PHARMACOGENOMICS OF ADVERSE DRUG REACTIONS (ADRS)

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PHARMACOGENOMICS OF ADVERSE DRUG REACTIONS (ADRS)

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Editorial: Pharmacogenomics of Adverse Drug Reactions

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Editorial on the Research Topic

Pharmacogenomics of Adverse Drug Reactions

WHY DO ADVERSE DRUG REACTIONS MATTER

Therapeutic adherence is necessary to achieve drug targets, improve outcomes, and reduce the occurrence of morbidity and preventable mortality. Adverse drug reactions (ADRs) are a major contributor to reduced compliance, adherence to treatments, morbidity, and mortality. ADRs are estimated to be the fourth leading cause of death in the United States—ahead of pulmonary disease (before the COVID-19 pandemic), diabetes, AIDS, pneumonia, accidents, and automobile deaths (Center for Drug Evaluation and Research, 2021). Individuals experiencing ADRs are more likely to seek follow up visits with healthcare providers, have decreased compliance, and a higher rate of drug failure. Therefore, understanding both the clinical and genetic risk factors for ADR is crucial in the delivery of precision medicine.

DATA SUBSTRATES FOR ADR RESEARCH

Randomised clinical trials provide a key resource for post-hoc pharmacogenetic studies. However, there is a substantial diversion between in-trial behaviours and real-world practices. RCTs usually have strict inclusion and exclusion criteria, meaning that the patients enrolled in RCTs typically do not represent the complexity of patients in real-world clinical practice. Moreover, the practice of conducting a run-in period results in the retention of a sub-set of individuals more likely to comply with therapy. RCTs are also typically run for a finite amount of time, typically one to a few years at the most, during which dose and therapies are not changed, and patients in the real-world are treated with drugs for much longer periods of time. On the other hand, observational data resources reflect real-world behaviours such as highly complex patients with multiple drug therapies and comorbidities, physicians adapting therapies based on drug response, and altered prescribing patterns due to changes in guidelines or expiring patents. In observational data resources, the longer follow up period allows researchers to better gauge patterns of adherence, and adverse drug reactions that are milder but perhaps more

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commonplace. While RCTs are the most reliable source of recorded severe ADRs, individual behaviours in the ambulatory setting shed light on long-term adherence, drug-response, and milder ADRS. This is reflected in the breadth of articles in this special issue, which are all based on observational data resources from across the world, including the United Kingdom, United States, India, and China.

WHAT DOES PHARMACOGENETICS ADD?

Pharmacogenetics and genomics allow researchers to assess the role of genetic variants in key genes such as those involved in pharmacokinetic and immune pathways. This can shed light on the functional role of variants and help tailor newer therapies. There are several examples in which candidate gene studies have identified and validated genetic variants associated with ADRs, which are now recommended in clinical practice guidelines and by regulatory agencies across the world to reduce patient risk of ADRs (PharmGKB, 2022). However, ADRs remain a major clinical problem, and thus more real-world evidence is needed. While a lot of studies have focussed on candidate gene studies, latterly research has suggested that most genome-wide signals are found in non-candidate regions–further highlighting the potential for discovery in the field of pharmacogenetics (Linskey et al., 2021). Another avenue of investigation are rare variants, which are poorly characterised due to the lack of sequencing-based genomic data. Recent studies have highlighted the added value of rare variants in drug response, and it stands to reason that they would have value in understanding ADRs (McInnes et al., 2021). The majority of pharmacogenetic research has also focused on patients of European ancestry; this is largely driven by the preponderance of trials and observational cohorts available on individuals of European descent. However, this leaves a substantial gap in our understanding of ADRs and pharmacogenetics in non-European ethnicities, a field with enormous potential.

