Clopidogrel	*CYP2C19*[2]	Actionable	A	–	–	Dominant	(Jonsson, 2009, *47*)
				$4,200/QALY	$100,000/QALY	CE	(Meckley and Neumann, 2010)
Crizotinib	*ALK*	Required	–	$136,000/QALY	$200,000/QALY	CE	(Blue Regence, 2019)
Erlotinib	*EGFR*	Required	–				
Erlotinib	*EGFR*	Required	–	$110,658/QALY	$100,000/QALY	Not CE	(*86*)
				$162,018/QALY	$150,000/QALY	Not CE	(*63*)
Panitumumab	*KRAS*	Required	–	–	–	Cost saving, same effectiveness	(*44*)
Phenytoin	*HLA-B*1502*[1]	Actionable	A	$85,697/QALY	$50,000/QALY	Not CE	(Stolk et al., 2004)
				$29,750/QALY	$50,000/QALY	CE	(De Lew, 2000)
Trastuzumab	*ERBB2 (HER-2)*	Required	–	–	–		
Vemurafenib	*BRAF*	Required	–	–	–		

*[1]HLA-B*1502 is regarded medically necessary and thus reimbursed only for patients of Asian ancestry. [2]CYP2C19 testing is regarded as medically necessary only for patients with ACS undergoing PCI who are initiating or reinitiating Clopidogrel therapy. CE, Cost-effective; DT, Dominant. ++Identification number of the article (**Supplementary Table 1**).*

that are genetically at-risk due to the high risk of serious and sometimes fatal dermatological reactions. CPIC guidance concurs with that of the FDA, strongly recommending the use of an alternative drug in case patients are *HLA-B*1502* carriers and carbamazepine-naïve. Similarly, both on the FDA phenytoin label and in the corresponding CPIC guideline, it is documented that consideration should be given to avoiding phenytoin as an alternative for carbamazepine in patients positive for

TABLE 3 | Genomic tests covered by Medicare.

Allele	FDA	CPIC	ICUR	Willingness-to-pay threshold	Type	Reference[++]
MMRgenes	–	–	$26,000/QALY	$50,000/QALY	CE	(*91*)
BRCA1/2[3]	–	–	$9,000/QALY	$50,000/QALY	CE	(Gavan et al., 2018)

*[3]BRCA1/2 testing is regarded as medically necessary only for patients with breast cancer and healthy individuals with a family history of breast cancer. [++]Identification number of the article (**Supplementary Table 1**).*

*HLA-B*1502*. Therefore, even though the economic results cannot lead to a definite conclusion as far as the reimbursement policy that should be applied, the clinical evidence that lies behind these pharmacogenomic correlations supports the broad clinical adoption of pre-emptive testing in patients of Asian ancestry (BlueCross Blueshield of Western New York, 2018; IGNITE Implementing GeNomics In practice, 2018; Local Coverage Determination (LCD)).

With the exception of those gene-drug correlations whose economic data are inconclusive, there are also pharmacogenomic biomarkers whose economic benefits remain to be assessed or whose economic results clearly discourage reimbursement. According to the FDA drug label, erlotinib is a tyrosine kinase inhibitor indicated for first-line treatment of patients with metastatic non-small cell lung cancer (NSCLC), whose tumors are characterized by epidermal growth factor receptor (*EGFR*) exon 19 deletions or exon 21 (p.L858R) missense mutations. The FDA indicates that patients being considered for erlotinib treatment should first be tested for the aforementioned mutations by means of an FDA-approved test as neither the safety nor the efficacy of erlotinib have been established in NSCLC patients whose tumors have other *EGFR* mutations. *EGFR* testing is part of the Medicare reimbursement fee schedule (Centers for Medicare & Medicaid Services, 2018; PGX Tests Determined to be Medically Necessary for Medicare Coverage; Local Coverage Determination (LCD); Hess et al., 2015), despite the fact that no economic evaluation analysis has been performed which shows that testing for over-expression of *EGFR* prior to erlotinib treatment can be cost-effective; in all analyses, the new interventions exceed the $100,000/QALY threshold (*63*, *86*). However, the decision to allow reimbursement appears to have been influenced by strong clinical evidence documented on the FDA drug label, suggesting that testing for overexpression of EGFR contributes to achieving an optimal therapeutic effect in both lung and colon cancers. The example of vemurafenib-*BRAF* should also be mentioned, as this pharmacogenomic test is also reimbursed by Medicare (Centers for Medicare & Medicaid Services, 2018; IGNITE Implementing GeNomics In practice, 2018; Local Coverage Determination (LCD); Hess et al., 2015) even in the absence of economic evaluation studies. Based on the mechanism of action of this tyrosine kinase inhibitor, the FDA drug label states that vemurafenib is only indicated for the treatment of patients with unresectable or metastatic melanoma with *BRAF* p.V600E mutation as detected by an FDA-approved test.

As far as genomic tests are concerned, we aimed to cross-correlate the Medicare reimbursement policies with results from cost-utility analyses from our systematic review. In general, Medicare covers genomic tests that are regarded as medically necessary by the Centers of Medicare & Medicaid Services. More specifically, screening for *MMR* variants in colorectal tumors is regarded as medically necessary only for colorectal cancer patients (and then only for whose family members meet specific criteria/the revised Bethesda guidelines) (Local Coverage Determination (LCD)). Precautionary *MMR* testing in individuals who are at-risk of developing colorectal cancer and/or Lynch syndrome is considered experimental and hence not medically necessary. It is encouraging though that there are economic evaluation results which suggest that precautionary testing for *MMR* mutations in unaffected individuals with a family history of colorectal cancer is cost-effective, with an ICUR of $26,000/QALY (*91*). Another example is testing for *BRCA1/2* genes, which constitute well-established pharmacogenomic biomarkers for breast cancer, as specific mutations in these genes have been associated with a greatly increased risk of developing breast cancer. *BRCA1/2* testing is covered mostly for affected individuals with a family history of breast cancer and occasionally for healthy individuals with suspected breast cancer and/or breast cancer history (Centers for Medicare & Medicaid Services, 2018; Local Coverage Determination (LCD); Beattie et al., 2012; Meckley and Neumann, 2010). However, economic evaluation results indicate that precautionary testing for *BRCA1/2* is cost-effective with an ICUR of $9,000/QALY) even in healthy/unaffected women with a family risk of breast cancer (*36*).

DISCUSSION

Personalized medicine targets health care interventions to subgroups of patients, who share specific biological and genetic characteristics. The most commonly used applications are genomic tests, which dominate the era of personalized medicine and, thus, constitute the main focus of this study, as far as their pricing and reimbursement are concerned. Pre-emptive genotyping leads to new individualized drug treatment interventions, where the appropriate drug is administered to each patient in an effort to minimize the incidence of drug toxicity or lack of efficacy. Hence, diseases are treated more effectively, while the quality of the patient's life improves. At the same time, national healthcare systems benefit from the expected reduction in expenditure on unnecessary medical procedures and/or the hospitalization of patients suffering from adverse drug reactions resulting from inadequate therapies. However, it should be noted that

personalized medicine also includes other forms of applications, such as algorithm-based prescribing, population-based screening programs etc., which were not taken into consideration in the present study (Gavan et al., 2018; Vizirianakis et al., 2019).

The heterogeneity of patients' specific characteristics (phenotype) due to genome-variants leads to the heterogeneity in patients' drug treatment response and/or development of adverse drug reactions. Researchers consider this patient-level heterogeneity, while conducting economic evaluation analysis, as it affects both total treatment costs and outcomes (Gavan et al., 2018). This is why many national health care agencies, for instance the National Institute for Health and Care Excellence (NICE) for England and Wales, suggest that sub-group analyses should be conducted in order to make decisions about implementation of new health technologies (including genomic tests) in clinical practice (Espinoza et al., 2014).

Genomic tests are also directed to particular populations, for example, patients suffering from rare diseases that cannot be easily treated with conventional interventions. Indicatively, in our systematic analysis, there were studies, in which pre-emptive genotyping for the diagnosis of rare diseases (hypertrophic cardiomyopathy, Cowden syndrome, neovascular macular degeneration, neurofibromatosis etc.) was evaluated. Moreover, there are applications of genomic tests even in unborn children. In our systematic review, we identified studies evaluating prenatal screening for spinal muscular atrophy, cystic fibrosis, and X-linked hemophilia.

Two essential parameters emerge as fundamental preconditions for a pharmacogenomic test to be broadly adopted in the clinic and for it to be reimbursed: concrete evidence of the relevant gene-drug correlation, as well as favorable economic evaluation results. As implied by our literature review, the strong classification of the pharmacogenomic information by the CPIC and/or FDA guidance constitutes a key factor in reimbursement decision making, even in the absence of favorable economic results as illustrated by the pharmacogenomic correlations of vemurafenib-*BRAF* and erlotinib-*EGFR*. Pre-emptive genotyping is often deemed crucial for a specific population, the so-called high-risk groups. In particular, treatment with carbamazepine is strongly associated with a high risk of developing Stevens-Johnson syndrome and toxic epidermal necrolysis (SJS/TEN) in carriers of the *HLA-B*1502* allele. Given that this inherited allelic variant is mainly observed in patients of Asian ancestry, the corresponding pharmacogenomic test is only reimbursed for patients with Asian ancestry (Centers for Medicare & Medicaid Services, 2018; Local Coverage Determination (LCD)). In parallel, although *BRCA1/2* constitutes a well-established genomic biomarker for the development of breast cancer, *BRCA1/2* genetic testing is considered medically necessary and, thus, uniquely covered for individuals with a family history of such a disease (Beattie et al., 2012).

As far as the economic evaluation results are concerned, it is of paramount importance that the pharmacogenomic tests are proven to be either "cost-saving" or at least cost-effective in economic terms, in other words, that the proposed therapeutic intervention is also cost-saving, apart from being more effective, than the already established one. Given that the inclusion of pre-emptive genotyping often increases the cost of the therapeutic recommendation, the eventual decision depends upon the willingness-to-pay thresholds, which serve to ensure the affordability of the new interventions. An indicative example of just such a cost-effective pharmacogenomic test which meets the aforementioned criteria is *HLA-B*5701* genotyping prior to the initiation of abacavir treatment in HIV patients. More specifically, the agreement of both FDA and CPIC about the necessity of pre-emptive *HLA-B*5701* genotyping, as well as the favorable economic evaluation results ($36,700/QALY at a willingness-to-pay threshold of $50,000/QALY), justifies the reimbursement of this pharmacogenomic test by Medicare (*60*).

In our study, we focused on the US Medicare program, as there are limited data regarding reimbursement of genomic testing in countries of the European or Asian region. More specifically, as far as the European Union is concerned, each member state has a different reimbursement policy, as each country spends a different amount of budget on the health sector. In addition, there is a different percentage of private and public insurance contribution in reimbursement of healthcare services. Some countries have approved reimbursement exclusively from public or private insurance funds, while others from a combination of them. As a result, there is no uniform regulatory framework providing precise instructions and provisions on the conditions and the exact procedure for reimbursement of genomic tests from the public funds (Vozikis et al., 2016).

Most economic evaluation studies in our systematic review were cost-utility analyses. However, the credibility of economic evaluation analysis is negatively affected by the lack of actual clinical utility data from genomic testing in real patients (Snyder et al., 2014). Indeed, 80 out of 96 studies in our systematic review were based on hypothetical cohorts, where hypothetical patients and simulated clinical data from older clinical trials were used. Furthermore, the use of retrospective cohorts also raises concerns about the quality of the results produced. There were 13 retrospective studies in our systematic review, which accounts for a significant proportion of the total number of publications. More specifically, a retrospective study design may be associated with poorer data quality, as it is based on data from healthcare databases that have already been collected. This increases the risk of selection bias, which refers to the selection of inappropriate individuals that are unrepresentative of the population that researchers wish to study. Moreover, inaccurate or incomplete recollections from the past of the cohort's individuals (recall bias) may also lead to questionable economic evaluation results.

Elaborating more on the economic evaluation method, economic evaluation from a societal perspective is considered more complete and more reliable, in comparison to the corresponding analysis from the health-payer perspective (Jonsson, 2009). It is well known that due to the lack of official societal cost recordings and the general targeting of reimbursement programs in the so-called direct medical cost, most studies are orientated from the health-payer perspective. This tendency, which is also confirmed by our systematic review results, indicates the need for more cost-utility analyses from the societal perspective, in order to allow optimal (societal) decisions to be made. It is encouraging that the methodology of economic evaluation has improved significantly with the development of

statistical models that enable forecast of the interventions' overall cost and clinical effectiveness over time, providing even life-time analysis (Naimark et al., 1997; Payne et al., 2018). More specifically, in 31 studies covered in our systematic review, both short-term and long-term analyses were achieved using a combination of decision trees and Markov models. Taking into account 11 additional studies in which only Markov models were used, long-term analysis was achieved in almost half of the studies under this systematic review.

In model-based economic evaluation analyses, uncertainty may also arise because of difficulties in estimating the true value of varying parameters (variables), which are used in the aforementioned models. Such variables usually constitute the cost of health care interventions or the age of patients. The models used in economic evaluation offer the opportunity to estimate the impact of parameter uncertainty using probabilistic sensitivity analysis. Taking into consideration the potentiality of long-term analysis of incremental costs and outcomes, it is of no doubt that model-based economic evaluation constitutes the preferred approach in decision making (Payne et al., 2018).

Conclusions and Future Perspectives

National healthcare systems are often unable to cope with the ever-increasing social needs for high-quality healthcare service provision. Over the decades, the rapid growth of the population, the increasing economic resources of national economies, and the increasing cost of healthcare provision have led to a marked increase in the annual health expenditure in Western countries (McFarland, 2014). However, funding for the public health sector has decreased since the 2008 financial crisis, and as a result, qualitative selection among different medical interventions has to be made.

In relation to the role of economic evaluation in public health policy-making, there is an urgent need for the establishment of national policies that favor the conduct of economic evaluation in state-owned research institutes and universities, as well as in the private sector. It is encouraging that in recent years, the number of published economic evaluation analyses in the field of genomic and personalized medicine has continued to increase. This tendency accords with our systematic review results, which highlight the increasing number of publications since 2011. However, and in accordance with our findings from this study, more cost-utility analyses should be conducted in various countries in order to cover as many populations and ethnic groups as possible. By contrast, only a few relevant analyses have been conducted in Asian and African countries. Given the high frequency of high-risk and actionable alleles in Asian and African populations, it might reasonably be expected that researchers would be especially interested in economic evaluation of genomic testing in these countries. Taking into consideration the recorded mortality rates in low income Asian and African countries, pharmacogenomic research would contribute to the mitigation/prevention of global health inequalities.

Another crucial issue to be investigated is the economic thresholds. Based on our findings, there are no strictly defined willingness-to-pay thresholds even for a specific country's national health system. Given existing social inequalities, health economists suggest that the commonly used thresholds should be expanded. The lack of concordance between the budgetary capability of the national health systems and the needs of local societies have led to the use of expanded willingness-to-pay thresholds suggested by health economists (Eichler et al., 2004; Neumann et al., 2014). In other words, it is likely that the persistence in strictly defined economic thresholds could lead to a fruitless controversy between public health providers and specific social groups or patients, while underestimating the scientifically proven clinical utility of genomic testing. Moreover, the interventions under evaluation are developed against diseases, which differ in terms of their severity, their pathophysiological mechanisms, and, consequently, their treatment regimens and cost. As a result, apart from social inequalities and other socio-economic factors, it would be scientifically inappropriate to use a strictly defined threshold for universal assessment of dissimilar interventions.

Many studies considered in this systematic review concluded that genomic-guided treatment may represent a cost-saving or cost-effective strategy against various diseases, including different types of cancer. Many of these strategies include genomic tests that are reimbursed by the US Medicare program, which is indicative of the leading role of economic evaluation results in determining reimbursement policymaking. Unsurprisingly, most of the non-cost-effective interventions are not covered by Medicare. Apart from the unfavorable economic evaluation results, which clearly do not provide a cogent argument for reimbursement, the decision not to cover the cost of the relevant genomic tests is mainly attributed to insufficient evidence supporting their clinical utility (Hess et al., 2015; Local Coverage Article: MolDX). It should be mentioned that this claim is more aligned with FDA regulations than with CPIC guidance.

Furthermore, our systematic review results emphasize the wide range of potential genomic testing applications, including interventions against colorectal and breast cancer, as well as acute coronary syndrome, cardiovascular disease, neonatal diabetes, and macular degeneration (**Table 1, Supplementary Table 5A**). Additional analyses could usefully be performed in order to enrich the already favorable economic evaluation data, in an effort to ensure positive reimbursement decisions by the national healthcare systems.

Last, but not least, the adoption of an appropriate universal legal framework is deemed necessary in order to determine the appropriate conditions for reimbursement of clinically valid tests. It should be noted that a basic precondition for achieving this goal is the foundation of a stable, effective, and transparent pricing system to avoid overpricing.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript/supplementary files.

AUTHOR CONTRIBUTIONS

GP and CM conceived the study, AV, SS and SK conducted the analysis. DC, CM and GP commented on the results. All authors have written, 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/fphar.2019.00830/full#supplementary-material

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ITPA, *TPMT*, and *NUDT15* Genetic Polymorphisms Predict 6-Mercaptopurine Toxicity in Middle Eastern Children With Acute Lymphoblastic Leukemia

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Background: Acute lymphoblastic leukemia (ALL) is the most common cancer seen in children worldwide and in the Middle East. Although there have been major advances in treatment approaches for childhood ALL, serious toxicities do occur but with significant inter-individual variability. The aim of this study is to measure the frequency of polymorphisms in candidate genes involved in 6-Mercaptopurine (6-MP) disposition in a combined cohort of Middle Eastern Children with ALL, and evaluate whether these polymorphisms predict 6-MP intolerance and toxicity during ALL maintenance therapy.

Methods: The study includes children treated for ALL on two treatment protocols from two cohorts; one from Lebanon (N = 136) and another from Kurdistan province of Iran (N = 74). Genotyping for the following six candidate genetic polymorphisms: *ITPA 94C > A* (rs1127354) and *IVS2+21A > C* (rs7270101), *TPMT*2 238G > C* (rs1800462), *TPMT*3B 460G > A* (rs1800460) and **3C 719A > G* (rs1142345), and *NUDT15 415C > T* (rs116855232) was performed and analyzed in association with 6-MP dose intensity and toxicity.

Results: As expected, *TPMT* and *NUDT15* variants were uncommon. As for *ITPA*, both polymorphisms were more common in the Lebanese as compared to the Kurdish cohort with a minor allele frequency of 0.05 for *94C > A* and 0.14 for *IVS2+21A > C* in the Lebanese only (N = 121), and of 0.01 for either *ITPA* polymorphism in Kurds. The most significant toxic effects were depicted with the *NUDT15* polymorphism with a median 6-MP dose intensity of 33.33%, followed by 46.65% for *TPMT*3A* polymorphism, followed by 65.33% for two *ITPA* risk allele carriers and 74% for one *ITPA* risk allele carriers, in comparison to a median of 100% for the homozygous wild type in the combined cohort

($P < 0.001$). In addition, the onset of febrile neutropenia was significantly higher in variant allele carriers in the combined cohorts.

Conclusions: These data confirm the predictive role of *TPMT*, *NUDT15*, and *ITPA* in 6-MP intolerance in Middle Eastern children with ALL. Given the relatively high frequency of *ITPA* variants in our study and their significant association with 6-MP dose intensity, we recommend that physicians consider genotyping for *ITPA* variants in conjunction with *TPMT* and *NUDT15* prior to 6-MP therapy in these children.

Keywords: acute lymphoblastic leukemia, pharmacogenetics, *ITPA*, *TPMT*, *NUDT15*

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common cancer seen in children worldwide and in the Middle East (Dabbous et al., 2003; An et al., 2017). Although there have been major advances in treatment approaches for childhood ALL, serious toxicities such as profound leukopenia frequently affect treatment and lead to life threatening consequences such as severe infections and even death (Muwakkit et al., 2012; An et al., 2017).

There are currently a number of treatment protocols for childhood ALL, almost all of which entail combination chemotherapy administered in three phases: induction, consolidation with or without re-induction and maintenance (Kato and Manabe, 2018). 6-Mercaptopurine (6-MP) is concomitantly given with Methotrexate (MTX) during consolidation and maintenance. It is a purine antimetabolite, and it is frequently associated with life threatening myelosupression, though with major individual variability (Al-Mahayri et al., 2017; Maxwell and Cole, 2017; Rudin et al., 2017; Koutsilieri et al., 2019).

Driven by this inter-individual variability, a number of investigators have extensively evaluated germline pharmacogenetic (PGx) markers with a focus on candidate pharmacokinetic and pharmacodynamic targets to predict 6-MP toxicity (Al-Mahayri et al., 2017; Maxwell and Cole, 2017; Rudin et al., 2017; Koutsilieri et al., 2019). The oldest and most robust evidence is currently for genetic variants in thiopurine-S-methyltransferase (TPMT), an enzyme that inactivates the drug. For instance, testing for specific decreased enzyme function polymorphisms prior to therapy, mainly *TPMT*2*, **3A*, **3B,* and **3C* has been included in several clinical guidelines and drug labels (PharmGKB, 2016; PharmGKB, 2018a). More recently, a low function variant in nucleoside diphosphate-linked moiety X motif (NUDT15) was also shown to be associated with decreased thiopurine metabolism (Yang et al., 2015) and, similarly to *TPMT* polymorphisms, it was clinically annotated as a level 1A variant by the pharmgkb (PharmGKB, 2018b).

These alleles are, however, limited by being relatively uncommon and sometimes confined to specific populations or ethnicities. For example, the *NUDT15* variant is rare in Europeans and most common in Asians and Hispanics (Moriyama et al., 2017; Zhou et al., 2018). In addition in the Middle East, we have shown that, although these *TPMT* and *NUDT15* variants are associated with significant 6-MP intolerance, they are also quite uncommon (Zgheib et al., 2017). Therefore, the contribution and ethnic variability of polymorphisms in other genes remains an important and active field of research.

An enzyme that is gaining momentum in the PGx of 6-MP is the inosine triphosphate (ITPA) (Simone et al., 2013). Several studies examined the role of essentially two variants in the *ITPA* gene (*94C > A* and *IVS2+21A A > C*) with 6-MP metabolism (Stocco et al., 2009), as well as toxicity in patients with inflammatory bowel disease (Zelinkova et al., 2006; Ansari et al., 2008; Ban et al., 2010) and children with leukemias of various ethnicities (Adam de et al., 2011; Chiengthong et al., 2016; Milosevic et al., 2018; Zhou et al., 2018; Khera et al., 2019), with promising results. To our knowledge, no data are yet available on the prevalence and role of *ITPA* genetic polymorphisms with 6-MP toxicity in Middle Eastern populations except for one from Turkey, though with a very small sample size and negative results (Eldem et al., 2018). In addition, although there are few reports on the frequency of *TPMT* polymorphisms and their association with 6-MP from this area of the world (Hakooz et al., 2010; Albayrak et al., 2011; Bahrehmand et al., 2017), *NUDT15* was only recently evaluated in our Lebanese cohort (Zgheib et al., 2017).

The aim of this study is to measure the frequency of polymorphisms in candidate genes involved in 6-MP disposition in a combined cohort of Middle Eastern Children with ALL, and evaluate whether these polymorphisms predict 6-MP intolerance and toxicity during ALL maintenance therapy.

METHODS

This study includes children treated for ALL on two treatment protocols from two cohorts; one from Lebanon and another from Kurdistan. Access to clinical data and collection of peripheral blood for DNA isolation was approved by the respective Institutional Review Boards (IRBs), and all subjects and parents signed an informed consent or assent, as applicable.

Patients and Data Collection

Lebanon

This study builds on a previously described cohort of children treated at the Children's Cancer Center of Lebanon for ALL. Subjects were recruited between 2010 and 2013 (Zgheib et al., 2014; Zgheib et al., 2017; Zgheib et al., 2018), the majority of whom received and finished treatment as per the St Jude

Children's Research Hospital (SJCRH) protocol TOTAL XV (Muwakkit et al., 2012). This protocol consists of an induction followed by consolidation therapy, then a maintenance phase that lasts up to 120 weeks for girls and 143 weeks for boys. The first 20 weeks of maintenance include 2 re-inductions between weeks 7 and 9 and between weeks 17 and 20. During weeks 20 till 100 of maintenance, low risk patients receive 6-MP and MTX with pulses of Dexamethasone, Vincristine, and MTX every 4 weeks. Patients with intermediate and high risk disease receive three rotating drug pairs as such: 2 weeks of 6-MP and MTX, 1 week of Dexamethasone plus Vincristine, and 1 week of Cyclophosphamide and Cytarabine every 28 days. After week 100, only weekly MTX and daily 6-MP are given with dosages being adjusted according to tolerance.

Retrospective chart review was performed for baseline characteristics and treatment information. Specifically for this study, the 6-MP dose intensity (%) was computed as the ratio of the final 6-MP dose to that of the prescribed 6-MP maintenance dose as per protocol (75 mg/m^2/day). The 6-MP dose are adjusted so as to maintain the white blood cell count between 1,500 and 3,000 per µl, the absolute neutrophil count (ANC) more than 300 per µl and the platelet count more than 50,000 per µl. Data were also collected on whether patients were admitted for febrile neutropenia during maintenance. In addition, the highest direct bilirubin values reached after week 100 of the maintenance phase were recorded, with hepatotoxicity defined as a value of more or equal to 1.5 mg/dl, a value that is clinically relevant.

Data on the role of *TPMT* and *NUDT15* genetic polymorphisms with 6-MP dose intensity during maintenance therapy were previously published (Zgheib et al., 2017), and this study adds data on the contribution of two polymorphisms in the *ITPA* gene.

Kurdistan

Seventy-four children with ALL were recruited between 2012 and 2018 at the Besat Hospital, Kurdistan University of Medical Sciences and Health Services, Sanandaj, Kurdistan. All recruited patients were uniformly treated according to the COG protocol (Carroll and Bhatla, 2016) and completed treatment. Similarly to the SJCRH protocol, treatment with the COG protocol starts with an induction phase followed by consolidation and maintenance. During maintenance, patients receive the same starting dose of weekly MTX (20 mg/m^2) and daily 6-MP (75 mg/m^2) until the end of therapy, accompanied by Vincristine and Prednisone or Dexamethasone pulses every 28 days until the end of maintenance phase. The dose of 6-MP and MTX are adjusted in order to obtain WBC between 2,000–3,000/µl and the ANC more than 500/µl. As such, the doses of 6-MP and MTX are reduced by 25% each time the WBC count is less than 2,000/µl in each visit during therapy.

Retrospective chart review was performed for baseline characteristics and treatment information. Specifically for this study, the 6-MP dose intensity (%) was computed as the ratio of the final 6-MP dose reached during maintenance therapy to maintain the WBC between 2,000 and 3,000 per µl and the ANC more than 500 per µl to that of the prescribed 6-MP maintenance dose as per protocol (75 mg/m^2/day). Data were also collected on whether patients were admitted for febrile neutropenia during maintenance. In addition, the highest SGPT/ALT values reached during the maintenance phase were recorded, with hepatotoxicity defined as values at least three times higher than the upper normal limit.

Genotyping

This study entails genotyping for the following six candidate genetic polymorphisms: *ITPA 94C > A* (rs1127354) and *IVS2+21A > C* (rs7270101), *TPMT*2 238G > C* (rs1800462), *TPMT*3B 460G > A* (rs1800460) and **3C 719A > G* (rs1142345) with *TPMT*3A* being the combination of the *TPMT*3B* and *TPMT*3C* genotypes, and *NUDT15 415C > T* (rs116855232).

Lebanon

Genomic DNA was isolated from 150 µl peripheral blood using the QIAmp Blood MINI kit from Qiagen (Germantown, MD, USA) and stored at -20°C until analysis. Genotyping for the three *TPMT* polymorphisms was performed using light SNP kits on a Lightcycler from Roche (Roche Diagnostics, Switzerland). The *NUDT15* polymorphism and the two *ITPA* variants were measured using TaqMan® allele discrimination kits (Thermofisher, Waltham, MA, USA) on a CFX384 real-time PCR instrument from Biorad (Hercules, CA, USA). Ten percent of the samples were genotyped twice for reproducibility.

Kurdistan

Genomic DNA was isolated from 300 µl peripheral blood using a commercial kit for isolation of DNA (GeneAll, Seoul, South Korea), according to the manufacturer instructions. Allele-specific PCR analysis was used to evaluate the genetic polymorphism in *TPMT* exon 5 (G238C; *TPMT*2* allele) using standard primer pairs published elsewhere (Yates et al., 1997). The exon 7 (G460A; *TPMT* 3B* allele) and exon 10 (A719G; *TPMT* 3C* allele) polymorphisms were determined by PCR-RFLP analysis using MwoI (HpyF10VI) and AccI (XmiI) restriction enzymes (Yates et al., 1997). Exon 7 gave a PCR amplicon of 442 bp, which was not digested in the presence of a variant allele, whereas wild-type allele was digested and was seen as 224 and 114 bp fragments. The 337 bp PCR amplicon from wild-type exon 10 remained undigested after enzyme treatment, whereas the variant allele was digested and was seen as 283 and 90 bp fragments. For *NUDT15* genotyping, PCR-RFLP was used using and TaaI (HpyCH4III) restriction enzyme and the specific primers according to Fong et al (Fong et al., 2017). *NUDT15* wild-type gave a 191 bp PCR product which remained undigested after enzyme treatment, whereas the variant allele was digested to 122 and 69 bp fragments. A mismatch PCR-RFLP method was used for the amplification and detection of *ITPA 94C > A* and *ITPA IVS2+21A > C* using PdmI (XmnI) restriction endonuclease and specific primer pairs (Mollaahmadi et al., 18 A.D). The 94A > C variant allele was seen as an undigested amplicon of 256 bp, whereas the wild-type created fragments of 228 and 28 bp after digestion. The 204 bp amplicon of wild-type *ITPA IVS2+21A > C* allele was not digested, whereas the

IV2+21A > C variant was digested to 175 and 29 bp fragments. The PCR conditions for all above described experiments were as follows: an initial denaturation at 95˚C for 5 min followed by 35 cycles of 30 at 95˚C, 25 at specified annealing temperatures (58˚C for amplification of exon 5 and exon 7 of *TPMT*, 60˚C for amplification of exon 10 of *TPMT* and of *NUDT15*, and 50˚C for amplification of *ITPA*), 30 at 72˚C, and a final extension for 5 min at 72˚C. The PCR amplicons and RFLP products were electropherized and visualized on 3% agarose gel. Twenty percent of the samples including all variant genotypes were analyzed by Sanger sequencing, and results showed complete compatibility with amplification and enzyme digestion methods.

Statistical Analysis

Data were entered and analyzed in SPSS v.24 (IBM, North Castel, NY, USA). They are presented as mean ± SD, median [Min–Max], or numbers (%) as applicable. Genotype frequencies were computed, and the Minor Allele Frequencies (MAFs) of the Lebanese and Kurds were tested for Hardy Weinberg Equilibrium (HWE) using chi-square test. Baseline characteristics, 6-MP related toxicities and genotypes were compared between the two cohorts using Student t-test and two-sided Fisher exact test for continuous and categorical data respectively.

The associations of the different genotypes with 6-MP related febrile neutropenia and hepatotoxicity were evaluated using the two-sided Fisher exact test. The Kruskall Wallis non-parametric test was used for the association with 6-MP dose intensity. Of note that for the *ITPA* genotypes, the number of risk alleles were entered in the association analysis. These data are visualized using PRISM software (GraphPad6, La Jolla, CA, USA).

A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Sample Characteristics

Baseline characteristics are shown in **Table 1**. The Lebanon cohort included 136 subjects almost all of whom were Lebanese except for 15: 7 Palestinians, 5 Syrians, and 3 Iraqis. They were of similar age and gender distribution when compared to the 74 Kurds; nevertheless there were significant differences in the immunophenotype distribution and the treatment protocol risk group allocation.

6-Mercaptopurine Related Toxicities

As shown in **Table 1**, the 6-MP dose intensity was significantly lower, and there was a significantly higher incidence of febrile neutropenia in the Lebanon cohort when compared to that of Kurdistan. This is to be expected since significantly more of the ALL children from Kurdistan were treated with the low or standard risk protocol. In addition during maintenance with the COG protocol in Kurdistan, MTX is given at a dose of 20 mg/m^2 weekly in contrast to 40 mg/m^2 with the SJCRH protocol in Lebanon.

Genetic Polymorphisms

Table 1 also shows the genotype frequencies. As expected, *TPMT* and *NUDT15* variants were uncommon. As for *ITPA*, both polymorphisms were more common in the Lebanon cohort as compared to Kurdistan with a MAF of 0.05 for *94C > A* and 0.14 for *IVS2+21A > C* in the Lebanese only (N = 121), and of 0.01 for either *ITPA* polymorphism in Kurds. All frequencies were in HWE ($P > 0.05$).

Associations Between Genetic Polymorphisms and 6-Mercaptopurine Related Toxicities

As shown in **Figure 1** for the combined and the individual Lebanon and Kurdistan cohorts, the evaluated variant alleles were significantly associated with 6-MP intolerance depicted as lower 6-MP dose intensities in carriers of variant alleles when compared to wild type. The most significant effects were depicted with the *NUDT15* polymorphism with a median 6-MP dose intensity of 33.33%, followed by 46.65% for *TPMT*3A* polymorphism, followed by 65.33% for two *ITPA* risk allele carriers and 74% for one *ITPA* risk allele carriers, in comparison to a median of 100% for the homozygous wild type in the combined cohort ($P < 0.001$).

As shown in **Supp. Table 1**, no significant differences in onset of febrile neutropenia emanated for the Lebanon cohort although the three patients with either *TPMT*1/*3A* or *NUDT15 CT* genotypes were admitted for febrile neutropenia. Interestingly, onset of febrile neutropenia was significantly associated with risk allele carriers in Kurds as all four children (two *CA* for *ITPA 94C > A*, one *AC* for *ITPA IVS2+21A > C*, and one *TPMT *1/*3A*) had this toxicity during maintenance ($P = 0.002$), an association that was also significant in the combined cohorts ($P < 0.001$). Notably, no significant associations appeared with hepatotoxicity in neither combined nor the two separate cohorts (**Supp. Table 2**).

DISCUSSION

In recent decades, there has been a lot of interest in inter-individual differences in drug metabolizing enzymes in order to better adjust drug dosage and therapy. In this regards, *TMPT* was the first pharmacogene that showed a substantial association with 6-MP maximum tolerated dose and 6-MP related toxicities leading to the implementation of *TPMT* genotyping before drug administration (Relling et al., 2013). Similarly, *NUDT15*, an enzyme involved in detoxification of 6-MP metabolites, showed a strong association with 6-MP intolerance in the maintenance phase of ALL therapy (Moriyama et al., 2016), and it has hence been recently integrated in the updated CPIC guidelines for thiopurine dosing (Relling et al., 2019). However, the frequency of these genetic polymorphisms is noticeably lower in some ethnic groups when compared to others (Hakooz et al., 2010; Albayrak et al., 2011; Bahrehmand et al., 2017; Moriyama et al., 2017; Zgheib et al., 2017; Zhou et al., 2018), hence the

TABLE 1 | Baseline characteristics, 6-mercaptopurine (6-MP)-related toxicities and genotypes of children with acute lymphoblastic leukemia (ALL) from 2 cohorts (N = 210).

Variables			Lebanon[1,2]	Kurdistan	P-value
Number of subjects			**136**	**74**	
Treatment protocol			**SJCRH XV**	**COG**	
		Characteristics			
Age	Years	Mean ± SD	6.63 ± 4.93	6.25 ± 3.07	0.495
Sex	Male	N (%)	77 (56.6)	43 (58.1)	
	Female	N (%)	59 (43.4)	31 (41.9)	0.884
Treatment risk group	Low/standard	N (%)	69 (51.1)	58 (78.4)	
	Mid/high	N (%)	66 (48.9)	16 (21.6)	< 0.001
ALL immunophenotype	Pre B	N (%)	107 (81.1)	70 (94.5)	
	T cell	N (%)	22 (16.6)	3 (4.1)	
	Pre-B with AML	N (%)	2 (1.5)	1 (1.4)	
	Early pre B	N (%)	1 (0.8)	0 (0)	0.015
		6-MP-related toxicities			
6-MP dose intensity[3]	%	Mean ± SD	77.39 ± 21.27	95.38 ± 16.03	< 0.001
Febrile neutropenia[4]	No	N (%)	44 (34.9)	58 (78.4)	
	Yes	N (%)	82 (65.1)	16 (21.6)	< 0.001
Hepatotoxicity[5]	No	N (%)	111 (90.2)	65 (87.8)	
	Yes	N (%)	12 (9.8)	9 (12.2)	0.638
		Genotypes			
ITPA 94C > A	CC	N (%)	126 (92.7)	72 (97.3)	
	CA	N (%)	9 (6.6)	2 (2.7)	
	AA	N (%)	1 (0.7)	0 (0)	0.481
ITPA IVS2+21A > C	AA	N (%)	103 (75.7)	73 (98.6)	
	AC	N (%)	30 (22.1)	1 (1.4)	
	CC	N (%)	3 (2.2)	0 (0)	< 0.001
*TPMT*3A*[6]	*1/*1	N (%)	133 (97.8)	73 (98.6)	
	*1/*3A	N (%)	3 (2.2)	1 (1.4)	1.000
NUDT15	CC	N (%)	135 (99.3)	0 (0)	
	CT	N (%)	1 (0.7)	0 (0)	1.000

P-values were generated by two-sided Fisher exact test or Student t-test as applicable.

SJCRH, St Jude's Children Research Hospital; COG, Children's Oncology Group.

[1]Numbers may not add up to 136 due to some unavailable data.

[2]Lebanese (121), Palestinian (7), Syrian (5), Iraqi (3).

*[3]**Lebanon:** ratio of the MP dose reached during maintenance therapy to maintain the WBC between 1,500 and 3,000 per µl and the ANC > 300 per µl to that of the maintenance prescribed MP dose as per protocol. **Kurdistan:** ratio of the MP dose reached during maintenance therapy to maintain the WBC between 2,000 and 3,000 per µl and the ANC > 500 per µl to that of the maintenance prescribed MP dose as per protocol.*

[4]Lebanon and Kurdistan: At least one episode of febrile neutropenia during maintenance therapy.

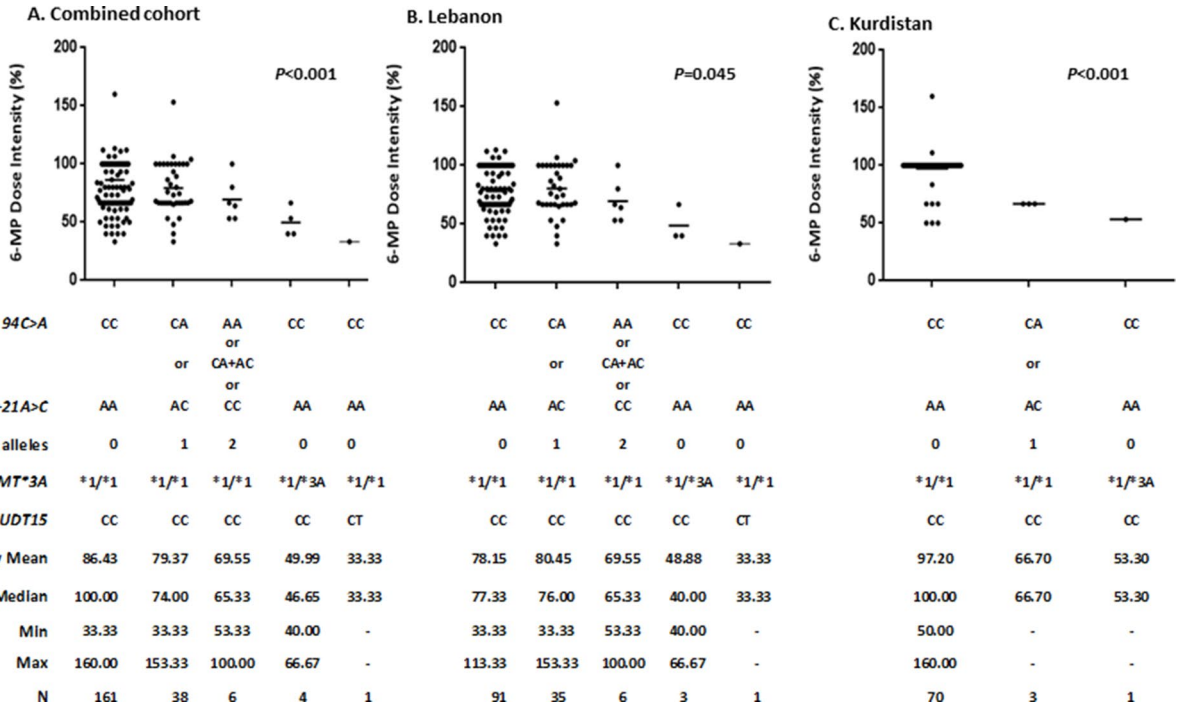
[5]Lebanon: Highest direct serum bilirubin level being ≥1.5 during the MP and Methotrexate combination therapy phase in maintenance (i.e. week 100 and on). Kurdistan: Highest serum SGPT(ALT) level being at least three times higher than the upper level of normal during the MP and Methotrexate combination therapy phase in maintenance.

*[6]TPMT*3A is a combination of the TPMT*3B and TPMT*3C genotypes.*

need to elicit the role of other variants in other genes that are more relevant to specific populations. This study reports on the frequency and role of *TPMT*, *NUDT15*, and *ITPA* polymorphisms with 6-MP dose intensity and toxicity in two cohorts from the Middle East, one from Lebanon and another from Kurdistan. We have shown that, while variants in the three genes are significant predictors of 6-MP intolerance, *TPMT* and *NUDT15* polymorphisms are quite infrequent, hence the importance of integrating *ITPA* genotyping in ALL PGx guidelines for this area of the world.

In term of allele frequency (**Table 1**), results showed 1.4% and 2.2% frequency for the *TPMT*3A* risk allele in the Lebanese and Kurdish population, respectively. This range is similar to that reported in other studies in West Asian populations (Collie-Duguid et al., 1999), and is far less than the mean global prevalence of *TPMT* genetic variations which is around 10% (Relling et al., 2013). Besides in our study, only one patient was a carrier for the risk allele of *NUDT15* gene, accounting for only 0.4% in the full cohort, a frequency that is very low when compared to Japanese, Taiwanese and Korean people (Tanaka et al., 2015; Liang et al., 2016; Kim et al., 2017). Notably, no patient had a homozygous form of *TPMT* or *NUDT15*. More importantly, patients who harbor defective alleles of *TMPT* and *NUDT15* genes required a significantly lower dose of the planned dose of 6-MP compared to the wild-type carriers of these alleles (**Figure 1**), with 6-MP dose intensity in the one child with the *NUDT15 CT* genotype being less than that reported in Asian patients with ALL (Yang et al., 2015; Zhou et al., 2018). Therefore, despite the low frequency of *TPMT* or *NUDT15* variant alleles, testing for them prior to therapy is still clinically warranted in this area of the world.

ITPA is another gene candidate involved in 6-MP detoxification with variants reported to be associated with 6-MP intolerance (Hawwa et al., 2008; Wan Rosalina et al., 2012).



	A. Combined cohort					B. Lebanon					C. Kurdistan		
ITPA 94C>A	CC	CA or AA or CA+AC or		CC	CC	CC	CA or AA or CA+AC or		CC	CC	CC	CA or	CC
ITPA IVS2+21A>C	AA	AC	CC	AA	AA	AA	AC	CC	AA	AA	AA	AC	AA
ITPA risk alleles	0	1	2	0	0	0	1	2	0	0	0	1	0
*TPMT*3A*	*1/*1	*1/*1	*1/*1	*1/*3A	*1/*1	*1/*1	*1/*1	*1/*1	*1/*3A	*1/*1	*1/*1	*1/*1	*1/*3A
NUDT15	CC	CC	CC	CC	CT	CC	CC	CC	CC	CT	CC	CC	CC
6-MP dose intensity Mean	86.43	79.37	69.55	49.99	33.33	78.15	80.45	69.55	48.88	33.33	97.20	66.70	53.30
Median	100.00	74.00	65.33	46.65	33.33	77.33	76.00	65.33	40.00	33.33	100.00	66.70	53.30
Min	33.33	33.33	53.33	40.00	-	33.33	33.33	53.33	40.00	-	50.00	-	-
Max	160.00	153.33	100.00	66.67	-	113.33	153.33	100.00	66.67	-	160.00	-	-
N	161	38	6	4	1	91	35	6	3	1	70	3	1

FIGURE 1 | Association[1] between *ITPA, TPMT, NUDT15* genetic polymorphisms and 6-mercaptopurine (6-MP) dose intensity[2] during maintenance therapy in **(A)** Combined cohorts (N = 210), **(B)** Lebanon cohort (N = 136), and **(C)** Kurdistan cohort (N = 74). [1]Kruskall Wallis test; Horizontal lines indicate the mean. [2]Lebanon: ratio of the MP dose reached during maintenance therapy to maintain the WBC between 1,500 and 3,000 per µl and the ANC > 300 per µl to that of the maintenance prescribed MP dose as per protocol. Kurdistan: ratio of the MP dose reached during maintenance therapy to maintain the WBC between 2,000 and 3,000 per µl and the ANC > 500 per µl to that of the maintenance prescribed MP dose as per protocol.

In this study, and similarly to other numbers reported in the literature (Adam de et al., 2011; Milosevic et al., 2018), the frequency of both evaluated ITPA variants was higher than those in *TPMT* or *NUDT15* for both cohorts, especially for the Lebanon cohort. More importantly, patients with one or two risk alleles of the ITPA gene tolerated a median 74% (33–153%) or 65.33% (53–100%), respectively, of the standard dose of 6-MP. Notably that these dose intensities of 6-MP in *ITPA* variant groups were higher than those in carriers of the *TPMT* or *NUDT15* variant alleles, but still significantly lower than individuals with no risk alleles ($P < 0.001$) (**Figure 1**). In order to evaluate further the relationship between 6-MP toxicity and the tested genotypes, we analyzed the onset of febrile neutropenia and hepatotoxicity among the cohorts in wild-type individuals compared to variant allele carriers. Results showed that none of the variant alleles was associated with hepatotoxicity during maintenance, a negative finding that may be explained by the study design being based on retrospective chart review, or confounded by other concomitant drugs such as MTX (Schmiegelow et al., 2014). Interestingly, the onset of febrile neutropenia was significantly higher in variant allele carriers in Kurds and the combined cohorts, a result that was previously published in other populations (Stocco et al., 2009; Adam de et al., 2011).

In conclusion, these data confirm the predictive role of *TPMT*, *NUDT15*, and *ITPA* in 6-MP intolerance in Middle Eastern children with ALL. Given the relatively high frequency of *ITPA* variants in our study and their significant association with 6-MP dose intensity, we recommend that physicians consider genotyping for *ITPA* variants in conjunction with *TPMT* and *NUDT15* prior to 6-MP therapy in these children.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

Lebanon: Study is approved by the American University of Beirut Institutional Review Board under protocol: PED.SM.05. All participants and parents signed an informed consent or assent as applicable. **Kurdistan:** Study is approved by the Kurdistan University of Medical Sciences Institutional Review Board under protocols IR.MUK.REC.1396/102 and IR.MUK.REC.1396/339. All participants and parents signed an informed consent or assent as applicable.