IN THIS SPECIAL ISSUE

The special issue advances the much-needed evidence base for pharmacogenetics of adverse drug reactions by covering a wide breadth of therapeutic areas, including cardiovascular, neurology, endocrinology and diabetes, as well as cancer. For clinical conditions with limited treatment strategies and with severe adverse outcomes, pharmacogenetics helps identify new drug targets and patients at risk of ADRs. In this issue, Tirozzi and Willcocks investigate ADRs in Parkinson's disease and schizophrenia that present such challenges (Tirozzi et al.; Willcocks et al.). This special issue also advances the evidence base for non-European populations such as East Asian and Asian Indians bringing a much needed global perspective (Shanbhag et al.). Evidence is also emerging to show that genetic risk for ADRs is polygenic, and the papers by Melhem et al. and Ooi et al. in this special issue applied advanced methods such as genetic scores and machine learning to further our understanding of the polygenic risk for ADRs (Melhem et al.; Ooi et al.). Moving forward, the field of pharmacogenetics and genomics for adverse drug reactions has three main lacunae: the use of data from under-represented ethnicities, methods to translate genetic risk to clinical practice, and the use of novel genetic methods that can help establish causal relationships such as Mendelian Randomisation.

AUTHOR CONTRIBUTIONS

MS developed the presented idea and drafted the manuscript. JL, MC, and SHM contributed to the manuscript content. All authors approved the submitted version.

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MINI REVIEW
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Genetic Markers for Stevens-Johnson Syndrome/Toxic Epidermal Necrolysis in the Asian Indian Population: Implications on Prevention

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This review attempts to collate all the studies performed in India or comprising a population originating from India and to find out if there is an association between the HLA (human leucocyte antigen) type of individual and development of Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN) subsequent to medication use. The authors performed a PubMed search of all articles published in English from 2009 to 2019 for articles that studied HLA type in patients who developed SJS/TEN after intake of a specific drug in the Asian Indian population or in individuals of Asian Indian origin. The selection criteria were satisfied by a total of 11 studies that reported HLA associations with specific drugs, which induced SJS/TEN, mainly anti-epileptic drugs, and cold medicine/non-steroidal anti-inflammatory drugs. These studies involved a small number of patients, and hence, there is limited evidence to conclude if these associations can be extrapolated to a larger population of the same ethnicity. Similar multi-center studies need to be conducted with a larger sample size to confirm these associations. This would have implications in policy making and for understanding the potential of using genetic markers as a screening tool before prescribing a drug to a patient, which might make them susceptible to developing a potentially life-threatening disease such as SJS/TEN. This is possibly the only mode of primary prevention for this potentially fatal severe cutaneous adverse drug reaction.

Keywords: human leucocyte antigen, genetic markers, India, carbamazepine, anti-epileptics, toxic epidermal necrolysis, Stevens-Johnson Syndrome, severe cutaneous adverse drug reaction

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INTRODUCTION

Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN) are diseases, which belong to a spectrum of immunological conditions affecting the skin and the mucosa. SJS/TEN are life-threatening conditions affecting multiple organ-systems, generally warrant intensive care unit or burn unit admission (Kohanim et al., 2016b; Shanbhag et al., 2020). Beginning as a skin rash and involvement of oral and ocular mucosa, it then evolves into necrolysis of the skin with involvement of mucosa of different organ systems (Kohanim et al., 2016a). Currently, supportive

care guidelines are available for the treatment of SJS/TEN in the acute phase (Creamer et al., 2016; Seminario-Vidal et al., 2020). Despite aggressive treatment in the acute phase, the morbidity and mortality associated with SJS/TEN is still high (Hsu et al., 2016). On survival of the acute episode of SJS/TEN, a multitude of chronic complications affecting different organs still occur (Yang et al., 2016), the most debilitating of which are chronic ocular complications leading to corneal blindness (Saeed and Chodosh, 2016; Lee et al., 2017). Hence, primary prevention is the best form of prevention for SJS/TEN.

SJS/TEN is categorized as a severe cutaneous adverse reaction (SCAR) and multiple drugs have been implicated in the pathogenesis (Nguyen et al., 2019). Prevention is possible if patients who are susceptible to this SCAR on being prescribed a certain medication are identified. If a strong association is identified between ingestion of a drug and an HLA (human leukocyte antigen) type, then genetic screening of all patients before prescribing this drug to prevent the onset of SJS/TEN can be instituted. However, preemptive genotype screening before prescribing such medications is not yet practiced in the Asian Indian population due to the disease being uncommon and sparse evidence of such associations. This review was undertaken with the sole intention of understanding the existing evidence linking HLA associations with drug-induced SJS/TEN in the Asian Indian population.

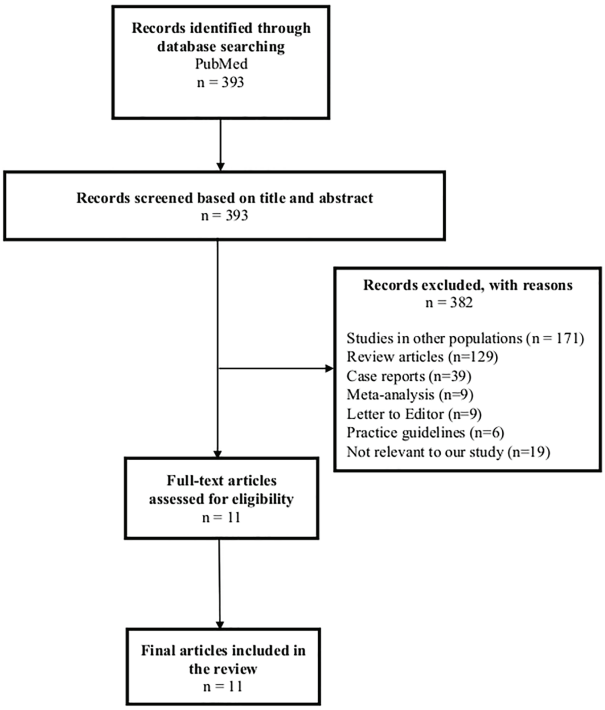

FIGURE 1 | Flowchart of literature search for studies on genetic markers for Stevens-Johnson syndrome/toxic epidermal necrolysis in the Asian Indian population.

DATA SOURCES AND SELECTION CRITERIA

A search was conducted in February 2020 on PubMed for articles between January 1, 2009 and December 31, 2019. The keywords used were "Stevens-Johnson syndrome," "toxic epidermal necrolysis," "human leucocyte antigen," "HLA," "association," "India," and "Indian." Articles in the English language were included. Letters, conference abstracts, case reports, review articles, editorials, and animal studies were excluded. A total of 393 articles were identified, out of which 382 articles did not fulfill inclusion criteria and were excluded after screening the titles and abstracts (**Figure 1**). A total of 11 studies met our criteria for inclusion and were further analyzed (Mehta et al., 2009; Chang et al., 2011; Aggarwal et al., 2014; Khor et al., 2014, 2017; Ueta et al., 2014; Ramanujam et al., 2016; Kannabiran et al., 2017; Srivastava et al., 2017; Devi, 2018; Ihtisham et al., 2019). Out of these 11 studies, three studies included a multi-ethnic population, including HLA associations in the Indian population in their countries (Chang et al., 2011; Khor et al., 2014, 2017).

Associations Between Specific Drugs and HLA in the Pathogenesis of SJS/TEN

Most cases of SJS/TEN worldwide are associated with prior drug exposure. Certain drugs are implicated in the causation more than others such as antibacterial sulfonamides, carbamazepine (CBZ), allopurinol, lamotrigine, phenobarbital, phenytoin, nevirapine, and oxicam-type non-steroidal anti-inflammatory drugs (NSAIDs; Roujeau et al., 1995). SJS/TEN usually occurs the first time the drug is ingested, without prior sensitization, and usually within the first 2 months of therapy (Mockenhaupt et al., 2008).