AUTHOR CONTRIBUTIONS

EM and NZ contributed to the conception and design of the study. BM and SM contributed to the study subjects. FZ and EM carried out the experiments. EM, NZ, and EG organized the database and performed the statistical analysis. EM and NZ wrote the first draft of the manuscript. All authors contributed to manuscript revision and 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.2019.00916/full#supplementary-material

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Variation in Actionable Pharmacogenetic Markers in Natives and Mestizos From Mexico

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The identification and characterization of pharmacogenetic variants in Latin American populations is still an ongoing endeavor. Here, we investigated SNVs on genes listed by the Pharmacogenomics Knowledge Base in 1284 Mestizos and 94 Natives from Mexico. Five institutional cohorts with NGS data were retrieved from different research projects at INMEGEN, sequencing files were filtered for 55 pharmacogenes present in all cohorts to identify novel and known variation. Bioinformatic tools VEP, PROVEAN, and FATHMM were used to assess, *in silico*, the functional impact of this variation. Next, we focused on 17 genes with actionable variants that have been clinically implemented. Allele frequencies were compared with major continental groups and differences discussed in the scope of a pharmacogenomic impact. We observed a wide genetic variability for known and novel SNVs, the largest variation was on *UGT1A* > *ACE* > *COMT* > *ABCB1* and the lowest on *APOE* and *NAT2.* Although with allele frequencies around 1%, novel variation was observed in 16 of 17 PGKB genes. In Natives we identified 59 variants and 58 in Mestizos. Several genes did not show novel variation, on *CYP2B6*, *CYP2D6*, and *CYP3A4* in Natives; and *APOE*, *UGT1A*, and *VKORC1* in Mestizos. Similarities in allele frequency, comparing major continental groups for VIP pharmacogenes, hint towards a comparable PGx for drugs metabolized by *UGT1A1*, *DPYD*, *ABCB1*, *CBR3*, *COMT*, *and TPMT*; in contrast to variants on *CYP3A5* and *CYP2B6* for which significant MAF differences were identified. Our observations offer some discernment into the extent of pharmacogenetic variation registered up-to-date in Mexicans and contribute to quantitatively dissect actionable pharmacogenetic variants in Natives and Mestizos.

Keywords: pharmacogenomics, population variation, next generation sequencing, pharmacodynamics, pharmacokinetics

Abbreviations: NGS, next generation sequencing; WGS, whole genome sequencing; WES, whole exome sequencing; MAF, minor allele frequency; PGKB, Pharmacogenomics Knowledge Base; SNVs, single nucleotide variant; PK/PD, pharmacokinetics/pharmacodynamics.

INTRODUCTION

Pharmacogenetic studies in Mexican populations have been directed towards the identification of markers previously reported with functional consequences for drug safety and efficacy (Contreras et al., 2011; Bonifaz-Pena et al., 2014; Marsh et al., 2015; Fricke-Galindo et al., 2016). The advent of faster and more accessible technologies has made feasible to investigate a broader swath of the pharmacogenome including the presence of novel variants. In most of the developed world pharmacogenetic testing is being implemented with the aid of consortia and institutions such as the PGRN, the PGKB, CPIC, and the FDA. These offer a list of genetic variants to guide drug selection, dose optimization, and to reduce the risk of adverse drug reactions. Moreover, implementation programs such as The electronic Medical Records and Genomics (eMERGE) initiative have started preemptive pharmacogenomics testing in over 10,000 patients, on the basis of the benefits of pharmacogenetic information, despite the so-called lack of cost–benefit evidence (van Driest et al., 2014). Nevertheless, in developing countries broad pharmacogenetic implementation is even more incipient, a curated collection of local variants has not been yet defined, and for many relevant markers there are significant differences for minor allele frequencies (MAF) when comparing to major continental groups. For example, Campos et al. reported lower *CYP2C19**17 rs1224856 allele frequencies in 346 Mexican Americans compared to CEU, MAF: 0.14 vs. 0.22, which may be indicative of lower frequency of bleeding with clopidogrel in the former (Claudio-Campos et al., 2015). In Mexicans higher allele frequencies have been reported for *CYP3A4* rs2750574 and *NQO1* rs1800566, which may be differentially affecting the efficacy of tacrolimus, fluorouracil, and anthracyclines, when compared to Europeans.

Mexico is home to 68 genetically different ethnic groups which suggests urgency for an adequate collection, classification, and characterization of variants on genes that affect drug efficacy and safety. Published studies collect the identification and determination of allele frequencies of at least all PGKB level 1 variants in Mestizos (Cuautle-Rodriguez et al., 2014; Fricke-Galindo et al., 2014; Fricke-Galindo et al., 2016; Gonzalez-Covarrubias et al., 2017). Fewer studies have included Natives, NGs techniques, or genotype-phenotype assessments. Results accede that for several makers, allele frequencies and its clinical impact differ among Natives, Mestizos and, major continental groups (Bonifaz-Pena et al., 2014; Fricke-Galindo et al., 2016; de Andrés et al., 2017) In addition, little is known of the presence and frequency of local private variation, which can only be investigated though sequencing (Moreno-Estrada et al., 2014). NGS studies have also shown that it is the interplay of several variants, rather than one maker, that explains drug response variability (Johnson et al., 2011; Beekman et al., 2013; Katsila and Patrinos, 2015; Yang et al., 2016). In this regard, Han S.M. et al. identified dozens of rare variants with functional consequences to drug response, but more importantly, it revealed that targeted sequencing enabled profiling of actionable and rare variants, some of yet unknown functional consequences (Han et al., 2017). Furthermore, *in vitro* studies of major pharmacogenes have confirmed that many rare variants do have a functional impact confirming NGS bioinformatic predictions (Matimba et al., 2009; Han et al., 2017). More limited in scope, our own work has documented the importance of variants, derived from NGS, to increase the predictive value of genotypes on the pharmacokinetics of atorvastatin (Cruz-Correa et al., 2017) and the pharmacodynamics of cumarins (Gonzalez-Covarrubias et al., 2017). Nevertheless, the collection of a comprehensive pharmacogenetic set of variation is still underway, mostly for understudied populations.

Here, we identified, and classified pharmacogene variation on 17 genes listed by the PGKB in 1284 Mestizos and 94 Natives from several institutional cohorts at the National Institute for Genomic Medicine in Mexico, from which NGS data were available. Allele frequency comparisons showed that pharmacogenetic variation is significantly different between Natives and Mestizos for several PGKB variants. We observed twice as many actionable variants in Mestizos vs. Natives, but 4× more novel variation *per* individual in Natives suggesting that the pharmacogenome in the latter is far from complete, and that current pharmacogenetic guidelines may prove to be more beneficial for certain populations within the country.

METHODS

Participants and Sequencing Files. We analyzed WES data from 1,284 Mexican Mestizos published at the ExAC platform (Lek et al., 2016; Karczewski et al., 2017) and three institutional cohorts sequenced with custom probes using different Haloplex v2, Agilent Technologies protocols, thus variant calling was performed independently for each cohort. In addition, sequencing files for 94 Natives were procured by the 100 Genome Consortium (INMEGEN, to be published) and the project Metabolic Analysis in an Indigenous Sample (MAIS) (Contreras-Cubas et al., 2016). All participants provided a blood sample after signing an informed consent and all research projects were approved by the Ethics and Research Committees at INMEGEN. Participants were self-reported as Natives and confirmed by AIMS analyses (to be published). DNA from Natives was sequenced by BGISEQ-500 (Cambridge, MA, USA). Samples from Natives belonged to 36 ethnic groups thus, further stratification and multiple comparisons within Natives or with Mestizos was not possible. Similarly, for Mestizos >90% of all DNA samples came from individuals from the center of the country limiting further stratification. In summary, vcf data came from five institutional cohorts: WGS (Natives = 94, to be published), WES (Mestizos = 968) (Flannick et al., 2019), NGS-targeted 1 (Mestizos = 110) (Gonzalez-Covarrubias et al., 2017), NGS-targeted 12 (Mestizos = 146) (Gonzalez-Covarrubias et al., 2016), and NGS-targeted 3 (Mestizos = 60) (Cruz-Correa et al., 2017). All data were processed according to the Broad Institute recommended best practices workflow and the Genome Analysis ToolKit (GATK) (Auwera et al., 2013).

To assess the functional impact of variants in coding and non-coding regions, we selected three algorithms. We utilized PROVEAN, VEP, and FATHMM which are well published and have been used by the scientific community (Devarajan et al., 2019). PROVEAN predicts whether a SNV affects the function of a protein by giving a score based on sequence alignment;

the lower the score, the more dissimilar is the substitution compared to the reference (Mizzi et al., 2014; Wendt et al., 2018). VEP (Variant Effect Predictor) seeks for genes/transcripts of a variant to determine its effect at the amino acid level (e.g. stop gained, missense, stop lost, altered splicing, frameshift, stop loss, and start loss), a high impact predicted by VEP is considered as deleterious (Oetting et al., 2016; Lakiotaki et al., 2017). FATHMM was utilized to predict functional consequences of non-coding variants, it integrates functional annotations from ENCODE and calculates a score (0–1). Scores >0.5 are indicative of deleterious variation (McInnes et al., 2019; Zhou et al., 2018), and although non-coding variants may not have a direct impact on a protein the overall regulation may render faulty. These tools have been utilized in PGx research (Devarajan et al., 2019), and we have reported their use to filter variants with potential effects on atorvastatin pharmacokinetics with acceptable results. PGx-oriented algorithms for the prediction of the functional impact of novel variants are being developed and validated, these new tools include some of the algorithms utilized here (Zhou et al., 2018; Zhou et al., 2019).

Genotypes of PGKB variants in **Table 2** were in complete concordance with previous genotyping experiments by RTPCR ex. *CYP2C9/VKORC1* (Villegas-Torres et al., 2015), DMET microarray (Gonzalez-Covarrubias et al., 2016) and by NGS (Gonzalez-Covarrubias et al., 2017).

Pharmacogenetic Analyses. Statistical analyses focused on 55 genes since these were identified in all samples. In-depth analyses were undertaken for variants listed by the PharmGKB with a level of evidence 1 and 2 (Relling and Klein, 2011). PGKB level 1 (1A/1B) represent variation with a strong evidence of PK/PD alteration, and these have been published as markers in CPIC dosing guidelines. Level 2 refers to variants with moderate evidence to be linked to a drug's safety or efficacy. We computed allele frequencies and performed comparisons for several populations, CEU, Northern Europeans from Utah (Caucasians); MXL, Mexicans from Los Angeles; YRI, Yoruba in Ibadan, Nigeria; CHB, Chinese Han from Beijing. Data analyses, descriptive statistics, allele frequency calculations, and variant inferences were performed with R (Team R Core, 2014), PLINK (Purcell et al., 2007), and PGKB resources (Sangkuhl et al., 2008).

RESULTS

NGS Summary. Sequencing files from 94 Natives and 1,284 Mestizos showed a depth-coverage between 50× and 600×, tNGS aimed for 100× coverage while WES and WGS for at least 50×. Depth coverage varied per genomic region regardless of the theoretical depth calculated per sequencing run. For example, tNGS showed a range of depth and coverage of 40×–600× and 80–100% for the selected genes, but after quality control some pharmacogenetic relevant regions including that of *VKORC1* rs9323231 and *CYP2C19**17 rs12248560 were not covered.

In Natives, preliminary analyses identified the largest number of variants as data came from WGS, NGS-targeted and WES in Mestizos yielded a lower count. Together, all cohorts gathered 436 pharmacogenes, on which we identified on average 102 novel variants *per* individual in Natives, a value almost three times higher than in Mestizos. Consistently, non-synonymous SNVs (single nucleotide variants) were five times more abundant in Natives, suggesting a genetic diversity uncovered by NGS, but these 436 genes were not identified in all individuals. Therefore, we directed further analyses on genes identified in all cohorts and genes listed by the PGKB with a validation level of 1 or 2, which are the focus of this report.

Pharmacogenetic Analyses

Fifty-five genes were identified in all in 1,378 individuals including most CYPs, FMOS, members of the *UGT1A* family, *SULT1A*, and 11 genes involved in pharmacodynamics. **Table 1** lists variation

TABLE 1 | Variation in 55 pharmacogenes shared among cohorts.[1]

	Natives		Mestizo	
	Novel	All[1]	Novel	All[1]
N, individuals	94		1,284	
Genes	55		55	
Total variants	528	3,092	1,809	7,627
Variants *per* individual	14	818	4	230
Average MAF of variants	0.043	0.117	0.004	0.029
Private variants	349	868	1,090	3,256
Private *per* individual	4	9	1	3
[2]Functional	228	1,511	865	4,224
Deleterious	128	621	280	1,828
Non-synonymous SNV	55	395	134	1,489
Synonymous SNV	80	348	44	861
Multiple AA Change	0	0	14	14
Frameshift	4	13	102	151
Nonsense	0	1	7	54
Others	89	754	557	1,644

[1]All refers to all variants known (rs identifier) and novel. [2]Functional variants are those with an in silico functional consequence. Pharmacogenes included: ADH1C1, CYP3A43, COMT, UGT1A9, ACE, CBR1, CYP3A5, NAT1, UGT2B7, APOE, CBR3, CYP3A7, NAT2, ABCB1, DRD2, CYP11B1, DPYD, SULT1A1, SLC16A1, F8, CYP11B2, FMO1, TPMT, SLC22A11, F9, CYP1A1, FMO2, UGT1A1, FTO, CYP1A2, FMO3, UGT1A10, LPA, CYP2B6, FMO4, UGT1A3, P2RY2, CYP2C18, FMO5, UGT1A4, TOMM40L, CYP2C19, MAOA, UGT1A5, VDR, CYP2C9, MAOB, UGT1A6, VKORC1, CYP2D6, NQO1, UGT1A7, CYP3A4, UGT1A8, SLCO1B1.

for these 55 genes many of which are listed by the eMERGE initiative and the Pharmacogenomics Research Network (Bush et al., 2016). On these, we observed 3.4× and 2.5× more variants in Mestizos vs. Natives (novel and known), as expected given the larger sample of Mestizos. However, the average number of variants per individual was 3.9× and 3.4× higher (novel and known) in Natives, reflecting the larger diversity unreported in the latter. Also, private variants, i.e., polymorphisms observed as heterozygous in only one individual, were more frequent in Natives (n = 868) vs. Mestizos (n = 3256, **Table 1**).

Next, we focused on 17 actionable genes with clinically validated variants to perform comparisons between populations. Genes were selected according to the PGKB classification considering only those with a validation level 1 and 2, included in published CPIC dosing guidelines also, considered actionable by the FDA. These 17 genes were, *ABCB1*, *ACE*, *APOE*, *CBR3*, *CYP2C19*, *CYP2C9*, *CYP2B6*, *CYP2D6*, *CYP3A5*, *CYP3A4*, *COMT*, *DPYD*, *NAT2*, *SLCO1B1*, *TPMT*, *UGT1A*, and *VKORC1*. On these, we observed 2,387 non-private, novel, and known variants, 34 of which were listed by the PGKB. The largest number of known variants were observed on *ACE* (251), *UGT1A* (318), *ABCB1* (182), and *COMT* (235), (**Figure 1**). Genes with the lowest number of variants were *NAT2* (33) and *APOE* (24), again with a higher count in Mestizos.

Next, we determined minor allele frequencies for these 17 PGKB-listed variants and compared them between Mexicans and those reported for major continental populations. In Natives, we observed a higher MAF, compared to Mestizos, for *VKORC1* rs8050894 (1.4×), *CYP2B6* rs2279343 (1.5×), and *CYP3A5* rs776746 (2×), with potential implications for the differential management in Natives for cumarins, efavirenz, nevirapine, methadone, and tacrolimus am ong several others. For most of these variants allele frequencies in Natives were higher compared to Mestizos, also when compared to CEU and CHB (**Table 2**).

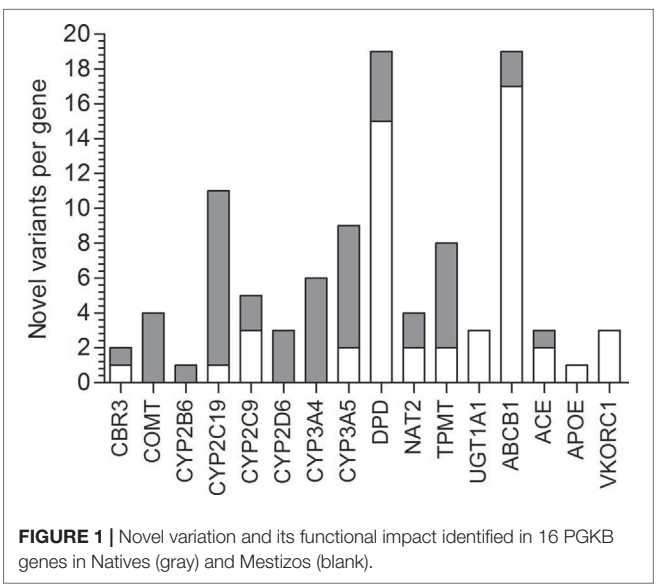

FIGURE 1 | Novel variation and its functional impact identified in 16 PGKB genes in Natives (gray) and Mestizos (blank).

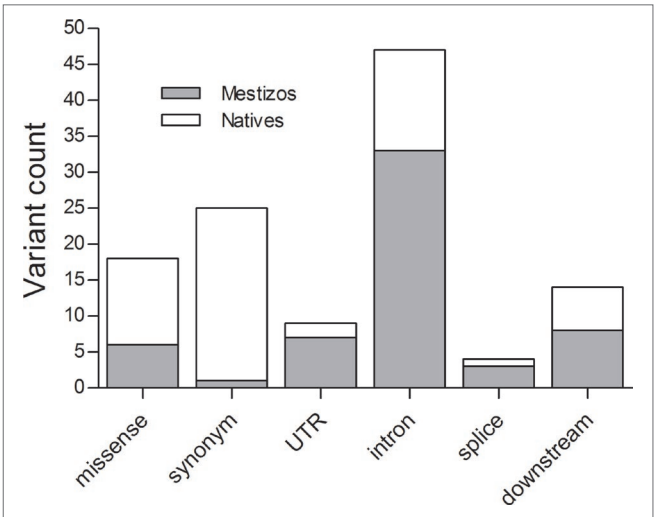

FIGURE 2 | Functional impact of novel variation in Mestizos (gray) and Natives (blank).

The opposite was also observed, i.e., lower allele frequencies in Natives were identified for *CYP2C19* rs4244285 (2×), *CYP2C9* rs1799853 (4.5×), rs1057910 (2×), *NAT2* rs179930 (2×), *SLCO1B1* rs4149015, and *APOE* rs7412. The latter two were not found in Natives. *CYP2C19* and *CYP2C9* lower allele frequencies in Natives may be indicative of a lower proportion of poor metabolizers potentially affecting the pharmacokinetics of over 18% of all drugs. For the remaining 26 variants on 8 genes, allele frequencies were comparable between Natives and Mestizos. A summary of variation on these 17 genes is presented in **Table 2**.

Novel Variants on Actionable PGKB Genes

Using NGS data we sought for novel variation, and identified 116 not-previously reported variants on 16 of the 17 PGKB genes (MAF ≥1%). We found some novel variation in all genes studied in Mestizos or Natives except for *SLCO1B1*. The functional impact of these variants was assessed by the algorithms, VEP, Provean, and FATHMM (Dong et al., 2015). **Table 3** lists novel variants that were predicted as deleterious by more than two algorithms, observed on *ABCB1* (4), *CYP2C19* (1), and *CYP3A4* (1), and although their frequency does not exceed 2% we believe their strongly predicted functional impact pinpoints them as worth investigating. A full list of novel variants annotated by the three algorithms is listed in **Supplemental Table 1**.

Lack of novel variation was detected for *APOE*, *UGT1A1*, and *VKORC1* in Mestizos, and for *CYP2D6*, *CYP3A4*, *CYP2B6, and COMT* in Natives (**Figure 1**). The largest count of novel variants in Natives was observed for *ABCB1* (25) and *DPYD* (15), and in Mestizos on *CYP2C19* (10) and *CYP3A4*/5 (13). Novel variation was on average comparable between population groups, but differed largely *per* gene **Figure 1** and **Supplemental Table 1**.

TABLE 2 | Allele frequencies for 17 actionable pharmacogenetic variants.

Variants	Natives	Mestizo	gMAF[1]	MXL[1]	CEU[1]	YRI[1]	CHB[1]	PGKB, minor allele impact, drugs
ABCB1 (182)	66	118						Decreased enzyme activity affecting toxicity
rs1045642	0.436	0.515	0.395	0.480	0.430	0.880	0.620	and efficacy of nevirapine, ondasteron,
rs2032582	0.410	0.539	0.334	0.550	0.560	1	0.440	methotrexate, fentanyl, digoxin, simvastatin
ACE (251)	72	273						No PGKB variants detected
APOE (24)	6	22						PGKB evidence level 2
rs7412	0	0.036	0.075	0.050	0.070	0.110	0.110	Atorvastatin efficacy
CBR3 (40)	10	34						PGKB evidence level 2
rs1056892	0.277	0.241	0.427	0.280	0.310	0.510	0.390	Anthracyclines PK
CYP2C19 (173)	62	170						PGKB evidence level 1
rs4244285	0.053	0.1	0.220	0.110	0.130	0.170	0.340	PM, decreased enzyme activity; drugs
rs4986893	0	0.001	0.1	0	0	0	0.040	affected: proton pump inhibitors, clopidogrel,
rs28399504	0	0.006	0.002	0.010	0	0	0.010	citalopram, imipramine, diazepam, mephenytoin
CYP2C9 (96)	37	72						PGKB evidence level 1
rs1799853	0.011	0.049	0.050	0.100	0.150	0	0	Poor metabolizers, decreased enzyme activity;
rs1057910	0.016	0.031	0.050	0.020	0.070	0	0.040	drugs affected: NSAIDs, phenytoin, cumarins
CYP2B6 (103)	50	89						PGKB evidence level 1 and 2
rs2279343	0.351	0.240	0.302	0.270	0.210	0.460	0.190	Altered enzyme activity; affecting toxicity and
rs28399499	0	0.003	0.023	0.01	0	0.120	0	pharmacokinetics of efavirenz, nevirapine,
rs3745274	0.351	0.318	0.295	0.31	0.280	0.400	0.160	methadone.
rs4803419	0.596	0.522	0.289	0.420	0.280	0.04	0.500	
CYP2D6 (76)	38	72						Other relevant PGKB genes
rs1065852	0.124	0.102	0.240	0.150	0.240	0.110	0.400	PM, decreased enzyme activity affecting,
rs28371706	0.005	0.007	0.060	0	0	0.750	0	loperidone, escitalopram, nevirapine, timolol
rs28371725	0	0.028	0.064	0.020	0.120	0.010	0.030	
rs16947	0.796	0.778	0.360	0.740	0.680	0.440	0.840	
CYP3A5 (90)	26	88						PGKB evidence level 1 and 2
rs776746	0.303	0.189	0.380	0.230	0.040	0.830	0.310	PM, decreased enzyme activity; drugs
CYP3A4 (76)	29	78						affected: tacrolimus.
rs2740574	0.069	0.071	0.230	0.070	0.020	0.210	0	
COMT (235)								
rs4680	0.399	0.391	0.370	0.400	0.470	0.310	0.320	Decreased activity affecting nicotine replacement therapy
DPYD (119)	48	105						PGKB evidence level 1
rs67376798	0	0.001	0.002	0	0.010	0	0	PM, decreased enzyme activity; affecting fluoropyrimidines
NAT2 (33)	15	26						PGKB evidence level 1
rs1041983	0.303	0.299	0.400	0.270	0.300	0.500	0.360	PM, decreased enzyme activity; affecting:
rs1799930	0.059	0.120	0.265	0.130	0.300	0.200	0.300	ethambutol, isoniazid, pyrazinamide, rifampin
SLCO1B1 (124)	60	98						PGKB evidence levels 1 and 2
rs4149056	0.117	0.091	0.090	0.080	0.150	0.010	0.140	Impaired transporter activity affecting statins
rs4149015	0	0.017	0.055	0.020	0.040	0	0.120	
TPMT (82)	27	54						Other relevant PGKB genes
rs1800462	0	0.002	0.002	0	0	0	0	Poor metabolizers, decreased enzyme
rs1800460	0.048	0.049	0.010	0.040	0.030	0	0	activity, affecting tiopurines capecitabine
rs1142345	0.048	0.052	0.040	0.050	0.030	0.060	0.010	
UGT1A (318)	68	268						PGKB evidence levels 1 and 2
rs887829	0.335	0.3	0.350	0.370	0.320	0.520	0.110	Decreased protein levels affecting irinotecan,
rs4148323	0.043	0.030	0.030	0.020	0.010	0	0.230	deferasirox, atazanavir
VKORC1 (66)	22	61						PGKB evidence level 1
rs7294	0.441	0.447	0.420	0.35	0.31	0.510	0.040	PM, decreased enzyme activity; drugs
rs8050894	0.537	0.381	0.420	0.510	0.430	0.230	0.960	affected: cumarins
rs9934438	0.537	0.431	0.360	0.470	0.430	0.030	0.960	
rs9923231	0.543	0.402	0.360	0.470	0.430	0.030	0.960	

[1] *Data from 1000 Genomes (Auton et al., 2015). In parenthesis, total number of variants identified per gene.*

DISCUSSION

As genetic variation is better defined within populations it is relevant for each country to assess whether current pharmacogenetic platforms can offer direct benefits for their people. There are 68 Native groups in Mexico representing around 10% of the population, but admixture proportions vary greatly throughout the country. Thus, we sought to determine and compare differences in gene variation related to pharmacokinetics and pharmacodynamics in Natives and Mestizos by consolidating different institutional cohorts. Sequencing data for all 1,378 DNA samples were obtained, by

TABLE 3 | Selected novel variants predicted as deleterious.[1]

Gene	Position	Nucleotide	Functional impact	MAF %
ABCB1	7:87165002	C/A	transcript	1.85
ABCB1	7:87160718	C/A	synonymous	1.60
ABCB1	7:87160786	T/A	missense	1.06
ABCB1	7:87179286	A/G	synonymous	1.06
CYP2C19	10:96609809	T/A	missense	1.15
CYP3A4	7:99377662	C/A	missense	1.47

[1]*For a full list see* ***Supplemental Table 2.***

various groups utilizing different NGS approaches, and our analyses focused on 17 PGKB genes. Despite the sample size difference between Natives (94) and Mestizos (1284) we were able to capture representative pharmacogenetic variation for both groups. Not unexpectedly, Natives showed almost 10× more variants, which hints towards a larger proportion of unaccounted variation (novel and known).

Comparative analyses among Mestizos, Natives, and continental populations, highlight the extent of our incomplete registry of pharmacogenetic variability within the country, but also indicate a closer completion for Mestizos than for Natives, because these have been much less studied (Cid-Soto et al., 2018; Sánchez-Pozos et al., 2018). For instance, Natives showed 2.4 functional novel variants on average per individual compared to 0.7 in Mestizos, which is in agreement with Romero-Hidalgo et al. (2017) showing that damaging variation is 2× higher for unreported variants in Native Americans. These observations indicate that genetics may underlie drug disposition differences not only between Mexico and major continental groups, but also within the country's populations.

PGx Markers in Guidelines for Clinical Implementation

CPIC dosing guidelines follow data curation by the PGKB and represent the most useful pharmacogenetic resource around the world. Therefore, local adoption of pharmacogenetics in developing countries requires identifying the frequency of already published actionable markers, and to assess if their presence warrants its application to local populations prior to implementation. Here, we compared allele frequencies for 34 PGKB variants in 17 genes with a validation level 1 and 2 to assess if they were similar or different within the studied populations, and were also compared to the major ancestral population (**Table 2**). First**,** we discuss variants with higher allele frequency in Natives vs. Mestizos vs. CEU. These were observed on *VKORC1, CYP3A5, and UGT1A1*. Cumarin-sensitivity variants *VKORC1* rs8050894, rs994438, and 9923231, all in LD, were 25–40% more frequent in Natives hinting for higher cumarin sensitivity. However, only 1.6% of Natives showed variants on *CYP2C9* vs. 5–10% of Mestizo and CEU. In Natives *VKORC1* variation may reflect higher sensitivity to cumarins, but the low CYP2C9*2/*3 frequencies a higher metabolism this particular *VKORC1–CYP2C9* interplay may abrogate cumarin dosing adjustment in Natives. It is relevant to consider that this is mostly the case for warfarin since other CYPs such as CYP2C8 are responsible for R-acenocumarol metabolism, the isomer to which the anticoagulant effect is ascribed.

*CYP3A5*3,* rs776746 affects the disposition of over 20 drugs, but it is particularly relevant for cyclosporine and tacrolimus. In Europeans the T allele has a frequency of 4%, significantly lower than the one observed in Natives (MAF: 0.303) and Mestizos (MAF: 0.189). It is possible that these differences may lead to more dose adjustments for tacrolimus in Mexicans compared to CEU (Zhu et al., 2011), but similar to that in Asians (MAF: 0.311) (Niioka et al., 2012).

Variants on *UGT1A1* significantly influence the pharmacokinetics of several drugs including irinotecan and atazanavir, lower enzyme levels are a consequence of several polymorphisms including, rs887829 and rs4148323, the former showed similar allele frequencies in Natives, Mestizos, MXL, and CEU, but rs4148323 was 4× and 3× more frequent in Natives and Mestizos compared to CEU, this may be indicative of low glucuronidation in a higher proportion of Mexicans. The overall impact of these differences cannot be conclusive, but it is relevant for many other drugs, for which its clearance rate is limited by UGT1A1. Our NGS data did not identify other key variants such as *UGT1A1*28* rs3064774 or rs4148323 which have been shown to be differently distributed in other Latin American countries (Marsh et al., 2015).

We identified 4 of the 24 *CYP2B6* variants listed by the PGKB, only rs4803419 (Level 2B) showed differential allele frequencies Natives > Mestizos > MXL > CEU, suggesting that Natives and Mestizos are 2× more likely to have decreased CYP2B6 metabolism due to rs4803419 which is in LD with rs3745274 a Level 1 variant. This is mostly relevant for efavirenz elimination, since CYP2B6 is its major metabolizing enzyme. A recently published guideline considers rs3745274 as the pharmacogenetic marker for efavirenz dosing assessment (Desta et al., 2019).

We identified five variants with lower allele frequency in Natives vs. Mestizos, CEU or MXL on, *CYP2C19**2 rs4244285, *NAT2 rs1799930*, *CYP2D6* rs28371725, *SLCO1B1* rs41419015, and *ABCB1* rs2032582. In addition to the already discussed *VKORC1–CYP2C9**2/*3 interplay, our results on *CYP2C9* validate previous reports showing that Mestizos are 3× and 4× more likely to show impaired CYP2C9 metabolism than Natives due to *CYP2C9**2 and *3 (Villegas-Torres et al., 2015). CYP2C9 is the second most expressed CYP in liver after CYP3A4, and impacts 15% of all drugs including losartan, NSAIDs, phenytoin, and hypoglycemic agents, (Sconce et al., 2005; Van Booven et al., 2010). Here, we hypothesize that Natives will show a lower probability of impaired CYP2C9 activity compared to Mestizos and both of them lower to CEU. These comparisons have been previously confirmed for *CYP2C9*, *ABCB1*, *CYP3A5*, and *CYP2C19* (Vargas-Alarcón et al., 2014; Zhou et al., 2017). For *CYP2C19*, we can infer that a higher proportion of Natives are normal/extensive metabolizers. Clopidrogrel dosing guideline lists around 40 variants on *CYP2C19*, we identified 3 of the 10 variants

reported for the Americas, *CYP2C19*3 and *4* were rare (<1%) in Mestizos and CEU, and we did not observe them in Natives. The CPIC reported these variants associated to ADRs and are relevant for dose estimation for clopidogrel, escitalopram, and voriconazole. It is possible that ADR or dose adjustments would be seen in a lower proportion in Mexicans. Similarly, for *SLCO1B1* rs4149015 and *ABCB1* rs2032582 frequencies were lower in Natives, from we could infer a higher efficacy of drugs such as statins, atazanavir, or sunitinib.

Similarities among Natives, Mestizos, MXL, and CEU were observed for several variants on *UGT1A1*, *DPYD*, *ABCB1*, *CBR3*, *CYP2B6*, *COMT*, *and TPMT* hinting towards a comparable pharmacokinetics between populations for the role variants observed. Maybe these variants could smoothly transition into clinical implementation, but these inferences should not only be validated, but should also account for unreported, rare, and novel variation to determine the overall pharmacogenetic impact for each gene-drug combination.

Novel Variants on PGKB Genes

Pharmacogenetic research in populations from Mexico has been actively increasing, however the number of publications by 2018 barely reached 0.3% of the >22,000 NCBI pharmacogenetic/pharmacogenomics reports. Here, we identified novel variants on 16 of the 17 studied PGKB genes, in Mestizos (58 variants) and Natives (67 variants, **Figures 1** and **2, Table 3** and **Supplemental Table 2**). For some genes novel variants were identified only in Natives, *APOE* (1), *UGT1A1* (3), and *VKORC1* (3) or only in Mestizos, *COMT* (4), *CYP2B6* (1), *CYP2D6* (3), and *CYP3A4* (6). Although these counts are influenced by sample size, it may offer an estimate of the completion in gene variation. Interesting differences in the number of novel variants arose for *ABCB1*, *ACE*, *DPYD*, *UGT1A1*, and *VKORC1*. For example, the transporter gene *ABCB1* showed 25 novel variants in Natives, and only 5 in Mestizos, the functional impact of most of these were confirmed by independent algorithms and 10 were validated as deleterious. Similar results were observed for variants on *DPYD* suggesting that variation on these genes is far from complete in Natives. In the previous section, we mentioned that the allele frequency of several PGKB variants in Natives were similar to that in other populations however, novel variation indicates that it is likely that the overall pharmacogenetic impact has not been fully described. This is in agreement with a few reports on *DPYD* and *ABCB1* novel variation in underrepresented populations, indicating that our current information of pharmacogenetic predictors remains to be thoroughly depicted (Mukonzo et al., 2009; Del Re et al., 2015; Elraiyah et al., 2017).

Variation on *CYP2C19* did not show novel variants in Natives, we may infer that most common variation has been recorded for this gene, and that further phenotypic variability may be imputed to rare mutations. In Mestizos, we found 10 novel variants, 2 were missense deleterious (POS.10:96522531 and POS 10:96609809) which may be relevant for dozens of drugs including, proton pump inhibitors, antiepileptics, antiplatelets, and antidepressants. We anticipated fewer novel variation on this gene since intense research has been done given its high importance in multiple drug-drug interactions and drug metabolism. Nevertheless, 10 novel variants were identified in Mestizos, which may accord with a couple of recent NGS studies, reporting novel, common, and rare variation on this gene in Asians and Africans (Matimba et al., 2009; Han et al., 2017; Dai et al., 2015).

Interestingly, we detected 14 novel variants with a MAF>3% on (4 SNVs MAF:8%), *DPYD* (1 SNV, MAF:3%), *CYP2C9* (1 SNV, 3%), *ACE* (1 SNV, MAF:5%), *APOE* (1 SNV, MAF:5%), *CYP3A4*/5 (5 SNVs MAF 3%), and *CYP2D6* (1 SNV, MAF:40%), although none of these were predicted as deleterious (**Supplemental Table 1**).

We are aware of the different sequencing techniques and size of the study, potentially affecting the number of variants and the inferences made. Also, PGKB variants not reported here reflect either lack of sequencing coverage or an allele frequency lower than 1%. Nevertheless, several of our observations have been confirmed by previous reports (Villegas-Torres et al., 2015) or paralleled those from Latin American countries such as Brazil with which we share pharmacogenetic similarities and differences ex. MAF for *CYP2B6* rs3745274, *CYP3A5* rs776746, *VKORC1* rs8050894, and *ABCB1* rs2032582, supporting the notion that pharmacogenetic diversity across the Americas ought to be consolidated.

CONCLUSIONS

Our observations summarize variation in 55 pharmacogenes in 1,378 Natives and Mestizos from Mexico, focusing on 17 PGKB genes. This is one of the largest collections of genetic variability related to pharmacogenomics in Mexicans. Our report offers a collection of variants in core pharmacogenes, confirming previous knowledge and contributing to the list of novel variants that can be further investigated and may become a part of a preliminary catalogue for PK/PD, and phenotype-genotype correlations. These results may also complement genotyping platforms with relevant pharmacogenetic variants with specific population background. Future studies will seek to validate this variation and to confirm its potential application in pharmacogenomics.

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study can be found at the databases: dbGAP: WES (Mestizos=968) (Flannick et al. 2019) [https://www.ncbi.nlm.nih.gov/gap/ phs001393 and phs001099], and at database, European Variation Archive (EVA) under project PRJEB343334 for: NGS-targeted 1 (Mestizos =110) (Gonzalez-Covarrubias et al. 2017) [EVA INMEGEN-ACOAG analysis: ERZ1079024], NGS-targeted 12 (Mestizos =146) (Gonzalez-Covarrubias et al. 2016) [INMEGEN-SCZ analysis: ERZ1079026], and NGS-targeted 3 128 (Mestizos = 60) (Cruz-Correa et al. 2017) [INMEGEN-ATV analysis: ERZ1079025]. WGS (Natives=94) Access to this dataset can be requested via email to Dr. Cristobal Fresno of 100G-Consortium, INMEGEN [cfresno@inmegen.gob.mx].

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comite de Etica e Investigacion, Instituto Nacional de Medicina Genomica #25/2016/I. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

VG-C conceived the analyses and wrote the paper. MM-F performed bioinformatics and statistical analyses. OC-C contributed to data collection. AM-H, HG-O, and FB-O contributed to patient recruitment and genetic experiments. AG-M contributed to patient recruitment and genetic experiments. JM-M recruited patients and performed genetic experiments. HN designed project, contributed to patient recruitment. LO conceived, coordinated, and executed the project. XS conceived and coordinated the project.

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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.2019.01169/full#supplementary-material

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Effect of Gene-Based Warfarin Dosing on Anticoagulation Control and Clinical Events in a Real-World Setting

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The *cytochrome P450 2C9* and *vitamin K epoxide reductase complex subunit 1* genotypes are associated with anticoagulation control and the clinical events in warfarin therapy. However, the clinical utility of gene-based warfarin dosing (GBWD) is controversial. We compared the anticoagulation control and clinical events related to warfarin with GBWD to those with clinically fixed dosing (CFD). A retrospective cohort study was conducted in a real-world setting. Of the 915 patients who were reviewed, 844 patients met the study-entry criteria; 413 cases were guided by GBWD using the International Warfarin Pharmacogenetic Consortium algorithm; 431 cases were guided by CFD (2.5 mg/day). The primary outcomes were the time needed to achieve the therapeutic International Normalized Ratio (INR) and the time in the therapeutic range (TTR) during a 3-month timeframe. The time needed to achieve the therapeutic INR (in days) for patients in the GBWD group was shorter than that for patients in the CFD group (10.21 ± 4.68 *vs.* 14.31 ± 8.26, P < 0.001). The overall TTR (Day 4-90) was significantly different between the GBWD group and CFD group (56.86 ± 10.72 vs. 52.87 ± 13.92, P = 0.007).In subgroup analysis, the TTR was also significantly different between the GBWD group and CFD group during the first month of treatment (Day 4-14: 54.28 ± 21.90 *vs.* 47.01 ± 26.25, *P* = 0.012; Day 15-28: 59.60 ± 20.12 *vs.* 51.71 ± 18.96, *P* = 0.001). However, no significant difference in the TTR was observed after 29 days of treatment. These data suggest that GBWD provided clinical benefits.

Keywords: pharmacogenetics, CYP2C9, precision medicine, warfarin, VKORC1

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INTRODUCTION

Despite the recent approval of direct oral anticoagulants (DOACs) (Zhang et al., 2014b; Arwood et al., 2016), warfarin remains the most commonly used oral anticoagulant. Warfarin is the only option for patients with artificial heart valves or atrial fibrillation with severe hepatic/renal insufficiency. In particular, warfarin is suitable for people on low incomes or intermediate incomes who cannot afford the high cost of DOACs. However, warfarin administration is hindered by a narrow therapeutic index and large variability among different individuals in the dose required to achieve therapeutic anticoagulation (Arwood et al., 2016).

Studies have suggested that the cytochrome *P450 2C9* (*CYP2C9*) and *vitamin K epoxide reductase complex subunit 1* (*VKORC1*) genotypes are associated with the time needed to achieve therapeutic anticoagulation, the dose requirements of warfarin, and risk of supra-therapeutic anticoagulation and major bleeding (Jonas and Mcleod, 2009; Johnson et al., 2011; Fung et al., 2012; Arwood et al., 2016). Therefore, in 2007, the United States Food and Drug Administration updated the drug label for warfarin to reflect the potential value of incorporating genetic information into dose selection. Patients with certain genetic variants of *CYP2C9* require a lower dose of warfarin and a longer time to reach a stable dose. They are also at higher risk of over-anticoagulation and serious bleeding (Higashi, 2002; Schwarz et al., 2008; Voora et al., 2010). Patients with the A/A haplotype of *VKORC1* have a reduced time to the first International Normalized Ratio (INR) within the therapeutic range and to the first INR > 4 (Higashi, 2002; Schwarz et al., 2008; Voora et al., 2010).

Some randomized controlled trials have evaluated the clinical efficacy of gene-based warfarin administration (Jonas et al., 2013; Kimmel et al., 2013; Pirmohamed et al., 2013; Belley- et al., 2015). However, the results were mixed, with some studies recommending gene-based warfarin therapy and others not supporting this strategy.

We explored the clinical efficacy of gene-based warfarin administration by comparing the anticoagulation control and clinical events related to warfarin with gene-based warfarin dosing (GBWD) to those with clinically fixed dosing (CFD) in a real-world scenario.

METHODS

Study Design and Eligibility

This was a retrospective cohort study designed to compare GBWD with CFD. The study protocol was approved by the Ethics Committee of China Fujian Medical University Union Hospital (Fujian, China). All patients who were newly prescribed warfarin between March 2014 and May 2019 were enrolled.

The inclusion criteria were people: (i) aged ≥18 years; (ii) with results for detection of *VKORC1* and *CYP2C9* polymorphism available; (iii) in whom anticoagulation with warfarin for ≥3 months had been achieved.

The exclusion criteria were individuals with: (i) a diagnosis of active cancer; (ii) severe infection or respiratory failure; (iii) severe hepatic/renal insufficiency; (iv) hematologic diseases; (v) abnormal thyroid function.

Data Collection and Follow-Up

The intervention was the initial warfarin dose. GBWD was calculated according to the International Warfarin Pharmacogenetic Consortium (IWPC) algorithm. The CFD of warfarin was 2.5 mg/day. For Chinese patients with mechanical heart valves, bleeding was the major complication rather than thromboembolism. Most clinicians apply low-intensity anticoagulation for patients undergoing heart valve replacement in China (Zhou et al., 2005). Therefore, the target therapeutic INR range was 1.7–2.5 for patients with valve replacement, and 2.0–3.0 for patients with atrial fibrillation or venous thromboembolism (Tao et al., 2018).

Patient interview, review of medical records, and telephone follow-up revealed the following data: age, sex, height, weight, indication for warfarin therapy, range of target INR, date of initiation of warfarin therapy, initial warfarin dose, concomitant medications, INR values, warfarin doses, smoker status, and thromboembolic and bleeding events.

Genotyping

Peripheral venous blood (2 mL) was collected from each patient. Genomic DNA was extracted using a DNA extraction kit according to manufacturer (Shanghai Baio, Shanghai, China) instructions. The *CYP2C9* and *VKORC1* genotypes were determined by DNA microarray hybridization reactions to a gene chip (Shanghai Baio) after initial polymerase chain reaction amplification of the target region with mutation sites using primers for the major variant alleles *CYP2C9*2 (rs1799853)*, *CYP2C9*3 (rs1057910)*, and *VKORC1 (rs9923231)*. The mutant allele or wild-type allele was identified using a biometric reader (BE 2.0; Shanghai Baio).

Outcomes

The primary outcomes were the time to achieve the therapeutic INR and time in the therapeutic range (TTR) during a 3-month timeframe. The time to achieve the first INR in the therapeutic range was defined as the time from the initiation of warfarin therapy until the first INR reached the treatment anticoagulation range. TTR was calculated based on the method developed by Rosendaal et al. (1993). The secondary outcomes were INR ≥4 events, major bleeding, minor bleeding, and thromboembolism events (TEs). Major bleeding events are those that result in death, are life-threatening, cause chronic sequelae or consume major health-care resources, as defined in the International Society on Thrombosis and Haemostasis classification (Schulman and Kearon, 2005).

Sample Size

The primary endpoint of this study is the goal attainment rate of the TTR index. The sample size was calculated according to the expected difference between the TTR goal attainment rate of the clinically fixed dosing group and gene-based dosing group. Based on previous research, the TTRs of the clinically fixed dosing group and gene-based dosing group were about 60.3% and 67.4% (Pirmohamed et al., 2013). If the requirement to meet is at least 80%, a class of errors will be 0.05. Calculation with PASS V.11 software shows that a study of the gene-based dosing group and clinically fixed dosing group at a 1:1 proportion needs at least 268 patients per group. Assuming a dropout rate or loss rate of 20%, each group needs at least 322 patients, with a total of 644 patients.

Statistical Analyses

Data were analyzed using SPSS v22.0 (IBM, Armonk, NY, USA) and Prism v7.0 (GraphPad, San Diego, CA, USA). Statistical significance was set at $P < 0.05$. Continuous data are given as the mean ± standard deviation. Categorical variables are described as

percentages. The chi-square test or Fisher's exact test (as appropriate) was used to identify the difference between two percentages. A two-tailed Student's *t*-test was used to compare two sets of continuous data. Time-to-event outcomes were shown with Kaplan–Meier curves.

RESULTS

Population Characteristics

Of the 915 patients who were reviewed, 71 were excluded from the analysis: 11 patients had abnormal liver function; 9 did not have an indication for warfarin therapy; five were undergoing chemotherapy; 14 had abnormal thyroid function; 32 switched to other anticoagulant drugs. These exclusions resulted in a final study population of 844 patients. In 413 cases, the initial dose of warfarin was guided by GBWD (IWPC algorithm). In 431 patients, CFD (2.5 mg/day) was employed.

The demographic characteristics of the patients are shown in **Table 1**. Patient characteristics and genotypic distributions were well-balanced between the two groups at baseline. The mean age of the recruited patients was 56.45 ± 11.46 years, and 57.8% of patients were female.

The indications for warfarin were artificial heart valves, atrial fibrillation, and venous thromboembolism. Also, 93.6% of patients were *CYP2C9* wild-type (*CYP2C9*1/*1*) and 87.2% were *VKORC1 AA*. The distributions of genotypes were consistent with a Han-Chinese population reported by Zhang and colleagues (Zhang et al., 2016).

TABLE 1 | Demographic characteristics of patients.

Characteristic	Gene-based dosing group (N = 413)	Clinically fixed dosing group (N = 431)	*P*
Age (years)	57.14 ± 11.02	55.80 ± 11.85	0.088
Male	43.1%	41.3%	0.597
Body surface area (m^2)	1.59 ± 0.17	1.61 ± 0.18	0.106
Current smoker	14.8%	14.4%	0.874
Current use of amiodarone	21.8%	21.1%	0.810
Indications for treatment			0.774
Heart-valve replacement	68.8%	71.0%	
Atrial fibrillation	26.4%	24.4%	
Treatment of DVT and/or PE	4.8%	4.6%	
Concomitant diseases			
Hypertension	24.0%	23.0%	0.732
Diabetes mellitus	9.9%	9.5%	0.839
***VKORC1* genotype**	86.7%	87.7%	0.657
AA	12.1%	11.1%	
AG	1.2%	1.2%	
GG			
***CYP2C9* genotype**	93.7%	93.5%	0.905
**1/*1*	6.3%	6.3%	
**1/*3*	0	0.2%	
**3/*3*			

DVT, deep venous thrombosis; PE, pulmonary embolism.
The "" in CYP2C9 genotype means an expression of a gene mutation site.*

Primary Outcomes

The time needed to achieve the therapeutic INR (in days) for patients in the GBWD group was shorter than that for patients in the CFD group (10.21 ± 4.68 *vs.* 14.31 ± 8.26, P < 0.001) (**Table 2**, **Figure 1**). The overall TTR (Day 4–90) was significantly different between the GBWD group and CFD group (56.86 ± 10.72 vs. 52.87 ± 13.92, P = 0.007).In subgroup analysis, the TTR was also significantly different between the GBWD group and CFD group during the first month of treatment (Day 4-14: 54.28 ± 21.90 *vs.* 47.01 ± 26.25, *P* = 0.012; Day 15-28: 59.60 ± 20.12 *vs.* 51.71 ± 18.96, *P* = 0.001). However, no significant difference (P = 0.206, P = 0.887) in the TTR was observed after 29 days of treatment. (**Table 2**, **Figure 2**).

Secondary Outcomes

Only five major bleeding events and five thromboembolic events were reported, and they occurred in the CFD group. One patient in the GBWD group had minor bleeding events, whereas 34 patients in the CFD group had minor bleeding events. There were significant differences in the prevalence of minor bleeding between the GBWD group and CFD group (0.2% *vs.* 7.9%, *P* < 0.001), but there were no significant differences in INR ≥4.0 events, major bleeding events, or thromboembolic events (*P* > 0.05 for all) (**Table 3**).