The basis behind the pathogenesis of SJS/TEN is believed to be immunological. Genetic factors influencing drug metabolism and the immune response, including HLA genotype, might increase the risk of drug hypersensitivity, thus causing SJS/TEN (Roujeau et al., 1986; Svensson et al., 2001). The specific HLA allele presents a drug/metabolite to the T-cell receptors on cytotoxic T-lymphocytes resulting in cell activation, clonal expansion, and extensive keratinocyte death in SJS/TEN (Chung et al., 2008). This immunological hypothesis was given further credence by a study in the European population by Roujeau et al. (1987), where they noted that HLA alleles could be the primary genetic factor for determining an individual's susceptibility to SJS/TEN. Further evidence for this finding was provided by Chung et al. (2004) in the Han-Chinese population, where presence of HLA-B*15:02 was strongly associated with CBZ-related SJS/TEN, and HLA-B*58:01 with allopurinol-related SJS/TEN (Hung et al., 2005b). However, HLA-B*15:02 was not significantly associated with CBZ-related SJS/TEN in the European population (Lonjou et al., 2006), which is explained by the low allele frequency of HLA-B*1502 of 1–2% in studies performed on European populations (Geer et al., 1998). This proves that HLA-B*15:02 is not a universal marker for CBZ-related SJS/TEN. Hence, it is important to study well-defined ethnic populations, identify the causative drug accurately, and test for specific HLA associations.

Successful Implementation of Preemptive Genotyping for Prevention of SJS/TEN

The strongest association between HLA type and a drug causing SJS/TEN has been found between HLA-B*15:02 and CBZ in the Han-Chinese, Thai, and Malaysian populations (Chung et al., 2004; Tassaneeyakul et al., 2010; Chang et al., 2011; Tangamornsuksan et al., 2013), and HLA-B*58:01 and allopurinol in the Han-Chinese population (Hung et al., 2005b; Somkrua et al., 2011). The United States Food and Drug Administration in 2007 issued an alert regarding package labeling and recommended genotyping in all East Asian patients prior to prescribing CBZ (Ferrell and McLeod, 2008). Certain Asian countries, such as Taiwan, Hong Kong, Singapore, and Thailand, have since then started HLA-B*15:02 screening programs before the prescription of CBZ. This has been incorporated in the electronic prescribing system to interface with laboratory records to ensure that CBZ is not started in patients untested or positively tested for HLA-B*15:02. If patients screen positive for this allele, they are provided alternative medications. Certain countries like Thailand, Taiwan, and Singapore have included the cost of this screening in their national health insurance schemes (Dong et al., 2012; Tiamkao et al., 2013). In Taiwan, this measure clearly translated into a decrease in the incidence of CBZ-related SJS/TEN (Chen et al., 2011). Another study from Thailand also showed a significant decrease in the number of cases of CBZ-related SJS/TEN if a universal HLA-B*15:02 screening policy is instituted (Rattanavipapong et al., 2013). Regulatory recommendations for HLA-B*15:02 genotyping combined with government subsidy for the test also contributed to a reduction in CBZ-related SJS/TEN in Singapore by >90%, with additional reductions in number of phenytoin-related SJS/TEN cases (Sung et al., 2020).

Cost-Effectiveness of Preemptive Genotyping for the Prevention of SJS/TEN

Calculating the cost-effectiveness of an intervention, such as preemptive genotyping, to reduce the incidence of SJS/TEN depends on several factors. These include the incidence and severity of the SCAR, the sensitivity and specificity of the marker, and the availability of inexpensive alternative medications with better safety profiles for individuals who screen positive for the marker (Chung et al., 2010). In the Han-Chinese population, the HLA-B*15:02 marker for CBZ-related SJS/TEN has been found to be 100% sensitive and 97% specific (Hung et al., 2005a). The average allele frequency of HLA-B*15:02 in the Han-Chinese population is 6% (1.9–12.4%; Gonzalez-Galarza et al., 2011). This is still relatively higher than other populations. Hence, screening for HLA-B*15:02 allele before starting treatment with CBZ in Asian countries is justified in view of high frequency of the allele, the seriousness of the consequences of SJS/TEN, high sensitivity and specificity of the marker as well as availability of alternative anti-epileptic drugs (AEDs; Chung et al., 2010). Whether screening prior to prescribing CBZ is financially viable depends on the extra cost of the test in a given population, whether the cost of the test is partially or fully covered by insurance, whether it outweighs the costs of SJS/TEN treatment, expense of alternative safer drugs, and subsequent loss of quality of life and due to sequelae of the ailment (Locharernkul et al., 2011). The second option is avoiding the use of CBZ altogether and prescribing alternative medications. However, CBZ use is still rampant in most South-East Asian countries since it is cheaper, effective, and physicians are experienced with its use (Chung et al., 2010). Alternative medications are more expensive preventing them from being cost-effective for the health-care system due to their long-term use (Locharernkul et al., 2011). Hence, countries in South-East Asia have consistently found that HLA-B*15:02 genotyping screening in the Han-Chinese population is less expensive than the cost of SJS/TEN treatment (both in the acute phase, in the chronic phase, including loss of quality-adjusted life years) or the cost of providing alternate drugs (Dong et al., 2012; Rattanavipapong et al., 2013; Tiamkao et al., 2013).

Morbidity and Mortality of SJS/TEN in the Asian Indian Population

SJS/TEN is considered to be a rare condition with an estimated annual incidence (cases/million population/year) ranging from 0.6 to 12 cases per million population in different countries (Naldi et al., 1990; Schöpf et al., 1991; White et al., 2015; Hsu et al., 2016). Although the incidence of SJS/TEN in India is not known, it is possible that the incidence could be higher. Sushma et al. (2005) noted that 19.5% of hospitalized patients with SCAR over a 9-year period were diagnosed with SJS/TEN. A systematic review conducted on SJS/TEN in India reported an overall mortality of 12.94% in SJS/TEN cases (Patel et al., 2013), with the most common culprit drugs being antimicrobials (sulfonamides being the most common – 37%), followed by AEDs (CBZ and phenytoin being the most common – 36%), followed by NSAIDs (16%; Patel et al., 2013; Singh et al., 2015).

SJS/TEN contributes to life-long complications in the chronic phase, affecting multiple organ systems, with published reports from India discussing ophthalmic sequelae of SJS/TEN including bilateral corneal blindness (Kompella et al., 2002; Basu et al., 2018; Vazirani et al., 2018), respiratory and gastrointestinal system complications such as bronchiolitis obliterans (Basker et al., 1997; Dogra et al., 2014), esophageal strictures, drug-induced liver injury (Agrawal et al., 2003; Misra et al., 2004; Devarbhavi et al., 2016). Owing to the morbidity and mortality secondary to SJS/TEN in the Asian Indian population, measures in reducing the incidence of SJS/TEN could be beneficial in reducing the overall disease burden. Since HLA associations are not universal and are ethnicity specific, there is definitely a need to study if strong HLA genotype-drug associations in the Asian Indian population exist, thus making them more susceptible to developing SJS/TEN. Hence, a review of the existing literature on studies from India of HLA genotype-drug association on patients from the Asian Indian population who developed SJS/TEN to a specific drug was undertaken. Due to the paucity of such studies, studies performed on patients of Indian origin in countries other than India were also included.