TABLE 2 | Primary outcomes (anticoagulation control).

Time	Gene-based dosing group (N = 413)	Clinically fixed dosing group (N = 431)	*P*
Time to reach therapeutic INR -days	10.21 ± 4.68	14.31 ± 8.26	<0.001
Day 4–90	56.86 ± 10.72%	52.87 ± 13.92%	0.007
Day 4–14	54.28 ± 21.90%	47.01 ± 26.25%	0.012
Day 15–28	59.60 ± 20.12%	51.71 ± 18.96%	0.001
Day 29–56	58.55 ± 23.24%	56.42 ± 25.47%	0.206
Day 57–90	52.95 ± 22.52%	53.17 ± 23.07%	0.887

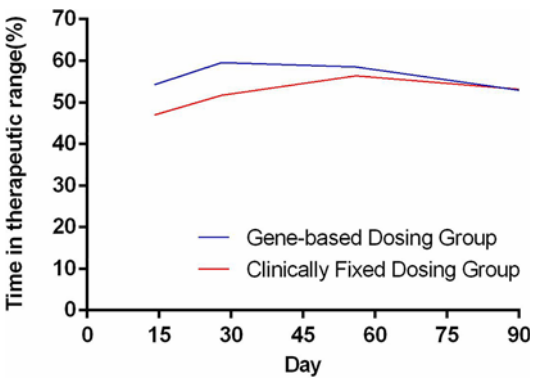

FIGURE 1 | Time needed to reach the therapeutic INR.

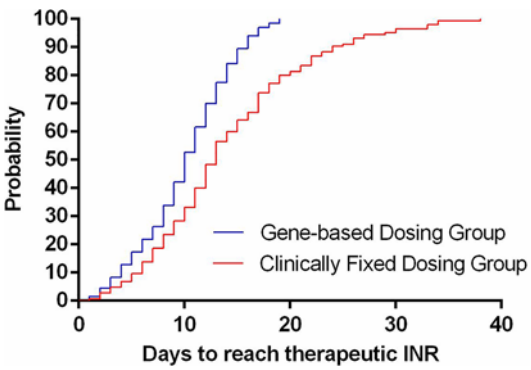

FIGURE 2 | Time in the therapeutic range during follow-up.

TABLE 3 | Secondary outcomes.

Time	Gene-based dosing group (N = 413)	Clinically fixed dosing group (N = 431)	*P*
INR ≥4.0	3.4%	5.1%	0.218
Major bleeding events	0	1.2%	0.062
Non-major bleeding events	0.2%	7.9%	< 0.001
Thromboembolic events	0	1.2%	0.062

DISCUSSION

Since 2013, warfarin-related gene testing has been carried out in increasing numbers of hospitals in China. The IWPC algorithm was introduced in China to recommend the initial dose of warfarin. The IWPC algorithm was based on a study involving the enrollment of >5000 patients from three major ethnic populations (Caucasian, African, and Asian), which was the largest-scale study on warfarin-dose prediction (Wen et al., 2017).

Prompt achievement of therapeutic anticoagulation is a major goal when initiating warfarin treatment (Arwood et al., 2016). Especially for patients undergoing implantation of artificial heart valves, the first postoperative month is a high-risk period for thromboembolism (Baumgartner and Helmut, 2017). The risk of thrombosis recurrence in patients with acute venous thrombosis in the first few months after the diagnosis is also very high (White, 2003; Arwood et al., 2016). The risk of major bleeding events is tenfold higher during the first month following warfarin initiation than for the remainder of therapy (Heit et al., 2003). In China, warfarin is usually started at a fixed dose of 2.5 mg/day, with dose titration based on the INR response (Zhang et al., 2014a). However, achieving the target anticoagulant treatment range is difficult and during this time, patients are at a high risk of thrombosis and bleeding (Pirmohamed et al., 2013; Hua et al., 2018).

We revealed that the use of GBWD improved primary outcomes (the time to achieve the therapeutic INR and TTR) significantly. The time to achieve the therapeutic INR in the GBWD group was shorter than that in the CFD group. The time to reach a therapeutic INR has been studied by several investigators. Our results are in accordance with those of other studies and suggest that a GBWD algorithm may shorten the time to reach a therapeutic INR (Johnson et al., 2017; Wen et al., 2017). However, Li and colleagues, (2013) showed no difference in the time to reach the target INR between a GBWD group and CFD group. The reason for this difference could be related to the loading dose (5 mg/day) used in the CFD group in the study by Li and colleagues.

We showed that the overall TTR (Day 4-90) in the GBWD group was higher than that in the CFD group. Subgroup analysis also revealed that the TTR in the GBWD group was higher than that in the CFD group in the first 28 days. However, the TTR did not differ between the two groups from 29 days to 90 days. These data may suggest that the benefits of GBWD over CFD are especially marked in the first month after anticoagulation initiation. Subsequently, GBWD had less of an effect on anticoagulation control, and multiple-dose titrations might have had a greater role. The finding that the TTR was shorter in the GBWD group is similar to that observed in the GIFT, EU-PACT, and COUMAGEN-II trials (Anderson et al., 2012; Wen et al., 2017) but different from that in the COAG study and other Asian-based studies (Kimmel et al., 2013; Gage et al., 2017; Syn et al., 2018; Zhe et al., 2018). Our study revealed that GBWD resulted in a lower prevalence of bleeding events related to anticoagulation therapy, which may have been due to superior primary outcomes.

All of the primary outcomes and some of the secondary outcomes strongly indicated that GBWD provided benefits. Also, the IWPC algorithm could be suitable for Chinese populations if a locally developed dosing algorithm is not available, a hypothesis that is in accordance with a study by Li and collaborators, (2013). This study suggests that the use of gene-based warfarin dosing deserves continued consideration, evaluation, and application (assuming that the cost of the warfarin plus the genotyping is less than the use of a DOAC) throughout the world.

Our study had three main limitations. First, as a retrospective study, participants were not assigned randomly to the GBWD group and CFD group, so selection bias and other potential confounding variables may have been present. Second, telephone follow-up may not collect all the bleeding and thrombosis-related events. Prospective, multicenter cohort studies are required to confirm our findings. Third, the sample size was too small to perform a subgroup analysis of the effect of CYP2C9 and VKORC1 composite genotypes on warfarin.

CONCLUSION

The GBWD group was superior to the CFD group in terms of anticoagulation control and the prevalence of minor bleeding, especially in the first month of initial anticoagulation. These data suggest that GBWD provides clinical benefits. The IWPC algorithm may be suitable for Chinese populations.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The study protocol was approved by the Ethics Committee of China Fujian Medical University Union Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JZ analyzed the data and wrote the article, LC designed the research, and TW and WC performed the research. XX and JF collected patient information. All the authors contributed to the final paper.

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Machine Learning Algorithm for Predicting Warfarin Dose in Caribbean Hispanics Using Pharmacogenetic Data

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Despite some previous examples of successful application to the field of pharmacogenomics, the utility of machine learning (ML) techniques for warfarin dose predictions in Caribbean Hispanic patients has yet to be fully evaluated. This study compares seven ML methods to predict warfarin dosing in Caribbean Hispanics. This is a secondary analysis of genetic and non-genetic clinical data from 190 cardiovascular Hispanic patients. Seven ML algorithms were applied to the data. Data was divided into 80 and 20% to be used as training and test sets. ML algorithms were trained with the training set to obtain the models. Model performance was determined by computing the corresponding mean absolute error (MAE) and % patients whose predicted optimal dose were within ±20% of the actual stabilization dose, and then compared between groups of patients with "normal" (i.e., > 21 but <49 mg/week), low (i.e., ≤21 mg/week, "sensitive"), and high (i.e., ≥49 mg/week, "resistant") dose requirements. Random forest regression (RFR) significantly outperform all other methods, with a MAE of 4.73 mg/week and 80.56% of cases within ±20% of the actual stabilization dose. Among those with "normal" dose requirements, RFR performance is also better than the rest of models (MAE = 2.91 mg/week). In the "sensitive" group, support vector regression (SVR) shows superiority over the others with lower MAE of 4.79 mg/week. Finally, multivariate adaptive splines (MARS) shows the best performance in the resistant group (MAE = 7.22 mg/week) and 66.7% of predictions within ±20%. Models generated by using RFR, MARS, and SVR algorithms showed significantly better predictions of weekly warfarin dosing in the studied cohorts than other algorithms. Better performance of the ML models for patients with "normal," "sensitive," and "resistant" to warfarin were obtained when compared to other populations and previous statistical models.

Keywords: pharmacogenetics, machine-learning, warfarin, Hispanics, prediction algorithms

INTRODUCTION

Warfarin is one of the most used anticoagulants worldwide. However, its use tends to be challenging, due to its narrow therapeutic window and dose variability requirements among patients (Liu et al., 2015; Ma et al., 2018). Side effects may result in bleeding for patients with an overdosing or thrombosis in case of under-dosing, both related with an inadequate dosage. Consequently, patients who are under treatment need to be continuously monitored to avoid further damage. Studies have been developed in order to improve the recommended dose for warfarin patients that present side effects related to bleeding or thrombosis (Liu et al., 2015; Ma et al., 2018).

Demographic variables, genetic variants, and clinical factors are largely responsible for the broad variability of warfarin dosing among patients. Previous studies have reported that non-genetic factors such as age, height, weight, race, and drug interactions can explain around 15–20% of such inter-individual variability (Liu et al., 2015). On the other hand, genetic factors are considered critical predictors of warfarin dose requirements in various populations worldwide, particularly polymorphisms in genes encoding cytochrome P450, family 2, subfamily C, polypeptide 9 (CYP2C9) and vitamin K-epoxide reductase complex 1 (VKORC1). These two genes may individually contribute to 6–18 and 15–30%, respectively, in warfarin dose variability. However, the combination of relevant polymorphisms in both pharmacogenes accounted for approx. 30% of observed inter-patient variability in warfarin dose requirements, affecting both pharmacodynamics and pharmacokinetics of this drug (Liu et al., 2015).

To improve patient quality of life, researchers have developed predictive pharmacogenetic dosing algorithms for warfarin in multiple ethnicities (Cosgun et al., 2011; Hu et al., 2012; Liu et al., 2015; Sharabiani et al., 2015; Li et al., 2015; Ma et al., 2018). Most of the algorithms integrate demographic, clinical, and genetic variants, based on multiple linear regression (MLR) methods. Previous studies have demonstrated a prediction accuracy of around 37–55% for the patients of warfarin stable dose. In addition, machine learning (ML) algorithms in pharmacogenetic warfarin dosing have been reported (Liu et al., 2015). Some of these algorithms have been compared in racially diverse groups, however Caribbean Hispanic populations have not been included. Thus, in this study we aim to compare seven ML methods to predict stable warfarin dosing in Caribbean Hispanic patients, using genetic and non-genetic clinical data.

MATERIALS AND METHODS

Patient Cohorts

This is a secondary analysis of genetic and clinical data collected from participants in an open-label, single-center, population-based, observational, retrospective cohort study (ClinicalTrial.gov identifier NCT01318057). Participants were recruited from the Veteran's Affairs Caribbean Healthcare System (VACHS)-affiliated anticoagulation clinic in San Juan, Puerto Rico, which serves a predominantly Caribbean Hispanic population. Participants self-reported as Caribbean Hispanic Puerto Ricans, were ≥21 years old and on a stable maintenance dose of warfarin. For the purpose of the study, a stable warfarin dose was defined as the average weekly amount of drug required to maintain stable anticoagulation levels (i.e., international normalized ratio (INR) values within therapeutic range defined as 2–3 for most indications on at least three consecutive visits). A full description of this cohort as well as detailed information on the patient's recruitment process can be found elsewhere (Duconge et al., 2016). The study was approved by the Institutional Review Boards (IRBs) of the VACHS (#00558) and the University of Puerto Rico Medical Sciences Campus (A4070109). Additional data from participants in a multicenter case–control study of Puerto Rican Hispanic patients receiving antiplatelet therapy with clopidogrel, who were recruited between January 2018 and March 2019, were also included in this secondary analysis (ClinicalTrial.gov identifier NCT03419325). This study was also IRB-approved (A4070416) by the corresponding institutional committee.

These two clinical studies were conducted according to the principles in the Declaration of Helsinki. Written informed consent was obtained from each participant prior to enrollment. Patients were divided into three major categories or classes based on their corresponding weekly warfarin dose requirements as "normal" (i.e., > 21 but <49 mg/weekly; a.k.a., intermediate dose subgroup), "sensitive" (i.e., ≤21 mg/weekly; a.k.a., low-dose subgroup), and "resistant" (i.e., ≥49 mg/weekly; a.k.a., high-dose subgroup) (Duconge et al., 2016).

Dataset Preparation

The study dataset was prepared using information from patients of the A4070109 study cohort (N = 95), but also included data from another 95 patients in the secondary cohort (A4070416 protocol), for a total of 190 patients. Only 95 warfarin patients from the original cohort (n = 275) had full genetic, ancestry, clinical, and demographic data available to run the corresponding ML methods. Pharmacogenetic variants previously found to be associated with warfarin dose requirements in Puerto Ricans (Ramos et al., 2012; Duconge et al., 2016; Claudio-Campos et al., 2017), individual ancestry proportions, as well as clinical and demographic data from all enrolled patients were considered in the corresponding analyses. The primary cohort, which corresponds to patients on warfarin, included 40 "normal," 38 "sensitive," and 17 "resistant" cases. All cases from the secondary cohort were assigned to the "normal" weekly warfarin dose category. Their doses were imputed as random values within ±20% of the average dose level in the "normal" subgroup of the primary cohort.

To develop and evaluate the models, the data was separated as approximately 80% for the training set (N of training = 154) and about 20% for the testing set (N of testing = 36). The training set had an imbalanced distribution for the number of "normal" cases ("normal" = 111), *versus* "sensitives" and "resistant" ("sensitive" = 30, "resistant" = 13). Then, a randomized oversampling

technique was used to balance the training dataset in order to develop the models (Ling and Li, 1998).

Genotyping and Ancestry Estimations

All DNA specimens from participants were tested following manufacturer's instructions. A full description of genotyping methods can be found elsewhere (www.illumina.com/genotyping). Briefly, the Infinium™ Human OmniExpress-24 v1.2 BeadChip by Illumina, which provides a broad coverage of relevant markers for genome-wide association studies (GWAS), was used to perform the genetic testing of 95 warfarin patient from the A4070109 study cohort in iScan® system (Illumina, San Diego, CA). Additionally, the Infinium™ Multi-Ethnic Global AMR/AFR BeadChip was used in the A4070416 cohort of patients on clopidogrel. Genotypes at relevant loci (i.e., *FMO2* c.107A > G, p.D36G, rs2020870 in chromosome 1; *ABCB1* c.1000-44G > A, rs10276036 in chromosome 7; *SLCO1B3* c.1833G > A, G611 =, rs3764006 in chromosome 12; *CYP2C9*, rs1856908 and *CYP2C9*2* c.430C > T, p.R144C, rs1799853 in chromosome 10; *VKORC1* c.1173C > T, rs9934438 in chromosome 16; *CYP4F2*3* c.1297G > A, p.V433M, rs2108622 in chromosome 19; *NQO1*2* c.4559C > T, p.P187S, rs1800566 in chromosome 16) were then retrieved from the corresponding Variant Call Format (VCF) files.

Individual proportions of each ancestry component in the study population were estimated by ADMIXTURE software (Alexander et al., 2009), with the corresponding parental references for the Native American (NAT), European (EUR), and African (AFR) contributions taken from the 1,000 Genomes Project (Auton et al., 2015). To this purpose, data from Iberian populations in Spain (IBS) and Yoruba population in Ibadan, Nigeria (YRI) were used to properly represent EUR and AFR ancestries in the analysis, respectively.

Machine Learning Algorithms

Seven ML algorithms were selected for generating the models and testing them using the data from the two Caribbean Hispanic cohorts. These algorithms were multivariate adaptive splines (MARS) (Klein et al., 2009), artificial neural networks (ANN) (Grossi et al., 2013), random forest regression (RFR) (Cosgun et al., 2011), support vector regression (SVR) (Suykens and Vandewalle, 1999), K-nearest neighbor for K from 1 to 3 (i.e., iBK1, iBK2, iBK3, respectively) (Aha et al., 1991), recursive partitioning (RPART) (Breiman, 1984), and reduces error tree classifier (REPT) (Mohamed et al., 2012). Weka—ML in Java software was used to both train the ML algorithms and obtain the predictive models, as well as evaluate and compare the models (Frank et al., 2016). For each ML algorithm tested, the model with the best predictability was chosen regardless of the number of added variants.

To evaluate and compare the model's predictability, we primarily computed the mean absolute error (MAE) and the percentage (%) of patients whose predicted warfarin dosage values were within 20% of the actual stable dosage found in the available data (Duconge et al., 2016). This 20% value represents a difference of 7 mg/week relative to the standard starting dose of 35 mg/week, a difference that clinicians define as clinically relevant. The MAE is the average of the absolute difference between two continuous values, in this case the actual and the predicted dose values. Both metrics (i.e., MAE and percentage within 20%) were compared among the ML models independently and after dividing patients into the above-mentioned three categories based on their warfarin dose requirements (i.e., "normal," "sensitive," and "resistant").

Statistical Analyses

All comparisons of mean values between training and test datasets were performed by using a two-sided unpaired t-test (Hsu, 1938) for continuous variables (e.g. warfarin dose, weight, ancestry estimates, etc.) and a proportion-test (Wilson, 1927) for frequencies or dichotomous variables (e.g. diplotypes, conditions, co-medications, etc.).

RESULTS

Basic Characteristics of the Study Cohort

Clinical and demographic variables of interest are summarized in **Table 1** for the 190 patients included in this study (i.e., 154 assigned to a training set and another 36 in the test set). **Table 1**

TABLE 1 | Relevant characteristics of the Caribbean Hispanic patients included in this study.

Variables	Groups		*p*-value	Total cohort (n = 190)
	Training set (n = 154)	Test set (n = 36)		
Warfarin dose (mg/week), mean (SD)	32.59 (8.99)	32.84 (7.68)	0.8627	32.64 (8.74)
Weight (kg), mean (SD)	81.27 (18.67)	81.42 (16.12)	0.9612	81.29 (18.17)
Height (cm), mean (SD)	167.45 (8.73)	168.94 (8.36)	0.3440	167.74 (8.66)
Ancestry proportions – mean (SD)				
NAT	0.11 (0.03)	0.11 (0.03)	0.9419	0.12 (0.05)
EUR	0.68 (0.13)	0.70 (0.10)	0.4789	0.68 (0.14)
AFR	0.20 (0.14)	0.19 (0.10)	0.5026	0.20 (0.13)
Population by age (%)				
≥50 years-old	151 (98.05)	33 (91.67)	0.1915	184 (96.84)
<50 years-old	3 (1.95)	3 (8.33)	0.1915	6 (3.16)
Conditions (%)				
DVT	12 (7.79)	6 (16.67)	0.1897	18 (9.47)
PE	4 (2.60)	4 (11.11)	0.1273	8 (4.21)
AF	50 (32.47)	16 (44.44)	0.1995	66 (34.74)
VR	5 (3.25)	2 (5.56)	0.5788	7 (3.68)
Stroke	9 (5.84)	5 (13.89)	0.1975	14 (7.37)
DM2	73 (47.4)	18 (50.0)	0.7826	91 (47.89)
CHF	9 (5.84)	5 (13.89)	0.1975	14 (7.37)
Smokers	15 (9.74)	5 (13.89)	0.5146	20 (10.53)
Others*	101 (65.58)	21 (58.33)	0.4331	122 (64.21)
Co-medications (%)				
Aspirin	56 (36.36)	10 (27.78)	0.3175	66 (34.74)
Statins	101 (65.58)	21 (58.33)	0.4331	122 (64.21)
Azoles	5 (3.25)	1 (2.78)	0.8813	6 (3.16)
Clopidogrel[#]	79 (51.3)	16 (44.4)	0.8700	95 (50)

*Mean refers to arithmetic mean. NAT, Native Americans; AFR, Africans; EUR, Europeans; DVT, Deep Vein Thrombosis; PE, Pulmonary Embolism; AF, Atrial Fibrillation; VR, Valve Replacement; DM2, Type-2 Diabetes Mellitus; CHF, Congestive Heart Failure. *Others means any other diagnosis of cardiovascular conditions (e.g., acute coronary syndrome, peripheral artery disease, chronic hypertension, etc.). [#]clopidogrel doses of 75mg/daily.*

also presents their corresponding ancestry proportions. Furthermore, diplotypes at each genetic locus of interest in this study are also shown in **Table 2**.

Among these 190 patients, 96.8% were aged 50 years or older. Their average warfarin dose was 32.6 mg/week with a standard deviation of 8.74. A total of 122 patients were using statins to lower their cholesterol levels. Of note is the relatively low prevalence of *CYP2C9*2* carriers in the study cohorts, with only 16% of single and double carriers combined (minor allele frequency (MAF) = 0.08). About 50–60% are homozygous for the major alleles (i.e., wild-types) across all other pharmacogenetic loci tested in this study; whereas, the percentage of heterozygous at each of these polymorphic sites ranged from 29.5 to 41.6%. Accordingly, a relatively low number of patients were homozygous for the variant allele and just a few of them had unknown genotypes at these loci and, therefore, were excluded from subsequent analyses.

TABLE 2 | Frequency distributions of relevant genotypes in the Caribbean Hispanic patients included in this study.

Genotypes	Groups		p-value	Total cohort (n = 190)
	Training set (n = 154)	Test set (n = 36)		
at locus 1, Chr1: *FMO2* c.107A > G, p.D36G, rs2020870 (%)				
A/A	84 (54.56)	23 (63.89)	0.3072	107 (56.32)
A/G	48 (31.17)	12 (33.33)	0.8068	60 (31.58)
G/G	20 (12.99)	1 (2.78)	0.0010	21 (11.05)
Unknown[#]	2 (1.30)	0	–	2 (1.05)
at locus 2, Chr7: *ABCB1* c.1000-44G > A, rs10276036 (%)				
G/G	81 (52.60)	28 (77.78)	0.9986	109 (57.37)
G/A	59 (38.31)	8 (22.22)	0.9748	67 (35.26)
A/A	13 (8.44)	0	–	13 (6.84)
Unknown[#]	1 (0.65)	0	–	1 (0.53)
at locus 3, Chr12: *SLCO1B3* c.1833G > A, G611 =, rs3764006 (%)				
G/G	82 (53.25)	27 (75)	0.9941	109 (57.37)
G/A	51 (33.12)	8 (22.2)	0.1781	59 (31.05)
A/A	20 (12.99)	1 (2.78)	0.9109	21 (11.05)
Unknown[#]	1 (0.65)	0	–	1 (0.53)
at locus 4, Chr10: *CYP2C9*, rs1856908 (%)				
A/A	94 (61.04)	26 (72.2)	0.1955	120 (63.16)
A/G	47 (30.52)	9 (25.0)	0.5043	56 (29.47)
G/G	13 (8.44)	1 (2.78)	0.1166	14 (7.37)
at locus 5, Chr10: *CYP2C9*2* c.430C > T, p.R144C, rs1799853 (%)				
C/C	129 (83.7)	30 (83.3)	0.9961	159 (83.5)
C/T	24 (15.6)	6 (16.6)	0.8102	30 (15.8)
T/T	1 (0.65)	0	–	1 (0.77)
at locus 6, Chr16: *VKORC1* c.1173C > T, rs9934438[§] **(%)**				
C/C	75 (48.70)	12 (33.33)	0.1388	87 (45.79)
C/T	62 (40.26)	17 (47.22)	0.5651	79 (41.58)
T/T	17 (11.04)	7 (19.44)	0.2765	24 (12.63)
at locus 7, Chr19: *CYP4F2*3* c.1297G > A, p.V433M, rs2108622 (%)				
C/C	69 (44.80)	25 (69.44)	0.0133	94 (49.47)
C/T	70 (45.45)	9 (25.0)	0.0399	79 (41.58)
T/T	15 (9.74)	2 (5.56)	0.6400	17 (8.95)
at locus 8, Chr16: *NQO1*2* c.4559C > T, p.P187S, rs1800566 (%)				
C/C	77 (50.0)	23 (63.39)	0.1878	100 (52.63)
C/T	60 (38.96)	11 (30.56)	0.4549	71 (37.37)
T/T	17 (11.04)	2 (5.56)	0.4973	19 (10.00)

[#]*unknown genotype indicates a missing or non-calling at this particular locus.* [§]*The* VKORC1*c.-1639G > A (rs9923231) and c.1173C > T (rs9934438) SNPs are in near complete linkage disequilibrium in individuals of European, Asian and African descent (Cavallari and Momary, 2013).*

The p-values in **Tables 1** and **2** correspond to the statistical comparisons of relevant characteristics between the training and test sets. Overall, no significant differences between both sets were found with regard to their pharmacogenetics, ancestry, clinical, and demographic variables. Accordingly, these two sets of data are comparable to each other as they were matched by all these relevant variables. Likewise, all genotypes and allelic frequencies of the genetic markers included in this study were in Hardy-Weinberg (HW) equilibrium, as no significant departure from HW assumptions were found.

Overall Comparison of Predictive Algorithms

As can be seen in **Table 3**, with a MAE of 4.73 mg/week and a percentage within 20% of 80.6, RFR was significantly better in predictability than the other developed models. Indeed, all these other models fell short in their performances to predict optimal doses (i.e., MAEs of 6.15–9.87 mg/week and predictions of 47.22–72.22% of ideal doses) when compared to RFR. The MAE values lie within the 6.00–7.00 mg/weekly range in five of these algorithms (i.e., SVR, RPART, iBK1, iBK2, and iBK3). Notably, REPT, ANN, and MARS had the worst performances as suggested by their corresponding MAE values and % predictions within 20% of the ideal doses (8.52–9.87 mg/week and 47.2–58.3%, respectively). Interestingly, the combination of novel and common variants across the pharmacogenes of interest improved model's predictability in all but SVR and REPT algorithms, with −5 and −18% of ideal dose predictions (i.e., within 20%) after adding common variants of previously demonstrated clinical relevance.

Comparison of Predictive Algorithms Within Warfarin Dose Range

In general, these ML algorithms performed better in the subgroup of patients with normal dosing requirements. Nonetheless, the RFR algorithm was again the best in terms of MAE (2.91 mg/week) and within 20% (100%) when compared to the other methods. In the subgroup with low dose requirements (sensitives), SVR and RFR significantly outperformed all other

TABLE 3 | Mean absolute error (MAE) and percentage within 20% of actual dose in the overall test set of the Caribbean Hispanic cohort.

Models	MAE (mg/week)	Within 20%
RPART	6.27 (4.16-8.38)	72.22
MARS	8.52 (5.92-11.12)	55.56
RFR	4.73 (3.24-6.21)	80.56
ANN	9.73 (6.53-12.93)	58.33
SVR[#]	6.86 (4.75-8.97)	61.11
iBK1	6.78 (4.41-9.15)	66.67
iBK2	6.30 (3.88-8.71)	69.44
iBK3	6.15 (3.72-8.57)	72.22
REPT[#]	9.87 (6.62-13.12)	47.22

[#]*best prediction model does not include common variants in* VKORC1 *(rs9923231),* CYP2C9 *(rs1799853),* CYP4F2 *(rs2108622) and* NQO1 *(rs1800566).*

Data are expressed as mean (95% CI) or percentage. MAE, mean absolute error; multivariate adaptive regression splines (MARS), artificial neural networks (ANN), random forest regression (RFR), support vector regression (SVR), K-nearest neighbor for K from 1 to 3 (iBK1, iBK2, iBK3), recursive partitioning (RPART) and reduces error pruning tree classifier (REPT).

methods in MAE (4.79–7.17 mg/week, respectively) and within 20% (75.00–50.00%, respectively). For the resistant patients, MARS was the best algorithm in both MAE (7.22 mg/week) and % values within 20% (66.67), though iBK2 and iBK3 also showed good results (7.58 mg/week and 66.67%). Overall, the models generated for the subgroup with normal warfarin dose requirements performed better than those used to predict dosing among sensitives and resistant patients (**Table 4**). Strikingly, when models included both common and novel variants combined their predictability improved in general, except for the sensitive subgroup where performances were as bad as −67% of ideal dose predictions (i.e., within 20%) in comparison to the models excluding the common variants. In the resistant subgroup, only MARS had a worse performance (−50%) after adding the common variants (**Supplementary Material S1**).

DISCUSSION

Overall, we found different performances of the nine ML-based algorithms that were used to predict warfarin dosing in the Caribbean Hispanic population (**Table 3**). When all the cases were considered, the RFR algorithm achieved the best performance. However, RFR, SVR, and MARS algorithms had the best performance when the patients were grouped by dose range as "normal," "sensitive," "resistant," respectively. There is no obvious explanation or a given reason why these specific models performed better than the others. It is because algorithms derived from ML techniques are based on choosing the best model as they learn from data (Brownlee, 2019). Therefore, it seems to be population dependent.

The model with best predictability was chosen for each of the ML-based algorithms tested, regardless of the number of added variants. However, we tried to keep the models as simple as possible (i.e., minimum number of parameters or variables) while preserving a reasonably great explanatory predictive power. Since models with low parsimony will likely be useless for predicting other datasets, we chose the models with the right balance between parsimony and goodness of fit.

Comparison to Previous Algorithms for Dose Predictions in Other Populations

The performance of similar ML methods applied to warfarin dose predictions have shown different results in a previous study (Liu et al., 2015). Of note is that no significant differences in overall performances of various ML-based algorithms were reported by others when used as a prediction tool for stable warfarin dose estimations in a multi-ethnic cohort. However, differences in model accuracy were indeed found after stratifying data by ethnicity (i.e., White *vs.* Asians *vs.* Blacks) or dose range subgroups (i.e., high *vs.* intermediate *vs.* low) (Liu et al., 2015; Ma et al., 2018). We have obtained better MAEs than this previous report for the analyses of data from all cases in most of the tested ML methods (e.g. RFR, SVR, RPART, iBK1). When datasets from Liu et al. (2015) and our study were compared, the best result for all cases was obtained with the use of the RFR technique in our dataset of Caribbean Hispanics (i.e. MAE = 4.73 mg/week and 80.56% cases within ±20% of ideal doses). We reason that these observed differences in performance may have arisen as a consequence of the unique genetic backgrounds, clinical characteristics of our study cohort (Caribbean Hispanics), and special attributes of the available dataset (e.g., genetic markers for resistance, ancestry metrics). Accordingly, such findings may be attributed to differences in the characteristics of participants from both studies and the fact that the previous one was conducted in a more heterogeneous cohort of individuals from the International Warfarin Pharmacogenetics Consortium (IWPC), without a proper representation of Caribbean Hispanics (Liu et al., 2015). It is important to mention that the IWPC cohort comprised a mixed sample from different countries, regions, and clinical sites that could lead to misclassification and large genetic variability. Finally, it may also be related to the unequal sample sizes between both studies.

Similarly, after grouping patients by dose requirements (i.e. "normal," "sensitive," and "resistant" to warfarin, **Table 4**), the ML prediction models in our study performed better than those in the published report (Liu et al., 2015). In those labeled as "normal," our best model (RFR) yielded a MAE = 2.91 mg/week

TABLE 4 | Mean absolute error and mean percentage within 20% of actual dose stratified by therapeutic warfarin dose requirements (i.e., sensitives, resistant and normal) in the test set of the Caribbean Hispanic cohort.

Models	Normal		Sensitive		Resistant	
	MAE (mg/week)	Within 20%	MAE (mg/week)	Within 20%	MAE (mg/week)	Within 20%
RPART	4.17 (2.60-5.73)	88.00	9.83 (3.53-16.12)	37.50	14.28 (7.19-21.37)	33.33
MARS	6.98 (4.44-9.52)	68.00	11.44 (6.55-16.33)[#]	25.00	7.22 (2.11-12.33)[#]	66.67
RFR	2.91(2.18-3.64)[#]	100.00	7.17 (3.48-10.86)[#]	50.00	13.45 (7.41-19.48)	33.33
ANN	8.03 (5.01-11.05)	68.00	10.32 (1.49-19.15)	50.00	16.75 (10.87-22.63)[#]	0.00
SVR	5.67 (3.82-7.53)[#]	68.00	4.79 (1.21-8.36)	75.00	19.44 (15.83-23.05)	0.00
iBK1	3.62 (2.01-5.24)	88.00	12.81 (6.15-19.48)[#]	25.00	12.83 (2.48-23.18)	33.33
iBK2	3.75 (1.94-5.55)	88.00	9.33 (2.17-16.49)[#]	37.50	7.58 (2.33-17.50)	66.67
iBK3	3.83 (2.01-5.64)	88.00	9.33 (2.17-16.49)[#]	37.50	7.58 (2.33-17.50)	66.67
REPT	6.89 (4.68-9.09)[#]	56.00	12.30 (2.60-22.01)[#]	37.50	15.08 (2.67-27.50)	66.67

[#]*best prediction model does not include common variants in* VKORC1 *(rs9923231),* CYP2C9 *(rs1799853),* CYP4F2 *(rs2108622) and* NQO1 *(rs1800566).*

Data are expressed as mean (95% CI) or percentage. MAE: mean absolute error; multivariate adaptive regression splines (MARS), artificial neural networks (ANN), random forest regression (RFR), support vector regression (SVR), K-nearest neighbor for K from 1 to 3 (iBK1, iBK2, iBK3), recursive partitioning (RPART) and reduces error pruning tree classifier (REPT).

to outperform the 5.53 mg/week from the study by Liu et al. (2015). For "sensitive" patients, the SVR is our best model with a MAE = 4.79 mg/week that resulted more accurate for predictions in this subgroup than the value of 8.68 mg/week from the previous report (Liu et al., 2015). Finally, those warfarin patients classified as "resistant" had a MAE = 7.22 mg/week with our best-performance model in this subgroup (MARS), which is far better than the reported 15.24 mg/week in the previous study by Liu et al. (2015).

As expected, our results indicate that both the MAEs and mean percentages within 20% of all algorithms under consideration differed across the dose range categories (i.e., "normal," "sensitive," and "resistant"), with best performance and accuracy (i.e., lower MAE and higher mean percentage within 20%) achieved in the "normal" dose group and "resistant" showing the worst predictions. In fact, the largest difference in the MAE and percentage within 20% were observed between "normal" and "resistant" subgroups. However, better predictors do not really translate into a real clinical utility to this "normal" subgroup as patients in this class are least likely to benefit from pharmacogenetics (Klein et al., 2009). Consequently, benefits are mainly for those at the extreme dose requirements. "Resistant" demonstrated to have the highest variability in warfarin dose requirements among patients at any dosing range, suggesting that either current ML-based methods are not yet robust enough to optimally predict dosing in patients with a resistant phenotype or the lack of information from all predictors of resistance to warfarin in the model. Since ML techniques learn from existing data, the insufficient number of "resistant" cases in available dataset and, therefore, the limited amount of relevant data that can inform the model, may in part explain the poorer performance at this dose range. Accordingly, efforts should be made in order to enhance the representation of this sub-group in future assessments.

Comparison to Previous Algorithms for Dose Predictions in Caribbean Hispanics

Our group has earlier published three previously developed pharmacogenetic algorithms to predict optimal warfarin dosing in Caribbean Hispanics of mostly Puerto Rican origin, which included ethno-specific alleles and adjustments by admixture measures in the derivation cohort (Ramos et al., 2012; Duconge et al., 2016; Claudio-Campos et al., 2017). All these models were based on multivariate linear regression analyses. Overall, they showed a good predictability in our patients to outperform prior genotype-guided algorithms derived from populations other than Hispanics. When using these regression pharmacogenetic models, up to 46% of their predictions in high risk individuals resulted in ideal doses (i.e., % of predictions within ±20% of the actual patient's stabilization dose) with MAE values that sit slightly over 5 mg/week. However, some ML-based models developed in this survey by using RFR, MARS, and SVR approaches showed even better results in predicting optimal warfarin doses in the study cohort as compared to the previously published regression methods. Particularly, the overall performance of the RFR model was better than published algorithms, as suggested by a MAE of less than 5 mg/week and 80.6% of ideal dose predictions. Among those at the highest risk of adverse events, both SVR and RFR showed superiority over the previously published regression algorithms with higher percentages of ideal dose predictions (i.e., 50 and 62%, respectively). Notably, the ML-based methods (RFR, SVR) performed better than previous linear regression models in both high- and low-dose subgroups (i.e., resistant and sensitives). Therefore, this analysis reflects the potential of ML techniques for predictions at extreme dose levels given their capabilities to assess patient characteristics under extreme dosing requirements. A possible explanation for this observed superiority of ML models over the conventional algorithms is given by the fact that these applications of artificial intelligence (AI) provides systems the ability to automatically learn and improve predictability from experience (i.e., available data).

The Missing Links for Global Pharmacogenomics

Most of the existing pharmacogenetic-driven algorithms such as the one developed by the IWPC project have been derived from findings in individuals of mostly European descent, and therefore they often include variants commonly found in white people only. Multiple ethno-specific variants occurring across warfarin-related pharmacogenes are generally overlooked and, consequently, the utility of existing prediction models is limited in patients with mixed ancestry. Healthcare disparities could be exacerbated when such models are not suitable to populations with ethno-geographic particularities.

White people of European ancestry make up the largest percent of participants in pharmacogenomic (PGx) studies, despite the fact that they only represent a fraction of the world's population. Furthermore, clinically relevant findings from such studies with Europeans do not generalize well to other ethnic groups. This overwhelming whiteness of pharmacogenetics research is holding back the new paradigm of precision medicine. One of the greatest promises of the Precision Medicine initiative is the opportunity to develop treatment plans that are tailored to an individual's genetic risk profile. Therefore, if individuals from underrepresented populations are not involved in these investigations, they will not benefit from the advances. Indeed, there is a paucity of data from studies recruiting minority, more diverse or admixed populations like Caribbean Hispanics who reside in Puerto Rico. Unfortunately, individuals from these populations are often excluded or marginally represented in these studies and this lack of representation tends to exacerbate existing healthcare disparities. It's adding to the long-standing problem of minorities being excluded from medical research, which preclude any opportunity to make them equitable.

MLR analysis routinely used to derive pharmacogenetic models is data driven and hence population dependent. There is promising research indicating that mathematical models other than linear regression may yield more predictive algorithms (Cosgun et al., 2011; Hu et al., 2012; Liu et al., 2015; Sharabiani et al., 2015; Duconge and Ruaño, 2018; Ma et al., 2018). AI, and particularly the use of ML techniques, offers new

avenues in the prediction of clinical outcomes (e.g., warfarin dose requirements) by accounting for relevant gene–drug interactions. Failure to account for ethno-specific genotypes and a better use of available predictive tools (e.g., ML) has raised some concerns about expected benefits of genotyping patients to guide pharmacotherapies and improve clinical outcomes, leading to a lack of full endorsement by medical organizations and payers. The more complete the PGx characterization and the more learned the prediction models, the larger the benefit.

This study has some limitations. Firstly, some data were retrospectively collected and, therefore, we were unable to control for such data variability and potential confounders. Given a relatively lesser representation of cases at the extreme dose levels with respect to those in the "normal" range, a potential bias may arise in the comparison after subgrouping by dosing requirements. For the purpose of the analyses in this paper, we considered "normal" responders as those without any obvious or given reason to make adjustments in their standard initial warfarin dose (i.e., 35 mg/week; range: 21–49 mg/weekly). Theoretically speaking, we reasoned that those from the clopidogrel study can be considered as "normal" because of a lack of any obvious reason for starting these patients with a different dosing (e.g., frail elderly, high risk of bleeding/thrombosis, etc.) had they been treated with warfarin. However, this assumption should be observed with caution and, hence, is another study limitation. Finally, our findings need further validation in a larger replication cohort before making any statement about the superiority of some of these algorithms over the others.

The metrics to assess the performance of algorithms developed in other studies are not comparable to those used in this study, whose methodology is mainly based on an early work by Liu and coworkers (Liu et al., 2015). Unlike previous reports, in this study we have included genetic markers for both sensitivity and resistance phenotypes, and admixture/ancestry estimates as critical covariates in model development (Klein et al., 2009; Ramos et al., 2012; Liu et al., 2015). Moreover, relevant data from a highly diverse admixed population of Caribbean Hispanics is used for the first-time to perform an ML prediction modeling of a pharmacogenetic trait. MAEs and percentage of predictions within ±20% revealed that models generated by using RFR, MARS, and SVR ML algorithms showed significantly better predictions of warfarin dosing in our cohort of participants than other algorithms. Better performance of the ML models for patients with "normal," "sensitive," and "resistant" to warfarin were obtained in our study as compared to other populations and previous statistics models.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the dbGaP, Study Accession: phs001496.v1.p1, https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs001496.v1.p1.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Research Subjects Protection Office (HRSPO) affiliated to the University of Puerto Rico Medical Sciences Campus, (IORG000223; Federal-wise Assurance #FWA00005561). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AR-L provided the infrastructure, supervised the analysis of the data using bioinformatics tools, and participated in the writing of the manuscript. AR-S and RF-M both performed most of the data analyses and drafted the original version of the manuscript. They also worked with the corresponding author in data collection and assembly. JR-M, BN, and KC-C performed part of the data analysis. CR and IS'A performed the statistics of this study and contributed to the manuscript preparation. SM contributed in the preparation of the manuscript. JD is the principal investigator, responsible of the study coordination, and contributed to the elaboration of the manuscript.

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SUPPLEMENTARY MATERIAL

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DPD Testing Before Treatment With Fluoropyrimidines in the Amsterdam UMCs: An Evaluation of Current Pharmacogenetic Practice

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Introduction: The fluoropyrimidines (FP) (5-Fluorouracil, capecitabine, and tegafur) are commonly used anti-cancer drugs, but lead to moderate to severe toxicity in about 10–40% of patients. DPD testing [either the enzyme activity of dihydropyrimidine dehydrogenase (DPD) or the *DPYD* genotype] identifies patients at higher risk for toxicity who may be treated more safely with a lower drug dose. The Netherland's National guideline for colon carcinoma was updated in 2017 to recommend *DPYD* genotyping before treatment with FP. Pretreatment *DPYD* genotyping identifies approximately 50% of the patients that will develop severe FP toxicity. The aim of the study was to assess the uptake of DPD testing in the Amsterdam University Medical Centers over time and to evaluate stakeholder experiences to indicate barriers and facilitators of implementation in routine clinical care.

Materials and Methods: We used a mixed-method approach involving electronic patient records of 753 unique patients and pharmacy information systems analyses and fifteen semi-structured interviews with oncologists, pharmacists, and patients. The constellation perspective was used to identify barriers and facilitators at the level of practice, culture and structure. The proportion of FP users who were DPD tested pretreatment showed an increase from 1% (1/86) in Q2-2017 up to 87% (73/84) in Q4-2018. Unlike a landmark paper published in 2015, the National guideline for colorectal carcinoma followed by meetings to achieve local consensus led to this steep increase in the proportion of patients tested.

Results: Facilitating factors for stakeholders to implement testing included the existence of clear protocols, (anecdotal) evidence of the utility, being aware that peers are adhering to standard practice and clear and simple procedures for ordering and reporting. Main barriers included the lack of clear divisions of responsibilities, the lack of consensus on a test approach, long turn-around times and non-user-friendly IT-infrastructures. More

professional education on the utility and limitations of pharmacogenetic testing was desired by most stakeholders.

Conclusion: While the evidence for DPD testing was sufficient, only after the update of a National guideline and local consensus meetings the proportion of FP users that were DPD tested pretreatment rose to 87%. The implementation of personalized medicine requires stakeholders involved to attune practice, culture and structure.

Keywords: DPYD gene polymorphism, fluoropyridine, pharmacogenomics and personalised medicine, toxicicity, electronic patient record, qualitative & quantitative analyses

INTRODUCTION

5-Fluorouracil (5-FU), its oral prodrugs capecitabine and tegafur (fluoropyrimidines; FP) are amongst the most frequently prescribed anti-cancer drugs in the treatment of common cancer types such as colorectal, gastrointestinal, and breast cancer. A subset of patients (10–40%) treated with FP experience moderate to severe toxicities, including vomiting, diarrhea, and hand-foot syndrome (Amstutz et al., 2011).

Administered FP is primarily (> 80%) eliminated by the liver enzyme dihydropyrimidine dehydrogenase (DPD) (Thorn et al., 2011). Deficiency in DPD, prevalent in about 3–5% of the Caucasian population, results in decreased inactivation of FP and can lead to severe and fatal toxicity (van Kuilenburg et al., 2010). DPD deficiencies are often related to genetic variants in the dihydropyrimidine dehydrogenase gene (*DPYD*) (Amstutz et al., 2011). The genetic variant *DPYD*2A* has been long reported to result in decreased DPD enzyme activity, but more recently for other variants similar effects have been described, including the variants *DPYD*13*, c.2846A > T and c.1236G > A (Amstutz et al., 2009; van Kuilenburg et al., 2010; Offer et al., 2014). Carriers of one *DPYD*-mutation comprise the majority of deficient patients, and homozygous or compound heterozygous carriers occur in 0.3% (Henricks et al., 2018), leading to complete deficiency.

Although not all DPD deficiencies and FP toxicity can be explained by known genetic variants (Deenen et al., 2011) (Terrazzino et al., 2013), pretreatment testing for *DPYD* variants is a well-known strategy to detect DPD deficiencies and improve patient safety (Deenen et al., 2016). However, because not all variation in DPD enzyme activity can be explained by genetic variants, other methods such as DPD phenotyping may be used to detect decreased DPD activity (van Staveren et al., 2013). Patients with a complete or partly deficient DPD enzyme can be more safely treated with a reduced dose of FP or an alternative drug (Deenen et al., 2012). Recently, the advice to perform pretreatment DPD testing to optimize treatment efficacy and avoid adverse effects has been added to the Netherland's National guideline for colorectal carcinoma (NVMO, 2017).

According to the results of a poll conducted among Dutch internist oncologists (n = 208) in 2016 by the editorial board of *Medische Oncologie* (Medical Oncology), 65% of the oncologists test their patients for DPD deficiencies prior to treatment with FP. Results of this poll also showed that the main reasons DPD testing is not yet standard of care are the low prevalence of DPD deficiency (mentioned by 23% of respondents), the minimal cost-effectiveness (15%), the poor availability (4%) and other reasons (58%), such as that no quick test results are possible, the test is not validated, toxicity is not only seen among DPD deficient patients, and the incidence of DPD induced toxicity is rather low (NVMO, 2016).

The National guideline for colorectal carcinoma was updated in September 2017 to recommend *DPYD* genotyping before treatment with FP. Whether and to what extent the recommendation to prospectively execute *DPYD* genotyping is followed up in patients treated with FP is unknown.

The aim of this study was to assess the uptake of DPD testing before the use of FP in the Amsterdam University Medical Centers [UMCs, locations VU University Medical Center (VUMC) and Amsterdam Medical Center (AMC)] over time, and to evaluate stakeholder experiences to ultimately indicate barriers and facilitators of DPD testing implementation as routine clinical care. The results of this study will hopefully inform colleagues elsewhere who also strive for 100% patient safety in the end, and now are implementing DPD testing step by step. Barriers and facilitators identified in Amsterdam may apply elsewhere too.

In discussions about optimal strategies for DPD testing, our AMC colleague Van Kuilenburg (van Kuilenburg et al., 2010; van Kuilenburg et al., 2010) has been quite active in test development. As he still works on improvement of the test sensitivity, at AMC DPD phenotyping by assessing the enzyme activity is performed (**Table 1**).

Furthermore, we apply the "constellation perspective", by structuring the influences on implementation, as mentioned by the stakeholders, in terms of changes in culture, structure, and

TABLE 1 | Specification of DPD tests used in the Amsterdam UMCs.

	VUMC	AMC
Test	*DPYD* genotyping	DPD phenotyping + successive genotyping for deviating enzyme activities
Variants	*DPYD*2A* (c.1905+1G > A)	Whole *DPYD* gene, including deletions and amplifications
	*DPYD*13* (c.1679T > G) c.2846A > T c.1236G > A	

practice (Rigter et al., 2014). By doing so we aimed to define lessons learned for implementation of other pharmacogenetic applications beyond oncology and beyond DPD.

MATERIALS AND METHODS

Design

A mixed methods approach was used involving patient records and pharmacy information systems analyses (quantitative analysis) and stakeholder interviews (qualitative analysis). All research was done within Amsterdam UMC, which comprises of location VUMC and AMC. This study was approved according to the national legislation. The Medical Ethical Committee of the VU University Medical Center Amsterdam evaluated the study design and decided that the Medical Research Involving Medical Subjects Act (WMO) does not apply to this study and that further official approval is not required (2019.069).

Quantitative Analysis

The Research Data Platforms of Amsterdam UMC contain retrospective data of different software systems. For location VUMC, data was extracted *via* this platform from EPIC (electronic patient information system) and GLIMS (laboratory information system). For location AMC, also EPIC was used *via* the Research Data Platform, but laboratory information was extracted from Genesis (a clinical genetics information system).

We selected all patients that started FP treatment (Anatomical Therapeutic Chemical (ATC) codes: L01BC53, L01BC02, and L01BC06) between the 4th quarter of 2016 up to and including the 4th quarter of 2018. For all patients we collected the following data: anonymously encrypted unique patient ID, ATC codes, medication name, start/stop date of administration, dose, medication status, administration route, and DPD analysis date.

The implementation of the pretreatment *DPYD* genotyping at Amsterdam UMC location VUMC was evaluated for subsequent quarters by determining the proportion of patients who started FP treatment and were registered as DPD tested. For AMC similar calculations for DPD phenotyping were made. Patients receiving topical 5-fluorouracil (part of ATC code L01BC02) were excluded from the analysis because the guideline applies to systemic use only. Side effects are less likely for topical application. The date of the first administration was used to determine the quarter. The DPD analysis date was used to determine if DPD testing was executed. Additionally, we compared the date of the first administration with the DPD analysis date. All analyses were performed using Microsoft Excel 2016.

Several key moments in relation to the introduction of DPD testing were considered. In 2015 Meulendijks et al. published a landmark paper (Meulendijks et al., 2015). In the 3rd quarter of 2017 the Netherland's National guideline for colon carcinoma was updated (NVMO, 2017). Finally, in the 4th quarter of 2017 local consensus was reached to test all patients receiving FP.

Qualitative Analysis

Theoretical Framework

The "constellation perspective" was applied in the development of the interview-guide, as well as the analysis of the results from the interviews (Rigter et al., 2014). This theory implies that a group of individuals or actors (professionals and patients) are used to working in a certain structure, culture, and practice (the constellation) and by this are defining and fulfilling a function in a larger societal system. As such, different ways of doing (practice), thinking (culture), and organizing (structure) by the actors are needed to achieve fundamental changes in the constellation.

Participants

Stakeholders were selected such that a comprehensive overview of the experiences around pretreatment DPD testing in Amsterdam UMC could be developed. Relevant stakeholders included oncologists who treated patients with colorectal, gastrointestinal, and/or breast carcinomas, hospital and outpatient pharmacists, and lab specialists involved in DPD testing at the Amsterdam UMC. DPD tested patients were identified and invited for an interview through the interviewed oncologists. In total 15 interviews were conducted in the Amsterdam UMC and outpatient pharmacies on location AMC and VUMC between February 2019 and June 2019, after which data saturation was reached. The interviews were held with 6 oncologists (2 AMC; 4 VUMC), two clinical hospital pharmacists (1 AMC; 1 VUMC), two outpatient pharmacists (1 AMC; 1 VUMC), one lab specialist (AMC), and four patients (1 AMC; 3 VUMC). All interviews were conducted face-to-face; 9 interviews by two interviewers (FM and DH) and the other 6 interviews by one interviewer (FM). Informed consent was signed by all participants before the start of the interviews.

Interview Guide

A semi-structured interview guide was developed based on the constellation perspective (with key concepts culture, structure, practice) and main themes from literature on barriers and facilitating factors for implementation of pharmacogenomics (Rigter et al., 2014). The guide covered the following topics for oncologists, pharmacists, and lab specialists: the current situation of *DPYD* genotyping and/or DPD phenotyping, the procedures around DPD testing, the reasons for and experiences with the current approach, and barriers and facilitating factors of implementing this test. Patients were asked about their experience and expectations about the information provision around DPD testing. The interview guides for patients and professionals are available as **Supplementary Material**. Depending on the background and expertise of the interviewee details of the interview guide have been adjusted and/or omitted.

Data Preparations and Analysis

Interviews were audiotaped and transcribed verbatim and content analysis was performed using Atlas.ti (Version: WIN 7.5). Transcripts were read and discussed by two researchers (FM, TR). First, recurring topics were labeled. Second, all labels

TABLE 2 | Number and percentage of patients using fluoropyrimidines who had been DPD-tested before the start of treatment.

Time period	DPD tested AMC				DPD tested VUMC			
	Yes	No	Total	*percentage*	Yes	No	Total	*percentage*
Q4-2016	0	37	37	*0*	2	43	45	*4*
Q1-2017	0	50	50	*0*	1	48	49	*2*
Q2-2017	0	49	49	*0*	1	37	38	*3*
Q3-2017	0	32	32	*0*	10	27	37	*27*
Q4-2017	4	42	46	*9*	15	32	47	*32*
Q1-2018	14	20	34	*41*	27	20	47	*57*
Q2-2018	21	10	31	*68*	22	19	41	*54*
Q3-2018	31	7	38	*82*	31	17	48	*65*
Q4-2018	32	4	36	*89*	41	7	48	*85*
Total	102	251	353	*29*	150	250	400	*37*

were clustered based on "current practice" and the elements culture, structure, and practice of the constellation perspective in order to identify main themes. Differences in coding were discussed until consensus was reached. Representative quotes were selected and member-checked and translated into English to illustrate findings.

RESULTS

Uptake of DPD Testing

For a total number of 753 unique patients FP was prescribed and 252 patients received DPD testing. In **Table 2** the results are specified per center and quarter. **Figure 1** shows the proportional results of the quantitative analysis. The chart shows a relative increase in the proportion of DPD tested patients starting FP treatment after the 2nd quarter of 2017. In Q2-2017 1/86 patients were tested. The start of the increase coincides with the updating of the National guideline for colon carcinoma and the local consensus meetings. In the 4th quarter of 2018, 87% of the initiated patients (73/84) was registered as DPD tested.

Additionally, we compared the date of administration to DPD analysis date. Ninety-one percent of patients (229 out of 252, 91%) received FP only after the result of the DPD test was known.

Barriers and Facilitators for Implementation

The interviews with key stakeholders revealed several themes, including the needs and barriers regarding the implementation of the DPD test in clinical practice. Relevant themes are discussed below, starting with the current situation in the Amsterdam UMCs, followed by the changes that were needed to achieve the current situation and that are needed to improve implementation of pretreatment DPD testing, clustered into the three levels of the constellation perspective (practice, culture, structure).

Clear Procedures (Practice)

Current Situation

In the interviews, oncologists and a pharmacist of the VUMC expressed that they follow the recommendation to conduct *DPYD* genotyping for all patients prior to receiving FP treatment.

> *"Yes. For everyone who will get treated with 5-FU or capecitabine we determine the DPYD gene activity." (#6, fellow medical oncology VUMC)*

The *DPYD* genotyping test on four variants (see **Table 1**) is outsourced and performed in the Erasmus Medical Center Rotterdam.

Oncologists of the AMC and a lab specialist expressed that their hospital uses a phenotypic test instead, which is performed in-house. If the results of the phenotypic test are aberrant, a

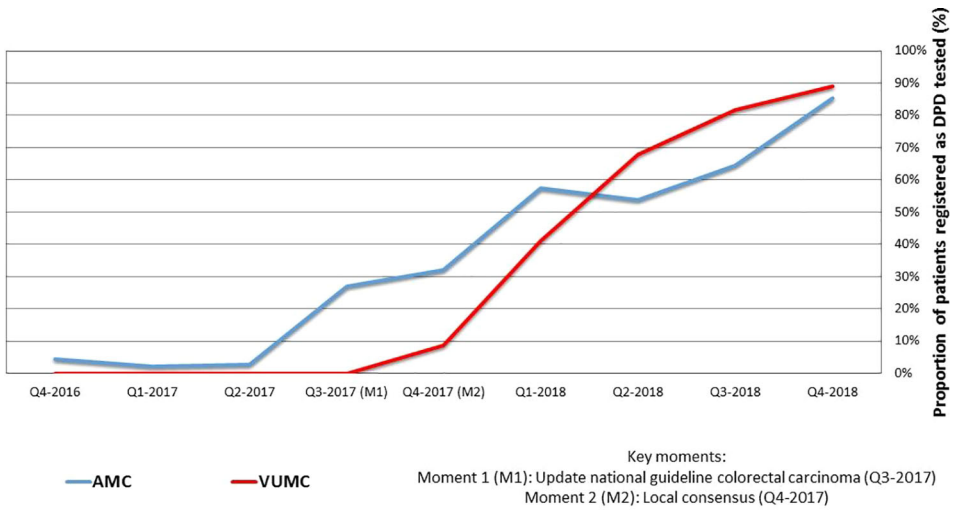

FIGURE 1 | Quarterly collected proportions of patients receiving fluoropyrimidines registered as DPD tested in VUMC and AMC.

successive genotyping test of the whole *DPYD* gene is performed at the VUMC.

> "It is standard procedure that we order the DPD *[phenotypic]* test in advance, before we start with therapy." *(#5, fellow medical oncology AMC)*
> "*In our hospital, performing a DPD test before the start of the therapy is a standard procedure."(#11, lab specialist AMC)*

Outpatient pharmacists and one hospital pharmacist of the AMC were unaware of the existence of a DPD test.

> *"Well, I rarely see it [DPD orders/test results]. I don't think it is standard procedure." (#9, hospital pharmacist AMC)*
> *"I don't know whether this is standard procedure in this hospital. We at least don't have a role in it." (#10, outpatient pharmacist AMC)*

Patients recalled that they were tested for DPD deficiency. Most of them needed some time and explanation before remembering undergoing the test.

> *"I can't remember exactly and I don't think it has been said emphatically. When they took a liver biopsy, they also analyzed that [DNA test]. That is when I received the gene passport, which was used to determine whether I could possibly receive different therapy. So, maybe I knew it [being tested] this way." (#13, patient)*

In general, oncologists and pharmacists did not know why either the genotyping or phenotyping DPD test was chosen.

> *"No, I have no idea. I have never looked into that [choice for genotyping]." (#3, oncologist VUMC)*

Opinions vary about the turnaround time of the test in the VUMC, but it perceived as too long by many. Some oncologist stated that it could take up to two weeks, another mentioned half a week, another 7–8 days and a pharmacist thought it would be a maximum of three days.

The duration of the phenotyping is 4–10 days according to a lab specialist and the successive genotyping may take up to two weeks, but is often done quicker. Oncologists of the AMC experienced that it may take up to two weeks, but approximately a week to 10 days when no further genotyping needs to be done.

> *"Then [when no aberrant values have been found]it takes approximately a week." (#4, oncologist AMC)*

The turnaround time is seen as an important barrier by AMC oncologists.

> *"Yes, I think that's [waiting a week] too long." (#4, oncologist AMC)*

Division of Roles and Responsibilities

All interviewees expressed that oncologists, as head practitioners, have the main responsibility for initiating the DPD test.

> *"I have the impression that this [oncologist is responsible for having DPD test result before start] view is shared by all. I simply cannot order treatment when I haven't seen it [DPD test result] […]." (#2, oncologist VUMC)*

According to a hospital pharmacist of the VUMC, they check whether DPD has been assayed. AMC pharmacists indicated that they are not involved in the process. Oncologists of the AMC, however, expressed that the nurses often check whether DPD results are known.

> *"The oncology nurses will always double check if it is safe to start with the DPD test included." (#5, fellow medical oncology AMC)*

Although outpatient pharmacists and the AMC pharmacist indicated that they are not involved, they expressed interest in the monitoring of medication and the need for clarity of the procedures and responsibilities.

> *"I think it is an important part of our responsibility to check this, yes." (#7, outpatient pharmacist VUMC)*

> *"Legally this is correct. In practice, both doctors as well as pharmacists and other healthcare providers are responsible for doing something with aberrant lab values or results, but this must always happen in full agreement between professionals. In the outpatient pharmacy we work with 2 systems (AIS CGM Pharmacy and ZIS EPIC), which makes it more complicated to properly check and record data as well as any follow up actions." (#10, outpatient pharmacist AMC)*

> *"[…], so I think it is important that we are getting involved with the implementation of such a project [DPD testing for patient receiving fluoropyrimidines] and if that hasn't been the case, then it's a bit disappointing." (#7, outpatient pharmacist VUMC)*

Communication Is Key

Oncologists and a lab specialist are not aware whether and in what way the pharmacists are involved and have generally no contact with each other. Only some oncologist indicated to have contact about logistics or to answer questions concerning adjusting the treatment dose. Hospital pharmacists are unaware of any involvement of the outpatient pharmacists and vice versa.

> *"There might be some uncertainty. […] Most of the time it [capecitabine] is provided by the outpatient*

pharmacy. I don't know whether they perform a check" (#8, hospital pharmacist VUMC)

Information Provision to Patients

All oncologists expressed that they informed their patients about the DPD test, most of them prior to the start of the therapy. The communication included information on why the test was done and if applicable how the dose of medication was reduced. One oncologist indicated that patients are only informed more extensively when the test results are aberrant.

"We communicate more extensively when it [DPD test result] is aberrant and we will give a lower dose of chemotherapy. […] When it [test results] is okay, I inform the patient that the DPD results are good and no adjustment of the therapy needs to be made." (#2, oncologist VUMC)

According to the patients the information provision was sufficient. Patients for whom the start of the therapy was longer ago did not remember exactly when the information was provided.

"Yes, she told me [enough information]." (#14, patient)

One patient who recently started therapy added that, although the information was sufficient, a simpler (in layman's terms) explanation of what the DPD test exactly is would have been favorable.

Convincing Stakeholders of Need and Utility (Culture)

The main themes identified as important for changing culture regarding pretreatment DPD testing were evidence (scientific and anecdotal), willingness to follow guidelines, shared views with coworkers and the perceived need to start treatment as quickly as possible after diagnosis.

Evidence For Clinical Utility/Usefulness

In the guideline *DPYD* genotyping is advised. Most participants were convinced of the importance of this test in order to prevent toxicity and FP related death. However, in general interviewees had no clear idea why the current approach (genotyping or phenotyping) was chosen.

In general, VUMC oncologists indicated that the test is clinically useful, however, some oncologists questioned the need and clinical utility of the test as they mentioned that the occurrence of toxicity due to DPD deficiency is low and the test not able to perform 100% accurately.

"Look, I think you have to test many people to really significantly reduce morbidity or mortality. It is worth a lot to prevent every death, that's the truth of course, but we actually encountered severe toxicity once a year at most. Very few. […] but recently there have been publications on routine screening to prevent toxicity, so there is [some] more evidence." (#1, oncologist VUMC)

"Well, I have always heard that it wasn't a good test and that you will still not find 50% of the people [with a DPD deficiency], or something like that." (#2, oncologist VUMC)

Another oncologist expressed the importance of having a realistic understanding of the limitations of the test.

"Yes, I think people are pretty aware right now, but I can imagine that in the future the story will be; you can have toxicity, but when you do that test then you won't have it. So I think it is very important to keep saying that this is only a small part of the possible gene abnormalities and apart from genetic [causes], you can also have toxicity due to other reasons." (#6, fellow medical oncology VUMC)

Also oncologists of the AMC expressed that they were convinced of the clinical usefulness of the DPD test.

"The DPD deficiency is proven by science and is very important, so we can treat our patients safe." (#5, fellow medical oncology AMC)

However, one of them questions whether the current approach in AMC is evidence-based.

"Well a barrier for me, or not particularly a barrier, but more a doubt why or to what extent it is evidence-based what we do with the phenotyping. Everyone knows about the evidence of cost effectiveness of the genotyping [4 variants], but we phenotype. So, that I find a bit hard." (#4, oncologist AMC)

Pharmacists are enthusiastic about DPD testing, even when they indicated that it is not yet standard procedure. One hospital pharmacist expressed that besides guidelines, the scientific evidence for DPD testing is important.

"But when there is scientific evidence and guidelines state that the pharmacy needs to monitor it, then I will definitely do so." (#9, hospital pharmacist AMC)

A lab specialist expresses that in order to provide the best possible patient care, not costs but rather the effectiveness of the test [in preventing toxicity] should be leading in future decisions about which DPD test (genotyping/phenotyping) to use.

"Having a DPD deficiency is a contraindication for being treated with 5-FU or capecitabine. So, providing a patient a suboptimal test, when knowing there is a better test, I think one doesn't act ethically.[…] So, I understand the [need for evidence on] cost effectiveness, but I also think it gets a bit exaggerated sometimes." (#11, lab specialist AMC)

Experiences With Relevant Cases

An experience with an aberrant genotype that potentially would have been missed with the current DNA test was described by a lab specialist:

> *"That was a patient who was diagnosed by a hospital as a carrier of one of the four pathogenic variants [c.1905 +1G > A] in the DPD gene. Fortunately, they also sent us a blood sample and we actually found a complete DPD deficiency when we analyzed the DPD enzyme activity. When we performed an extensive analysis of the DPD gene (DPYD) we discovered that the patient was heterozygous for an amplification of part of the DPYD gene. Such an amplification is very rare and this patient was eventually treated with only 0.8% of the normal dose and would have died if treated with 50% of the normal dose, which is the recommended dose for carriers of this particular variant".(#11, lab specialist AMC)*

Adhering Guidelines

In general, oncologists expressed that they simply follow the guideline. However, they also indicated that prior to the implementation of the DPD test they were already able to monitor patients adequately. They expressed that the DPD test is a helpful tool, but remaining critical on what is best for the patient is important.

> *"I think it can contribute, but may also give false security […] I don't see it as a holy grail." (#6, fellow medical oncology VUMC)*

> *"[…] before [implementation of the DPD test] we haven't done it for very long. Back then we dosed on the basis of how the patient was doing in the first weeks, and since it is not that prevalent, there is something to say for that as well […]. On the other hand, the impact of DPD deficiency can be huge, with serious morbidity and mortality that can be avoided by a relatively simple DPD test." (#3, oncologist VUMC)*

However, they do not always follow the protocol. According to an oncologist (VUMC) they do not order *DPYD* genotyping, when patients have been treated successfully during a previous treatment cycle of 5 FU.

> *"Then we have proof that it is well tolerated." (#2, oncologist VUMC)*

Starting Before Results

Many oncologists indicated that waiting for test results could take (too) long, which causes an unnecessary delay. As a solution they mentioned that they start treatment on a lower dose, before test results are available.

> *"The DPD test will take 2–3 weeks to be known. If it is necessary we will start our therapy 50% lower dose until the results are to be known." (#5, fellow medical oncology AMC)*

> *"Certainly, when it causes a hassle and when you have to postpone, then you think I'll just start and will raise the dose later, or I call [the patient] after three days and then I'll still be able to reduce the pills you know, they take it every day, you have some room to play a bit." (#6, fellow medical oncology VUMC)*

Shared Views With Peers And the Need for a Convinced Supervisor

A facilitating factor to implement DPD genotyping is that views were shared with their co-workers, according to the interviewees.

> *"Well, in the same way. Everyone is convinced I think, that one way or the other you have to test on such a lowered function of the DPD enzyme and whether that is genetic or phenotypic, that doesn't really matter. […], but everyone is convinced that a test has to be performed I think." (#4, oncologist AMC)*

> *"Yes, they[my colleagues] support that." (#11, lab specialist AMC)*

One oncologist indicated that a possible reason for why DPD testing was not part of standard procedures before the update of the guideline in 2017, was the fact, that the lead oncologist lacked personal belief in the added value of DPD testing.

> *"I think it is the policy of the head of the department, whom is then not convinced." (#2, oncologist VUMC)*

Facilitating Safe and Effective Procedures (Structure)

The three main themes identified as important for changing structure were: logistics (process automation)/infrastructure, protocols and education.

Logistics and Infrastructure

Most oncologist of the VUMC indicated that the process of ordering a DPD test is cumbersome and hence expressed that the non-automated process is a disadvantage.

> *"A very inconvenient method; with a lab form from the Erasmus [Medical Center, Rotterdam] that we fill out by hand and sent with the patient to the blood test [facility], which will then be sent [to the Erasmus]. (#2, oncologist VUMC)*

> *"[…], I think actually the fact that I have to print out and then fill out, that I actually find that the most annoying." (#3, oncologist VUMC)*

AMC oncologists indicated that the DPD test is ordered digitally in EPIC (hospital information system) and expressed that they do not see any logistic barriers. Pharmacists perceived the lack of a link between several software-systems as a barrier for

implementing the test and further implementation of pharmacogenetic testing and expressed that a prerequisite for proper functioning of the process is that all systems communicate.

> *"Definitely, but a barrier is that not all support and systems work optimal. […] Logistics are kind of a challenge and that may be why it [DNA medication pass] isn't used that much. […] So, I think it [optimal systems and support] is a prerequisite for how this will work." (#9, hospital pharmacist AMC)*

Pharmacists also expressed that only ten contraindications can be registered in the outpatient pharmacy system, which requires expansion when more pharmacogenetic tests will be performed.

Protocols

A main prerequisite for implementation of the DPD test are the protocols and agreements. Most oncologist expressed that they perform the test, because it is the protocol.

> *"[…] Since we have made the decision to not start before [having a DPD test result], we adhere to this." (#2, oncologist VUMC)*

> *"Yes, it is an obligation. So it is seen as a fault to not test." (#2, oncologist VUMC)*

> *"Yes. Before [the guideline update] we did not do that [DPD genotyping], but since the recommendation has been included in the guideline, we adhere to it." (#1, oncologist VUMC)*

This opinion is also shared by a lab specialist, who indicated that including the instruction to perform DPD testing in a protocol promotes compliance.

> *"I think that in general healthcare is very protocol driven, so when something is not in a protocol, you will not see changes so quickly." (#11, lab specialist AMC)*

The reports with the test results were perceived as adequate and stakeholders indicated to have enough knowledge to change the treatment dose appropriately.

> *"That [the dose advice] is included [in the VUMC report], we don't need to look it up." (#3, oncologist VUMC)*

However, one oncologist of the AMC acknowledged that it would be of great support when the test result report could include instructions on how to interpret the test results.

> *"Well, no. I often have to call, because it is unclear to me. So, yes, I think this could be better. I think when you have results that state it is normal or reduced that it also immediately says, in this range the advice is to start with this much of a percentage of the normal dose. That is not included." (#4, oncologist AMC)*

Education

Generally, oncologists and pharmacists indicated that knowledge was sufficient. However, some mentioned more professional education is needed; that education could possibly benefit doctors in training.

> *"Yes [education is a prerequisite], especially when we will be involved." (#9, hospital pharmacist AMC)*

> *"Well, it is, I think for people in training it will be good to know why we do it." (#3, oncologist VUMC)*

DISCUSSION

Over a two-and-a-quarter years' time period, 753 patients started FP treatment at Amsterdam UMC. The proportion that was DPD tested before the start of the treatment started to increase around the time of the publication of National guideline for colorectal carcinoma, which was also discussed at local meetings to achieve consensus between oncologists and pharmacists at a local level. The publication of a landmark paper two years before had no effect in terms of implementation. The increase of the proportion of patients tested continued to the fourth quarter of 2018, when 87% was achieved. Guidelines clearly are very important for implementation, as well as multidisciplinary local meetings to achieve consensus at a local level.

According to our data, around 13% of patients were not tested against the end of the study period. Perhaps some of these had received FP treatment before, without experiencing side effects, or had been tested in other hospitals. The possibility, however, that DPD-testing could have been "forgotten" for some patients led to renewed discussions in 2019. Since the goal is to achieve 100% pretreatment DPD testing in order to maximize patient safety, additional checks have been built recently in the medical protocols, the electronic ordering system, and dispensing protocol by both the clinical as well as the out-patient pharmacists. Protocols for these different sites were attuned. Also on the oncology wards, nurses started to check DPD status as part of their standard protocol.

The 2017 National guideline applies to colon cancer, but apparently the uptake of DPD testing increased overall. From a biological point of view evidently similar toxicity is at stake. From an implementation point of view it is remarkable to see that a protocol in one field may stimulate implementation of innovation overall.

Since the update of the guideline to conduct *DPYD* genotyping for all patients prior to receiving FP treatment, in the VUMC the patients are tested for 4 genetic variants of the *DPYD* gene, while at the AMC a conscious decision to use a phenotypic test first was made, followed by genotyping for aberrant results.

Since genotyping of a limited number of *DPYD*-variants explains only part of the variance, potentially the sensitivity of the test could be improved with phenotyping. In theory the use of assays that determine enzyme activity first, could be more predictive.

Although no agreement exists on the best test-approach, in general stakeholders are convinced of the clinical utility of DPD testing prior to FP treatment. Multiple stakeholders seem to realize that cost-effectiveness for genotyping is demonstrated, but some are convinced that phenotyping first is a better (more sensitive) method. A large prospective head-to-head comparison would be needed to identify the optimal algorithm, either one assay or a combination. Studies comparing (cost-)effectiveness of different approaches therefore seem to be warranted.

Another pressing issue that arose from the interviews was the need for a more clear division of responsibilities. Although, when asked, most stakeholders expressed that it was the oncologist's responsibility that the test was performed, no clear division of roles seemed to have been agreed upon. Especially the role of the (outpatient) pharmacist could be more formalized, at least as having a responsibility to check whether standard procedures have been followed to ensure drug safety: in this case preventing toxicity in patients with potential aberrant DPD geno- and/or phenotypes.

Patients appreciated being tested for *DPYD*-variants for reasons of medication safety. They mentioned that information in simple language was needed. For the patients it was not relevant whether or not the assay was a DNA test. They liked the idea of having possession of their own DNA test results, as well as data sharing of these results between health care professionals.

In general, facilitating factors for stakeholders to implement pretreatment testing included the existence of clear protocols, (anecdotal) evidence of the utility, being aware that peers are adhering to standard practice and clear and simple procedures. Main barriers included the lack of clear divisions of responsibilities, the lack of consensus on a test approach, long turn-around times and non-user-friendly IT-infrastructures. More education about the utility of pharmacogenetic testing, but also the limitations of such tests was desired by most stakeholders.

While we describe the situation in Amsterdam UMC only, the process we undertook to study the ongoing implementation of DPD testing before FP treatment can hopefully inspire others. While competencies required by pharmacists and other health care professionals have often mentioned knowledge and academic skills, we here illustrate the importance of the successful integration of pharmacogenomics into health and public policy. Training efforts should also include the development of implementation skills. Should other researchers repeat this study, we hope that more than 87% pretreatment testing is found, since we strive for 100% patient safety. The barriers and facilitators that we identified can hopefully contribute to optimal implementation.

Strengths and Limitations

The study reports data from two Amsterdam University Medical Centers only. Whether the proportion of patients who have been DPD tested before the start of FP treatment increased to the same extent in other centers needs to be investigated. Local lessons on barriers for implementation, however, can inform other centers on the implementation of DPD and other pharmacogenetic tests. We have also shown that more clarity can be achieved on roles and responsibilities, to achieve optimal patient safety.

Future Perspectives

Personalized medicine is gaining ground. In terms of implementing new tests to give the right dosage of the right medication to the right person at the right time, it is needed to have clear evidence, professional guidelines, local consensus on the practical implications of guidelines and a clear division of roles and responsibilities. Patients want to be informed about pharmacogenetic testing in simple wording. Research has to show the pros and cons of genotyping vs. phenotyping after which the two locations of Amsterdam UMC will choose one approach. The evaluation of the test has to take both test properties (sensitivity, predictive value) and cost-effectiveness into account. DPD testing is an opportunity to improve patient safety.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

PB, MC and MB developed the project, FM and DH performed interviews, TR provided methodological input and analysed the interviews, DH took care of the quantitative analyses. All authors discussed and reviewed the draft versions of the manuscript and agreed on the final text.

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SUPPLEMENTARY MATERIAL

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Implementation of Pharmacogenetics in Primary Care: A Multi-Stakeholder Perspective

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Introduction: Aberrant pharmacogenetic variants occur in a high proportion of people and might be relevant for the prescription of over 26 drugs in primary care. Early identification of patients who metabolize these drugs more rapidly or slowly than average could predict therapeutic effectivity and safety. Yet implementation of pharmacogenetics is progressing slowly. A high public health impact can potentially be achieved by increasing the proportion of people tested, when and where eligible according to clinical validity and utility.

Methods: In this study we defined actions, roles, and responsibilities for implementation of pharmacogenetics in primary care in consultation with stakeholder groups, by using a three-step mixed-methods approach. First, to define barriers and facilitators, public pharmacists (n = 24), primary care physicians (n = 8), and patients (n = 21) participated in focus groups and face-to-face interviews. Second, a multidisciplinary expert meeting (n = 16) was organized to define desired actions, roles, and responsibilities. Third, an online Delphi Study (n = 18) was conducted to prioritize the designated actions.

Results: For the integration of pharmacogenetics in primary care guidelines and practice, lack of evidence for clinical utility was mentioned as a main barrier. Furthermore, reimbursement, and facilitation of data registration and sharing were considered as key elements for future routine application of pharmacogenetic testing. Moreover, the division of roles and responsibilities, especially between general practitioners and pharmacists, is currently perceived as unclear. Sixteen actions in these four areas (clinical utility, reimbursement, data registration and sharing, and roles and responsibilities) were formulated and assigned to specific actors during the expert meeting. After ranking these 16 actions in the Delphi Study, nine actions remained pertinent, covering the four areas with at least one action. However, participants showed low agreement on the prioritization of the different actions, illustrating their different perspectives and the need to attune between them.

Discussion: Stakeholders together were able to formulate required actions to achieve true integration of pharmacogenetics in primary care, but no consensus could be achieved on the prioritization of the actions. Coordination of the current independent initiatives by the different stakeholders could facilitate effective and efficient implementation of useful pharmacogenetics in primary care.

Keywords: pharmacogenetics, primary care, implementation, stakeholder perspectives, qualitative research

INTRODUCTION

Pharmacogenetics (PGx) can help identify patients who metabolize certain drugs more rapidly or slowly than average in the population. Application of pharmacogenetics thereby could have substantial impact on the safety and efficacy of drugs prescribed in primary health care. In the Netherlands, more than 80 potential gene–drug pairs have been reviewed by the Dutch Pharmacogenetics Working Group (DPWG), of which 47 guidelines provide therapeutic recommendations for one or more aberrant phenotypes (Bank et al., 2018). It has been estimated that more than 95% of people have a relevant gene-variant for at least one of these drugs (Van Driest et al., 2014; Dunnenberger et al., 2015). Twenty-six of these drugs for which pharmacogenetic guidelines are available are prescribed in the primary health care setting to relatively large groups of patients (see **Supplementary Table 1**) (Houwink et al., 2015). It is therefore expected that many patients would benefit from PGx-based prescription policy (Alshabeeb et al., 2019).

Although expectations of PGx are high, limited application is observed in routine health care, especially in primary care (Bartlett et al., 2012; Swen and Guchelaar, 2012; Mills et al., 2013; St Sauver et al., 2016). If PGx testing is performed, it is usually done when side effects arise or when a drug lacks effectivity (i.e., reactive testing; see **Figure 1**). In secondary care sometimes testing is done before prescribing, as a companion diagnostic (CDx), for example in oncology and treatment of HIV. In a few of these cases, the Summary of Product Characteristics (SmPC) requires a PGx test to be performed before the first delivery of the medication (Weda et al., 2014). Panels are increasingly available where the most frequent and relevant variants can be tested at once (van der Wouden et al., 2017). This would allow for future prescription according to genotype for a large number of drugs. Preemptive testing, without any specific indication however, is very rare (van der Wouden et al., 2017).

Barriers and facilitators of implementation of pharmacogenetics into health care have been widely studied (Deverka et al., 2007; Swen et al., 2007; Haga and Burke, 2008; Altman et al., 2011; Ieiri, 2012; Bell et al., 2014; Perry et al., 2016; Frick et al., 2016; Hicks et al., 2016; Abbasi, 2016; Kapoor et al., 2016). Main hurdles that are described include the need for improvement in physician and pharmacist awareness and education about PGx, more insight in relevant measures for clinical validity and utility of (preemptive) PGx testing (Tonk et al., 2017; Jansen et al., 2017), and a proper infrastructure to integrate pharmacogenetics into the workflow of physicians and pharmacists (van der Wouden et al., 2017; Slob et al., 2018).

Shared initiatives to carefully plan how to overcome these barriers and draw on facilitators in Dutch primary care are limited. With this study we aimed to define actions, roles, and responsibilities for implementation of pharmacogenetics by conducting a multi-phased stakeholder study. Stakeholders such as pharmacists, primary care physicians, patients, scientists, and policy makers were invited to discuss thresholds and opportunities for next steps in the implementation of pharmacogenetics in primary care in the Netherlands. Input was collected from all relevant actors in the implementation process, from research to policy and health care. By including this range of actors, a complete view of different perspectives and expectations and broad consensus on priorities was strived for. These insights might help to formulate a strategy to progress large-scale implementation of relevant pharmacogenetics applications in routine health care, and thus contribute to a roadmap for the future.

MATERIAL AND METHODS

The research consisted of three phases (see **Figure 2**): 1) (focus group) interviews with end users to define barriers and

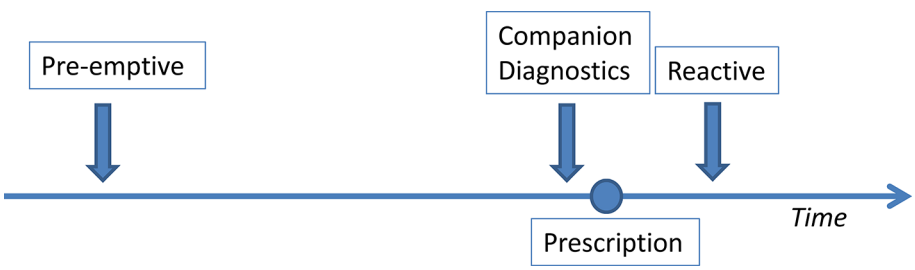

FIGURE1 | Possible timing of pharmacogenetic testing in relation to prescription.

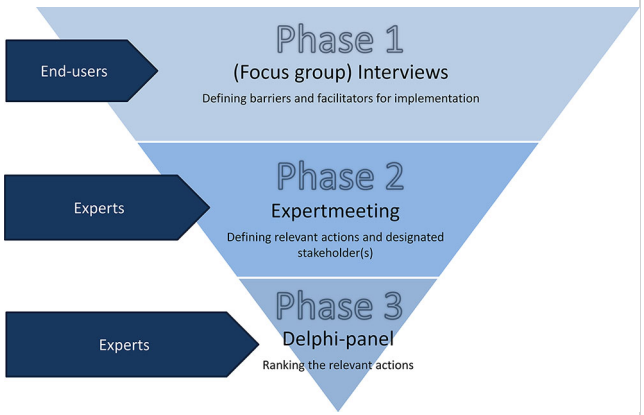

FIGURE 2 | Methods and aim of the three phases of the study.

facilitators for implementation of PGx in primary care; 2) an expert meeting to define necessary actions, roles, and responsibilities for responsible implementation; and 3) an online Delphi panel to prioritize these actions.

This study was approved according to the national legislation. The Medical Ethical Committee of the VU University Medical Center Amsterdam evaluated the study design and decided that the Medical Research Involving Medical Subjects Act (WMO) does not apply to this study and that further official approval is not required (2017.074).

Phase 1: Interviews and Focus Group Interviews

To elicit perceived barriers and facilitators for implementation of pharmacogenetics in primary care, individual and focus group interviews (FGIs) were conducted with the end users: general practitioners (GPs), patients, and pharmacists.

Six FGIs were conducted: three groups with patients and three groups with pharmacists. Although a similar approach was intended for studying the views of GPs, recruitment of these participants proved unsuccessful (see **Datasheet 1** for details on recruitment and response rate of GPs). We therefore conducted eight interviews with individual general practitioners.

Purposive sampling was used to recruit the GPs, patients, and pharmacists for this study. All three key stakeholder groups were recruited from an urban environment (Amsterdam), a rural environment (Northern Limburg), and in a "mixed" region (Utrecht). The division in urban, rural, and mixed region groups was made to attract a variety of participants who would contribute to the diversity of the sample. We attempted to include community GPs and pharmacists and preferred non-experts in the PGx field to represent the average situation in current primary health care. Furthermore, we purposively invited patients who visited their GP in the last year. We did not look for specific patient groups, but for representation of GP's patients in general. Participation was voluntarily, but when present at the (focus group) interviews, all stakeholders groups were expected to share complete perspectives, opinions, and participate actively. All stakeholders were reimbursed for travel and other expenses made for this study.

Both the interviews and focus groups were conducted using a similar semi-structured interview-guide (see **Datasheet 2**), designed to collect input on all aspects of change needed for implementation of pharmacogenetics. The interview guide followed the constellation perspective of van Raak et al. (Van Raak, 2010) [adapted by Rigter et al. (Rigter et al., 2014)], which describes that transitions in health care require new ways of doing (changes in practice), new ways of thinking (changes in culture), and new ways of organizing (changes in structure) by the actors involved. In this case, the topics included: views and expectations, required structural changes, when and whom to test, and roles and responsibilities.

The completed interviews and focus groups were anonymously transcribed verbatim and inductive content analysis was performed using thematic coding, supported by the qualitative software program: AtlasTI, version 7.5.10. The coding process was a joint effort between multiple researchers. All transcripts were individually read and coded by at least two researchers (JMdG, TR, and MJ). The findings were consistently evaluated throughout the process until consensus was reached on the coding strategy.

The official language for the interviews was Dutch; therefore, the participants' statements were translated for use in this report.

Phase 2: Expert Meeting

Main barriers and facilitators from the interviews were grouped into themes, which were used to organize an expert meeting to further define needed actions, roles, and responsibilities of relevant stakeholder groups. Thirty-two stakeholders with expertise in different aspects of PGx or primary care were purposively selected and invited to take part in an interactive expert meeting. Twenty-three experts accepted the invitation and 16 participated in the meeting. The following expertise were represented: health technology assessment, health care insurance and reimbursement, clinical pharmacology, clinical research, primary health care policy, patient advocacy, psychiatry, biomarker development, pharmacy, information technology in primary health care, and pharmacogenetics.

After a plenary introduction to the project and the results of the focus groups, participants were assigned to a group based on their expertise and asked to discuss a specific topic (division of responsibilities, data registration and sharing, generating evidence for guideline development, and reimbursement). Each group was chaired by a project-member who posed some pre-formulated questions (see **Datasheet 3**) to discuss and define all relevant actions and one or more designated stakeholder(s). Outcomes were summarized on a flip-over by each chair and shared between groups after the workshops to initiate a plenary discussion and formulate conclusions. Furthermore, the experts were asked to give written input if specific topics or actions were found relevant, but had not been discussed at the meeting. Based on the concluding remarks, a list of actions was formulated, serving as input for the Delphi panel.

Phase 3: Delphi Panel

The defined actions from the expert meeting were prioritized through an online Delphi process. The Delphi technique has been a widely accepted method for data collection and reaching consensus among respondents within their domain of expertise (Dalkey and Helmer, 1963; Hsu and Sandford, 2007; Burke et al., 2009).

We aimed to obtain consensus of a heterogeneous Delphi panel on the prioritization of actions for implementation of PGx in primary care (see **Figure 3**). Twenty-seven experts were purposively selected and invited with similar expertise fields as the expert meeting.

Twenty experts accepted the invitation and 18 experts completed all rounds (response rate: 74.1%). Each expert e-mailed their prioritizations with arguments in three separate rounds between April and July 2017. Between rounds, all participants received an anonymized overview of answers and arguments in the next questionnaire.

The initial Delphi questionnaire contained 16 actions and suggestions for designated stakeholder(s). Participants were asked to score each action on importance on a five-point Likert scale, give a rationale for their score, and could suggest additional or different designated stakeholder(s). The questionnaire was finalized with a question to prioritize a top 3 of the actions for implementation of PGx in primary care. Criteria for consensus for each round were applied as described by Houwink et al. (Houwink et al., 2012) and Kendall's *W* was calculated as a coefficient for concordance in the final prioritization by participants. A p value of ≤0.05 was considered statistically significant.

To analyze if certain experts within a group showed higher correlation in ranking the actions, participants were stratified. Each participant was allocated based on self-reported expertise. The groups were: scientists, pharmacists, policy experts, patient representatives, and GPs.

RESULTS

Phase 1: Interviews and Focus Groups

Focus group interviews (FGIs) were conducted with in total 24 pharmacists and 21 patients. Unfortunately, GPs initial response rate for the focus groups was only around 1% and did not result in successful planning of a group interview (see **Datasheet 1** on recruitment and response rate of GPs), after which it was decided to conduct interviews with individual GPs. Eight GPs were interviewed. Although this approach did not allow for interactive discussions among GPs, we were able to evaluate the reasons for the low response. General lack of interest and

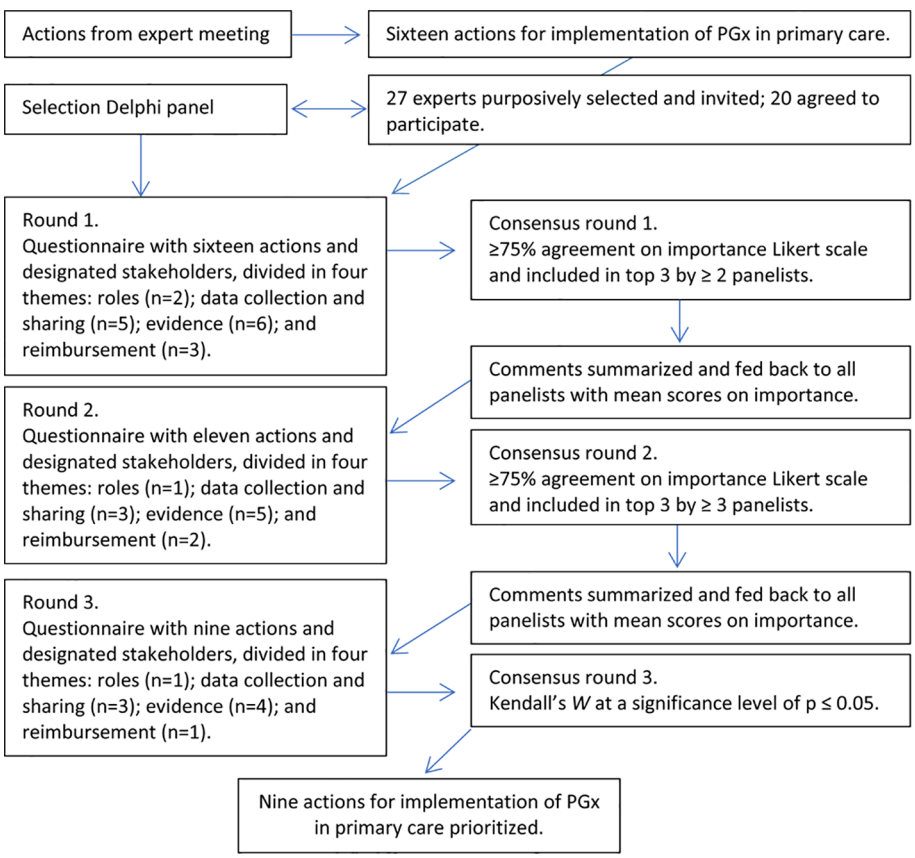

FIGURE 3 | Steps of the Delphi procedure including cutoff values.

knowledge about the topic made the input from eight individual GPs satisfactory because data saturation was reached for all three stakeholder groups. We specifically recruited non-experts from primary care (community GPs and pharmacists), but a high percentage of patients included in our study reported to have a chronic disease. For general demographics (e.g., age and years of experience in their field) of participants to phase 1, see **Supplementary Table 2**.

Relevant and recurrent themes describing barriers and facilitators for implementation of pharmacogenetics in primary care are discussed below, under headings following the main themes from the interview guide (views and expectations, organizational changes, when and whom to test, and roles and responsibilities). We have selected quotes to illustrate the views and arguments within these themes.

Views and Expectations

In the (focus group) interviews, GPs and pharmacists expressed that pharmacogenetics is currently rarely considered or used by GPs.

> "To me it [PGx testing] is all very new […], I don't think about it [PGx testing]. This totally isn't something that I am considering as a GP." GP5, 5:54

Patients themselves said to generally be unaware of (potential usefulness) of the influence of genes on drug response.

> "I am surprised by the list of drugs [you just showed] for which they know they could work differently for certain groups of people." Patient FG5, 1:30

Some pharmacists said to have experience with pharmacogenetics in their practice, either by being involved in a pilot study or responding to (anecdotal) evidence of utility of PGx testing for specific drugs.

> "[In the context of a PGx implementation study] it is a small group of patients still, fifty now, that we have genotyped." Pharmacist, FG3, 3:3

> "[after a year of raising awareness of PGx for clopidogrel] I have to say: […] it has been more than a year, we only have five contra-indications registered on 40,000 patients." Pharmacist, FG3, 4:37

Although most participants of the interviews seemed to recognize the potential of pharmacogenetics—to reduce adverse drug reaction, increase effectiveness of treatment, and possibly indirectly increase adherence—not all seemed convinced of the urgency to press large-scale implementation. Especially general practitioners were perceived as reluctant to change their current practice of "trial-and-error" when prescribing drugs.

> "In [current] practice they [GPs] will just play with the [medication] dose: we will increase it and see what happens, decrease it and if drug A doesn't work, we will try drug B […]. It never really comes to the test. Even though that is the most likely cause of the problem." Pharmacist, FG1, 3:35

> "In general our profession is relatively conservative when it comes to new developments: first seeing what the effects are and what we gain from it and what the outcomes are and then getting on board. There are few people who then are pioneers […]" GP8, 10:3

Especially pharmacists seemed supportive of the use of pharmacogenetics and were expecting more applications to be developed to optimize treatment for the patient. It was also expressed that it could be an opportunity to expand the current job responsibilities and accompanying funding structure of pharmacists. Consequently, most pharmacists showed disappointment about the current lack of use of the potential of PGx in primary care.

> "I think that more should be done with it [PGx] and that you should not wait until people develop all sorts of, euhm, just muddle along with their drugs. That we should be more pro-active." Pharmacist, FG3, 5:2

Although most participating pharmacists said to have both the knowledge and infrastructure available to increasingly start applying pharmacogenetics in daily practice, there was doubt as to whether their peers would be as well-equipped.

It was acknowledged by both GPs and pharmacists that there currently was a lack of knowledge and clear protocols for effective implementation of pharmacogenetics in primary care, in particular for GPs.

Organizational Changes

Lack of evidence on clinical utility was mentioned as a general barrier to include pharmacogenetic dosing advices in guidelines for general practitioners.

> "[…] As long as you don't know the effectiveness, but also the costs and benefits in primary care. I would think, that as a GP, you should be very careful in this matter." GP5, 5:16

Besides lack of evidence and easily accessible guidelines, other main structural prerequisites were mentioned, such as reimbursement of the test and subsequent therapy, user-friendly software systems, and data sharing infrastructures.

> "It should be clear, practical and applicable, otherwise it won't happen." Pharmacist, FG3, 5:29

Another impediment to the routine application of pharmacogenetics surfaced when discussing reimbursement. It was expressed that potentially investments are required in a different silo of health care than where the return on investment will appear.

Efficient data exchange was mentioned by all participant-groups as a prerequisite for effective implementation. This

included exchange of guidelines, since participants expressed that existing pharmacogenetic dosing advices are only included in routine health care guidelines for pharmacists, but are not easily accessible for general practitioners. Moreover, exchange of test results between GPs and pharmacists, but also between professionals in primary and secondary care, was requested to prevent unnecessary repeated testing.

Furthermore, protocols when to test a patient (see **Figure 1**) are considered essential to implement pharmacogenetics successfully.

> "You should know: when do you want a test? Do you want it before therapy or when the therapy doesn't work or when adverse reactions occur? Who will you test?" Pharmacist, FG1, 4:97

When and Whom to Test

When discussing the best timing of testing, there seemed to be a tendency to prefer preemptive testing because of the direct usefulness of the information at the moment of prescription of a relevant drug.

> "I think something is going to change […] and that you will advise more proactively instead of reactively. Because that is a profile that is established since moment zero […], then you already know for the coming years what your patient is allowed to have and what not." Pharmacist, FG2, 1:166

> "The moment of testing… I think in the future we will go towards the moment a baby is born, that immediately a DNA-profile is made." Patient, FG6, 4:2

However, there was no consensus about the target population (e.g., newborns or specific subgroups later in life) and questions arose about the (cost)-effectiveness of preemptive testing. Therefore, some participants preferred companion diagnostic or reactive testing.

> "But if they are not going to use drugs, then there is no need to know it. You can also wait until the moment someone is going to use drugs." Pharmacist, FG3, 5:62

> "I would still argue to do it on indication alone […], so if you expect problems, but not standard with everybody." GP6, 7:56

Deciding on most appropriate timing of testing proved complex and therefore participants expect it to be resolved at policy level, as well as clearly described in protocols.

Roles and Responsibilities in Applying PGx

Disagreement exists about the best division of responsibilities between general practitioners and pharmacists, and the patient's role. GPs generally expressed the desire to be able to request the test themselves and want to remain end-responsible for the correct dosing of drugs. GPs mainly see the role of the pharmacist as signaling and advising on drug-prescription, including pharmacogenetic influences.

> "[…] I expect the pharmacist to know more than I know from pharmacokinetics and that sort of things and that he could advise me better in: this combination should be avoided in any case and this can go together." GP8, 10:2

Pharmacists themselves seem to picture a more central role in pharmacogenetics for their profession; some even as party responsible for all prescription of drugs in general.

> "But in that case I would actually want the doctor to only write down the diagnosis. […]. And that I come up with the pills for that." Pharmacist, FG2, 1:167

However, pharmacists generally also seem to acknowledge that this role should be granted by GPs as well as patients.

Patients explicitly prefer the GP as having the final responsibility and being the contact person when it comes to applying pharmacogenetics, mainly because of familiarity and trust.

> "But I think a pharmacist in itself, is too commercial to do such things [order a PGx test and adjust treatment accordingly]. A blood drawing station or so [could do that], okay, or the GP himself, but a pharmacist absolutely not." Patient, FG6, 10: 6

All participants emphasize that there is a need for cooperation and explicitness about roles and responsibilities between GPs and pharmacists.

> "Together [the GP and the pharmacist] we can make sure that the chosen therapy gets a very good chance of success when it, ehm, when the genotypes of the patient are known." Pharmacist, FG1, 4:20

To maintain the relationship of trust and give all stakeholders the time to become acquainted with the new division of roles and responsibilities, participants mentioned that it would be wise to not act precipitately and implement pharmacogenetics in phases.

In order to list all required actions for implementation of PGx, output from the interviews was used to organize an expert meeting in the next phase of the study.

Phase 2: Expert Meeting

Based on the interview data, four themes were defined and discussed in an expert meeting: 1) division of responsibilities; 2) data registration and sharing; 3) generating evidence for guideline development; and 4) reimbursement. During the expert meeting, actions within these themes were formulated, with an indication of the responsible stakeholders for the action (see **Table 1**).

Phase 3: Delphi Panel

The formulated actions and responsibilities were prioritized by a heterogeneous Delphi panel in the third phase of the study.

TABLE 1 | Actions, roles, and responsibilities as discussed in the expert meeting.

Themes	Actions	Responsible stakeholder(s)
Division of responsibilities	**Develop a national guideline on collaboration.*	*Health care provider organizations of pharmacists and GPs (KNMP/NHG).*
	Make agreements on a regional level about when and who can request PGx tests.	*Regional groups for pharmacotherapeutic consultation (local organization of GPs and pharmacists).*
Data registration and sharing	**Define relevant data that should be registered and shared between health care professionals for effective use of PGx.*	*Health care provider organizations of pharmacists and GPs (KNMP/NHG).*
	**Standardize patient data that needs to be registered with regard to PGx.*	*Health care provider organizations of pharmacists and GPs (KNMP/NHG) and NICTIZ (National IT Institute in Health Care).*
	Further develop the National Link Point to enable easy exchange of PGx data between health care professionals.	*VZVZ (Association of health care providers for health communication) at the initiative of the health care provider organizations (KNMP/NHG) in collaboration with NICTIZ (National IT Institute in Health Care).*
	Facilitate aligned registration for the reason of adjusting a patient's treatment regime, to monitor and evaluate effectiveness of applying PGx.	*NICTIZ (National IT Institute in Health Care), in collaboration with software developers HIS/AIS (information systems for GPs/pharmacists), at the initiative of the Dutch GP association (LHV)/Royal Dutch Pharmacists Association (KNMP).*
	Adjust or develop software systems to facilitate applying PGx.	*Software developers HIS/AIS (information systems for GPs/pharmacists), at the initiative of the Dutch GP association (LHV)/Royal Dutch Pharmacists Association (KNMP), in collaboration with NICTIZ (National IT Institute in Health Care).*
Generating evidence for guideline development	**Gather data on the number of prevented ineffective or adverse drug responses through PGx.*	*Funders for research/independent research institutes/scientific organizations.*
	**Validate the predictive value of PGx tests through prospective or observational research.*	*Scientific organizations.*
	**Assess the cost saving of PGx test through pharmaco-economic studies.*	*Scientific organizations.*
	**Collect data on the impact on clinical outcomes by assessing the patient experience of the severity of ineffective or adverse drug response.*	*Scientific organizations, together with patient organizations.*
	**Develop aligned patient information on the benefit of PGx tests. Monitor data on the frequency of genetic variants that are tested with PGx.*	*Health care provider organizations (KNMP/NHG) of pharmacists and GPs together with patient organizations Independent research institutes.*
	Monitor data on the frequency of genetic variants that are tested with PGx.	*Independent research institutes.*
Reimbursement	**Include PGx tests as an optional test for general practitioners in their guideline.*	*Dutch organization for general practitioners (NHG).*
	Develop aligned patient information on the costs of PGx test and the impact on their health care insurance reimbursement.	*Health care provider organizations of pharmacists and GPs (KNMP/NHG), in collaboration with ZN (Dutch Health Care Insurers) and patient organizations.*
	Define and prioritize disease areas eligible for reimbursement based on data on clinical utility.	*Health insurers and ZINI (Dutch Health Care Institute).*

Statements preceded by an asterisk (*) remained after three iterations of the Delphi procedure. PGx pharmacogenetics, GP general practitioner.

Eighteen out of 20 experts in the Delphi panel completed all rounds. Ten experts were female (50%), and the mean age was 48.5 years (SD = 9.9). We aimed to include representatives of key stakeholders and similar expertise as in the expert meeting, but, mainly due to time constrains, some expert groups allocated this task to another colleague. Ten of the panelists also participated in the expert meeting. From the 16 actions suggested during the expert meeting, nine remained after the three iterations of the Delphi procedure (see **Table 1**).

In the overall analysis, results showed low agreement between participants on the ranking of the remaining nine actions (W = 0.14, p = 0.011; see **Supplementary Table 3**) While not statistically significant, the highest correlations in ranking were seen between respondents within the expertise pharmacists (W = 0.617, p = 0.275) and GPs (W = 0.842, p = 0.097). The participants within these two groups show moderate agreement on the ranking of the actions, but—as can be deducted from the overall analysis—the ranking differs between the groups. For example, on average, action 15 "Include PGx tests as an optional test for general practitioners in their guideline" was ranked third of nine by pharmacists and 8.5 (i.e., almost last place) by GPs.

High consensus on a topic's importance did not always translate into many experts putting it in their top 3, and vice versa (see **Supplementary Table 4**). In round 1 for example, only 75% of the panelist scored the statement "*Validate the predictive value of PGx tests through prospective or observational research*" as (very) important, while 7 of the 20 panelists put the statement in their top 3. In contrast, while 95% of panelist scored the statement "*Standardize patient data that needs to be registered with regard to PGx*" as (very) important, only three put the statement in their top 3. In support of this last statement, some panelist argued that "From my point of view, this is one of the major barriers" and "Without standardized data management, appropriate and useful application of pharmacogenetics is not possible.", while others also mentioned that they thought "Are all patient data not already standardized? Seems logical to do so.", suggesting that some panelist scored statements lower because they assumed the action was already in place.

Looking at statements that were accepted in the first round, but then rejected in the second round (n = 2), the statement "*Define and prioritize disease areas eligible for reimbursement based on data on clinical utility*" dropped from 75% consensus on importance to 56%. While in round 1 supportive panelists mentioned "Start with diseases that have the most impact and/or prevalence" and "Start with disease areas that seemingly will have the highest clinical utility," others stated that "To be able to prioritize, you need the research data mentioned in the other statements." or "Patient characteristics and individual response or type of medication are more important than disease areas.", which may have led to other participants changing their scores.

The nine statements that remained after three iterations of Delphi procedure also had differing arguments from panelists why an action was or was not important. For example, the action "*Develop a national guideline on collaboration*" was considered important because "It is essential that it will become clear who will lead the way, who is responsible in daily practice, and how it will be implemented.", while another panelist stated that PGx should be "included in general collaboration guidelines, not a specific one for pharmacogenetics." While many of the panelist considered the actions under evidence important, because "If there is no clinical utility, then the other actions also become less important" and "First research, then implementation," one panelist was skeptical "Gathering data on prevented ADRs is wrongly considered as very important, it should be less prominent." and considered collecting data on the patient experience from side effects "Unethical. We have a classification system for ADRs." Informing the patient was also considered highly relevant action, as one panelist stated "Honest and independent patient information that is also available online seems necessary to me." Some panelist fed back that they missed an action to educate GPs.

Overall, the Delphi procedure helped to define nine actions that were considered important by most experts. The majority of the actions (five out of nine) fall within the category of generating evidence for guideline development, indicating that this is currently perceived as a main barrier. However, no consensus on which of the actions should be top priority was reached among the Delphi panelists.

DISCUSSION

With this study, we aimed to define actions, roles, and responsibilities for implementation of pharmacogenetics in primary care. Based on a qualitative inventory of perceived barriers and facilitators for responsible implementation of pharmacogenetics among primary care end users in the first phase of this study, experts formulated and ranked actions to achieve effective application in the two later stages (see **Figure 2**). The (focus group) interviews, as well as the input from expert meeting, indicate that currently the main barrier for implementation is the lack of insight into clinical utility of pharmacogenetics testing. Some stakeholders express they are convinced of the need to use pharmacogenetic information in primary care, but others state that necessary evidence for preemptive testing in primary care is lacking. Current publications give little insight in the actual (cost-)effectiveness of a structural offer of pharmacogenetics testing and in what context it could prove most beneficial to patients. Although evidence on what to do in case of specific phenotypes has been translated into guidelines, evidence of how to generate and use these genotypes in primary care is lacking. This is partly due to uncertainty which patients to test at which time point. This issue is subject to recent discussions: if there is no clear view of the actual context of testing, researchers will keep failing at providing insight into relevant measures for policy decisions and stick to reporting associations between drugs and genotypes (Tonk et al., 2017; Jansen et al., 2017).

If clinical utility is established however, for example from results from current studies on implementation of preemptive pharmacogenetics panels [e.g., the uPGx project: (van der Wouden et al., 2017)], experts involved in this study acknowledge that there are still other barriers to overcome. The required actions involve making clear arrangements for collaboration between different stakeholders, data registration and sharing, and reimbursement of testing and follow-up.

It is noteworthy that awareness and education among (primary) healthcare professionals on PGx has not surfaced as a main topic requiring action in our study. Many recent publications have described awareness and education as important prerequisites for implementation. Different efforts have therefore focused on developing (continuous) education programs for professionals (Just et al., 2017). When asked to formulate actions, experts in our study, however, expressed other prerequisites instead of awareness and education as such, perhaps because other actions are considered more urgent. A clear example is the prerequisite to construct guidelines and protocols on when and whom to test, and the need for evidence which could be incorporated in professional guidelines. Creating awareness and effective education will have to build on these guidelines and protocols.

Although this study provides insight into the actions required by different stakeholders to achieve true integration of pharmacogenetics in primary care, there was no consensus on the priority of each action. This might be due to a lack of a collective sense of urgency to adopt this innovation in daily practice and/or the multitude of stakeholders that are expected to take action. In spite of the fact that some stakeholders did seem to perceive their actions as urgent, collaboration between stakeholder groups was scarce. Furthermore, the incentives for the different stakeholders to undertake the actions described seem to be unclear or perhaps even lacking. There seems to be no (independent) coordination of the initiatives that contribute to the required actions for effective and efficient integration of pharmacogenetics in primary care, perhaps leading to suboptimal attuning between stakeholders.

Strengths of this study include the fact that the stakeholders themselves defined actions and priorities. This contributes to the likelihood that relevant and feasible actions towards implementation of PGx in primary care were defined and could help in raising awareness about the required steps. Eventually, this could perhaps motivate the professionals to take action. Furthermore, the different methods used in this study provided a platform for the different stakeholders to share their views on how to take the use of pharmacogenetics in primary care to a next level. For the Dutch health care setting, and potentially other countries as well, this might therefore be a good model towards finding consensus on who is expected to undertake which responsibilities.

To ensure internal validity of the study, researcher triangulation was adopted for the coding and interpretation of the data: multiple researchers from different backgrounds were involved.

It is possible that outcomes of this study cannot be fully translated to other countries because of the Dutch context, involving specific data infrastructures and, e.g., the particular role of the GP as a gatekeeper in the Dutch health care system. Although we attempted to include non-expert GPs, patients, and pharmacists from different regions in the Netherlands (both from cities and more rural areas) to increase transferability of the results, it should be noted that especially the pharmacists and GPs included in the (focus group) interviews expressed that they might be more interested or knowledgeable about PGx than the general pharmacist/GP and/or patient. This might imply even more thresholds in real life, such as a high proportion of stakeholders who are unknowingly unable.

GPs proved difficult to motivate to participate in our study, with a response rate for the intended focus groups of around 1%.

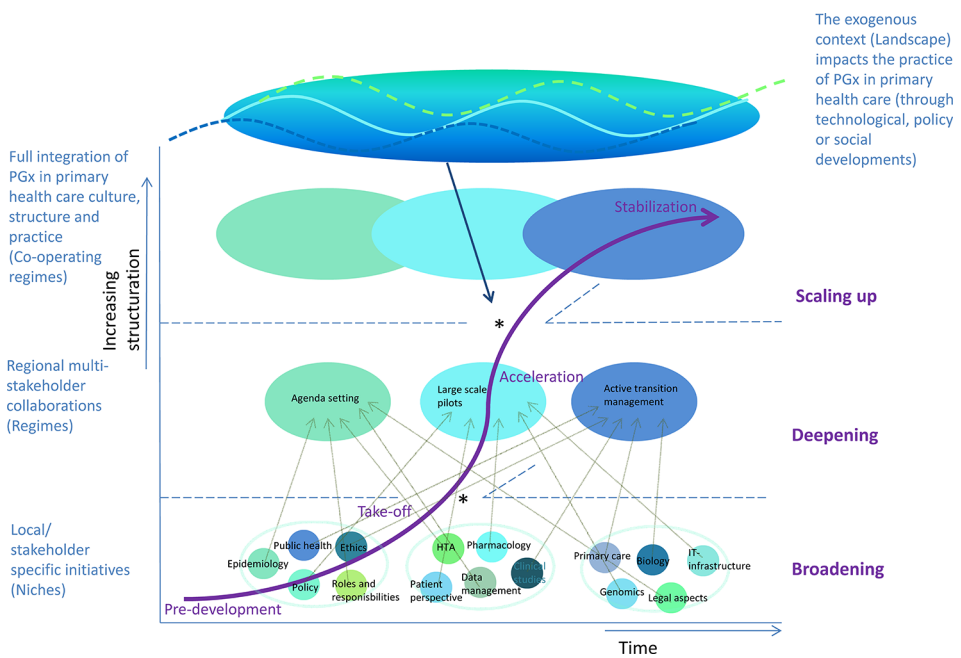

FIGURE 4 | True integration of pharmacogenomics in primary health care requires different transitions [adapted from (Rotmans et al., 2001; Geels and Schot, 2002; Geels, 2007)]. Local or stakeholder-specific initiatives will need to transform to a patchwork of multi-stakeholder collaborations which could create pressure to sustainably change the existing health care culture, structure, and practice. This could be achieved by joint efforts to 1) broaden implementation: transitioning from the pre-development to take-off phase of transitions requires effective learning processes on multiple dimensions; 2) deepening implementation: transitioning from the take-off phase to acceleration of transitions requires attunement and collaboration between stakeholders to align (lessons) from earlier niche applications; and 3) scaling-up implementation: transitioning from the acceleration to stabilization phase of transitions requires true changes in thinking, organizing, and doing of stakeholders. Furthermore, taking advantage of windows of opportunities (*) to next phases in transition (often achieved by alignment of different initiatives and/or stakeholders) could facilitate structuration and thereby integration of new ways of thinking, doing, and organizing.

This is comparable with response rates from GPs in other studies on (pharmaco)genetics [e.g., a focus group study with response rate of 0.45% by Jans et al. (Jans et al., 2013) and a questionnaire survey with a response rate of 3% by Stanek et al. (Stanek et al., 2012)]. GPs that participated to our interviews explained that the lack of interest most likely relates to the unfamiliarity and lack of knowledge on the topic.

CONCLUSION AND FUTURE PERSPECTIVE

For innovations to be sustainably integrated in health care, it is known that changes in culture, structure, and practice are required (Van Raak, 2010; Rigter et al., 2014; Holtkamp et al., 2017). The stakeholders in this study were able to define specific actions on all these levels to pave the road for integration of pharmacogenetics into primary care. Participants showed low agreement on the ranking of priorities for the different actions.

Different stakeholder groups have taken initiative (to prepare) for some of the prerequisites that have been formulated in this study, but there is still a lack of a collective driver of change. From a transition management perspective, it seems some aspects of implementation are deepened in the current niche initiatives (at a micro-level), but these are not substantially broadened to eventually achieve scaling-up to full implementation in primary care (Rotmans et al., 2001; Geels, 2002; Geels FW, 2007). This might be due to a lack of coordination of the different actions in the field and eventually might lead to stagnation of structuration of initiatives. **Figure 4** shows an overview of the implementation process for PGx in primary care, from a transition management perspective. The model summarizes general transition phases and aspects. Based on existing transition management models, the figure provides insight into the needs for full integration of PGx in primary health care culture, structure, and practice.

As shown in **Figure 4**, there seems to be a window of opportunity in the current awareness of the potential of pharmacogenomics under researchers, policy makers, and health care professionals, as well as the eagerness of public pharmacists to use PGx information in their prescription practice. Without a collective effort to substantially change curr ent culture, structure, and practice however, implementation of PGx in primary care might not answer to the needs of stakeholders, resulting in fading enthusiasm and potentially even decreasing trust in effectiveness of PGx. Missing this window of opportunity might thereby lead to premature plateau in the curve representing "lock-in" or even a backlash in transition (v.d. Brugge and Rotmans, 2007).

If stakeholders want national adoption of pharmacogenetics testing in primary care to be a success, we suggest that champions with good examples of effective application engage the field, including funding agencies (in science as well as care). Probably recent initiatives in secondary care could be used for this purpose: e.g., applications of PGx in psychiatric care and oncology, but also opportunistic screening for PGx variants in exomes sequenced for diagnostic purposes. This requires early involvement of stakeholders from primary care to discuss implications for their practice. Furthermore, developments in the data infrastructure in (primary) health care could facilitate adoption of PGx information in patient care. An alternative suggestion is to allocate top-down funding at a policy level for resources for clinicians and scientists to support collaboration and stimulate implementation of PGx in health care, similar to the IGNITE Initiative (funded by the NIH) in the USA (Geels FW, 2007) or embedded in a national initiative to foster implementation of genomic medicine, similar to the Genomics England (mainly funded by NHS England and the National Institute of Health Research) in the UK (Website Genomics England,). This could facilitate national cooperation and more efficient broadening and scaling up of initiatives that are currently undertaken mostly at regional or professional-subgroup level. Perhaps most importantly, a collective drive to collect evidence of clinical utility of PGx testing will have to be achieved to substantiate (ethical) evaluation of the impact of PGx and ensure its responsible and sustainable implementation.

DATA AVAILABILITY STATEMENT

Anonymized data generated for this study is available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

TR, WR, MJ, and MC worked together in setting the study objectives and designing the project. SJ and MC suggested the main concept about the project and highlighted its importance. TR and MJ took the lead in collecting the data, with help from JG in the interview phase and regular feedback of WR and MC. TR drafted the main part of the manuscript, with specific input of MJ for the parts on the Delphi Study. All co-authors reviewed and modified the paper.

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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.2020.00010/full#supplementary-material

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ORIGINAL RESEARCH
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Two Novel Loci of *RELN* Associated With Antipsychotics Response in Chinese Han Population

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Background: There are great individual differences in the drug responses; however, there are few prognostic drug response biomarkers available. *RELN* is one of the more extensively examined schizophrenia candidate genes. The purpose of this study was to determine whether *RELN* can affect antipsychotics response in the Chinese population. This may lead to the discovery of relevant novel drug response markers.

Methods: The unrelated 260 Chinese Han inpatients with schizophrenia were enrolled in the present study. The enrolled subjects have been prescribed antipsychotic medication during the study. A total of 15 SNPs of *RELN* were genotyped by MassARRAY® platform. The association of the *RELN* gene with therapeutic response to antipsychotics was analyzed based on sex and age at onset.

Results: Two novel SNPs of *RELN* were found to be associated with antipsychotic treatment response (rs155333, p = 0.010 and rs6465938, p = 0.049) at nominal significance threshold, but not after multiple correction. Our study also revealed highly significant association of a haplotype consisting of three SNPs (rs362814-rs362626-rs2237628) with antipsychotic treatment response. Even after permutation, the *p*-value indicated significant association (rs362814-rs362626-rs2237628: ACT, χ^2 = 6.353, *p* = 0.0117, permuted *p* = 0.04). Furthermore, a novel SNP, rs2535764, was found to be associated with antipsychotic response under overdominant genetic model at a marginal significant level of 0.046 (C/T vs. C/C + T/T: *p* = 0.046, AIC = 314.7, BIC = 321.6).

Conclusion: Our data indicated that *RELN* can affect antipsychotic treatment outcomes in the Chinese population. SNPs of *RELN* could be used as predictive biomarkers for future personalized medicine of antipsychotic drug treatment. However, none of the three novel SNPs (rs155333, rs6465938, and rs2535764) remained significant after Bonferroni correction. Therefore, validation is needed in larger pharmacogenetic studies.

Keywords: schizophrenia, RELN, antipsychotics, drug response, pharmacogenetics

INTRODUCTION

Schizophrenia is a widespread mental disorder with periods of remission and relapses over a patient's lifetime (van Os and Kapur, 2009; Jann and Penzak, 2018), and long-term treatment with antipsychotic drugs is often required (Kahn et al., 2015). The lifetime prevalence of schizophrenia in Chinese population is 0.9% (Huang et al., 2019). There are great individual differences in antipsychotic drug treatment responses, in terms of both therapeutic effects and adverse effects (Xu et al., 2013). For example, recent studies have shown that response rates for currently available antipsychotic drug treatment of first-episode psychosis are usually only about 50%–60% (Arranz and de Leon, 2007; Kahn et al., 2008; Robinson et al., 2015). Treatment failure of schizophrenia not only could increase economic costs but also could cause severe adverse drug reactions in patients.

In clinical practice, there is a lack of reliable predictors for antipsychotic drug responses, and currently no biomarker is available to guide medication. Risperidone, quetiapine, aripiprazole, olanzapine, and perphenazine are the most commonly used antipsychotic drugs in China. As with all antipsychotics, there are considerable individual differences in response to these drugs, in terms of both therapeutic effects and adverse effects. It has been reported that genetic polymorphism plays an important role in individual differences. Pharmacogenomics aims to identify biomarkers to maximize medication efficacy and minimize potential adverse events (Kirchheiner et al., 2005). In recent years, many studies have investigated the association between genetic variation and different antipsychotic drug responses (Xing et al., 2006; Fijal et al., 2009; Lee et al., 2012). However, the pharmacogenetics study of schizophrenia is still in its infancy. Most of the previous studies focused on genes related to the mechanisms of drug action. Many of these studies mainly focused on genes encoding drug targets (*e.g.*, dopamine or serotonin receptors), drug transporters, and cytochrome P450 genes. This may omit other potentially significant antipsychotic drug response markers which are beyond these candidate genes. More exploratory studies that investigate other genes of interest likely represent a superior strategy for discovering relevant novel antipsychotic drug response markers. Moreover, these newly discovered antipsychotic treatment markers have the potential to be novel drug targets for schizophrenia.

RELN is an extensively studied schizophrenia candidate gene (Luo et al., 2019; Sozuguzel et al., 2019). Its relationship with schizophrenia is well supported by linkage or association studies in different populations (van Schijndel et al., 2009; Liu et al., 2010; Wedenoja et al., 2010; Alkelai et al., 2012; Zhou et al., 2016; Tang et al., 2017; Xiao et al., 2017; Sobue et al., 2018). *RELN* is located at 7q22. It encodes the glycoprotein Reelin, which is secreted mainly from the Cajal-Retzius cells and a subpopulation of GABAergic interneurons in the developing cerebral cortex and hippocampus. *RELN* is known as a crucial molecule in brain development, acting as a key regulator in neuronal migration, cell aggregation, dendrite formation, microtubule function, and cell-cell interactions. Animal studies have revealed that *RELN* is an essential molecule for proper cortical neurons migration (Kubo et al., 2010; Franco et al., 2011; Jossin and Cooper, 2011; Kupferman et al., 2014; Sekine et al., 2014; Kohno et al., 2015), and it acts as the final regulator for the cell positioning in the cortex during embryonic and early postnatal stages. However, the expression patterns and distribution of *RELN* in the postnatal period are dramatically changed as compared to those during embryonic period. Intriguingly, evidence has proven that Reelin signaling modulates synaptic function in the adult brain (Alcantara et al., 1998; Herz and Chen, 2006) suggesting *RELN* also plays an important role in postnatal brain. *RELN* is also involved in signaling pathways related to neurotransmission, memory formation, and synaptic plasticity. Studies have shown that Reelin signaling plays a role in the processes of dendrite development (Olson et al., 2006; Jossin and Goffinet, 2007). *RELN* is essential for proper functional and behavioral development of juvenile prefrontal circuits through modulating the N-methyl-D-aspartate receptor (NMDAR) mediated signaling pathway (Iafrati et al., 2014; Lane-Donovan et al., 2015). Furthermore, Reelin signaling is also involved in the presynaptic functions. RELN acts presynaptically in mature neurons to rapidly enhance neurotransmitter release. It has been reported that the Reelin pathway controls learning and memory through activation of the transcriptional factors (Telese et al., 2015). Moreover, it has been shown that variants of *RELN* are closed associated with increased risk of schizophrenia (Shifman et al., 2008). Evidence has implicated the etiology of schizophrenia with regard to the crucial role of *RELN* in neurodevelopment (Fatemi, 2005). Both the mRNA and protein levels of *RELN* have been shown to be markedly reduced in schizophrenia patients (Impagnatiello et al., 1998).

Population studies have indicated that *RELN* may contribute to the genetic etiology of schizophrenia, and the known functions of *RELN* have implicated its involvement in etiology of schizophrenia with regard to a disruption in neurodevelopmental processes (Fatemi, 2005). As mentioned above, most pharmacogenetics studies mainly focus on drug targets genes, drug transporters genes, or cytochrome P450 genes. Therefore, in this study we aim to determine whether *RELN* affects antipsychotic drug treatment outcomes in the Chinese population. This study has the potential to identify novel antipsychotic drug response markers. Moreover, since *RELN* is crucial in the genetic etiology of schizophrenia, our study could find new possibilities for novel drug targets for schizophrenia.

MATERIALS AND METHODS

Subjects

Male and nonpregnant, nonlactating female subjects 18 to 65 years of age with a DSM-IV diagnosis of schizophrenia inpatients, were eligible for the study. All the patients were recruited from Shanghai Mental Health Center. In total, 260 subjects were recruited in this study with a Diagnostic and Statistical Manual of Mental Disorders-IV criteria for schizophrenia. The enrolled subjects were prescribed single antipsychotic medication during the study, including risperidone (150), quetiapine (37), aripiprazole (34),

olanzapine (10), and perphenazine (29). The enrolled patients were required to undergo drug cleaning if they were taking antipsychotics medication or withdrawal time is less than the required drug cleaning period. The inclusion criteria were drug naïve or drug cleaning period longer than five metabolic half-life periods and physically healthy with all laboratory parameters within normal limits. Major exclusion criteria were: physical complication or other substance abuse; history suggesting resistance to antipsychotic treatment; significant risk of suicidal or violent behavior, clinically significant abnormal vital signs or laboratory values; uncontrolled major medical illnesses, ischemic heart disease, history of myocardial infarction, coronary bypass surgery, and coronary angioplasty. The study protocol was reviewed and proved by the Shanghai Ethical Committee of Human Genetic Resources. Statement of informed consent was obtained from all subjects after full explanation of the procedure.

Variant Selection

We selected potential polymorphisms which may be involved in antipsychotic drug responses and were previously associated with schizophrenia. The SNPs were selected based on the following criteria: (1) SNPs previously reported associated with disease pathogenesis; (2) minor allele frequency of >0.1. In total, 15 variants were selected based on systematical literature and database search (dbSNP, HapMap, 1000 Genome). The genomic information of these 15 selected SNPs is listed in **Table 1**.

Clinical Assessment

For the risperidone, quetiapine, aripiprazole, olanzapine, and perphenazine subjects, the initial dosages were 1, 100–200, 10, 5, and 4 mg/day, respectively. The dosages were gradually increased to 2–6, 400–800, 10–30, 5–20, and 20–60 mg/day within the first week, respectively. The dosages were maintained until the end of week 2. After that, the dosages were adjusted according to individual tolerance. For all the participants, medication compliance was closely monitored and confirmed by the nursing staff, and no other medication was given except trihexylphenidy for extrapyramidal side effects, clonazepam or lorazepam for insomnia, and sennoside for constipation during the 6-week study period.

Clinical effect was assessed on the Positive and Negative Syndrome Scale (PANSS), including the positive, negative and general psychopathology subscales. For all the recruited patients, clinical assessments were conducted on the day of admission, as well as in the treatment process. All PANSS ratings were conducted independently by two qualified psychiatrists who were blind to the genotype of patients. The inter-rater reliability between the two psychiatrists was good. Measurements of psychiatric efficacy, safety, and tolerability were performed at screening, baseline, and the end of weeks 1, 2, 4, and 6, and their PANSS scores were recorded. In this study, 6-week PANSS score was used as the PANSS endpoint score as a measure of response.

TABLE 1 | Genomic information of selected SNPs of *RELN* genotyped in this study.

SNPs	Allele		Frequency[a] (1000 genome)	Position[b]	Information
	Major	Minor			
rs362719	C	A	0.417	chr7:103545430	Intron variant
rs11496125	C	T	0.405	chr7:103777110	Intron variant
rs155333	T	A/C	0.250	chr7:103798667	Intron variant
rs2237628	T	C	0.478	chr7:103581859	Intron variant
rs2535764	C	G/T	0.270	chr7:103552085	Intron variant
rs362626	A	C/T	0.264	chr7:103576772	Intron variant
rs362726	T	C	0.474	chr7:103566787	Intron variant
rs362731	T	C	0.474	chr7:103568581	Intron variant
rs362814	T	A	0.262	chr7:103574673	Intron variant
rs3808035	A	C/T	0.466	chr7:103513015	Intron variant
rs3819479	T	A/C	0.251	chr7:103756635	Intron variant
rs6465938	T	C	0.415	chr7:103851896	Intron variant
rs7341475	G	A	0.149	chr7:103764368	Intron variant
rs12705169	T	C/G	0.178	chr7:103936441	Intron variant
rs362813	T	A/C	0.474	chr7:103574493	Intron variant
rs39339	T	G	0.118	chr7:103819488	Intron variant

[a]*On the basis of 1000 genome.* [b]*On the basis of GRCh38.p12.*

DNA Extraction and Genotyping

Genomic DNA was extracted from leukocytes in venous blood using a QiaAmp® Isolation system (Qiagen, Basel, Switzerland) according to manufacturer's instructions. SNP genotyping was performed using the MassARRAY® SNP IPLEX platform (Agena Bioscience™, San Diego, CA, USA). Detailed information about the primers design and PCR (polymerase chain reaction) conditions is available upon request. Quality control was performed by excluding individual SNPs or samples with genotype call rates less than 95% and SNP assays with poor-quality spectra or cluster plots. Ten percent of samples were randomly tested on the same platform and no inconsistency was found, which ensured the reliability of further data analyses.

Statistical Analyses

As classifications based on PANSS could reduce sensitivity and the power of statistical tests, we used percentage change on PANSS to assess treatment responses to antipsychotic medications. PANSS is an interval scale ranging from 1 to 7 and does not have a zero point. To avoid incorrect calculations, we subtracted the theoretical minimum (30 for the total score) from the baseline score, resulting in a score range including zero. In this study, 6-week PANSS score was used as the PANSS endpoint score.

$$\text{PANNS percentage change} = (\text{PANNS baseline score} - \text{PANNS endpoint score})/(\text{PANNS baseline score} - 30) \times 100$$

The statistical power of this study was calculated online (https://clincalc.com/) using evidence-based clinical decision support tools and calculators for medical professionals. The Student's t-test was carried out using SPSS v20.0 software (IBM, Armonk, NY, USA) to examine clinical variables. In order to control the nongenetic confounding factors, we have investigated the associations of sex, age, baseline PANSS score, drug-exposure status, and duration of the disorder with response to the antipsychotics. We applied univariate general linear model analyses using SPSS v20.0 software to assess the associations. The association of genotype with antipsychotic response was assessed

using general linear model with age as covariate using SPSS v20.0 software. Haploview v4.0 was used to conduct the Hardy–Weinberg equilibrium test and the haplotype analyses. All tests were two-tailed and statistical significance was assumed at $p \leq 0.05$. Multivariate interactions were analyzed on multifactor dimensionality reduction (MDR) software. During the MDR process, the data were randomly divided into 10 equal parts, and the MDR was developed on 9/10 of the data (training set) and then tested on 1/10 of the remaining data (testing set). Statistical significance of testing-balanced accuracy of each selected multifactor model was determined by comparing the average prediction error from the observed data with the distribution of average prediction errors under the null hypothesis of no association derived empirically from 1000 permutations. The null hypothesis was rejected when the upper-tail Monte Carlo P-value derived from the permutation test was ≤0.05. SNPStats (Institut Català d'Oncologia, 2006) (https://www.snpstats.net) was used to evaluate the risk under five inheritance models, namely, codominant, dominant, recessive, overdominant, and additive models. All the statistical tests were two-sided, and $p < 0.05$ was defined as statistically significant.

RESULTS

Patient-Related Demographic Information and Clinical Parameters

Of the 260 subjects that formed the study cohort, the duration of the disorder was between 0–42 years. The average duration of the disorder for male was 7.73, and for female was 10.13. In order to assess whether duration of the disorder would influence patients' response to the antipsychotic drugs, we applied univariate general linear model to assess the association with response to the antipsychotic treatment. No significant difference was noted ($p = 0.610$). Thus, duration of the disorder was not counted as a confounding factor in our study. We also included drug-exposure status as a covariate to investigate the association with response to the antipsychotics. The percentage of patients who were drug naive was 27.78%. No significant difference between association of drug-exposure status with response was noted ($p = 0.586$) in our study. Descriptive statistics for patient-related variables such as age and PANSS scores with regard to response in antipsychotic drugs are summarized in **Table 2**. No significant differences in age and baseline PANSS score between the two groups were noted. However, there was a significant difference in age between these two groups. In order to control this confounding factor, we applied general linear model setting age as a covariate to assess the association of genotype with antipsychotic treatment response (PANSS percentage change) in the subsequent analysis. The Hardy–Weinberg equilibrium test showed no significant deviation in the cohort.

A reduction in total PANSS scores ≥50% were classified as good responders, while others were poor responders. Among the 260 subjects, 40% were good responders (100 patients), and the remaining 60% were poor responders (160 patients). The proportion of good responders for risperidone (150), quetiapine (37), aripiprazole (34), olanzapine (10), and perphenazine (29) were 59.33%, 62.16%, 52.94%, 80%, and 75.86%, respectively.

Statistical Power

Given the parameters from this study, a power calculation indicated that the study was sufficiently powered (96.5%) to detect many of the SNPs with the present sample size. Since it is commonly used for evaluating statistical power of an existing study, post-hoc Power Calculator model was chosen for this study. The incidence of good response in our study was 38.46%. With the study statistical parameters, we had 96.5% power to detect SNPs at a significance level of 0.05.

Effects of *RELN* Gene Polymorphisms on Antipsychotic Treatment Response

General linear model analyses setting age as a covariate were carried out to investigate the association of percentage change on PANSS scores with different genotypes. Genotype frequencies of the 15 SNPs and the associations with antipsychotic treatment response are listed in **Table 3**. The analysis showed that two SNPs had significant associations with reduction in PANSS scores, specifically, rs155333 at a significant level of 0.010 and rs6465938 at a borderline significance of 0.049. However, neither of the associations remained significant after Bonferroni correction (p-value×15>0.05).

Case-control study based on the reduction in the PANSS score was also performed to investigate the association of single polymorphism with antipsychotic drug response. At the end of 6-week treatment, 100 patients showed a reduction of ≥50% in total PANSS scores and they were classified as good responders. The remaining 160 patients were classified as poor responders. However, no significant association of antipsychotic drug response was identified using case-control study.

Association of Haplotypes With Antipsychotic Treatment Response

We performed linkage disequilibrium analysis between each pair of all the 15 SNPs of *RELN* in our cohort and defined the blocks

TABLE 2 | Descriptive statistics for patient-related variables with regard to response.

Response	Sex	p^a	Age	p^b	Baseline PANSS score	p^b
Good responders	Male (54%) Female (46%)	0.405	33.60 ± 11.07	**0.001**	84.65 ± 14.39	0.412
Poor responders	Male (56.87%) Female (43.12%)		39.15 ± 14.06		82.82 ± 17.02	

[a]*Chi-square test, p-value from Fisher's.* [b]*Two-tail t-test. p-values in bold indicate significant for the association.*

TABLE 3 | Association of *RELN* gene polymorphisms with risperidone response.

SNP	Genotype			MAF[a]	MS[b]	F	P[c]
rs362719	AA	CC	CA	A: 0.33	0.081	0.313	0.732
	22 (0.088)	107 (0.428)	121 (0.484)				
rs7341475	GG	AA	GA	A: 0.10	0.006	0.023	0.978
	185 (0.811)	1 (0.005)	42 (0.184)				
rs155333	CC	TT	TC	T: 0.22	1.173	4.754	**0.010**
	135 (0.595)	10 (0.044)	82 (0.361)				
rs39339	TT	GG	GT	G: 0.07	0.046	0.173	0.841
	220 (0.870)	1 (0.004)	32 (0.126)				
rs6465938	CC	TT	CT	T: 0.39	0.774	3.045	**0.049**
	88 (0.367)	33 (0.138)	119 (0.495)				
rs3819479	TT	AA	AT	A: 0.22	0.168	0.639	0.529
	145 (0.599)	8 (0.033)	89 (0.368)				
rs3808035	AA	CC	CA	A: 0.38	0.091	0.365	0.695
	88 (0.368)	29 (0.121)	122 (0.511)				
rs2535764	CC	TT	CT	T: 0.18	0.444	1.731	0.179
	161 (0.685)	11 (0.047)	63 (0.268)				
rs12705169	TT	GG	GT	G: 0.16	0.137	0.519	0.596
	180 (0.711)	10 (0.040)	63 (0.249)				
rs2237628	TT	CC	CT	C: 0.48	0.464	1.847	0.160
	50 (0.208)	58 (0.242)	132 (0.550)				
rs362626	AA	CC	CA	A: 0.34	0.198	0.934	0.394
	28 (0.120)	103 (0.442)	102 (0.438)				
rs362814	TT	AA	AT	T: 0.34	0.007	0.027	0.973
	29 (0.120)	105 (0.436)	107 (0.444)				
rs362813	CC	TT	CT	T: 0.47	0.022	0.102	0.903
	66 (0.263)	50 (0.199)	135 (0.538)				
rs362731	TT	CC	CT	C: 0.49	0.475	2.028	0.134
	52 (0.226)	58 (0.252)	120 (0.522)				
rs362726	TT	CC	TC	C: 0.44	0.038	0.149	0.862
	77 (0.231)	49 (0.204)	114 (0.475)				

[a]*MAF, minor allele frequency.* [b]*MS, mean square.* [c]*Analysis control for age. p-values in bold indicate significant for the association.*

to evaluate haplotype association with the criteria of minor allele frequency >5% and D' >0.75. Two blocks were identified under the definitions (**Figure 1**). High LD was observed between SNPs rs362726-rs362731 and there was a block consisting of three SNPs (rs362814, rs362626, and rs2237628) ranging 7KB in the chromosome. The subjects were classified into two groups according to reductions in the PANSS scores: good responders and poor responders. Furthermore, comparison of overall frequency differences across all possible haplotypes between good and poor responder groups were carried out (**Table 4**). To further assess haplotype association with treatment response, association analysis of the two haplotypes between good and poor responders was also performed. The results revealed significant association of a haplotype consisting of three SNPs rs362814-rs362626-rs2237628 with antipsychotic treatment response (**Table 4**). Even after 1000 times permutations, the *p*-value indicated significant association (rs362814-rs362626-rs2237628:ACT, χ^2 = 6.353, p = 0.0117, permuted p = 0.04. Haplotype ACT (rs362814-rs362626-rs2237628) was more prevalent in poor responders than in good responders.

Multivariate Interaction Analysis of Antipsychotic Drug Response

Multifactor dimensionality reduction (MDR) analyses were used to investigate probable multivariate interactions (including all SNPs, sex, age, weight, *etc.*) associated with antipsychotic drug treatment response. MDR is a model-free and non-parametrical approach method that can identify high dimensional gene-gene or gene-environment interactions in populations. Two-locus interactions through four-locus interactions were analyzed in the present study. Training-balanced accuracy and testing-balanced accuracy were obtained for each selected model. The multifactor model with the best testing-balanced accuracy and cross-validation consistency was selected. The best models are summarized in **Table 5**. However, the permutation testing showed that the two-, three- and four-locus best model was not significant associated with antipsychotic drug response, suggesting that there were no interactions between the 15 SNPS.

Five Genetic Models Analysis

We further investigated the 15 SNPs relations to antipsychotic drug response in five genetic models while controlling for confounding factors. The subjects were classified into two groups according to reductions in the PANSS scores: good responders and poor responders. The adjustment for age and gender factors in the case-control samples was executed using unconditional logistic regression under five inheritance models. Notably, rs2535764 was found to be associated with antipsychotic drug response under overdominant genetic model at a marginal significant level of 0.046 (C/T vs. C/C + T/T: p = 0.046, AIC = 314.7, BIC = 321.6). Genotype C/T were more prevalent in good responders than in poor responders.

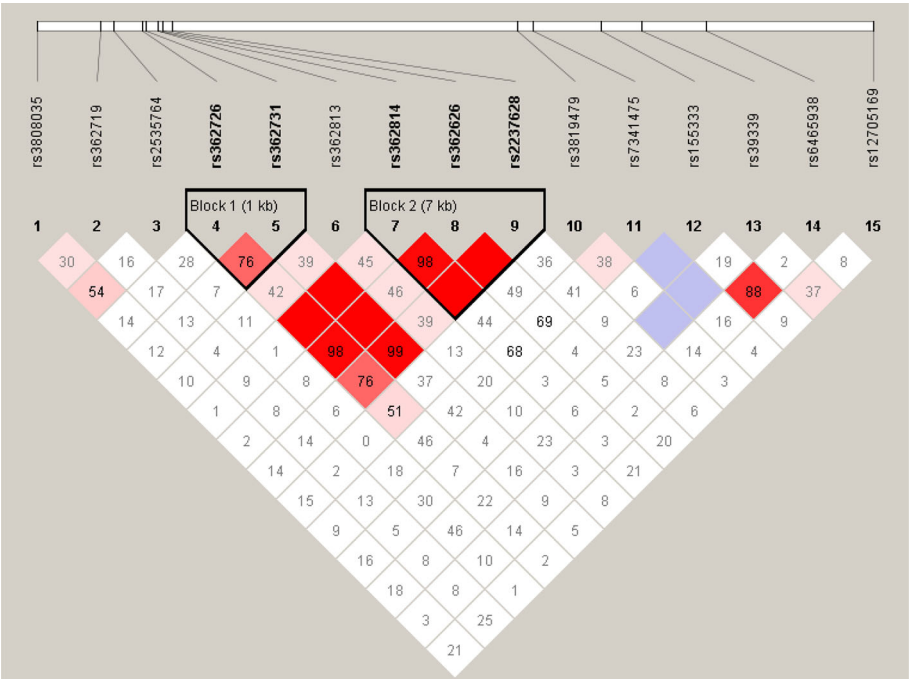

FIGURE 1 | Linkage disequilibrium block structure across RELN gene. The figures show the output of Haploview (version 4.0) LD Plot where each square (with D´ values written within the box) represents a pair-wise LD relationship between the two SNPs. Red squares indicate statistically significant LD between the pair of SNPs as measured by the D' statistic. Darker colors of red indicate higher values of D', up to a maximum of 1. White squares indicate pair wise D' values less than one with no statistically significant evidence of LD.

TABLE 4 | Frequency distribution and association analysis of haplotypes of *RELN* gene with antipsychotics response.

Haplotype			Haplotype frequency	Good responder (%)	Poor responder (%)	χ^2	*p*-value[a]	*p*-value[b]
rs362726	rs362731							
T	T		0.440	0.411	0.457	1.003	0.3167	0.913
C	C		0.394	0.415	0.382	0.541	0.462	0.978
T	C		0.115	0.128	0.108	0.461	0.4973	0.984
C	T		0.050	0.045	0.053	0.132	0.7164	0.999
rs362814	rs362626	rs2237628						
A	C	C	0.519	0.548	0.501	1	0.3173	0.914
T	A	T	0.339	0.357	0.328	0.44	0.5072	0.985
A	C	T	0.134	0.084	0.164	6.353	**0.0117**	**0.04**

[a]*Uncorrected p-value.* [b]*Permuted p-value, number of permutations: 1000. p-values in bold indicate significant for the association.*

TABLE 5 | Results of the MDR analysis of the dataset.

SNPs included in the best candidate model	Training Bal. Acc.	Testing Bal. Acc.	Cross-validation consistency	*p*-value[a]
rs3819479, rs362626	0.6146	0.4594	3/10	0.9880
rs362719, rs3808035, rs362726	0.6806	0.4775	5/10	0.9570
sex,rs362719, rs3808035, rs362726	0.7453	0.3938	3/10	0.9990

Acc, accuracy; Bal, balanced; MDR, multifactor dimensionality reduction. [a]*p-value based on 1000 permutations.*

Genotype C/C and T/T was associated with a poor response risk in the study population (OR = 0.55, P value = 0.046). Even after adjusting for age and sex, the associations remained significant in this model (**Table 6**).

DISCUSSION

RELN plays an important role in the etiology of schizophrenia. Few studies have investigated whether variants of *RELN* affects antipsychotic drug treatment outcomes. In the present study, we

TABLE 6 | Logistic regression analysis of associations between the genotypes of *RELN* rs2535764 with antipsychotic response.

Model	Genotype	Good responder (%)	Poor responder (%)	OR (95% CI)	P-value	AIC	BIC
Codominant	C/C	56 (62.9%)	106 (71.1%)	1.00	0.069	315.3	325.7
	C/T	31 (34.8%)	34 (22.8%)	0.58 (0.32–1.04)			
	T/T	2 (2.2%)	9 (6%)	2.38 (0.50–11.38)			
Dominant	C/C	56 (62.9%)	106 (71.1%)	1.00	0.19	316.9	323.9
	C/T-T/T	33 (37.1%)	43 (28.9%)	0.69 (0.39–1.20)			
Recessive	C/C-C/T	87 (97.8%)	140 (94%)	1.00	0.16	316.6	323.6
	T/T	2 (2.2%)	9 (6%)	2.80 (0.59–13.25)			
Overdominant	C/C-T/T	58 (65.2%)	115 (77.2%)	1.00	**0.046**	314.7	321.6
	C/T	31 (34.8%)	34 (22.8%)	0.55 (0.31–0.99)			
Log-additive	—	—	—	0.87 (0.55–1.38)	0.56	318.3	325.3

AIC, Akaike Information Criterion. BIC, Bayesian information criterion. p-values in bold indicate significant for the association.

focused on this well-known candidate gene to evaluate the role of *RELN* in response to antipsychotic treatment response. Fifteen SNPs were selected to investigate their potential as genetic markers to predict antipsychotic treatment efficacy. The major findings were that two novel SNPs of *RELN* (rs155333 [p = 0.010] and rs6465938 [p = 0.049]) were identified to be associated with antipsychotic treatment response. Our study revealed highly significant association of a haplotype consisting of three SNPs rs362814-rs362626-rs2237628 with antipsychotic treatment response. A novel SNP, rs2535764, was also found to be associated with antipsychotic treatment response under the overdominant genetic model. Our data indicates that *RELN* can affect the outcomes of antipsychotics therapies in the Chinese Han population. Our findings suggest that these SNPs have the potential to be used as antipsychotic treatment markers. Combined with previous studies, our findings may provide useful information for future designs of clinically useful predictive biomarkers of antipsychotic drug response.

Our results support our hypothesis that the risk gene of schizophrenia, *RELN,* can affect antipsychotic treatment outcomes in the Chinese population, suggesting that the susceptibility genes might be potential therapeutic targets. These findings have certain clinical significance. Firstly, our findings may provide novel drug response markers to be used by medical stuff to identify if the patients will have generally satisfactory or unsatisfactory treatment responses. Secondly, our findings may lead to the further study of the mechanism of antipsychotics response affected by *RELN* variants. This may lay the foundation for novel drug response markers discovery.

In the present study, we have identified two novel SNPs rs155333 and rs6465938 that were associated with antipsychotic response. One novel SNP rs2535764 was also found to be associated with antipsychotics response under the overdominant genetic model. To the best of our knowledge, this is the first study to report the associations of three SNPs associated with antipsychotic response. Rs155333 is an intronic locus within *RELN*. Han et al. have reported that rs155333 was significantly associated with cognitive impairment at a level of conventional genome-wide significance ($P_{adjusted} = 1.3 \times 10-^{8}$) (Han et al., 2017). Kahler et al. performed an association study on 839 schizophrenia cases and 1,473 controls of Scandinavian origin. Their study showed that rs155333 of *RELN* attained nominal significant *p*-values ($p < 0.05$) in both genotypic and allelic association test (Kahler et al., 2008). Rs6465938 was also reported to be significantly associated with the risk of schizophrenia in the Scandinavian origin population. The combined rs262355, rs155333, and rs6465938 haplotype was also significantly associated with schizophrenia in that study ($p = 0.031$) (Kahler et al., 2008). These previous findings indicated that rs155333 and rs6465938 genetically contribute to the risk of cognitive function and involve in schizophrenia pathology. Furthermore, our study suggests that rs155333 and rs6465938 could be novel drug targets for schizophrenia.

Previous researches have suggested haplotype-based association methods are more powerful than single locus-based methods (Shifman et al., 2002), therefore, in the present study, haplotype-based association analyses were performed to investigate the effects on antipsychotic treatment response. A single SNP is not sufficient to predict drug response (Drysdale et al., 2000; Luo et al., 2019), however, the interaction of several SNPs in a haplotype can affect the physiological reaction and response to treatment. In this study, the association of *RELN* with antipsychotics response was further supported by the results of haplotype analysis. A haplotype consisting of rs362814-rs362626-rs2237628 in *RELN* showed highly significant association with antipsychotic treatment response (**Table 4**). Even after permutation, the *p*-value indicated significant association ($p = 0.0117$, permuted $p = 0.04$. Haplotype A-C-T was more prevalent in poor responders than in good responders, suggesting that patients with haplotype A-C-T have a high risk of suffering poor antipsychotic treatment response. Several studies have also reported the associations of haplotypes with the risk of schizophrenia (Kahler et al., 2008; Li et al., 2011; Li et al., 2013; Luo et al., 2019). A recent study reported a haplotype consisting of rs362814, rs39339, rs540058, and rs661575 was found to be significantly associated with schizophrenia even after Bonferroni correction (chi(2) = 29.024, $p = 6.42E-04$, p Bonf = 0.017), and the T-C-T-C haplotype was a protective factor for schizophrenia (OR = 0.050, 95% CI = 0.004–0.705) (Luo et al., 2019). Li et al. have identified that the haplotypes incorporating the SNPs (rs2237628, rs362626, rs362814, rs362813, rs362731, and rs362726) (Li et al., 2013) were significantly associated with schizophrenia. Our study is the first one to report that a haplotype of rs362814-rs362626-rs2237628 in *RELN* showed highly significant association with antipsychotic treatment response. In clinical practice, the effect size of one SNP as a

response-related factor might be too small to predict treatment response. Therefore, the significant haplotype in our study may serve as a better marker to guide clinical individual medication in the future than a single SNP marker.

We also conducted a combined analysis between all the selected SNPs using MDR analysis in the present study. However, no model showed significant association with antipsychotic treatment response, suggesting there was no interaction among these SNPs.

In order to fully mine the data and provide systematic and comprehensive analysis, we applied different strategies for data analysis. They have different algorithms and different merit. In total, we applied five different analysis methods, three for single SNP association analysis (general linear model analyses, chi-square tests, and five genetic models analysis), one for haplotype-based association analysis (linkage disequilibrium analysis), and one for multivariate interactions analysis (MDR model). Using general linear model analyses, we can control the non-genetic confounding factors as covariates to investigate the association of percentage change on PANSS scores with different genotypes. This method is more rigorous. Chi-square tests is another different method for single SNP association analysis. It's case-control study strategy. The subjects were classified as two groups. Five genetic models analysis is also a method for single SNP association analysis, but the merit is that the data can be analyzed using unconditional logistic regression under five inheritance models (codominant, dominant, recessive, overdominant, and log-additive). Because haplotype-based association methods are more powerful than single locus-based methods, so we also applied linkage disequilibrium analysis to investigate the haplotype consisting of several SNPs associated with drug response. Multifactor dimensionality reduction (MDR) analysis is a more powerful model to investigate probable multivariate interactions, including all SNPs and the non-genetic clinical factors. It can identify high dimensional gene–gene or gene–environment interactions in population.

This study reported the association of *RELN* with antipsychotic response. Our findings may provide novel drug response markers. The potential of using *RELN* as a novel drug target has been investigated. Several studies have directly administrated RELN protein into the mouse brain to evaluate this possibility and examine the effects. Ishii et al. demonstrated that RELN had a preventive effect on phencyclidine-induced behavioral deficits (Ishii et al., 2015). Another group demonstrated that *in vivo* injection of RELN into the mouse cerebral ventricle affected the synaptic and cognitive functions in wild-type mice and heterozygous reeler mice (Rogers et al., 2011; Rogers et al., 2013). This group further showed that RELN administration ameliorated both the synaptic plasticity and the cognitive behavioral deficits in a mouse model of Angelman syndrome, which is characterized by mental retardation, absence of speech, seizures, and motor dysfunction (Hethorn et al., 2015).

Our study also had some limitations. Although we have considered underlying non-genetic factors such as sex, age, baseline PANSS score, drug-exposure, and duration of illness as covariates, other potential factors, such as treatment history, dosage of different antipsychotics, smoking, baseline weight, and concomitant medication should also be considered in future clinical information collection. Our makers were only identified in the Chinese Han population. It needs validation in other ethnicities and in larger sample sizes.

CONCLUSION

In summary, the aim of this study was to determine whether risk gene of schizophrenia *RELN* affects antipsychotic treatment outcomes in the Chinese Han population. We have identified two novel genetic loci of *RELN* associated with response to antipsychotic treatment in patients with schizophrenia. Future research should extend these findings to larger samples and different populations to confirm their potential use in the development of personalized medicine.

DATA AVAILABILITY STATEMENT

The datasets generated for this study will not be made publicly available: the original human genetic resources including genotype is not permitted to share, it is illegal.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Shanghai Ethical Committee of Human Genetic Resources. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

QX carried out the experiments and wrote this manuscript. ML and YL contributed to the data processing. WY, YF, DZ, DW, SQ, AN and HL contributed to the collection of materials. SY helped to revise the manuscript. QX, SY and WY designed and revised the manuscript.

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No Major Effect of Innate Immune Genetics on Acute Kidney Rejection in the First 2 Weeks Post-Transplantation

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Background: Innate immunity contributes to acute rejection after kidney transplantation. Genetic polymorphisms affecting innate immunity may therefore influence patients' risk of rejection. *IL2* -330T > G, *IL10* -1082G > A, -819C > T, and -592C > A, and *TNF* -308G > A are not associated with acute rejection incidence in Caucasian kidney transplant recipients receiving a calcineurin inhibitor, ciclosporin or tacrolimus (TAC). However, other important innate immune genetic polymorphisms have not yet been extensively studied in recipients and donors. In addition, innate immunogenetics have not been investigated in kidney transplant cohorts receiving only TAC as the calcineurin inhibitor.

Objective: To investigate the effect of recipient and donor *CASP1*, *CRP*, *IL1B*, *IL2*, *IL6*, *IL6R*, *IL10*, *MYD88*, *TGFB*, *TLR2*, *TLR4*, and *TNF* genetics on acute kidney rejection in the first 2 weeks post-transplant in TAC-treated kidney transplant recipients.

Methods: This study included 154 kidney transplant recipients and 81 donors successfully genotyped for 17 polymorphisms in these genes. All recipients were under triple immunosuppressant therapy of TAC, mycophenolate mofetil, and prednisolone. Recipient and donor genotype differences in acute rejection incidence within the first 2 weeks post-transplantation were assessed by logistic regression, adjusting for induction therapy, human leukocyte antigen mismatches, kidney transplant number, living donor, and peak panel-reactive antibody scores.

Results: A trend (Cochran-Armitage P = 0.031) of increasing acute rejection incidence was observed from recipient *IL6* -6331 T/T (18%) to T/C (25%) to C/C (46%) genotype [C/C versus T/T odds ratio (95% confidence interval) = 6.6 (1.7 to 25.8) (point-wise P = 0.017)]. However, no genotype differences were significant after Bonferroni correction for multiple comparisons.

Conclusions: This study did not detect any statistically significant effects of recipient or donor innate immune genetics on acute rejection incidence in the first 2 weeks post-transplantation. However, the sample size was small, and future larger studies or

meta-analyses are required to demonstrate conclusively if innate immune genetics such as *IL6* influence the risk of acute rejection after kidney transplantation.

Keywords: tacrolimus, immune genetics, kidney transplantation, acute rejection, *IL6* -6331

INTRODUCTION

Acute rejection is the major short-term challenge following kidney transplantation and it also increases long-term graft loss (McDonald et al., 2007). Although induction therapy, human leukocyte antigen (HLA) mismatches, number of kidney transplants, living donor, and peak panel-reactive antibodies (PRAs) have been studied as potential acute rejection predictors (Hammond et al., 2010; Lim et al., 2012; Lim et al., 2015; Zhu et al., 2016), these factors only contribute partially to acute rejection incidence.

While the T-cell driven adaptive immune system is essential to acute rejection, the innate immune system also plays a key role. Extracellular damage-associated molecular patterns from transplantation surgery and ischemia/reperfusion injury can induce the translocation of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) into T-cell nuclei *via* activation of the myeloid differentiation primary response 88 (MyD88)-dependent Toll-like receptor (TLR) signaling pathway (Li and Verma, 2002; Liew et al., 2005). Translocated NF-κB activates pro-inflammatory cytokine secretion [e.g. pro-interleukin (IL)-1β, IL-2, and tumor necrosis factor-α (TNF-α)] (Li and Verma, 2002). Caspase 1 (encoded by *CASP1*) is an inflammatory response initiator and converts pro-IL-1β into mature IL-1β (Kostura et al., 1989; Thornberry et al., 1992). These pro-inflammatory mediators can assist T-cell activation, proliferation, and differentiation, and intensify kidney tissue damage (Watson et al., 1980; Nankivell and Alexander, 2010; Anders and Schaefer, 2014). In contrast, anti-inflammatory cytokines (e.g. IL-10) can decrease pro-inflammatory cytokine release (Walsh et al., 2004) and therefore have the potential to attenuate rejection risk, whereas transforming growth factor β (TGF-β) and IL-6 have both pro- and anti-inflammatory action (Saxena et al., 2008; Scheller et al., 2011). Notably, IL-6 trans-signaling *via* soluble IL-6 receptor (IL-6R) is pro-inflammatory as it can enhance the expansion and activation of T- and B-cells and induce several acute phase reactants such as C-reactive protein (CRP) (Wolf et al., 2014).

Single nucleotide polymorphisms (SNPs) in *CASP1*, *CRP*, *IL1B*, *IL2*, *IL6*, *IL6R*, *IL10*, *TGFB*, and *TNF* can increase or decrease the protein production and/or function of these pro- and anti-inflammatory mediators *in vitro* (Kroeger et al., 1997; Turner et al., 1997; Awad et al., 1998; Hoffmann et al., 2001; Dunning et al., 2003; Hall et al., 2004; Trompet et al., 2008; Wang et al., 2009) or serum/plasma concentrations *in vivo* (Fishman et al., 1998; Grainger et al., 1999; Galicia et al., 2004; Smith et al., 2008; Lacruz-Guzmán et al., 2013). In addition, SNPs in the MyD88-dependent TLR signaling pathway affect innate immune responses to vaccines (Ovsyannikova et al., 2011) and susceptibility to infection or disease *in vivo* (Taniguchi et al., 2013; Santos-Martins et al., 2014). Therefore, these innate immunogenetic markers may serve as predictors of acute rejection post-kidney transplantation.

Meta-analyses have shown that recipient and/or donor *IL2* -330T > G (rs2069762), *IL10* -1082G > A (rs1800896), -819C > T (rs1800871), and -592C > A (rs1800872), and *TNF* -308G > A (rs1800629) SNPs do not affect acute rejection incidence in Caucasian kidney transplant recipients receiving immunosuppressive therapy (Hu et al., 2011; Hu et al., 2015; Xiong et al., 2015; Hu et al., 2016). However, none of the cross-sectional studies included in these meta-analyses was carried out in recipients treated with tacrolimus (TAC) as the sole calcineurin inhibitor (CNI). Since TAC has potent immunosuppression 100 times greater than ciclosporin (Kino et al., 1987), with fewer rejection complications (U.S. Multicenter FK506 Liver Study Group, 1994), most kidney transplant recipients in Europe and Australia have been treated with TAC as the first-choice CNI for immunosuppression therapy since 2003 (Wadström et al., 2017) and 2009 (ANZDATARegistry, 2010), respectively. Therefore, it is worthwhile exploring the innate immunogenetic impact on kidney transplant recipients treated with only TAC as the CNI.

Only one study has investigated the impact of *IL1B* 3954C > T (rs1143634) on acute rejection incidence in kidney transplant recipients and found recipient 3954C/T genotype had higher rejection incidence than C/C genotype (point-wise $P = 0.045$) but without multiple comparison adjustment (Manchanda and Mittal, 2008). In terms of *TLR4* 896A > G (rs4986790) and 1196C > T (rs4986791), it is still controversial if these two SNPs affect acute rejection incidence in kidney transplant recipients (Ducloux et al., 2005; Palmer et al., 2006; Nogueira et al., 2007). Limited sample size, low minor allele frequency of the *TLR4* SNPs, different criteria for acute rejection [biopsy-proven acute rejection (BPAR) versus clinical evidence, e.g. serum creatinine change], varied recipient/donor ethnicities, and different time of rejection post-transplantation between cross-sectional studies may contribute together to the inconsistent findings of *TLR4* genetics on acute rejection incidence. In addition, adjustment for multiple statistical comparisons was not conducted. Notably, SNPs in *CASP1*, *CRP*, *IL6R*, *MYD88*, and *TLR2* have not been examined for their impact on acute rejection in kidney transplant recipients.

To bridge these research gaps, this study aimed to explore the impact of recipient and donor *CASP1*, *CRP*, *IL1B*, *IL2*, *IL6*, *IL6R*, *IL10*, *MYD88*, *TGFB*, *TLR2*, *TLR4*, and *TNF* genotypes on BPAR incidence in a cohort of predominantly Caucasian kidney transplant recipients treated with TAC as the only CNI (Hu et al., 2018; Hu et al., 2019a; Hu et al., 2019b). We hypothesized that these recipient and donor innate immunogenetics would affect BPAR incidence in kidney transplant recipients in the first 2 weeks post-transplantation.

MATERIALS AND METHODS

Study Participants and Data Collection

This study was approved by the Central Adelaide Local Health Network Human Research Ethics Committee (Protocol number 2008178). All procedures complied with the Declaration of Helsinki and/or institutional research committee ethical requirements.

As described previously, 165 kidney transplant recipients and 129 donors were recruited (Hu et al., 2018; Hu et al., 2019a; Hu et al., 2019b). All recipients and living donors gave written informed consent before participation. For deceased donors, their respective recipients gave informed consent to use excess donor tissue blood vessels for genotyping. Recipient inclusion and exclusion criteria, demographics, anti-CD-25 induction therapy, immunosuppressant regimen (TAC, mycophenolate mofetil, and prednisolone), the number of HLA mismatches (HLA-A, -B, and -DR antigens) between recipients and donors, number of kidney transplants, donor type (living or deceased), PRA scores (%), and BPAR based on Banff classification of Solez et al., 2008 (as the transplants were performed between 2005 and 2011) have been described previously (Hu et al., 2018; Hu et al., 2019a; Hu et al., 2019b).

Genotyping

Genomic DNA was extracted from blood, buccal swab, and kidney tissue (Hu et al., 2018; Hu et al., 2019a). A panel of 21 SNPs in 15 genes described previously (Mulholland et al., 2014; Barratt et al., 2015; Coller et al., 2015; Somogyi et al., 2016; Coller et al., 2019) were assayed using Agena Bioscience (formerly known as Sequenom) MassARRAY at the Australian Genome Research Facility (Brisbane, Australia). This panel included SNPs in the MyD88-dependent TLR signaling pathway—*TLR2* 1350T > C (rs3804100), *TLR4* 896A > G and 1196C > T, and *MYD88* 1593A > G (rs6853); pro- and anti-inflammatory mediators—*CASP1* 5352G > A (rs580253) and 10643G > C (rs554344), *CRP* -717T > C (rs2794521), *IL1B* -511C > T (rs16944), -31T > C (rs1143627), and -3954C > T, *IL2* -330T > G, *IL6* -6331T > C (rs10499563), *IL6R* -48892A > C (rs8192284), *IL10* -1082G > A and -819C > T, *TGFB* -509C > T (rs1800469), and *TNF* -308G > A. The panel also included *BDNF* 196G > A (rs6265) and *OPRM1* 118A > G (rs1799971) that were considered outside the scope of this study, and *TGFB* -1287G > A (rs11466314) and *LY96* 379C > T (rs11466004) that are known to be of very low frequency in Caucasians; these four SNPs were therefore not included in the analyses described below.

Statistical Analyses

Hardy-Weinberg Equilibrium (HWE) tests for all genotypes, linkage disequilibrium (LD) between SNPs and haplotype inference within genes, and logistic regression analyses, were as described previously (Hu et al., 2018; Hu et al., 2019a). Due to the relatively limited sample size, only SNPs with minor allele frequencies >5% were included in logistic regression analyses. For SNPs in perfect or near-perfect ($r^2 > 0.9$) LD, only 1 of the linked SNPs in that gene, instead of haplotypes/diplotypes, was analyzed in logistic regression analysis.

Genotype differences in BPAR incidence were analyzed for each SNP separately by logistic regression, adjusting for induction therapy [yes/no (Y/N)], living donor (Y/N), HLA mismatches (<3 or ≥3), kidney transplant number (1 or ≥ 2), and peak PRA scores (≤10% or >10%). Statistical significance was assessed by the likelihood-ratio test, and effects described by odds ratios (OR) with 95% confidence intervals (CI). Genotype differences in BPAR without adjusting for non-genetic variables were tested by Cochran-Armitage test for trend in GraphPad Prism v8 (GraphPad Software, San Diego, CA, USA), or Fisher's exact test for SNPs with rare homozygous genotypes (n < 5) combined with heterozygotes, and OR with 95% CI.

P-value thresholds for significance were corrected for multiple testing by Bonferroni-adjustment ($\alpha = 0.05/N$, where N is the number of SNPs analyzed in the recipient or donor cohort, respectively).

RESULTS

One hundred and fifty-four recipients and 81 (57 living, 24 deceased) donors had sufficient DNA for genotyping. In total, 23% (n = 35) of recipients with genotype data developed BPAR in the first 2 weeks post-transplantation. The impact of induction therapy, HLA mismatches, kidney transplant number, living donor, and peak PRA scores on BPAR incidence has been reported (Hu et al., 2019a); none were statistically significant (likelihood-ratio test P-value > 0.1).

Genetic Variability in Kidney Transplant Recipients and Donors

All recipient and donor allele and genotype frequencies are summarized in **Table 1**. Six recipients each received a kidney from three deceased donors (two kidneys per donor), therefore, these three donors were counted only once for HWE tests but were treated independently for logistic regression analyses. For some SNPs, one to four recipients and/or donors had missing genotypes due to genotyping failure. All recipient and donor genotypes were in HWE (P ≥ 0.2). *CASP1*, *IL1B*, *IL10*, and *TLR4* haplotype and diplotype frequencies are summarized in **Supplementary Table 1**. Recipient and donor *CASP1* 10643G and 5352G, *IL1B* -511C and -31T, and *TLR4* 896A and 1196C were in perfect or near-perfect LD (D' > 0.99; $r^2 \geq 0.96$) while *IL10* -1082G and -819C were in complete but not perfect LD [D' = 1.0; $r^2 = 0.30$; resulting in six observed diplotypes (**Supplementary Table 1**)]. Therefore, only 5352G > A in *CASP1*, -511C > T and 3954C > T in *IL1B*, and 896A > G in *TLR4*, along with all SNPs (including *IL10* -1082G > A and -819C > T separately) in other innate immune genes, were included in the subsequent analyses.

Rare homozygous genotypes (n < 5) were combined with heterozygous genotypes for logistic regression and Fisher's exact test as follows: recipient *MYD88* rs6853 A/A genotype versus G allele carriers (A/G + G/G), *TLR4* 896A/A genotype versus G allele carriers (A/G + G/G); donor *IL6* -6331T/T genotype versus C allele carriers (T/C + C/C); recipient and donor *CASP1* 5352G/G genotype versus A allele carriers (G/A + A/A), *TLR2* 1350T/T

TABLE 1 | Recipient and donor genotype and allele frequencies of SNPs in pro- and anti-inflammatory mediators and MyD88-dependent TLR signaling pathway genes.

Genes & SNPs		Recipients[#] (n = 153–154)			Donors* (n = 77–81)		
		Genotypes (n, %)	Alleles (n, %)	HWE P	Genotypes (n, %)	Alleles (n, %)	HWE P
CASP1	5352G > A	G/G (107, 69) G/A (44, 29) A/A (3, 2)	G (258, 84) A (50, 16)	0.8	G/G (58, 72) G/A (21, 26) A/A (2, 2)	G (137, 85) A (25, 15)	1
	10643G > C	G/G (107, 69) G/C (44, 29) C/C (3, 2)	G (258, 84) C (50, 16)	0.8	G/G (58, 72) G/C (21, 26) C/C (2, 2)	G (137, 85) C (25, 15)	1
CRP	-717T > C	T/T (77, 50) T/C (61, 40) C/C (16, 10)	T (215, 70) C (93, 30)	0.4	T/T (33, 41) T/C (37, 46) C/C (10, 13)	T (103, 64) C (57, 36)	1
IL1B	-511C > T	C/C (76, 49) C/T (63, 41) T/T (15, 10)	C (215, 70) T (93, 30)	0.7	C/C (41, 51) C/T (32, 40) T/T (8, 10)	C (114, 70) T (48, 30)	0.8
	-31T > C	T/T (74, 48) T/C (63, 41) C/C (16, 10)	T (211, 69) C (95, 31)	0.7	T/T (41, 51) T/C (32, 40) C/C (8, 10)	T (114, 70) C (48, 30)	0.8
	3954C > T	C/C (84, 55) C/T (61, 40) T/T (9, 6)	C (229, 74) T (79, 26)	0.5	C/C (52, 64) C/T (24, 30) T/T (5, 6)	C (128, 79) T (34, 21)	0.5
IL2	-330T > G	T/T (70, 45) T/G (63, 41) G/G (21, 14)	T (203, 66) G (105, 34)	0.3	T/T (39, 48) T/G (36, 44) G/G (6, 7)	T (114, 70) G (48, 30)	0.6
IL6	-6331T > C	T/T (80, 52) T/C (61, 40) C/C (13, 8)	T (221, 72) C (87, 28)	0.8	T/T (50, 62) T/C (28, 35) C/C (3, 4)	T (128, 79) C (34, 21)	1
IL6R	48892 > C	A/A (50, 33) A/C (78, 51) C/C (25, 16)	A (178, 58) C (128, 42)	0.6	A/A (27, 34) A/C (39, 49) C/C (14, 18)	A (93, 58) C (67, 42)	1
IL10	-1082G > A	G/G (31, 20) G/A (79, 52) A/A (43, 28)	G (141, 46) A (165, 54)	0.6	G/G (16, 20) G/A (36, 44) A/A (29, 36)	G (68, 42) A (94, 58)	0.5
	-819C > T	C/C (88, 58) C/T (54, 35) T/T (11, 7)	C (230, 75) T (76, 25)	0.5	C/C (42, 52) C/T (35, 43) T/T (4, 5)	C (119, 73) T (43, 27)	0.4
MYD88	1593A > G	A/A (123, 80) A/G (29, 19) G/G (2, 1)	A (275, 89) G (33, 11)	0.7	A/A (64, 79) A/G (17, 21) G/G (0, 0)	A (145, 90) G (17, 10)	0.6
TGFB	-509C > T	C/C (81, 53) C/T (60, 39) T/T (13, 8)	C (222, 72) T (86, 28)	0.8	C/C (45, 56) C/T (29, 36) T/T (7, 9)	C (119, 73) T (43, 27)	0.6
TLR2	1350T > C	T/T (133, 86) T/C (19, 12) C/C (2, 1)	T (285, 93) C (23, 7)	0.2	T/T (74, 91) T/C (6, 7) C/C (1, 1)	T (154, 95) C (8, 5)	0.2
TLR4	896A > G	A/A (137, 89) A/G (16, 10) G/G (1, 1)	A (290, 94) G (18, 6)	0.4	A/A (71, 88) A/G (10, 12) G/G (0, 0)	A (152, 94) G (10, 6)	1
	1196C > T	C/C (136, 88) C/T (17, 11) T/T (1, 1)	C (289, 94) T (19, 6)	0.4	C/C (70, 88) C/T (10, 13) T/T (0, 0)	C (150, 94) T (10, 6)	1
TNF	-308G > A	G/G (113, 73) G/A (35, 23) A/A (6, 4)	G (261, 85) A (47, 15)	0.2	G/G (50, 62) G/A (30, 37) A/A (1, 1)	G (130, 80) A (32, 20)	0.2

HWE P, Hardy-Weinberg Equilibrium P-value; n, number; SNP, single nucleotide polymorphism.

Donors: donor numbers may differ from those in **Table 2**, as 3 deceased donors each provided kidneys for 6 different recipients, these 3 donors were not counted twice in HWE; also, donor numbers may differ within **Table 1** due to genotyping failure.*

*Recipients [#]: recipient numbers may differ within **Table 1** due to genotyping failure.*

genotype versus C allele carriers (T/C + C/C), *TNF* -308G/G genotype versus A allele carriers (G/A + A/A).

Consequently, a multiple testing-adjusted P-value threshold for significance was determined at 0.0036 ($\alpha = 0.05/14$).

Innate Immunogenetic Impact on BPAR Incidence

Table 2 summarizes the associations between recipient and donor genotypes and BPAR incidence in the first 2 weeks

post-transplantation, adjusting for induction therapy, HLA mismatches, kidney transplant number, living donor, and peak PRA scores. Although recipients with *IL6* -6331C/C genotype had a higher incidence of BPAR compared to T/T genotype recipients [OR (95% CI) = 6.6 (1.7–25.8), likelihood-ratio test P-value = 0.017], none of the genetic factors (including *IL6* -6331T > C) statistically significantly affected BPAR incidence after correction for multiple comparisons (P-value threshold = 0.0036).

In univariate analysis, there was a trend of increasing BPAR incidence for recipient *IL6* -6331T > C (18% in T/T, 25% in T/C, and 46% in C/C; Cochran-Armitage P = 0.031), although it was non-statistically significant after correcting for multiple comparisons (P-value threshold = 0.0036). Similar trends of increasing BPAR incidence were observed in recipient *CRP* -717T > C (16% in T/T, 30% in T/C, and 31% in C/C; Cochran-Armitage P = 0.048), recipient *CASP1* 5352G > A (18% in G/G, 34% in G/A, and 33% in A/A; Cochran-Armitage P = 0.033) and donor *IL6R* -48892A > C (15% in A/A, 28% in A/C, and 47% in C/C; Cochran-Armitage P = 0.019). Point-wise Cochran-Armitage and Fisher's exact test P-values were > 0.05 for all other recipient and donor SNPs.

Supplementary Table 2 summarizes recipient and donor genotype differences in BPAR incidence in the first 2 weeks post-transplantation for all 21 SNPs included in the genotyping panel.

DISCUSSION

To our knowledge, this is the first innate immunogenetic study retrospectively investigating both recipient and donor genetics of pro- and anti-inflammatory mediators for their impact on BPAR incidence in kidney transplant recipients receiving only TAC as the CNI.

The *IL6* -6331 T/T genotype was associated with up to 6-fold higher plasma IL-6 concentrations than C allele carriers in acute inflammatory-status patients post-coronary artery bypass grafting surgery and in patients requiring intensive periodontal therapy, whereas no significant impact was found in healthy volunteers (Smith et al., 2008). However, the relationship between -6331T > C genotypes and plasma IL-6 concentration has not previously been examined post-kidney transplantation, nor the impact of these genotypes on BPAR incidence in kidney transplant recipients. Our results indicate that recipient C/C genotype is associated with 6.6-fold higher odds of BPAR, and with a genotype trend of increasing BPAR incidence from T/T (18%) to T/C (25%) to C/C (46%). However, probably due to a limited sample size (see **Table 2**), the impact of -6331T > C on BPAR incidence was not statistically significant after adjusting for multiple comparisons. Although a recent liver transplant study also failed to show a significant relationship between -6331T > C and BPAR incidence, its sample size was even smaller (liver transplant recipient and donor n = 29; BPAR n = 8), and there were no recipients with the -6331 C/C genotype (Coller et al., 2019). Therefore, the impact of the *IL6* -6331T > C on inflammation and BPAR incidence is still uncertain, and more studies with larger sample sizes are needed to elucidate if this SNP affects BPAR incidence in kidney transplant recipients.

In terms of the impact of *IL2* -330T > G, *IL10* -1082G > A, and *TNF* -308G > A on BPAR incidence, our results are in accordance with previous meta-analyses (Hu et al., 2011; Hu et al., 2015; Xiong et al., 2015; Hu et al., 2016) indicating these SNPs are not significant determinants of BPAR incidence in Caucasian kidney transplant recipients receiving TAC or ciclosporin. Our study also supports cross-sectional studies in which *IL1B* -511C > T did not affect BPAR incidence in kidney transplant recipients receiving TAC or ciclosporin (Marshall et al., 2000; Marshall et al., 2001; Manchanda and Mittal, 2008; Seyhun et al., 2012; Ding et al., 2016). Some studies reported that *IL1B* 3954C > T and *TLR4* 896A > G and 1196C > T affected BPAR incidence but without multiple comparison adjustment (Ducloux et al., 2005; Palmer et al., 2006; Manchanda and Mittal, 2008). These findings were not reproduced in our cohort and in another kidney transplant study exploring the relationship between *TLR4* genetics and BPAR incidence (Nogueira et al., 2007). We are not aware of any other kidney transplant studies investigating the impact of these three SNPs on BPAR incidence in kidney transplant recipients. Recipient and donor *CASP1*, *CRP*, *IL6R*, *MYD88*, and *TLR2* genetics were expected to be important for any innate immune contribution to BPAR incidence in kidney transplant patients, however, common variants in these genes had no significant impact on BPAR incidence in our study. Overall, these results suggest that the innate immunogenetic SNPs investigated (except for *IL6*-6331T > C) are not likely to contribute greatly to BPAR incidence in the first 2 weeks following transplantation in Caucasian kidney transplant recipients receiving immunosuppressive therapy.

Our study has several limitations to consider when interpreting the results. Firstly, as a retrospective study, the limited sample size (recipient and donor n = 151 and 81, respectively) may have been insufficient to support the findings of no major innate immunogenetic impact on BPAR incidence. However, the data presented in this study, along with other innate immunogenetic studies may together provide valuable information for future meta-analyses investigating the impact of innate immunogenetics on BPAR incidence. Secondly, it was necessary to combine some rare homozygous genotypes for statistical purposes; thus the effect of certain rare homozygous genotypes is unknown. Thirdly, some additional SNPs, e.g. *IL6* -174G > C (rs1800795) and *IL10* -592C > A (Lv et al.,2012; Xiong et al.,2015) were not included in this study because of incompatibility with the genotyping array, and insufficient DNA was available to carry out separate genotyping of these SNPs. In addition, other important innate immune genes, e.g. *NFKB1* (encoding for the NF-κB1 subunit) (Misra et al., 2016), were not included in the gene panel design and are worthwhile exploring in the future for their impact on BPAR incidence.

In conclusion, this study did not detect any statistically significant impact of recipient and donor innate immune genetics on BPAR incidence in the first 2 weeks post-kidney transplantation. However, due to the limited sample size, future immunogenetic studies and/or meta-analyses are still required to demonstrate conclusively if innate immune genetics such

TABLE 2 | Recipient and Donor Innate Immune Genotype Differences in BPAR Incidence in the first 2 Weeks Post-Transplantation, Adjusting for HLA Mismatches, Induction Therapy, Kidney Transplant Number, Living Donor and Peak PRA Scores.

Genes & SNPs		Recipients# (n = 153–154)				Donors* (n = 83–84)			
		Genotypes (n)	BPAR (n, %)	OR [95% CI]	P	Genotypes (n)	BPAR (n, %)	OR [95% CI]	P
CASP1	5352G > A	G/G (107)	19, 18	Ref	0.07	G/G (60)	16, 27	Ref	0.9
		G/A + A/A (47)	16, 34	2.2 [0.9–5.2]		G/A + A/A (24)	7, 29	1.0 [0.3–2.9]	
CRP	-717T > C	T/T (77)	12, 16	Ref	0.05	T/T (34)	6, 18	Ref	0.1
		T/C (61)	18, 30	3.0 [1.2–7.6]		T/C (39)	15, 38	3.1 [1.0–10.5]	
		C/C (16)	5, 31	2.1 [0.5–7.8]		C/C (10)	2, 20	1.3 [0.2–7.5]	
IL1B	-511C > T	C/C (76)	18, 24	Ref	0.9	C/C (41)	13, 32	Ref	0.5
		C/T (63)	13, 21	0.8 [0.3–1.9]		C/T (34)	9, 26	0.7 [0.2–2.2]	
		T/T (15)	4, 27	0.9 [0.2–3.6]		T/T (9)	1, 11	0.3 [0.01–1.9]	
	3954C > T	C/C (84)	16, 19	Ref	0.2	C/C (54)	13, 24	Ref	0.07
		C/T (61)	18, 30	2.0 [0.9–4.6]		C/T (25)	10, 40	2.3 [0.8–6.6]	
		T/T (9)	1, 11	0.6 [0.03–4.1]		T/T (5)	0, 0	NA	
IL2	-330T > G	T/T (70)	12, 17	Ref	0.3	T/T (41)	10, 24	Ref	0.09
		T/G (63)	16, 25	1.5 [0.6–3.6]		T/G (37)	9, 24	1.1 [0.4–3.2]	
		G/G (21)	7, 33	2.4 [0.7–7.2]		G/G (6)	4, 67	8.1 [1.2–78.5]	
IL6	-6331T > C	T/T (80)	14, 18	Ref	0.02	T/T (52)	11, 21	Ref	0.09
		T/C (61)	15, 25	1.6 [0.7–4.0]		T/C + C/C (32)	12, 38	2.4 [0.9–6.9]	
		C/C (13)	6, 46	6.6 [1.7–25.8]					
IL6R	48892A > C	A/A (50)	12, 24	Ref	0.9	A/A (29)	4, 14	Ref	0.09
		A/C (78)	16, 21	0.8 [0.3–2.1]		A/C (39)	11, 28	2.3 [0.6–10.1]	
		C/C (25)	6, 24	0.9 [0.3–3.2]		C/C (15)	7, 47	5.4 [1.2–27.5]	
IL10	-1082G > A	G/G (31)	8, 26	Ref	0.7	G/G (18)	3, 17	Ref	0.4
		G/A (79)	19, 24	1.0 [0.4–2.9]		G/A (37)	11, 30	2.3 [0.6–11.8]	
		A/A (43)	8, 19	0.7 [0.2–2.3]		A/A (29)	9, 31	2.5 [0.6–13.3]	
	-819C > T	C/C (88)	22, 25	Ref	0.4	C/C (44)	9, 20	Ref	0.05
		C/T (54)	10, 19	0.6 [0.2–1.4]		C/T (36)	14, 39	2.7 [1.0–7.9]	
		T/T (11)	3, 27	1.2 [0.2–4.6]		T/T (4)	0, 0	NA	
MYD88	1593A > G	A/A (123)	28, 23	Ref	0.6	A/A (66)	17, 26	Ref	0.5
		A/G + G/G (31)	7, 23	0.7 [0.2–2.0]		A/G (18)	6, 33	1.5 [0.4–4.7]	
TGFB	-509C > T	C/C (81)	18, 22	Ref	0.7	C/C (47)	14, 30	Ref	0.5
		C/T (60)	13, 22	1.0 [0.4–2.3]		C/T (29)	6, 21	0.5 [0.2–1.7]	
		T/T (13)	4, 31	1.7 [0.4–6.1]		T/T (8)	3, 38	1.3 [0.2–6.2]	
TLR2	1350T > C	T/T (133)	33, 25	Ref	0.07	T/T (77)	22, 29	Ref	0.5
		T/C + C/C (21)	2, 10	0.3 [0.04–1.1]		T/C + C/C (7)	1, 14	0.5 [0.02–3.4]	
TLR4	896A > G	A/A (137)	31, 23	Ref	0.7	A/A (74)	20, 27	Ref	0.9
		A/G + G/G (17)	4, 24	1.3 [0.3–4.3]		A/G (10)	3, 30	0.9 [0.2–3.8]	
TNF	-308G > A	G/G (113)	21, 19	Ref	0.04	G/G (53)	13, 25	Ref	0.5
		G/A + A/A (41)	14, 34	2.4 [1.0–5.7]		G/A + A/A (31)	10, 32	1.4 [0.5–3.8]	

BPAR, biopsy-proven acute rejection; HLA, human leukocyte antigens (HLA-A, -B, and -DR) mismatches; n, number; NA, not available; OR, odds ratio; P, likelihood-ratio P-value; peak PRAs, peak panel-reactive antibodies scores assessed by serum lymphocytotoxicity assay; Ref, reference group; SNP, single nucleotide polymorphism; 95% CI, 95% confidence interval. Donors, donor numbers may differ from those in* ***Table 1****, as each of the 3 deceased donors provided kidneys for 6 different recipients, these 3 donors were counted only once for HWE tests but they were treated independently when associated with BPAR for the individual recipients. In addition, donor numbers may differ within* ***Table 2*** *due to genotyping failure. Recipients#, recipient numbers may differ within* ***Table 1*** *due to genotyping failure.*

as *IL6* -6331T > C influence the risk of BPAR incidence post-kidney transplantation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservations, to any qualified researcher.

ETHICS STATEMENT

This study was approved by the Central Adelaide Local Health Network Human Research Ethics Committee (Protocol number 2008178). The patients provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AS, BS, and JC contributed to the conception and design of the study. JC performed the DNA extraction and collation of genotyping results for the panel. RH and BS collected the clinical dataset. RH and DB performed the statistical analyses. RH 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.2019.01686/full#supplementary-material

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A Pharmacogenetically Guided Acenocoumarol Dosing Algorithm for Chilean Patients: A Discovery Cohort Study

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Background: Vitamin K antagonists (VKA) are used as prophylaxis for thromboembolic events in patients with cardiovascular diseases. The most common VKA are warfarin and acenocoumarol. These drugs have a narrow therapeutic margin and high inter-individual response variability due to clinical and pharmacogenetic variables.

Objective: The authors aim to develop an algorithm comprised of clinical and genetic factors to explain the variability in the therapeutic dose of acenocoumarol among Chilean patients

Methodology: DNA was obtained from 304 patients as a discovery cohort with an international normalized ratio (INR) range of 2.0–3.0. The non-genetic (demographic and clinical) variables were also recorded. Genotype analyses were performed using real-time PCR for *VKORC1* (*rs9923231*), *VKORC1* (*rs7294*), *GGCx* (*rs11676382*), *CYP4F2* (*rs2108622*), *ABCB1* (*rs1045642*), *CYP2C9*2* (*rs1799853*), *ApoE* (*rs429358*), and *CYP2C9*3* (*rs1057910*).

Results: The clinical variables that significantly influenced the weekly therapeutic dose of VKA were age, sex, body mass index (BMI), and initial INR, collectively accounting for 19% of the variability, and the genetic variables with a significant impact were *VKORC1* (*rs9923231*), *CYP2C9*2* (*rs1799853*), and *CYP2C9*3* (*rs1057910*), explaining for another 37% of the variability.

Conclusion: We developed an algorithm that explains 49.99% of the variability in therapeutic VKA dosage in the Chilean population studied. Factors that significantly

affected the dosage included *VKORC1*, *CYP2C9*2*, and *CYP2C9*3* polymorphisms, as well as age, sex, BMI, and initial INR.

Keywords: acenocoumarol, coumarins, algorithm, pharmacogenetics, pharmacogenomics, anticoagulation

INTRODUCTION

Cardiovascular diseases (CVD) have been highlighted as a health priority by several institutions worldwide, including the World Health Organization (WHO) through its Global Action Plan for the Prevention and Control of Non-communicable Diseases 2013–2020. Oral anticoagulants are indicated for patients who survive a cardiovascular disease in order to prevent new thromboembolic conditions (WHO, 2013).

Coumarin anticoagulants, also called vitamin K antagonists (VKA), include drugs such as warfarin, acenocoumarol, and phenprocoumon. VKA are highly effective antithrombotic agents used to prevent complications associated with atrial fibrillation, artificial heart valves, and thromboembolic diseases (such as deep venous thrombosis and pulmonary embolism). The most commonly used VKA are warfarin and acenocoumarol (Pengo et al., 2006; Guyatt et al., 2012; Verhoef et al., 2013; Quiñones et al., 2015; Nieto et al., 2019).

To calibrate weekly VKA doses, physicians use prothrombin time [expressed as international normalized ratio (INR) value] empirically to make adjustments at each visit until the patient reaches therapeutic range. Time in therapeutic range (TTR) is then used to assess the quality of anticoagulation. TTR should be above 65% to protect the patient against thrombotic or hemorrhagic risk, according to the National Institute for Health and Care Excellence (NICE) guidelines, or above 70%, according to the European Consensus (NICE, 2014; Esteve-Pastor et al., 2018). Major bleeding is the most concerning adverse effect of anticoagulant therapy, but the risk of bleeding associated with VKA is difficult to estimate. Risk estimates vary according to study design, with an approximate annual incidence of 0.6% for fatal hemorrhage, 3% for major hemorrhage, 9.6% for minor hemorrhage, and 0.2–0.4% for intracranial bleeding depending on the series (Ageno et al., 2012; Fitzmaurice et al., 2016; Haas et al., 2016; Bosch et al., 2017).

Few studies have characterized Chilean patients treated with coumarin derivatives; however, the GARFIELD-AF study, which assessed Chilean patients with atrial fibrillation (AF), reported in 2017 that the median TTR was 40% for 971 patients treated in several public hospitals and private clinics. In that sample, 36 patients (3.6%) had a cerebrovascular accident as an adverse event (Corbalán et al., 2017). According to the same study, the average number of days to reach the desired therapeutic range was 301.6 days, with a median of 204 days, among the patients treated at the Western Metropolitan Health Service (WMHS) in Santiago, Chile. The physicians at the facilities studied relied exclusively on INR values for dose adjustment. Furthermore, the median TTR was only 50% in these AF patients; this low value is worrisome as these patients are at an elevated risk of a new thrombotic pattern or a hemorrhagic complication while out of the therapeutic range (Nieto et al., 2019).

In recent decades, pharmacogenetic research has addressed the relationship between the genetic factors and the required doses of VKA. The most-studied polymorphisms include *CYP2C9*, *VKORC1*, *CYP4F2, GGCx*, and *ABCB1* (Leschziner et al., 2007; Caldwell et al., 2008; De Oliveira Almeida et al., 2011; Johnson et al., 2011; Sun et al., 2015; Shendre et al., 2016). Several algorithms with genetic and non-genetic variables have been developed to calculate VKA dosage, improving the efficacy and safety of the treatment according to TTR results (Wu et al., 2008; Ramos et al., 2012; Borobia et al., 2012; Pirmohamed et al., 2013; Wypasek et al., 2014; Santos et al., 2015; Johnson et al., 2017; Galvez et al., 2018). These algorithms seem to be more precise when developed and applied in specific populations as the frequency of the polymorphisms described depends on ethnicity. Furthermore, consumption of green vegetables, which are rich in vitamin K, also varies by geographical location (Visser et al., 2005; Kocael et al., 2019).

Several algorithms have been published for acenocoumarol in diverse populations. Verde et al. (2010) constructed an "acenocoumarol-dose genotype score" based on the number of alleles associated with a higher required acenocoumarol dosage carried by each patient for each polymorphism. In addition, two algorithms that include demographic, clinical, and genetic factors have been published for Indian populations, with coefficients of determination of 41 and 61.5% (Rathore et al., 2012; Krishna-Kumar et al., 2015).

Two algorithms have been developed for European populations. The first, designed for a mixed European population, includes *CYP2C9* and *VKORC1* polymorphisms and clinical variables (age, sex, weight, height, and amiodarone use). The genetic components in the algorithm explained 52.6% of the dosage variance, and the non-genetic variables explained 23.7% (van Schie et al., 2011). The second algorithm was developed in a cohort of 973 patients undergoing anticoagulation therapy and includes clinical factors [age and body mass index (BMI)] and genetic variants (*VKORC1*, *CYP2C9*, and *CYP4F2* polymorphisms). The genetic and clinical variables explained 50 and 16% of the variance in acenocoumarol dosage, respectively (Cerezo-Manchado et al., 2013).

The aim of this study was to generate a preliminary algorithm with clinical and genetic factors that explains the variability in the therapeutic dose of VKA in Chilean patients. In order to achieve that, we have investigated the association of relevant single nucleotide polymorphisms (SNPs) (**Table 1**) with acenocoumarol dosage.

TABLE 1 | Genetic variants and their potential effect on vitamin K antagonists (VKA) dosage (modified from Visser et al., 2005; Kocael et al., 2019).

Enzyme	Gene	SNP	Change	Effect on VKA dose
MDR1	*ABCB1*	rs1045642	c.3435C > T, exon 26 p.Ile1145Ile silent	Decrease
CYP4F2	*CYP4F2*	rs2108622	c.1297 C > T, exon 11 p.Val433Met missense	Increase
CYP2C9	*CYP2C9*2*	rs1799853	c.3608C > T, exon 3 p.Arg144Cys missense	Decrease
	*CYP2C9*3*	rs1057910	c.42614 A > C, exon 7 p.Ile359Leu missense	Decrease
GGCx	*GGCX*	rs11676382	c.2084+45 C > G Intron 14	Decrease
VKORC1	*VKORC1*	rs9923231	-1639 G > A promotor	Decrease
	VKORC1	rs7294	3730 G > A 3′UTR	Increase
APOE	*ApoE*	rs429358	T > C, exon 4 p.Arg176Cys missense	Decrease

MATERIALS AND METHODS

Study Design

A retrospective cohort study was carried out between March and December of 2018 among patients treated with acenocoumarol (Coarol, Andrómaco, Santiago, Chile) as an antithrombotic therapy at WMHS in the Santiago and Melipilla provinces of Chile. INR measurements were performed in a capillary sample using CoaguChek pro II® equipment (Roche Mannheim, Germany). The sample size was determined according to the frequency of the carriers with the variant allele carriers in the population under study using PS Power and Sample Size Calculations Version 3.0, January 2009, considering 80% power, $\alpha = 0.05$, OR = 2.0, and the less frequent minor allele frequencies (MAF) SNP CYP2C9*3 (rs1057910), according to literature (EMBL-EBI, 2019). The minimum patient number obtained was of 284 patients.

Initial Dosage and Dose Adjustment and Frequency of INR Monitoring

The initial dose was one 4-mg tablet of acenocoumarol on day 1. On day 2, the dose was decreased to 50% (half a tablet). The INR $[(PT^{test}/PT^{normal})^{ISI}]$ (Riley et al., 2000) was controlled on day 3; thus, if the INR was higher than 1.8, the dose was again reduced by 50%, and the patients were checked in 2 days for medical control to adjust the dose according to the INR results. The weekly therapeutic dose of acenocoumarol was modified at each control according to the INR value of the patient. The dosage for patients with INR ≤1.5 was increased by 20%, those of patients with INR >1.5– < 2.0 was increased by 5%, those of patients with INR >3.0–3.5 was decreased by 5%, those of patients with INR >3.5– < 6.0 had their dose discontinued and decreased by 15%, while patients with INR ≥6 had their dose suspended and controlled in 3 days to start again. For INR values within the therapeutic range, the patients were seen in 4 weeks for control (Ansell et al., 2008; Marcatto et al., 2018). Patients having three consecutive INR values in the therapeutic range (2.0–3.0) at the same dose of acenocoumarol were included in this study.

Ethics Statement

The research was authorized by the Ethics Committees of the University of Chile Faculty of Medicine, Project 222-2015, and the WMHS, Protocol No. 027/2016.

Patient Data

Data were obtained from clinical centers and managed with the statistical module of the anticoagulant treatment dosing software TAONet (Roche Diagnostics, Mannheim, Germany).

Genotypic Analysis

Genomic DNA was isolated from the peripheral blood samples of the subjects using the High Pure PCR Template Preparation Kit (catalog number 11796828001; Roche Diagnostics GmbH, Mannheim, Germany). *VKORC1* (*rs9923231*), *VKORC1* (*rs7294*), *GGCx* (*rs11676382*), *CYP4F2* (*rs2108622*), *ApoE* (*rs429358*), *ABCB1* (*rs1045642*), *CYP2C9*2* (*rs1799853*), and *CYP2C9*3* (*rs1057910*) were analyzed using the *TaqMan*® SNP Genotyping Assay (catalog number 4362691, Thermo Fisher Scientific, Waltham, MA, United States) in a Stratagene Mx3000p real-time PCR system. For quality assurance purposes, we randomly choose 20% of the samples for (a) repetition of the analysis and (b) PCR-RFLP analysis for coincidence. When the analyses were not coincident, we excluded the samples.

Statistical Analyses

Data analysis was performed with STATA 15.0® software. The Shapiro–Wilk test was used to determine whether the sample

had a normal distribution for the continuous variable weekly therapeutic dose (WTD; mg/week), that is, the acenocoumarol dosage at which patients were in the therapeutic range. The ladder command from STATA 15.0® was used to choose the best normal distribution expression. Finally, a linear regression analysis was performed among genetic (SNPs) and non-genetic variables with the logarithm of the WTD in the therapeutic range (2.0–3.0), incorporating adjustment variables (p-value > 0.05). The performance of the algorithm was evaluated by calculating the adjusted coefficient of determination (R^2) that represents the variability explained by the model. Hardy–Weinberg equilibrium (HWE) was tested for all SNPs using chi^2 test.

RESULTS

Characteristics of the Study Population

We enrolled 377 patients on oral anticoagulant treatment with acenocoumarol. A total of 72 patients were excluded for not having three consecutive INR values in the therapeutic range (2.0–3.0) at the same dose of acenocoumarol, and one was excluded for concomitant treatment with amiodarone (**Figure 1**). The final sample included 304 patients. As shown in **Table 2**, 47.4% of the patients were women, and 52.6% were men. The average age was 65.01 ± 13.99 years. The Caucasian–Amerindian admixture was 9.8% Amerindian and 90.2% Caucasian for this population (Acuña et al., 2000). No patient had bleeding nor myocardial infarction/stroke during the study.

Genotype Distribution in the Study Population

The analysis of the HWE showed that only CYP2C9*3 (rs1057910) is in HWE (chi^2 = 4.67). All other SNPs, VKORC1 (rs9923231) (chi^2 = 0.09), VKORC1 (rs7294) (chi^2 = 0.62), GGCx (rs11676382) (chi^2 = 1.2), CYP4F2 (rs2108622) (chi^2 = 1.02), ApoE (rs429358) (chi^2 = 0.08), ABCB1 (rs1045642) (chi^2 = 0.68), and CYP2C9*2 (rs1799853) (chi^2 = 2.33), are not in HWE.

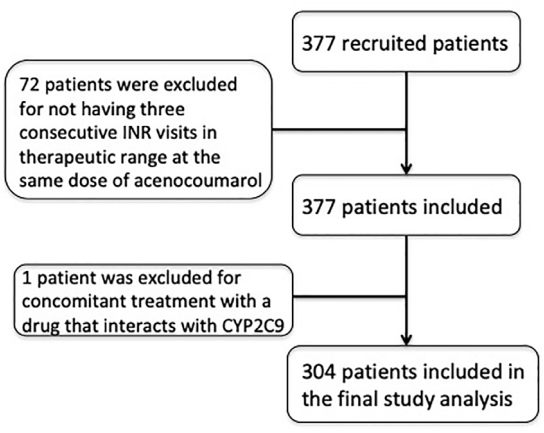

FIGURE 1 | Patient recruitment and selection flowchart.

The MAF were as follows: 0.467 for *VKORC1* (rs9923231), 0.311 for *VKORC1* (rs7294), 0.229 for *CYP4F2* (rs2108622), 0.081 for *CYP2C9*2* (rs1799853), 0.041 for *CYP2C9*3* (rs1057910), 0.036 for *GGCx* (rs11676382), 0.092 for *APOE* (rs429358), and 0.627 for *ABCB1* (rs1045642). In **Table 3**, it is possible to see that the MAF of *VKORC1* (rs9923231) is similar to that of the Colombian population, slightly higher than those of Spain, Puerto Rico, and Europe, higher than the African-American, and lower than East-Asian populations. *VKORC1* (rs7294) MAF is similar to Spain, Puerto Rico, Colombia, and European populations, lower than the African-American, and higher than East Asia. *CYP2C9*2* (rs1799853) MAF is slightly lower than Spain, Puerto Rico, Colombia, and Europe and higher than African-American and East Asians. *CYP2C9*3* (rs1057910) MAF is similar to Puerto Rico and East Asia, lower than Spain, Colombia, and Europe and higher than African-Americans; *CYP4F2* (rs2108622) MAF is similar to Puerto Rico, Colombia, Europe, and East Asia but lower than Spain and higher than African-Americans. *ABCB1* (rs1045642) MAF is higher than all the populations described but near to the European MAF. *GGCx* (rs11676382) MAF is similar to Puerto Rico and Colombia, lower than Spain and Europe, and higher than African-American and East Asian. Finally, *APOE* (rs429358) MAF is similar to Puerto Rico and East Asia and lower than Spain, Colombia, African-American, and European.

Genotype and VKA Dose Ratio in the Study Population

The continuous variable WTD did not have a normal distribution. The logarithm of the WTD was determined to be the optimal transformation for this analysis. The clinical variables that influenced the logarithm of WTD were sex ($p << 0.0001$), age ($p < 0.0001$), BMI ($p < 0.0002$), and INR at the beginning of treatment ($p < 0.0011$). The pharmacogenetic variables, that is, the polymorphisms analyzed which influenced the logarithm of the WTD, were *CYP2C9*2* (rs1799853) ($p < 0.0342$), *CYP2C9*3* (rs1057910) ($p < 0.0001$), and *VKORC1* (rs9923231) ($p < 0.0001$).

Algorithm for Acenocoumarol Dosing in the Chilean Population

After the clinical and pharmacogenetic variables that influenced the logarithm of WTD were selected, a linear regression was performed using only the clinical variables, resulting in a model with an R^2 of 0.2013, adjusted R^2 of 0.19, and model p-value of <0.0001. A linear regression was then performed using only the pharmacogenetic variables *VKORC1* (*rs9923231*), *CYP2C9*2* (*rs1799853*), and *CYP2C9*3* (*rs1057910*), producing a model with a R^2 of 0.3790, adjusted R^2 of 0.3685, and model p-value <0.0001. Finally, a linear regression was performed using the genetic factors *VKORC1* (*rs9923231*), *CYP2C9*2* (*rs1799853*), and *CYP2C9*3* (*rs1057910*) and the clinical variables age, sex, BMI, and initial INR to produce a single model explaining the variability in the logarithm of acenocoumarol WTD, with R^2 of

TABLE 2 | Characteristics of the study patients.

Characteristic	*N* (%)
Female	144 (47.4%)
Male	160 (52.6%)
Total	304 (100%)
% mixed Caucasian–aboriginal ethnicity	9.80%
Age ± SD [range] (years)	65.01 ± 13.99 [22–95]
Body mass index (BMI) (kg/m^2) ± SD (median)	29.2 ± 5.7 (28.4)
INR range	2.0–3.0
Acenocoumarol	100%
Weekly therapeutic dose of acenocoumarol (mg/week) ± SD	14.6 ± 2.2
Acenocoumarol dosage, mg/week; range (median)	3.5–46 (13)
Time to reach therapeutic range (days); average ± SD	308 ± 343
Time to reach therapeutic range (days); range (median)	3–353 (206)
Primary diagnosis	*N* (%)
Rhythm disorder	156 (51.3%)
Venous thrombosis with/without pulmonary thromboembolism	64 (21.1%)
Occlusive arterial disease	24 (7.9%)
Stroke	17 (5.6%)
Others	43 (14.1%)
Total	304 (100%)
Secondary diagnosis	*N* (%)
Arterial hypertension	64 (25.6%)
Diabetes mellitus	27 (10.8%)
Cardiomyopathy	26 (10.4%)
Other	88 (42.9%)
Total	205 (100%)

SD, standard deviation.

0.5147, adjusted R^2 of 0.4999, and model p-value <0.0001 (**Table 4**). Therefore, the algorithm equation developed in this study is the following:

Log WTD = 3.081 + (0.167 × men) - (age × 0.081) - (initial INR × 0.55) + (BMI × 0.013) - (CYP2C9*1/*2 × 0.107) - (CYP2C9*1/*3 × 0.323) - (CYP2C9*3/*3 × 0.746) - (VKORC1 G/A × 0.270) - (VKORC1 A/A × 0.701).

DISCUSSION

The patients enrolled in this study are a representative sample of the Chilean population, which is predominantly Amerindian–Caucasian admixture (9.8% of this sample) (Acuña et al., 2000). The group had an average BMI of 29.2 and median BMI of 28.4, classifying these patients as overweight (BMI 25–29.9). These data are consistent with the results of the most recent National Health Survey 2016–2017 (NHS, 2018), which indicated that 39.8% of the population was overweight (43.3% of males and 36.4% of females) and that among Chilean people in the age range of our study patients (65 years or older), 41.2% were overweight and 34.5% were obese. Notably, the time to reach therapeutic range in these patients was 308 ± 343 days on average, with a median of 206 days (**Table 2**). The patients are at high risk for stroke or hemorrhage while out of the therapeutic range. As relying exclusively on INR for dose adjustment is known to delay the time to reach the therapeutic range, the proposed pharmacogenetic dosage algorithm might be quite useful for the Chilean population.

As noted above, other published algorithms differ in the number of variables included and the weight of these variables, as well as in the type of population and methods used to develop the predictive model. The clinical variables included in these algorithms differ essentially in terms of inclusion of non-genetic variables such as sex, BMI, and use of amiodarone or enzyme-inducer drugs. In terms of the genetic variants, all algorithms published to date have included *CYP2C9* and *VKORC1* polymorphisms, whereas *CYP4F2* and *ApoE* are used only in some models. In addition, several models have been designed exclusively for patients with deep vein thrombosis and/or pulmonary embolism (Borobia et al., 2012), while others have included patient cohorts with phenprocoumon and acenocoumarol treatment. The genetic variables included CYP2C9 and VKORC1 genes and the clinical variables include weight, height, sex, age, and amiodarone use and explained up to 76% of stable dose (van Schie et al., 2011). Another algorithm for acenocoumarol included clinical factors (age, body mass index, and body surface area) and genetic variants (VKORC1, CYP2C9*, and CYP4F2 polymorphisms) and explained up to 50% of stable dose (Cerezo-Manchado et al., 2013).

The original Clinical Pharmacogenetics Implementation Consortium (CPIC) algorithm published in the United States accounted for 47% of warfarin dose variability and included the clinical variables age, amiodarone use, weight, height, use of CYP2C9 inducers, and race/ethnicity as well as the pharmacogenetic factors *VKORC1* (*rs9923231*), *CYP2C9*2* (*rs1799853*), and *CYP2C9*3* (*rs1057910*). CPIC suggests using *CYP2C9*5* (*rs28371686*) if the patient is African-American and added *CYP4F2* as an optional

TABLE 3 | Comparison of minor allele frequencies obtained in this study and those conducted among in Spain, Puerto Rico, Brazil, Colombia, and African American population (http://www.ensembl.org/Homo_sapiens/Info/Index; modified from Visser et al., 2005; Kocael et al., 2019).

Polymorphic variant	Chile (this study)	Spain	Puerto Rico	Colombia	African-American	European	East Asia	Effect on VKA dose
VKORC1 (rs9923231)	0.467	0.360	0.389	0.420	0.054	0.388	0.885	Decrease
VKORC1 (rs7294)	0.311	0.355	0.341	0.356	0.454	0.366	0.112	Increase
*CYP2C9*2 (rs1799853)*	0.081	0.140	0.139	0.122	0.008	0.124	0.001	Decrease
*CYP2C9*3 (rs1057910)*	0.041	0.084	0.043	0.064	0.002	0.073	0.034	Decrease
CYP4F2 (rs2108622)	0.229	0.355	0.288	0.282	0.082	0.290	0.214	Increase
ABCB1 (rs1045642)	0.627	0.463	0.428	0.441	0.150	0.518	0.398	Decrease
GGCx (rs11676382)	0.036	0.093	0.034	0.037	0.002	0.094	0.000	Decrease
APOE (rs429358)	0.092	0.140	0.106	0.154	0.268	0.155	0.086	Decrease

TABLE 4 | Linear regression including genetic factors and clinical variables in a single model.

				N observed	**287**
				Model *p*-value*	**< 0.0001**
				R^2	**0.5147**
				Adjusted R^2	**0.4999**
Variable	**Coefficient**	**Standard error**	***p*-value***	**CI (95%)**	
Sex (men)	0.1668786	0.0407027	0.000	0.0867528	0.2470045
Age	-0.008101	0.001472	0.000	-0.0109987	-0.0052034
Initial INR	-0.0547186	0.0168253	0.001	-0.0878404	-0.0215969
BMI	0.0125554	0.0035861	0.001	0.0054959	0.0196149
CYP2C9*2 (***rs1799853***)					
**1/*2*	-0.1067491	0.0538426	0.048	-0.2127418	-0.0007565
CYP2C9*3 (***rs1057910***)					
**1/*3*	-0.3227895	0.0806461	0.000	-0.4815465	-0.1640324
**3/*3*	-0.7465348	0.2416193	0.002	-1.222178	-0.2708915
VKORC1 (***rs9923231***)					
G/A	-0.2704925	0.0479039	0.000	-0.3647945	-0.1761906
A/A	-0.7008277	0.0583063	0.000	-0.8156074	-0.586048
Constant	3.080551	0.1622701	0.000	2.761112	3.33999

**P < 0.05 is considered as statistically significant.*
BMI, body mass index; INR, international normalized ratio.

factor in the most recent update in 2017 (**Table 4**) (Johnson et al., 2017). Three algorithms have been published for Latin American populations. The algorithm for the population of Puerto Rico (Ramos et al., 2012), which explained 51% of the variability in warfarin dosage, was performed only in men, includes non-genetic variables such as age, initial INR, and use of amiodarone and the genetic variables *VKORC1* (*rs9923231*), *CYP2C9*2* (*rs1799853*), *CYP2C9*3* (*rs1057910*), and *CYP2C9*5* (*rs28371686*). The *CYP2C9*5* (*rs28371686*) polymorphism was included due to the presence of a large African-American component in this population (**Table 5**). The Brazilian algorithm, in turn, accounted for 40% of the variability in warfarin dosage and includes the non-genetic variables age, sex, use of amiodarone or CYP2C9 inducers, and self-declared race, which, according to the Brazilian census criteria, includes white, mixed race, or black. The genetic variables included were *VKORC1* (*rs9923231*), *CYP2C9*2* (*rs1799853*), and *CYP2C9*3* (*rs1057910*) (Botton et al., 2011; Santos et al., 2015), similar to our model. Finally, the Colombian model explained 45.9% of the variability in warfarin dosage. This model included non-genetic variables (age, use of amiodarone, weight, height, use of CYP2C9 inducers, and race/

TABLE 5 | Comparison between the present study and the vitamin K antagonists (VKA) dosage algorithms published for other populations.

Algorithm		**Chile (this study)**	**Spain (Borobia et al., 2012)**	**Germany (van Schie et al, 2011)**	**CPIC (Johnson et al., 2017)**	**Puerto Rico (Ramos et al., 2012)**	**Brazil (Santos et al., 2015)**	**Colombia (Galvez et al., 2018)**
Drug	VKA	Acenocoumarol	Acenocoumarol	Acenocoumarol	Warfarin	Warfarin	Warfarin	Warfarin
Clinical variables	Age	X	X	X	X	X	X	X
	Sex	X		X			X	
	Initial INR	X				X		
	Amiodarone use		X		X	X	X	X
	Weight			X	X		X	X
	Height			X	X			X
	Body mass index	X	X					
	CYP2C9 inducer use		X	X	X		X	X
	Race/ethnicity				X		X	X
	% contribution to the final model	19%	22%	23.7%	N.D	19%	N.D	15.9%
Genetic variables	*VKORC1* (*rs9923231*)	X	X	X	X	X	X	X
	*CYP2C9*2* (*rs1799853*)	X	X	X	X	X	X	X
	*CYP2C9*3* (*rs1057910*)	X	X	X	X	X	X	X
	*CYP2C9*5* (*rs28371686*)				African-Americans	X		
	ApoE (rs429358)		X					
	CYP4F2 (*rs2108622*)		X		Optional			
	% contribution to the final model	36,9%	39%	52.6%	N.D	32%	N.D	30%
Percentage of variability in VKA dosage explained		49.99%	60.6%	76.3%	47%	51%	40%	45.9%

N.D., no data published.

ethnicity), and the genetic variables were the same as those in our model (**Table 4**) (Galvez et al., 2018).

All of the above models were developed for warfarin. In Chile, however, as in Spain, the Ministry of Health indicates that acenocoumarol should be used in preference to any other coumarin. The Spanish model included the non-genetic variables sex, age, BMI, and initial INR value, and the genetic variables were the *VKORC1* (*rs9923231*), *CYP2C9*2* (*rs1799853*), and *CYP2C9*3* (*rs1057910*) polymorphisms. The model explained 60% of the variability in acenocoumarol dosage, similar to our results (49.99%). Our model showed no association between WTD and *CYP4F2* or *ApoE* polymorphisms, which are included in the Spanish study (Borobia et al., 2012). Therefore, the final algorithm for this Amerindian-Caucasian admixture includes the genetic factors *VKORC1* (*rs9923231*), *CYP2C9*2* (*rs1799853*), and *CYP2C9*3* (*rs1057910*) and non-genetic variables age, sex, BMI, and initial INR, explaining almost 50% of the variability in WTD in the Chilean population studied. The algorithm equation developed for the group of Chilean patients explains a similar percentage of dose variability as the Puerto Rican, Brazilian, and Colombian algorithms (**Table 5**).

There are a number of limitations in this study. A number of parameters that affect coumarin dosage were not included, such as smoking status and use of other concomitant medications. These are important factors to keep in mind when establishing a stabilized dosage of acenocoumarol. Moreover, as this is a discovery cohort (also called a derivation or retrospective cohort), the next step is to perform a clinical application of this algorithm in a well-designed prospective validation cohort (also called a test cohort) to obtain sensitivity, specificity, and, of course, predictive values (Tong et al., 2016) before the algorithm is used routinely for acenocoumarol dose adjustment in Chilean patients.

CONCLUSION

Establishing appropriate coumarin dosage is challenging due in part to significant inter-individual variability in the dose required to achieve a stable range of anticoagulation (INR 2.0–3.0). Various genetic and non-genetic factors have been associated with coumarin dosage requirements, and pharmacogenetic-guided dosing algorithms for warfarin and acenocoumarol have been developed for mixed populations with different predictive values. Here, we have developed the first acenocoumarol dosage algorithm for this Chilean mixed Amerindian–Caucasian population, which explains about 50% of dose variability. After clinical validation, this algorithm could provide a new tool for adjusting VKA dosage, considerably improving TTR, and thereby reducing thrombotic and hemorrhagic risks in Chilean patients.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The research was authorized by the Ethics Committees of the University of Chile Faculty of Medicine, Project 222-2015, and the WMHS, Protocol No. 027/2016. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AR participated in the conception of the research, analysis of data, and writing of the manuscript. EN participated in the conception of the research and writing of the manuscript. MS, MR, GV, and FT conducted the experimental analyses. MB, AA, DC, JM, GB, PS, FM, GG, and PV facilitated the enrolment of patients. LQ participated in the conception of the research, the analysis of data, and the writing of the manuscript.

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Usage Pattern of Carbamazepine and Associated Severe Cutaneous Adverse Reactions in Singapore Following Implementation of *HLA-B*15:02* Genotyping as Standard-of-Care

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In April 2013, the Ministry of Health and Health Sciences Authority of Singapore jointly issued recommendations for *HLA-B*15:02* genotyping before starting carbamazepine (CBZ) in new patients of Asian ancestry as standard of care. The Ministry of Health also approved a 75% subsidy for *HLA-B*15:02* genotyping to all patients on subsidy at public healthcare institutions. To understand the impact of these regulatory decisions, we researched the usage patterns for CBZ and levetiracetam, the trend of Stevens–Johnson syndrome/toxic epidermal necrolysis [Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN)] reports associated with antiepileptic drugs and the take-up rates of *HLA-B*15:02* tests in Singapore. In the 5-year post-policy period, we found that the annual number of reported SJS/TEN cases associated with all antiepileptic drugs was significantly decreased by 57% ($p = 0.015$); SJS/TEN cases associated with CBZ and phenytoin reduced by 92% and 42% respectively. New CBZ users decreased by 31% while new levetiracetam users approximately doubled. The annual number of *HLA-B*15:02* tests conducted increased from 444 to approximately 1,200. Regulatory recommendations for *HLA-B*15:02* genotyping as standard of care coupled with government subsidy for the test had contributed to a reduction in CBZ SJS/TEN in Singapore by >90%, in line with that observed in other Asian countries with similar policies. Additionally, the number of phenytoin-SJS/TEN cases also declined. Taken together, this represents a successful example of precision medicine through implementation of a genotyping program to reduce a rare but serious adverse drug reaction among at-risk individuals, while preserving the availability of an effective and low-cost medicine for the broader population.

Keywords: *HLA-B*15:02*, carbamazepine, levetiracetam, Steven–Johnson syndrome, toxic epidermal necrolysis (Stevens–Johnson syndrome/toxic epidermal necrolysis), serious cutaneous skin reactions

INTRODUCTION

Carbamazepine (CBZ) is indicated in Singapore for the treatment of epilepsy and other conditions such as diabetic neuropathy, trigeminal neuralgia and bipolar disorders. While CBZ is an effective drug and the drug of choice for several conditions, Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) have been reported with its use. These serious adverse reactions are associated with significant mortality and long-term morbidity. (Pirmohamed et al., 2011).

Published studies had documented a strong association between the carriage of *HLA-B*15:02* allele and risk of CBZ-induced SJS/TEN among Han Chinese in Taiwan, Hong Kong, Malays, Indians, and Thais (Chung et al., 2004; Man et al., 2007; Locharernkul et al., 2008; Mehta et al., 2009; Chang et al., 2011). A large prospective study in Taiwan also found *HLA-B*15:02* screening prior to the initiation of CBZ therapy to be successful in preventing CBZ-induced SJS/TEN (Chen et al., 2011). Among Han Chinese in Taiwan, *HLA-B*15:02* was not associated with CBZ-related drug reaction with eosinophilia and systemic symptoms (DRESS) or maculo-papular erythema (Hung et al., 2006) which are other important phenotypes of severe cutaneous reactions. *HLA-B*15:02* also was observed to confer risk to phenytoin SJS–TEN in Han Chinese in Hong Kong and Taiwan, although the association was not as strong as with CBZ (Man et al., 2007; Hung et al., 2010).

Singapore is an island city-state in Southeast Asia with a population of 5.7 million. The three major ethnic groups among the 4.0 million residents are Chinese (74.4%), Malays (13.4%), and Indians (9.0%). The Health Sciences Authority (HSA), in its role as a national pharmacovigilance center, receives spontaneous reports of adverse drug reactions (ADR) related to marketed health products, with the vast majority (94.2%) of cases reported directly by healthcare professionals at public hospitals and primary care clinics. (HSA, 2019). As dermatological reactions comprised the largest category of ADRs received by HSA and local data were deemed necessary to assess the applicability of the *HLA-B*1502* association to CBZ-induced SJS/TEN in Singapore, HSA embarked on a program in 2009 to develop infrastructure for collection, storage, and analysis of DNA samples from patients who had experienced serious skin rash, and to capture phenotypic data associated with those samples. For the CBZ-induced SJS/TEN cases collected, all 13 were *HLA-B*15:02* positive, as were 3 of the 26 drug-tolerant controls. Hence, the odds ratio (OR) for *HLA-B*15:02* association with CBZ SJS–TEN was 181 (95% confidence interval: 8.7–3785, $p = 6.9 \times 10^{-8}$), validating a significant association for *HLA-B*15:02* in Singapore Chinese and Malays, as has been observed in a number of other Southeast Asian countries. (Toh et al., 2014).

On 30 April 2013, the Singapore Ministry of Health and HSA issued a joint "Dear Healthcare Professional Letter (DHCPL)" advising that genotyping for the *HLA-B*15:02* allele before the initiation of CBZ therapy in new patients of Asian ancestry would be standard of care. (HSA, 2013b) The letter further elaborated that "CBZ should not be prescribed prior to the return of *HLA-B*15:02* test results" due to the possible development and progression of SJS/TEN in susceptible patients even after prompt discontinuation of the drug. It was advised that patients who were found to be positive for *HLA-B*15:02* should not be prescribed CBZ or phenytoin, and treatment alternatives were recommended. Genotyping is not required for patients who have been taking CBZ for three months or longer with no adverse reactions. The Ministry of Health also approved a 75% subsidy for *HLA-B*15:02* genotyping to all patients on subsidy at public clinics and hospitals. A few months later, the 2013 CPIC guideline was published advising that CBZ should not be used when it is known that a patient is positive for *HLA-B*15:02* (Leckband et al., 2013).

To understand the impact of these regulatory decisions, we conducted this research on the trend of SJS/TEN reports associated with CBZ and other anti-epileptic drugs (AEDs), usage patterns for CBZ, in comparison with levetiracetam (LEV), an alternative AED that is commonly used in Singapore as well as the take-up rates of the *HLA-B*15:02* tests in Singapore.

METHODS

Retrieval of Local SJS and TEN Reports Associated With AEDs

Cases of SJS and TEN associated with the use of CBZ and other AEDs that had reported onset dates between May 2008 and April 2018 were retrieved from HSA's ADR report database and included in the analysis. The AEDs included in this analysis were CBZ, clobazam, gabapentin, lamotrigine, LEV, phenobarbitone, phenytoin, topiramate, and valproate.

Local Exposure and New Users for CBZ and LEV

Based on consultations with practicing neurologists in Singapore, LEV is the preferred alternative AED. National sales data of CBZ and LEV were used as a proxy for usage of these drugs. Unit sales of all formulations of these products were retrieved from the IQVIA database Singapore National Sales Audit, 2013–2017. The total number of daily defined doses (DDDs) sold annually were calculated, using a DDD of 1.0 g for CBZ and 1.5 g for LEV (WHO, 2019).

The number of new CBZ and LEV users from 2012 to 2017 were tabulated from the Singapore Ministry of Health's prescription database of de-identified prescription orders. Orders for CBZ and LEV issued from 2011 to 2017 were extracted and sorted by order date and pseudo-id. The data were further filtered to retain only the first prescription order tagged to each unique pseudo-id. Thereafter, the number of unique pseudo-ids were sorted by year to tabulate the annual number of new CBZ and LEV users. In order to account for new users who had not been prescribed the drug for at least one year preceding the first order, only data from 2012 onward were used.

*HLA-B*15:02* Genotyping Test

The *HLA-B*15:02* genotyping test is offered at four laboratories in Singapore: three public hospital laboratories, namely the

National University Hospital Molecular Diagnosis Centre, the Tan Tock Seng Hospital Molecular Diagnostic Laboratory, the DNA Diagnostic & Research Laboratory at Kandang Kerbau Women's and Children's Hospital, and the Tissue Typing Laboratory at Health Sciences Authority. The number of *HLA-B*15:02* genotyping tests performed by these laboratories were provided to HSA as part of post-recommendation surveillance. Genotyping was performed by the laboratories using clinically-validated assays developed in-house or using commercially available kits.

Data Analysis

Descriptive statistics were employed to summarize the data collected, while the Mann–Whitney test was employed to evaluate the differences in the annual number of AED-associated SJS/TEN cases received in the pre- and post-policy periods.

Ethics Statement

This study was granted approval of exemption by the National Healthcare Group's Domain Specific Review Board which determined that the study qualified for exemption as the research involved analysis of datasets without identifiers.

RESULTS

Trends in the Reported SJS–TEN Cases Associated With AEDs

The annual number of reported SJS/TEN cases associated with AEDs was significantly decreased by 57% from a 5-year pre-policy period (May 2008 to Apr 2013; median 16, range 11–24) as compared to that in the 5-year post-policy period (May 2013 to Apr 2018; median 7, range 5–11; p = 0.015; **Figure 1A**). In addition, the number of reported cases of SJS/TEN associated with CBZ use decreased sharply by 92% from 50 cases in the pre-policy period to 4 cases in the post-policy period (**Figure 1B**). Genotyping status was reported in 2 cases, of which one was negative for *HLA-B*15:02*. Moreover, the number of phenytoin-SJS/TEN cases also reduced by 42% from 24 cases to 14 cases in the same time-periods (**Figure 1B**). The numbers of SJS/TEN reports associated with the other AEDs were either stable or slightly increased/decreased, but the numbers were too low for meaningful interpretation.

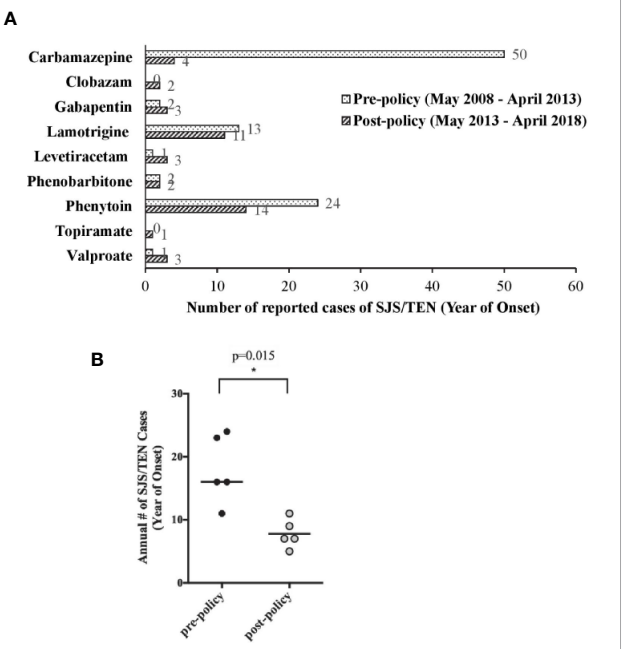

FIGURE 1 | Local spontaneous Stevens–Johnson syndome (SJS)/toxic epidermal necrolysis (TEN) reports associated with anti-epileptic drug (AED) use. **(A)** Comparison of SJS/TEN reports associated with the use of individual AEDs in the pre- and post-policy periods. **(B)** Comparison of annual number of AED-associated SJS/TEN reports in the 5 pre-policy years (May 2008 to April 2013) and 5 post-policy years (May 2013 to April 2018). Horizontal line is the median value, *p = 0.015 by Mann-Whitney test.

Trends in Usage of CBZ and LEV

Between 2013 and 2017, the annual usage of CBZ had decreased slightly by 16% from 1.19 million DDD to 1.00 million DDD while that of LEV increased by 182%, from 0.64 million to 1.16 million DDD (**Figure 2A**).

From 2013 to 2017, the number of new CBZ users in public sector healthcare institutions decreased by 31% from 715 to 495 patients while the number of new LEV users approximately doubled from 1,481 to 3,085 patients (**Figure 2B**).

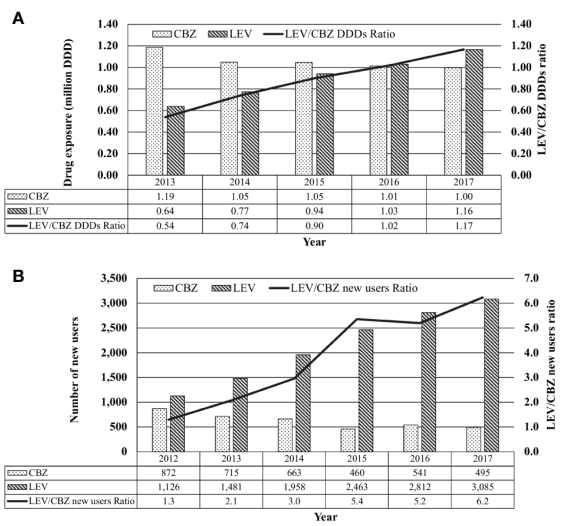

FIGURE 2 | Local usage of carbamazepine (CBZ) and levetiracetam (LEV). **(A)** Total use of CBZ, LEV or CBZ/LEV ratio from 2013 to 2017 based on daily defined dose (DDD), tabulated from IQVIA database Singapore National Sales Audit, 2013–2017. **(B)** New CBZ and LEV users and CBZ/LEV ratio form 2012 to 2017, tabulated from the Singapore Ministry of Health's prescription database.

Trends in *HLA-B*15:02* Screening Rates

From May 2013 to December 2017, a total of 4,595 samples had been sent for *HLA-B*15:02* screening at the four laboratories.

The number of samples sent per year increased steadily from 444 samples in 2013 to approximately 1,000 cases in 2015, and tapered toward almost 1,200 samples per year in 2016 and 2017. Of all the samples tested for the allele, 11.2% (n = 514) carried the *HLA-B*15:02* allele (**Figure 3A**). With the rising number in tests, the number of CBZ-SJS/TEN cases dropped sharply, with only a modest decrease in total sales and new prescriptions of CBZ (**Figure 3B**).

DISCUSSION

Impact on SJS/TEN Reports Associated With AEDs

Overall, we observed a significant reduction (57%) in the median annual number of AED-associated SJS/TEN cases received in the post-policy period. This reduction was mainly driven by 92% and 42% reduction in the total number of CBZ- and phenytoin-associated SJS/TEN cases, respectively, during the post-policy period as compared to the pre-policy period. Comparatively, the number of CBZ-associated SJS/TEN cases decreased remarkably by 87.1% in Taiwan (Lin et al., 2018). In Hong Kong, after HLA-B*1502 screening was implemented, the incidence of CBZ-induced SJS/TEN was reduced to zero. However, there was a reciprocal increase in phenytoin-associated SJS/TEN cases in Hong Kong, resulting in non-statistically significant reduction in the overall incidence of AED-associated SJS/TEN after policy implementation (Chen et al., 2014). The authors observed a slight but statistically significant increase (8%) in phenytoin prescription in the post-policy period, and speculated that the policy led to channeling of high-risk patients from CBZ to phenytoin. The changes in the number of SJS/TEN cases associated with AEDs other than CBZ in Taiwan were not discussed in the study by Lin et al. While *HLA-B*15:02* is associated with an increased risk of phenytoin–SJS–TEN in Han Chinese, a recent study reports that it is not a risk allele in a Thai population. (Sukasem et al., 2018).

In the Singapore DHCPL issued in April 2013, healthcare professionals were also informed of the suspected association between *HLA-B*15:02* and phenytoin-induced SJS/TEN, and advised to consider prescribing drugs other than CBZ and phenytoin for patients tested positive for *HLA-B*15:02* allele. This may explain why phenytoin-associated SJS/TEN cases also declined in the post-policy period, unlike the situation in Hong Kong. In addition, as part of our continual effort to maintain healthcare professionals' awareness of the recommendations for *HLA-B*15:02* genotyping and early signs of SJS/TEN, we published several articles in the HSA ADR News Bulletin on *HLA-B*15:02* genotyping as well as a guide on severe cutaneous adverse reactions and implicated drugs in end–2013 and 2016 (HSA, 2013a; HSA, 2013b; HSA, 2013c; HSA, 2016a; HSA, 2016b). These may have been helpful in reinforcing messaging about genotyping tests and importance of prompt withdrawal of drugs implicated in severe cutaneous skin reactions.

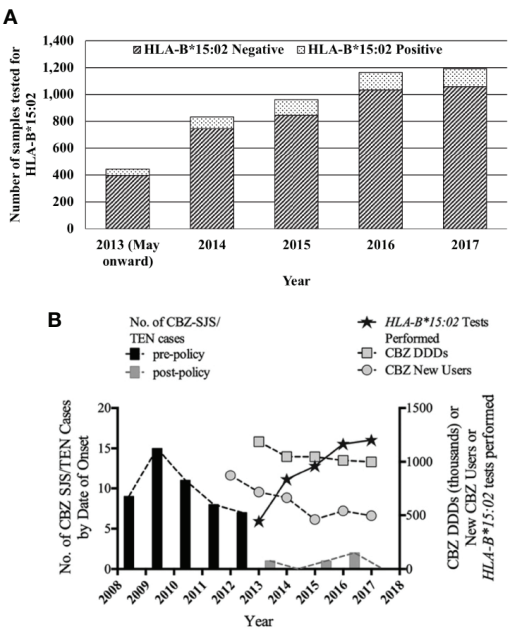

FIGURE 3 | (A) *HLA-B*15:02* genotyping tests in Singapore in the post-policy period. **(B)** Comparison of CBZ-SJS/TEN cases and trends in genotyping tests. CBZ daily defined doses from national sales data and new users of CBZ from the MOH prescription order database.

Impact on Local Usage of CBZ and LEV

Consistent with the findings of Chen et al. and Lin et al., we observed a dramatic decline in the number of new CBZ users during the post-policy period. Nonetheless, when extrapolated to the total usage of CBZ, the decline in new CBZ users was observed to have had minimal impact on the overall usage of CBZ. This could be attributed to continual usage by existing CBZ users who are not affected by the genotyping recommendations and HLA-B*1502 negative patients who are able to use CBZ with very low risk of SJS/TEN. In addition, Chen et al. observed an increase in prescriptions of other AEDs in patients prescribed first-ever AED, with a 3.2-fold increase in the prescription of LEV which was the highest among all the studied AEDs. Likewise, we observed an increasing trend in the number of new LEV users (LEV/CBZ new users ratios of up to 6.2; **Figure 1B**) as well as the overall usage of LEV (LEV/CBZ DDDs ratios of up to 1.2; **Figure 1A**). Notably, the number of new LEV users had begun to increase from 2012 to 2013, prior to the issuance of the local recommendations for pre-treatment *HLA-B*15:02* genotyping. LEV is indicated in Singapore as monotherapy and adjunct therapy for the treatment of epilepsy. From our consultation with neurologists, we gathered that LEV had been a favored alternative for epilepsy patients and that the usage of LEV had increased in the recent years. Apart from the availability of generic formulations in recent years, the favorable side effect profile, and ease of use have been cited as reasons for the higher take-up rate for LEV. Moreover, the ability to initiate treatment

promptly without the need to wait for pre-treatment genotyping results, had also been seen as a factor favoring its use over CBZ.

Impact on *HLA-B*15:02* Testing

On average, more than 900 tests were ordered per year since 2013. 11.2% of the tests were positive. The proportion of positive tests was in line with the local population *HLA-B*15:02* frequency of 11%–18.74% (Dong et al., 2012).

While 4,081 samples were tested negative for *HLA-B*15:02* from 2013 to 2017, there were only 2,874 new users of CBZ in the same time period. There could be patients who were not started on CBZ, despite not being found to carry the *HLA-B*15:02* allele. Chen et al. and Lin et al. reported that up to 47.2% of patients tested did not have any AED commenced after the test results became available. Another possible reason could be the healthcare professionals' decision to start on other AEDs, instead of CBZ, and continue on the same therapy even after the test results became available, partly due to the inconvenience of added waiting time for the test results. Also, it should be noted that this study was not designed to assess the adherence to *HLA-B*15:02* genotyping prior to treatment with CBZ. In Hong Kong and Taiwan, the adherence to *HLA-B*15:02* genotyping prior to CBZ therapy was observed in only up to 26.4% of the patients. Further studies are required to assess this issue in the local context.

Other Considerations

One limitation of our evaluation was the use of different databases for drug sales, drug prescriptions, and genotyping test orders, making it infeasible to trace the intention for genotyping, i.e. whether genotyped patients were those who had intended CBZ use, and the direct impact of these test results on the decision to use CBZ. First-time user data was based on public-sector healthcare system only, and may not be representative of the entire national usage. However, epilepsy is usually treated at specialist and tertiary centers, and approximately 70 to 80% of the overall healthcare demands in Singapore are addressed by the public sector. (Seng et al., 2019). Spontaneous adverse event reporting to HSA is associated with an unknown and variable degree of under-reporting, which is a limitation of our interpretation of SJS/TEN cases reported to this system. In spite of the above, the trend of pre- and post-policy CBZ use in new patients and the reduction in the number of CBZ-associated SJS/TEN cases post-policy were comparable to those observed in other countries that had implemented genetic screening policies. Apart from the number of new users of CBZ and LEV, we were not able to elucidate further information on the characteristics of the new users, such as the patient demographics, the prescribers' medical specialties and the indications for which the medicines were prescribed.

When new clinical care guidelines are published, it often can be difficult to predict the consequences, intended and unintended. When the joint MOH/HSA policy on *HLA-B*15:02* genotyping was issued, one concern was that implementation of *HLA-B*15:02* genotyping prior to new CBZ use may result in physicians avoiding the use of CBZ, which was considered an effective and low-cost drug of choice for several conditions based on consultations with clinicians. Another concern was that the delay through waiting for the test result would encumber clinical practice and drive physicians to prescribe alternative AEDs. Hence, as a follow-up to issuance of the DHCPL, we have been tracking *HLA-B*15:02* test orders and medication usage in addition to the usual pharmacovigilance role of monitoring the number of CBZ-associated SJS/TEN cases. This paper presents the results of that follow-up, namely (1) there has been a >90% decrease in the number of SJS/TEN cases associated with CBZ, (2) *HLA-B*15:02* genotyping test orders steadily increased for the first three years and now appears to have reached a steady-state, (3) CBZ continues to be used in clinical practice, albeit at a slightly lower rate, (4) first-time use of CBZ has declined by less than half, and (5) LEV, another AED, has gained in popularity, especially among new users.

CONCLUSION

The regulatory recommendations for genotyping for *HLA-B*15:02* as "standard-of-care" coupled with government subsidy of 75% for the test has contributed to a reduction in the number of CBZ- and phenytoin-associated SJS/TEN cases in Singapore. CBZ continues to be used in clinical practice though for new AED users, drug utilization of CBZ has decreased while that for LEV has increased. Taken together, this represents a successful example of precision medicine through implementation of a genotyping program to reduce a rare but serious ADR among at-risk individuals, while preserving the availability of an effective and low-cost medicine for the broader population.

DATA AVAILABILITY STATEMENT

The datasets generated for Figures and statistical analyses are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

CS, DT, and CLC conceived the idea for the research study. CS analyzed the national sales data. ML conducted the data analysis for local ADR reports and *HLA-B*15:02* genotyping tests performed. CS and GG retrieved and analyzed data from the Ministry of Health. LT, ML, and CS interpreted the analysis and wrote the manuscript. All authors read and approved the final manuscript.

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Influence of Genetic Admixture Components on *CYP3A5*3* Allele-Associated Hypertension in Amerindian Populations From Northwest Mexico

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CYP3A5 metabolizes endogenous substrates and ~30% of prescription drugs. The *CYP3A5* gene contains an active *CYP3A5*1* allele, and a non-functional version, the *CYP3A5*3* (rs776746), with consequences for drug therapeutic responses and side effects. Both *CYP3A5*1* and **3* have been associated with hypertension. The frequency of *CYP3A5*3* varies between populations of different ancestries, with Europeans having the highest allele frequency (> 90%). Given the importance of *CYP3A5*3* in drug response and hypertension development, the aim of the present study was to evaluate the frequency of this polymorphism and its association with hypertension in vulnerable indigenous populations in Mexico. A total of 372 subjects were recruited from eight ethnic groups in Northwest Mexico. Systolic (SBP), diastolic (DBP), and median (MBP) blood pressures as well as body mass index (BMI) were measured. Ancestry was evaluated through STR analysis, and the *CYP3A5*1/*3* polymorphisms were identified using real-time PCR with TaqMan® probes. Higher frequencies of *CYP3A5*1* and **3* were observed in groups with higher (>90%) and lower (<90%) Amerindian ancestry, respectively. The *CYP3A5*3/*3* genotype was more frequent in indigenous women with higher SBP and DBP values. On the other hand, the **1* allele showed a protective effect against both high SBP (OR, 0.38; 95% CI, 0.17–0.83, p = 0.001) and DBP (OR 0.38, 95% CI 0.18–0.81, p = 0.007) in women. This association remained significant after adjusting for BMI and age for diastolic (OR, 0.38; 95% CI, 0.17–0.84, p = 0.011) and systolic BP (OR, 0.33; 95% CI, 0.15–0.76, p = 0.005) BP levels in women. Thus, the frequency of *CYP3A5*3* varies between groups and seems to depend on ancestry, and *CYP3A5*1* decreases the risk of hypertension in Mexican indigenous women. This population analysis of *CYP3A5*1/*3* has profound implications not only for the susceptibility to diseases, such as hypertension, but also for

safer drug administration regimens, assuring better therapeutic responses and fewer side effects.

Keywords: CYP3A5, polymorphisms, Amerindian, Mexican, hypertension, ancestry

INTRODUCTION

The CYP3A5 enzyme is a member of the cytochrome P450 (CYP) 3A subfamily, which metabolizes ~30% of the drugs used in clinical practice (Zanger and Schwab, 2013). CYP3A5 protein is expressed mainly in the liver (Zhang et al., 2016) with an extrahepatic expression predominantly at the level of the renal proximal tubule (Givens et al., 2003; Bolbrinker et al., 2012). In kidney cells, CYP3A5 catalyzes the 6b-hydroxylation of corticosterone and cortisol (Grogan et al., 1990; Schuetz et al., 1992), increasing renal retention of Na^+ and influencing blood pressure (Watlington et al., 1992; Ghosh et al., 1995). The renal expression of CYP3A5 is variable (Haehner et al., 1996) and depends mainly on a non-functional polymorphism in intron 3 called *CYP3A5*3* (6986A > G, rs776746), which causes RNA splicing, resulting in protein termination at amino acid 109 (Kuehl et al., 2001). The frequency of *CYP3A5*3* varies considerably across populations, with the highest frequencies in Europeans (94%) and admixed Americans (80%), and the lowest in Africans (18%) (Zhou et al., 2017). Thompson et al. (2004) reported that the frequency of *CYP3A5*3* shows an unusual geographic distribution and increases significantly with distance from the equator. This could be because the functional reference allele *CYP3A5*1* may confer a selective advantage in dry weather by increasing Na^+ and water retention (Kuehl et al., 2001).

In several studies, CYP3A5 has been associated with hypertension in humans, although the results have been controversial, as evidenced by the review described by Bochud et al. (2009) and other recent studies (Fisher et al., 2016; Li et al., 2017). Ethnicity of study subjects may help explain these inconsistencies, as it has been demonstrated that the association of the *CYP3A5*1* allele with higher blood pressure occurs mainly in individuals of African descent, while in Caucasians it has only been observed in older individuals.

In Mexico, seven million inhabitants speak an indigenous language. In the northwest of the country, the main ethnic groups are distributed in two well-defined geographical regions. Five indigenous groups, the Coras, Huicholes, Tepehuanos, Tarahumaras, and Mexicaneros inhabit the Sierra Madre Occidental where the mean annual temperature is 19°C. On the other hand, the Seris, Guarijíos, and Mayos are located in semi-desert regions with a mean annual temperature of 30°C.

Currently, the distribution of the *CYP3A5*3* polymorphism in Mexican indigenous groups is unknown, and the association of the *CYP3A5* gene with hypertension has not been reported. Thus, the aim of the present study was to determine the frequency of *CYP3A5* polymorphisms, and their association with hypertension in Mexican Amerindians.

MATERIALS AND METHODS

Subjects

A total of 372 unrelated volunteers belonging to 8 different indigenous ethnicities of Northwest Mexico were studied. The sample included 94 Tepehuanos, 62 Huicholes, and 34 Mexicaneros from the state of Durango, 66 Tarahumaras from the state of Chihuahua, 58 Coras from the state of Nayarit and 14 Seris, 14 Guarijíos, and 30 Mayos from the state of Sonora. The study protocol was approved by the Ethics and Research Committee of the Durango General Hospital of the Mexican Health Ministry (Number 031/007). All subjects signed an authorized informed consent form after being informed of the nature of the study, in accordance with the Declaration of Helsinki.

Participants were recruited from their respective communities between 2010 and 2013. All individuals self-reported as Amerindians, and their ancestry was confirmed by analyzing 15 short tandem repeat (STR) loci (Sosa-Macías et al., 2013). Based on the results, the population was divided into high (HAA, > 90%) and low (LAA, < 90%) Amerindian ancestry. Medical histories and physical examinations were obtained from adult men and non-pregnant women to confirm that they were healthy. Volunteers diagnosed with diabetes, hypertension (HT), or undergoing anti-hypertensive treatment were excluded.

Measurements

Height and weight were measured in the standing position without shoes using a standard stadimeter. Body mass index (BMI) was calculated as weight (kg) divided by height (m^2). Overweight participants were classified based on a BMI ≥ 25 kg/m^2.

Blood pressure (BP) was measured using a mercurial sphygmomanometer in triplicate to obtain the mean value as the final BP after the participant had been sitting for at least 5 min. A diagnosis of HT was defined as systolic blood pressure (SBP) ≥ 140 mm Hg, diastolic blood pressure (DBP) ≥ 90 mm Hg, and median arterial pressure (MAP) ≥ 105 mm Hg, based on the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (Jones and Hall, 2004).

Admixture Analysis

The analysis of 15 STRs was performed using the AmpFISTR® Identifiler Kit (Applied Biosystems). The amplified PCR products were analyzed using capillary electrophoresis in an ABI PRISM® 3130 Genetic Analyzer, and genotypes were obtained using allelic ladders provided by the kit and the GeneMapper® software 3.1 (Applied Biosystems).

CYP3A5 Genotyping

A total of 5 mL of peripheral blood was drawn from an antecubital vein into a tube with EDTA and kept on ice during transportation to the laboratory. Genomic DNA was extracted using the QIAGEN Blood DNA Isolation Kit (QIAGEN, Hilden, Germany), and evaluated for integrity and concentration through 1% agarose electrophoresis and spectrophotometry, respectively. Genotyping was performed with quantitative real-time PCR using a TaqMan® assay in a StepOne equipment (Applied Biosystems, Carlsbad, CA, USA). PCR amplification was performed in a 20 mL final volume containing 20 ng of template DNA, 1X TaqMan® Genotyping Master Mix (Applied Biosystems), 1X specific TaqMan® probe, and water. Thermal cycling conditions were as follows: an initial denaturation step of 10 min at 95°C, followed by 40 cycles of denaturation at 92°C for 15 s and annealing at 60°C for 1 min. Genotype identification was carried out using allelic discrimination software (Applied Biosystems). The TaqMan® probe used to recognize *CYP3A5*3* (rs776746) was C_26201809_30. Genotypes were evaluated in duplicate, and the results were confirmed through Sanger sequencing in 40 randomized samples (~10% of the total population).

Statistical Analyses

Anthropometric parameters in the Mexican-Amerindian populations are presented as mean ± standard deviation, and comparisons were made using the Mann–Whitney *U* test. The inter-ethnic *CYP3A5* allele and genotype frequencies were compared using the χ^2 and Fisher's exact tests. The *CYP3A5* allele and genotype frequencies between normotensive and hypertensive subjects were performed using the Mann–Whitney *U* and Pearson's χ^2 tests. Statistical analyses were carried out using the statistical package SPSS® version 25 for Windows (SPSS Inc., Chicago IL). Hardy–Weinberg equilibrium (HWE) was calculated using the χ^2 goodness-of-fit test. The association between polymorphisms and HT was determined using multivariate logistic regression analysis, and the model was adjusted for age and BMI. These analyses were carried out using the SNPStats program (Solé et al., 2006). Statistical significance was established with a 95% confidence interval (CI) and a p value < 0.05.

RESULTS

A total of 372 Amerindian volunteers were enrolled, 120 (32.3%) men and 252 (67.7%) women. The anthropometric parameters for all populations investigated are summarized in **Table 1**. There were significant differences in BMI, SBP, DBP, and MAP between populations, which were higher in the Seris, Guarijios, and Mayos than in the other groups.

Previously, we studied 15 STRs to estimate non-Amerindian ancestry in all the Native American populations evaluated here

TABLE 1 | Anthropometric parameters among Mexican-Amerindian populations.

	Seris, n = 14	Guarijíos, n = 14	Mayos, n = 30	Tarahumaras, n = 66	Mexicaneros, n = 34	Huicholes, n = 62	Coras, n = 58	Tepehuanos, n = 94
Age (years)	55.9 ± 13.9	58.1 ± 14.5	45.1 ± 18.2	42.8 ± 13.2	41.5 ± 13.9	40.1 ± 19.0	47.4 ± 20.9	36.6 ± 14.6
BMI (kg/m^2)	27.3 ± 4.9*	27.6 ± 9.8*	27.8 ± 6.7*	24.1 ± 4.7	24.4 ± 3.9	23.5 ± 4.7	25.7 ± 5.3	22.3 ± 5.2
Systolic BP (mm Hg)	142.1 ± 27.8*	145.6 ± 19.6*	128.8 ± 20.1*	122.3 ± 19.0	117.1 ± 16.2	112.5 ± 21.2	120.6 ± 15.3	105.1 ± 16.4
Diastolic BP (mm Hg)	92.1 ± 12.5*	92.1 ± 7.8*	83.6 ± 11.7*	78.9 ± 9.4	74.7 ± 10.2	71.6 ± 12.4	78.4 ± 11.0	68.9 ± 11.6
MAP (mm Hg)	108.8 ± 16.4*	110.0 ± 10.4*	98.7 ± 14.0*	93.4 ± 12.0	88.8 ± 11.7	85.2 ± 14.6	92.5 ± 11.8	81 ± 12.8

Data are expressed as mean ± standard deviation.
**Mann–Whitney U test.*
MAP, median arterial pressure; BMI, body mass index; BP, blood pressure.

TABLE 2 | *CYP3A5*3* allele and genotypic frequencies among Mexican-Amerindian populations.

Population	N	*CYP3A5*3*							
		Ancestry (%)[1]		Allele frequencies (%)		Genotypic frequencies (%)			HWE
		Amerindian	European	**1 (A)*	**3 (G)*	**1/*1 (A/A)*	**1/*3 (G/A)*	**3/*3 (G/G)*	*p value*[α]
Tepehuanos	94	96.4	3.1	28.7	71.3	12.8	31.9	55.3	0.04
Huicholes	62	96.3	3.1	11.3	88.7	3.2	16.1	80.6	0.16
Mexicaneros	34	94.5	4.3	57.4	42.6	38.2	38.2	23.5	0.29
Coras	58	93.9	4.7	33.6	66.4	15.5	36.2	48.3	0.15
Tarahumaras	66	92.1	7.0	28.0	72.0	10.6	34.8	54.5	0.36
Seris	14	88.0	11.0	3.6	96.4	0	7.14	92.9	1.00
Guarijíos	14	81.6	16.8	7.1	92.9	0	14.3	85.7	1.00
Mayos	30	65.6	32.6	3.3	96.7	0	6.7	93.3	1.00

HWE, Hardy–Weinberg equilibrium; [α]χ^2 goodness-of-fit statistic. [1]Sosa-Macías et al., 2013.

(Sosa-Macías et al., 2013). The highest European component was observed in the Mayo group, while the Tepehuano group had the highest indigenous component (**Table 2**). The distribution of *CYP3A5* genotypes deviates from HWE only in the Tepehuano group. The wild-type allele *CYP3A5*1* was most frequent in the groups with higher indigenous ancestry: Mexicaneros (57.4%), Coras (33.6%), Tepehuanos (28.7%), Tarahumaras (28%), and Huicholes (11.3%). The highest frequencies of *CYP3A5*3* were observed in the Mayos (96.7%), Seris (96.4%), and Guarijíos (92.9%) groups with higher European admixture. In these groups, no homozygote status (*CYP3A5*1/*1*) was detected, and the **1/*3* genotype frequency was lower than in groups with higher indigenous components. Higher homozygosity for the allele *CYP3A5*3* was found in the Mayos (93.3%), Seris (92.9%), Guarijíos (85.7%), and Huicholes (80.6%).

In order to determine relationships between blood pressure levels and *CYP3A5*3* allele and genotypic frequencies, an analysis of the total population was carried out (**Table 3**). The frequency of allele **3* was significantly higher (84%) in Amerindians with diastolic BPs ≥ 90 mm Hg ($p < 0.001$). The **1/*3* genotype was the most frequent in subjects with diastolic BPs <90 mm Hg (29.8%), but only occurred in 17.8% of subjects with diastolic BPs ≥90 mm Hg ($p = 0.04$). A higher percentage of subjects with genotype *3/*3 was observed in the groups with the highest diastolic (75.3%) and systolic (74.6%) BP figures (*p* values 0.01 and 0.02, respectively). There were no significant differences in the allele and genotype frequencies of *CYP3A5*3* in terms of MAP values.

Gender analysis revealed significant differences only in Amerindian women (**Table 4**). The frequency of allele *CYP3A5*3* was significantly higher in women with diastolic BPs ≥90 mm Hg and systolic BPs ≥140 mm Hg than in the groups with lower BP values ($p = 0.01$). In women with diastolic BPs <90 mm Hg the *1/*3 genotype was significantly more frequent (29.3%) than in females with diastolic BPs ≥90 mm Hg (14.9%) ($p = 0.04$). The *3/*3 genotype frequency was higher in the groups with higher diastolic (78.7%) and systolic (79.1%) BPs than in groups with lower figures (58.5% and 58.9% respectively, $p = 0.01$).

Table 5 shows the results of the logistic regression analysis. After a crude and adjusted analysis under a dominant inheritance model, a significant negative association was found between *CYP3A5*1* and high diastolic, systolic, and MAP values in the whole population. A similar association was also observed in the female groups, but not in terms of MAP values. The analysis of male groups did not reveal any association.

DISCUSSION

Our results show that the frequency of the *CYP3A5*3* allele is higher in indigenous groups with lower Amerindian ancestry, while *CYP3A5*1* decreases the risk of HT in Mexican indigenous women.

CYP3A5 metabolizes a great variety of drugs and endogenous compounds that regulate physiological processes including blood pressure. The expression of CYP3A5 depends in part on polymorphisms whose frequencies vary in different populations.

In the current study, the highest frequencies of *CYP3A5*3* were observed in the three groups with the LAA (92.9 – 96.7%), which is similar to those reported for Europeans, Asians, and admixed Americans (> 90%) (Zhou et al., 2017). In the Tepehuano group exclusively, the observed genotype distributions deviated from HWE, likely resulting from the geographic isolation and endogamy present in the indigenous community from which most subjects were recruited. It is worth mentioning that the total number of participants from the different ethnic groups depended on both the number of inhabitants per community as well as the number of individuals who agreed to participate in the study.

On the other hand, the observed frequencies of *CYP3A5*1* in the five groups with HAA and the groups with LAA ranged from 11.3% to 57.4% in the former, and 3% to 7% in the latter. **Table 6** shows the frequencies of *CYP3A5*1* in each population. The frequencies in the groups with HAA coincide with those observed in Mexican Amerindians (30.3%) and Mestizos (18.9%) (Gonzalez-Covarrubias et al., 2019), and with those

TABLE 3 | *CYP3A5*3* allele and genotype frequencies in normotensive and hypertensive Mexican-Amerindian population.

	Diastolic BP, mm Hg		*p* value	Systolic BP, mm Hg		*p* value
	<90	≥90		<140	≥140	
	n=299	n=73		n=307	n=65	
BP	71.6 ± 9.5	94.6 ± 6.3	**0.000**	110.5 ± 14.2	151.8 ± 14.2	**0.000**
Age (years)	39.9 ± 15.6	53.9 ± 15.3	**0.000**	39.5 ± 15.4	57.4 ± 13.2	**0.000**
BMI (kg/m^2)	23.8 ± 4.2	26.8 ± 5.6	**0.000**	24.1 ± 4.5	25.5 ± 5.5	**0.043**
Alleles						
*1 (A)	165 (27.6)	23 (15.8)	**0.002**	165 (26.9)	23 (17.7)	**0.029**
*3 (G)	433 (72.4)	123 (84.3)		449 (73.1)	107 (82.3)	
Genotypes						
*1/*1 (A/A)	38 (12.7)	5 (6.8)	0.160	38 (12.4)	5 (7.7)	0.283
*1/*3 (A/G)	89 (29.8)	13 (17.8)	**0.040**	89 (29.1)	13 (20.0)	0.140
*3/*3 (G/G)	172 (57.5)	55 (75.3)	**0.005**	180 (58.6)	47 (72.3)	**0.040**

n (%). BP, blood pressure; BMI, body mass index.
Mann–Whitney U test, Pearson χ^2, p < 0.005 in bold.

TABLE 4 | *CYP3A5*3* allele and genotype frequencies in normotensive and hypertensive women and men.

	Diastolic BP, mm Hg		*p* value	Systolic BP, mm Hg		*p* value
	<90	≥90		<140	≥140	
Women	n = 205	n = 47		n = 209	n = 43	
BP	71.4 ± 9.4	93.8 ± 5.6	**0.000**	109.7 ± 13.9	148 ± 9.2	**0.000**
Age (years)	38.8 ± 14.8	51.4 ± 15.2	**0.000**	37.9 ± 14.1	56.9 ± 13.1	**0.000**
BMI (kg/m^2)	24.3 ± 4.3	27.5 ± 5.8	**0.000**	24.7 ± 4.5	25.7 ± 5.9	0.373
Alleles						
*1 (A)	110 (26.8)	13 (13.8)	**0.009**	112 (26.8)	11 (12.8)	**0.011**
*3 (G)	300 (73.2)	81 (86.2)		306 (73.2)	75 (87.2)	
Genotypes						
*1/*1 (A/A)	25 (12.2)	3 (6.4)	0.253	26 (12.4)	2 (4.7)	0.185
*1/*3 (A/G)	60 (29.3)	7 (14.9)	**0.044**	60 (28.7)	7 (16.3)	0.093
*3/*3 (G/G)	120 (58.5)	37 (78.7)	**0.010**	123 (58.9)	34 (79.1)	**0.013**
Men	n=94	n=26		n=98	n=22	
BP	72 ± 9.7	96 ± 7.5	**0.000**	112.2 ± 14.8	159.3 ± 18.8	**0.000**
Age (years)	42.4 ± 17	58.5 ± 14.9	**0.000**	43.2 ± 17.4	58.4 ± 13.5	**0.000**
BMI (kg/m^2)	22.7 ± 4	25.5 ± 5.1	**0.006**	22.9± 4.2	25.2 ± 4.5	**0.007**
Alleles						
*1 (A)	55 (29.3)	10 (19.2)	0.150	53 (27)	12 (27.3)	0.257
*3 (G)	133 (70.7)	42 (80.8)		143 (73)	32 (72.7)	
Genotypes						
*1/*1 (A/A)	13 (13.8)	2 (7.7)	0.519	12 (12.24)	3 (13.6)	1.00
*1/*3 (A/G)	29 (30.9)	6 (23.1)	0.440	29 (29.6)	6 (27.3)	0.653
*3/*3 (G/G)	52 (55.3)	18 (69.2)	0.203	57 (58.2)	13 (59.1)	0.508

n (%). BP, blood pressure; BMI, body mass index.
Mann–Whitney U test, Pearson χ^2. p < 0.05 in bold.

TABLE 5 | Association of *CYP3A5*1* and hypertension.

Population/variable	OR (95% CI)	*p* value
Whole cohort/DBP crude analysis	0.44 (0.25–0.79)	**0.004**
Whole cohort/DBP adjustment age, BMI	0.44 (0.23–0.83)	**0.009**
Whole cohort/SBP crude analysis	0.54 (0.30–0.98)	**0.036**
Whole cohort/SBP adjustment age, BMI	0.51 (0.27–0.97)	**0.034**
Whole cohort/MAP crude analysis	0.51 (0.27–0.96)	**0.029**
Whole cohort/MAP adjustment age, BMI	0.49 (0.24–0.97)	**0.033**
Women/DBP crude analysis	0.38 (0.18–0.81)	**0.007**
Women/DBP adjustment age/BMI	0.38 (0.17–0.84)	**0.011**
Women/SBP crude analysis	0.38 (0.17–0.83)	**0.001**
Women/SBP adjustment age/BMI	0.33 (0.15–0.76)	**0.005**
Women/MAP crude analysis	0.50 (0.23–1.12)	0.082
Women/MAP adjustment age/BMI	0.46 (0.19–1.11)	0.071
Men/DBP crude analysis	0.55 (0.22–1.39)	0.200
Men/DBP adjustment age/BMI	0.51 (0.18–1.43)	0.190
Men/SBP crude analysis	0.71 (0.26–1.94)	0.500
Men/SBP adjustment age/BMI	0.75 (0.25–2.27)	0.610
Men/MAP crude analysis	0.50 (0.18–1.40)	0.170
Men/MAP adjustment age/BMI	0.48 (0.16–1.47)	0.190

OR, odds ratio; DBP, diastolic blood pressure; SBP, systolic blood pressure; MAP, median arterial pressure; BMI, body mass index. p < 0.05 in bold.

TABLE 6 | Comparison of *CYP3A5 *1* frequency in different populations.

CYP3A5 *1 (rs776746)	Frequency (%)	Ref
Indigenous HAA	11.3 - 57	This study
Indigenous LAA	3 - 7	This study
Mexican Amerindian	30.3	Gonzalez-Covarrubias et al., 2019
Mexican Mestizo	18.9	Gonzalez-Covarrubias et al., 2019
MXL	23	Data from 1000 Genomes
CEU	4	Data from 1000 Genomes
YRI	83	Data from 1000 Genomes
CHB	31	Data from 1000 Genomes

HAA, high Amerindian ancestry; LAA, low Amerindian ancestry.

reported by Hustert et al. (2001) in three Asian populations (~30%). Meanwhile, the groups with LAA had similar frequencies as Caucasians (5%) (Hustert et al., 2001). Roy et al. (2005) evaluated Caucasian Canadians and reported frequencies of 7% for *CYP3A5*1*, an identical frequency to that found in our group with LAA. The frequency of *CYP3A5*1* in French Caucasians, Gabonese, and Tunisian subjects was 0%, 5%, and 3%, respectively (Quaranta et al., 2006). In the Iranian population, the frequency of *CYP3A5*1* was 8% (Azarpira et al., 2011), which is similar to that observed in Jordanians (7%) (Yousef et al., 2012). The low frequency in both populations was similar to that in the LAA groups in the present study. The evaluation of *CYP3A5*1* in the Mexican Mestizo population revealed a frequency of 9% (Vargas-Alarcón et al., 2014), almost the same as that in the LAA Amerindian groups with the highest levels of European admixture (Seris, Guarijios, and Mayos).

It is well known that the functional allele *CYP3A5*1* is involved in sodium reabsorption and influences blood pressure (Eap et al., 2007). In 2003, Givens et al. demonstrated that the heterozygous genotype *CYP3A5*1/*3* gave rise to a higher expression of the CYP3A5 enzyme in the kidneys compared to the inactive *CYP3A5*3* homozygous form. The same study

showed high systolic blood pressure values in African-American women with the *CYP3A5*1/CYP3A5*1* genotype (Givens et al., 2003). Similar results have been found in other studies. In 2005, Ho et al. compared hypertensive vs. normotensive Caucasian and black subjects and found higher baseline DBP and SBP in the black group with *CYP3A5*1/*1* or *CYP3A5*1/*3* genotypes. The same results were observed after saline infusion and furosemide administration, but these results were not observed in white subjects (Ho et al., 2005). In African descendants, an age-dependent significant increase in BP values was observed only in those carrying the *CYP3A5*1* allele (Bochud et al., 2006).

In Japanese men, the homozygous genotype **1/*1* was observed in subjects with high DBP, while no differences between genotypes were observed for SBP (Zhang et al., 2010). Conversely, in the present study the frequency of the homozygous genotype *CYP3A5*1* was higher in normotensive subjects, although these results were not significant. A similar result was demonstrated in Caucasian women and men with low SBP values carrying the homozygous *CYP3A5*1* genotype, although such an effect was not observed for DBP (Kreutz et al., 2005). In contrast, a German Caucasian population showed no association between high SBP or DBP and the *CYP3A5*1* allele (Lieb et al., 2006).

On the other hand, in this study, the frequency of *CYP3A5*1/*3* was significantly higher in women with lower DBP, while the *CYP3A5*3/*3* genotype was over-represented in women with higher DBP and SBP. Such differences were not found in men.

The distributions of genotypes in the present study agree with those reported by Fromm et al. (2005), who found higher SBP in young Caucasian men with *CYP3A5*3/*3*, compared with subjects carrying *CYP3A5*1/*3* genotype, suggesting a **3* allele dose-effect which could be associated with high systolic blood pressure (Fromm et al., 2005).

The functional allele *CYP3A5*1* contributes to salt avidity (sodium retention) and hence HT. It is more frequent in populations closer to the equator and is considered an ancestral allele in African populations before diaspora (Thompson et al., 2004). We observed a protective effect of the *1 allele in women against high diastolic and systolic BP, which remained after adjusting for BMI and age [diastolic (OR 0.38, 95% CI 0.17–0.84, $p = 0.011$) and systolic BP (OR 0.33, 95% CI 0.15–0.76, $p = 0.005$) BP]. Conversely, no association was found in the male group, presumably because of the lower number of samples in this group (**Table 5**). In this study, we observed that populations with HAA showed the highest frequencies of the *1 allele and were the most normotensive groups. These populations are settled in mountainous communities with low environmental salt availability, so the presence of the *1 allele is beneficial, similar to what may have occurred in ancient populations in Africa. This phenomenon could explain the apparently paradoxical observation. On the other hand, the observed association in women could be the result of the higher number of women in this study.

Scarce reports exist about the prevalence of HT in indigenous Mexican populations. In 2008, Rodríguez-Moran et al. in the search for cardiovascular risk factors, evaluated two ethnic groups from Northwestern Mexico (Tepehuanos and Yaquis), who presented with HT in 3.3% and 6.3% of the study subjects, respectively (Rodríguez-Morán et al., 2008). The same authors showed in a follow up study an increase in the prevalence of HT from 1.7% in 1996 to 3.5% in 2006 in Tepehuano's communities (Rodríguez-Morán et al., 2009). These data reveal that indigenous groups are adopting westernized habits, which is supported by the three groups in our study with LAA, who presented the highest values of BP and were located in more accessible and warmer communities with higher salt intakes. In this case, the presence of **3* was not sufficient to eliminate the high amount of salt, giving rise to the high BP values observed in these groups.

The distribution of **1* and **3* alleles in these populations and the influence of environmental factors, such as the use of traditional herbal remedies, can have repercussions in drug responses, making the evaluation of genetic profiles in indigenous groups more relevant.

This is the case in the Huichol group, which has the highest frequency of homozygous **3/*3* among the groups with the highest indigenous ancestry, which can be explained by the geographical differences between these groups. Huichols inhabit a region with little water availability, which could be an environmental pressure to maintain the **3* allele, promoting water retention without the development of hypertension. In addition, it is a community with little interaction with other groups, which has allowed them to maintain their habits and customs and could explain why they are among the groups with normal blood pressure.

Some limitations of this study deserve mention. Only one blood pressure measurement was performed. Because of the geographic isolation of these studied groups, a high rate of endogamy cannot be ignored. There were a high number of women compared to men. No measures of dietary salt content or urine salt elimination were performed. CYP3A5-interacting genes such as *AGT* were not evaluated.

To the best of our knowledge, this is the first study to evaluate the distribution of *CYP3A5 *1* and **3* alleles and its association with HT in a Mexican indigenous population confirmed by molecular ancestry.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics and Research Committee of the Durango

General Hospital of the Mexican Health Ministry (Number 031/007). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CG-H: collection of samples, determination of polymorphisms, analysis of results, and wrote the paper. BL-R: collection of samples, biostatistical analyses. IL-A: preliminary analyses of results, critical review of manuscript. AM-S: determination of polymorphisms. MO-C: determination of polymorphisms. HR-V: determination of ancestry. MS-M: design of the study, collection of samples, final analysis of results, critical review of manuscript.

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ORIGINAL RESEARCH
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CYP3A5 Gene-Guided Tacrolimus Treatment of Living-Donor Egyptian Kidney Transplanted Patients

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Background: Tacrolimus is an approved first-line immunosuppressive agent for kidney transplantations. Part of interindividual and interethnic differences in the response of patients to tacrolimus is attributed to polymorphisms at CYP3A5 metabolic enzyme. *CYP3A5* gene expression status is associated with tacrolimus dose requirement in renal transplant recipients.

Materials and Methods: In this study, we determined the allelic frequency of *CYP3A5*3* in 76 renal transplanted patients of Egyptian descent. Secondly, we evaluated the influence of the *CYP3A5* gene variant on tacrolimus doses required for these patients as well on dose-adjusted tacrolimus trough-concentrations.

Results: The *CYP3A5*3* variant was the most frequent allele detected at 85.53%. Additionally, our results showed that, mean tacrolimus daily requirements for heterozygous patients (*CYP3A5*1/*3*) were significantly higher compared to homozygous patients (*CYP3A5*3/*3*) during the first year after kidney transplantation.

Conclusion: This is the first study in Egypt contributing to the individualization of tacrolimus dosing in Egyptian patients, informed by the *CYP3A5* genotype.

Keywords: CYP3A5, kidney transplantation, living donor, tacrolimus, Egyptian population, dose requirements, C/D ratio, tacrolimus blood levels

INTRODUCTION

Chronic Kidney Disease (CKD) is a long-term, progressive, and irreversible condition characterized by functional and structural kidney damages lasting for at least 3 months (Levin et al., 2013; Webster et al., 2017). Kidney transplantation is the optimal kidney replacement therapy for patients who have reached end-stage renal disease (ESRD) (Thongprayoon et al., 2020). Transplant recipients require life-long immunosuppression to prevent allograft rejection. Tacrolimus, a calcineurin inhibitor, is the most frequently used drug in kidney transplantation recipients. The impressive

results of tacrolimus treatment, however, are offset by its side effects, narrow therapeutic index and variable and unpredictable pharmacokinetics (Tang et al., 2016). For this reason, therapeutic drug monitoring (TDM) is crucial in daily practice. Renal transplant recipients usually receive standard weight-based dose which is then adjusted according to TDM to maintain tacrolimus blood concentrations within the therapeutic range. However, using TDM do not guarantee optimal treatment efficacy or lack of rejections and adverse reactions (Birdwell et al., 2015; Yanik et al., 2019). Genetic factors are considered to play important role in the interindividual and interethnic variability in pharmacokinetics of tacrolimus (Ghafari et al., 2019).

CYP3A5 is an enzyme responsible for the metabolism of tacrolimus. Single nucleotide polymorphisms in *CYP3A5* gene explain 40–50% of the variability in tacrolimus metabolism and clearance (Woillard et al., 2017). The A to G transition at position 6986 in intron 3 of the *CYP3A5* gene is the most well-studied genomic variant which contributes to dose requirement of tacrolimus (Prasad et al., 2020). *CYP3A5*3* allele results in alternative spicing of the mRNA which leads to absence of CYP3A5 protein activity and is associated with reduced tacrolimus dose requirement (Ferraris et al., 2011). The presence of the wild-type allele (*CYP3A5*1*) contributes significantly to the increase of CYP3A activity associated with recovery of renal function after transplantation (Suzuki et al., 2015). Two more variant alleles, *CYP3A5*6* and *CYP3A5*7*, result also at loss of expression of the functional protein in homozygotes (Birdwell et al., 2015).

Several studies in different populations have shown that *CYP3A5* expressors, who carry at least one *CYP3A5*1* allele require 50% (1.5–2-fold) higher tacrolimus doses compared to *CYP3A5* non-expressors those who are homozygous for the variant alleles (*CYP3A5*3*, *CYP3A5*6*, or *CYP3A5*7*) (Birdwell et al., 2015; Chen and Prasad, 2018). However, this association between *CYP3A5* genotypes and tacrolimus dose requirement has not yet been studied in Egyptian kidney transplantation recipients.

In this study, we aimed to determine the allelic frequency of *CYP3A5*3* among Egyptian patients that have undergone transplantation and to evaluate the influence of this polymorphism on tacrolimus daily dose and on metabolism rate in adult patients during the first year after kidney transplantation.

MATERIALS AND METHODS

Study Population

For the present study, 76 unrelated kidney transplanted adult patients were enrolled in Urology and Nephrology Center at Mansoura University Hospital in Egypt. All patients underwent renal transplantation from living donors and were under tacrolimus immunosuppressive treatment for at least one year. Recipients received a standard bodyweight-based tacrolimus initial dose (day -1 before transplantation) of 0.1 mg/kg twice per day. Blood samples were collected into EDTA tubes and stored at -80°C till analyzed. Therapeutic drug monitoring was applied to all samples for dose adjustment. The target whole-blood concentration in early period after transplantation is 10–20 ng/ml and in the maintenance period (after 3 months) 5–10 ng/ml. Tacrolimus daily dose, tacrolimus blood levels, demographic, and clinical data were obtained from medical files of the patients at the beginning of the post-transplant period and at 12 months after transplantation. Patients with diarrhea or vomiting, liver disease, advanced renal dysfunction, or other disorders that could have altered the absorption of tacrolimus or patients that will be co-prescribed drugs that affect the pharmacokinetics of tacrolimus and its pharmacological effect (antifungals, antiepileptics, macrolide antibiotics) were excluded from the study.

The study was conducted in compliance with the declaration of Helsinki and was approved by the Ethics Committee of the Mansoura University Hospital and written informed consent was obtained from all subjects.

DNA Extraction and Genotyping

Total genomic DNA was extracted from the peripheral blood, followed by determination of its concentration and purity. The *CYP3A5* single nucleotide polymorphism (SNP) – *CYP3A5*3* (rs776746) was genotyped by PCR-restriction fragment length polymorphism (RFLP), using the SspI restriction endonuclease as previously described (Mendrinou et al., 2015).

Statistical Analysis

Estimation of allele and genotype frequencies was performed using gene counting method and their deviation from Hardy-Weinberg equilibrium was assessed by Pearson's goodness of fit chi-square test (degree of freedom = 1). Continuous variables are shown as mean and standard deviation and qualitative data are expressed as frequency and percentage.

Continuous data were tested for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests ($p = 0.05$) and visualized with Q-Q plots. Depending on the distribution, comparisons for variables between two groups were performed with two-tailed test or Wilcoxon test for related samples and with unpaired t-test or Mann-Whitney test for independent samples. The categorical data were analyzed using two-tailed Fisher's exact test.

In the present study patients were divided into two groups according to their genotype [CYP3A5 expressors (*1/*1 or *1/*3) and CYP3A5 non-expressors (*3/*3)]. Both groups were examined for statistically significant difference in dose requirements, tacrolimus blood levels, and C/D ratio (dose corrected trough concentration of Tac). These data were compared at different time points among related samples (patients with the same genotype) and at the same time points among independent samples (patients with different genotype).

Statistical analysis was performed using SPSS Statistics 25.0 (IBM SPSS software) and GraphPad Prism 8.0. The significance level was set at $p<0.05$.

RESULTS

Demographic Characteristics of the Patients

A total of 76 kidney transplant recipients were included in this study and they all were adults and self-reported Egyptians. According to the date of the transplantation, there were missing data for 17 of the patients regarding tacrolimus dose. The characteristics of 59 recipients according to their CYP3A5 genotype are shown in **Table 1**. There were no statistically significant differences between the two groups with respect to sex, family history, age of CKD, age at transplantation, time waiting for transplantation, incidence rejection, or donor type.

Frequency of the *CYP3A5*3* Variant in Kidney Transplant Recipients

Of the 76 kidney transplant recipients, the *CYP3A5*3/*3* genotype was observed in 55 (72.37%) cases, *CYP3A5*1/*3* in 20 (26.32%) cases, and *CYP3A5*1/*1* in 1 (1.32%) case. Total allelic frequency was 85.53% for *CYP3A5*3* and 14.47% for *CYP3A5*1* (**Figure 1**). No deviation from Hardy-Weinberg equilibrium was observed for the genotype frequencies ($\chi^2 = 0.58323 < 3.841$).

TABLE 1 | Comparison of the clinical characteristics, tacrolimus daily dose, tacrolimus blood levels, and C/D ratio of the study population between *CYP3A5* expressors and non-expressors.

Characteristics	Non-expressors (*3/*3) n = 41	Expressors (*1/*3, *1/*1) n = 18	P value
Gender, n (%)			
Male	35 (85.37%)	13 (72.2%)	0.2841
Female	6 (14.63%)	5 (27.8%)	
Onset of CKD, years, mean (range) (SD)	27.2 (9–55)	31.2 (14–65)	0.2403
Onset at transplantation, years, mean (range) (SD)	29.2 (10–55)	32.8 (14–67)	0.2983
Time waiting for transplant, years, mean (range) (SD)	2 (0–6)	1.56 (0–4)	0.1965
Graft rejection, n (%)			
Yes	8 (19.5%)	6 (33.3%)	0.3224
No	33 (80.5%)	12 (66.7%)	
Family history, n (%)			
Yes	3 (7.3%)	2 (11.1%)	0.6359
No	38 (92.7%)	16 (88.9%)	
Donor type, n (%)			
Living Related	33 (80.5%)	14 (77.8%)	1.0000
Living unrelated	8 (19.5%)	4 (22.2%)	
Initial Tac D, mg/day, mean (range) (SD)	6.76 (2–11)	9.86 (6–14)	**<0.0001**
1-year Tac D, mg/day, mean (range) (SD)	4.21 (1.5–10.5)	7.81 (2.5–13)	**<0.0001**
Initial Tac C, ng/ml, mean (range) (SD)	7.09 (2–22.6)	5.89 (2–13.5)	0.3035
1-year Tac C, ng/mL, mean (range) (SD)	7.39 (3.3–11.7)	7.15 (4.9–9.9)	0.6373
Initial C/D ratio, ng/ml per mg/day, mean (range) (SD)	1.50 (0.2–9.4)	0.64 (0.18–1.5)	0.0586
1-year C/D ratio, ng/ml per mg/day, mean (range) (SD)	2.10 (0.6–5.8)	1.10 (0.63–2.84)	**0.0003**

D, tacrolimus daily dose; C, tacrolimus blood concentration; SD, standard deviation. Bolded data are those which are statistically significant.

Association of the *CYP3A5* Genotype With Tacrolimus Dose, Tacrolimus Blood Levels, and C/D Ratio

For the 59 patients, tacrolimus initial doses (mean ± standard deviation) for *CYP3A5*1* carriers and *CYP3A5*3/*3* groups were 9.861 ± 2.182 (range: 6.0–14.0) and 6.756 ± 2.478 mg/day (range: 2.0–11.0), while doses one year after transplantation were 7.806 ± 3.158 (range: 2.5–13.0) and 4.207 ± 2.083 mg/day (range: 1.5–10.5), respectively. This shows a significant reduction of the dosage for both genotypic groups, 20.84% for expressors (*CYP3A5**1/*3 or *1/*1) (P = 0.0017) and 37.73% for non-expressors (*CYP3A5*3/*3*) (P < 0.0001). Differences between initial and first-year doses are shown in **Figure 2**.

Comparing the starting daily dose between *CYP3A5*3/*3* and *CYP3A5*1* carriers, mean dose for *CYP3A5*1* carriers was significantly higher (45.96%) than for *CYP3A5*3/*3* (P < 0.0001). One-year mean tacrolimus dose for *CYP3A5*1* carriers was 85.55% higher than for *CYP3A5*3/*3* (P < 0.0001) (**Figure 3**).

Average tacrolimus blood concentrations in *CYP3A5* non-expressors was higher in both time points compared with *CYP3A5* expressors. However, there was no significant differences between the two groups neither at the early post-transplant period (p = 0.3035) nor at the maintenance period (p = 0.6373).

*CYP3A5*1* recipients exhibited significantly lower C/D ratios (47.89% lower) than those homozygous for the variant allele (*3/*3) at one year of treatment (1.097 ± 0.5829 and 2.105 ± 1.030 ng/ml per mg/day, respectively, p = 0.0003). However, there was no significant difference between the two groups at the early post-transplant period (p = 0.0586). Significant increase was observed at C/D ratios comparing the two time points among *CYP3A5*1* carriers (p = 0.0003) and among *CYP3A5*3/*3* recipients (p = 0.0123) (**Figure 4**).

DISCUSSION

The biggest challenge for clinicians is the long-term maintenance of renal grafts after a kidney transplantation. Tacrolimus is one of the currently used immunosuppressive therapies, but its administration may be the causative factor of many side effects and graft rejection (Thishya et al., 2018). In addition to the highly variable oral bioavailability, pharmacokinetics of tacrolimus is characterized by diversity among individuals in the first-pass metabolism and systemic clearance. These differences are largely due to *CYP3A5* polymorphisms and their effect on the metabolism of tacrolimus.

Pharmacogenomics studies have reported significant association between the *CYP3A5* genotype and the daily doses required for kidney transplant recipients. Most of them noticed that tacrolimus doses were significantly higher in patients carrying *1 allele (*CYP3A5*1/*1* + *CYP3A5*1/*3*) compared to recipients homozygous for *3 allele (*CYP3A5*3/*3*) (Tang et al., 2016). Our study aimed to analyze the distribution of *CYP3A5* allele frequency in the Egyptian population. In the study

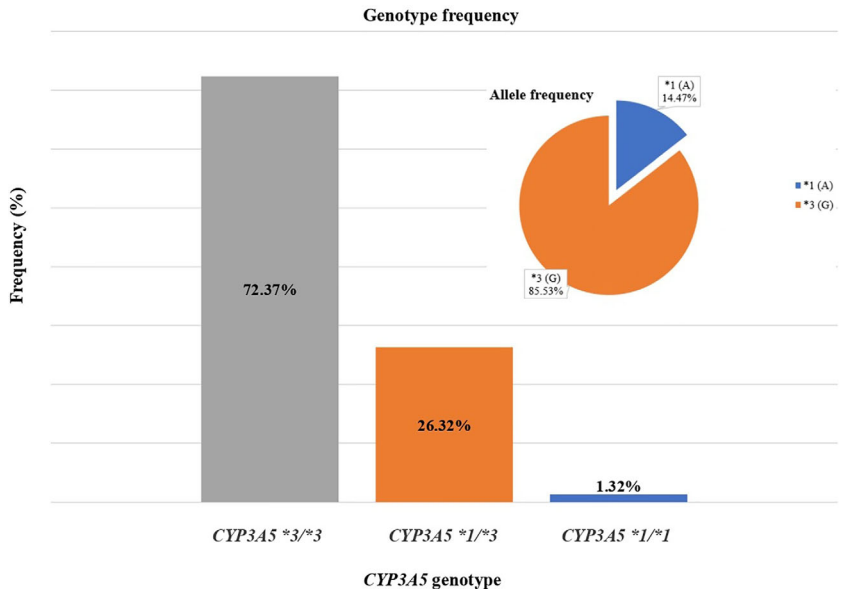

FIGURE 1 | Genotype and allelic frequencies of 76 renal transplant recipients for *CYP3A5* gene.

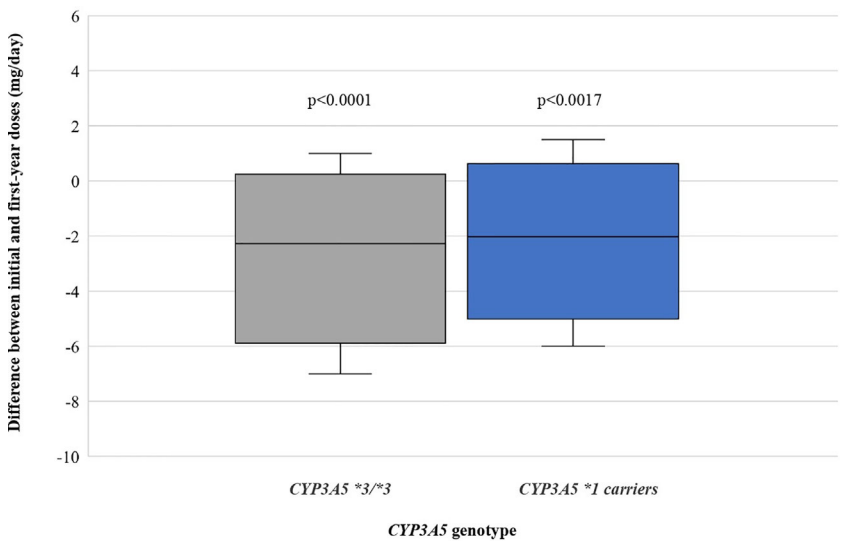

FIGURE 2 | Differences between initial and first-year doses as stratified by *CYP3A5* genotype.

population (n = 76) the three genotypic groups, *CYP3A5*1/*1*, *CYP3A5*1/*3*, and *CYP3A5*3/*3* were observed in 1.32, 26.32, and 72.37% respectively. The distribution of *CYP3A5* gene showed that the *CYP3A5*3* allele was 85.53%. In previous studies in the Egyptian population, different frequencies were reported for the *CYP3A5*3* allele, ranging from as low as 11% to as high as 78% (Zayed and Mehaney, 2015; Abo El Fotoh et al., 2016; El Wahab et al., 2017). Studies published in other North African populations (Algerians, Morocco, Tunisians, Libyans) showed that the *CYP3A5*3* allele was the most prevalent with a frequency that reaches even 90% (Novillo et al., 2015; Fernández-Santander et al., 2016), whereas in the African population as a whole is observed great diversity from 4 to 95% (Zhou et al., 2017).

Several studies have been conducted in North Africans in order to evaluate the effect of *CYP3A5* variants on tacrolimus dosage and on tacrolimus blood concentrations normalized by the dose and proved that there is significant difference between

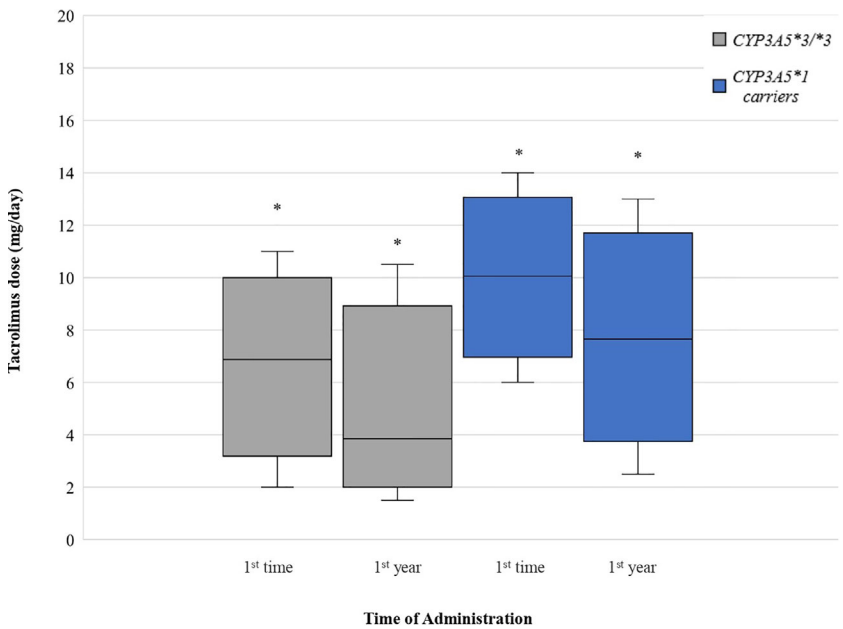

FIGURE 3 | Tacrolimus dose for *CYP3A5* genotypes. Each genotype appears as paired blots (first blot for initial dose-second blot for first-year dose). *p < 0.05.

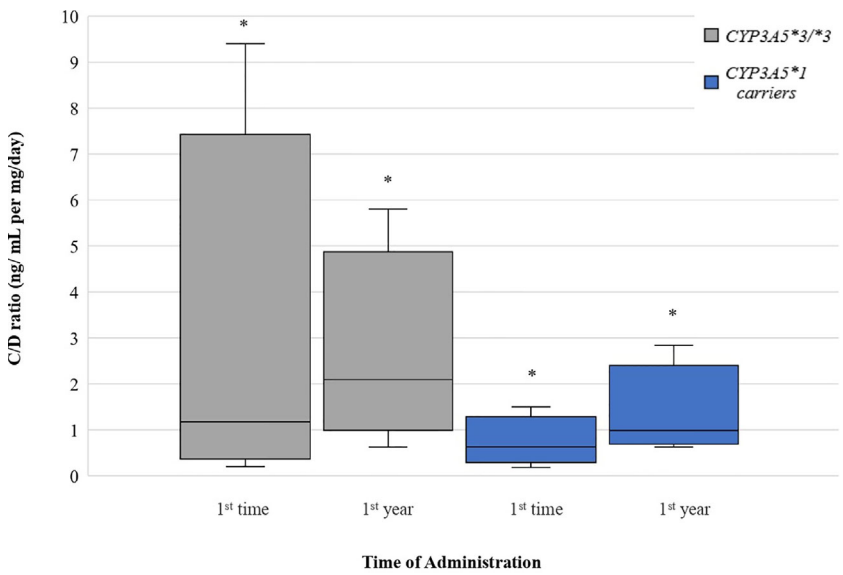

FIGURE 4 | Dose-Adjusted Tacrolimus Trough-Concentrations for *CYP3A5* genotypes. Each genotype appears as paired blots (first blot for initial dose-second blot for first-year dose). *p < 0.05.

renal transplant patients with the *CYP3A5*1* allele compared to homozygotes for the *CYP3A5*3* allele, especially during the early post-transplant phase (Elmachad et al., 2012; Aouam et al., 2015). To our knowledge, this is the first study to examine the association of the *CYP3A5*3* allele with tacrolimus dose requirements and C/D ratios in Egyptian kidney transplant recipients. To date, in the Egyptian population, some studies have been conducted examining the correlation of the *CYP3A5* genotype but in liver transplant patients (Fathy et al., 2016; Helal et al., 2017). Our results showed that tacrolimus doses were reduced between the first administration and one year after transplantation, regardless of genotype. Additionally, individuals homozygous for the *CYP3A5*3* allele need significantly lower tacrolimus daily dose than those carrying *1 allele ($p < 0.05$). Concentration/dose ratio was significantly lower

in *CYP3A5*1* expressors. All these indicate that *CYP3A5* expressors require a larger tacrolimus dose in order to maintain the same blood concentration.

Although there are minor limitations in our study, single center and small cohort, our results showed that frequency of the *CYP3A5*3* variant seems to be higher as compared with previous studies in the Egyptian population and in agreement to that reported prevalence of this allele for other North African or Caucasian populations. Furthermore, comparison of tacrolimus dose requirement for renal transplant patients showed statistically significant difference among genotypes. It is important to draw up different treatment plan for different recipients. As *CYP3A5* shows great heterogeneity in African population, there is a need for pharmacogenomic testing prior to tacrolimus administration after kidney transplantation to achieve genotype-guided dose and contribute to a better-individualized immunosuppressive therapy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Mansoura University Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EM, MMa, and GP conceded the study. MMa, AA, MMo, AR, EE, HHS, and HS provided samples and clinical data. EM performed the analysis. EM and GP compiled the draft manuscript. GP and HS provided funding. All authors contributed to the article and approved the submitted version.

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