RESULTS OF HLA GENOTYPING IN THE ASIAN INDIAN POPULATION WITH SJS/TEN

Descriptive information for each study is shown in **Table 1**. Out of the 11 studies, eight studies were from India, while three were from Malaysia. Among these eight studies, three included populations predominantly from North India (Aggarwal et al., 2014; Ramanujam et al., 2016; Ihtisham et al., 2019), and one study each included populations predominantly from South-India (Devi, 2018) and North-west India (Mehta et al., 2009). The studies conducted in Malaysia included a small cohort of Indian origin patients, predominantly from South India (Chang et al., 2011; Khor et al., 2014, 2017). The most commonly studied HLA genotype-drug association was HLA-B*15:02 and AEDs, specifically CBZ. Two studies focused on cold-medicine (CM) related SJS/TEN and studied HLA-A*02:06, HLA-A*33:03, and HLA-B*44:03. The number of patients tested in each study was small, ranging from 2 to 9 patients for the AED-related SJS/TEN, and 20–80 patients for CM-related SJS/TEN. All studies enrolled controls except one. Six studies enrolled controls that were drug-tolerant and had not developed SJS/TEN to AED's, while four studies enrolled normal controls with no drug exposure. All studies performed polymerase chain reaction with sequence-specific primers for HLA antigens.

Results of Studies of Associations Between HLA Alleles and Anti-Epileptic Drugs in the Indian Population With SJS/TEN

The studies performed in India in **Table 1** show that HLA-B*15:02 remains a significant risk predictor of CBZ-related SJS/TEN. The average allele frequency of HLA-B*15:02 in the Indian population among different communities evaluated primarily from the North Indian population is 2.5% (0–6%; Rajalingam et al., 2002; Rani et al., 2007). Since the carrier frequency in the Indian population is lower than the Han-Chinese population, there is a need to test for other susceptibility genes for CBZ-related SJS/TEN in the Indian population. In a study by Khor et al. (2017), HLA-A*31:01 was found to be associated significantly with CBZ-related SJS/TEN in Indians. In another study by Ihtisham et al. (2019), HLA-B*57:01 was found to be associated significantly with CBZ-related SJS/TEN in Indians. The allele frequency of HLA-A*31:01 and HLA-B*57:01 in the Indian population among different communities is 3.52% (primarily from South India) and 2–8% (from both North and South India; Gonzalez-Galarza et al., 2011), respectively. Although the frequencies of these alleles are similar to the allele frequency of HLA-B*15:02 in the Indian population, the utility of testing these alleles have not been otherwise studied widely for CBZ-related SJS/TEN. Collection of data from multiple centers will be useful as samples collected from individual centers may prove to be too small to attain a statistical significance. Also, the carrier frequency for HLA-B*15:02 may not be homogenously distributed in the Indian population, and multiple studies across the length and the breadth of the country are required to establish this, in the normal population.

CBZ is a commonly used AED in India because of affordability and easy availability and most studies on HLA type-drug association in SJS/TEN have focused on this drug. However, few studies have tested for susceptibility genes for SJS/TEN caused by other aromatic AED's (phenytoin and lamotrigine) in the Indian population (Aggarwal et al., 2014; Srivastava et al., 2017; Devi, 2018). However, the prescribing patterns for these drugs in India are not yet known, and hence, it is unclear if preemptive genotyping for these will prove to be useful and cost-effective. Also, it is essential to enroll controls that are tolerant to the drug in these studies to enable the study of cost-effectiveness of using such a test for screening.

Guidelines are available, provided by the Clinical Pharmacogenetics Implementation Consortium (CPIC) for appropriate usage of drugs like CBZ, oxcarbazepine, and phenytoin, which are some of the main culprit drugs for SJS/TEN (Leckband et al., 2013; Caudle et al., 2014; Phillips et al., 2018; Karnes et al., 2020). These guidelines provide therapeutic recommendations on how these drugs need to be utilized when genotyping results are available. Since these guidelines greatly assist clinicians in applying genetic information to patient care, thus optimizing the therapeutic usage of these drugs, physicians who routinely prescribe these drugs should be aware of these guidelines.

Results of Studies of Associations Between HLA Alleles and Cold Medicines (NSAIDs) in the Indian Population

Two studies studied associations between CM-related SJS/TEN and HLA-A*02:06, HLA-A*33:03, HLA-B*44:03, and HLA-C*07:01 in the Asian Indian population (Ueta et al., 2014; Kannabiran et al., 2017). Patients with the specific phenotype of severe ocular complications (SOC) in the chronic phase were selected for these studies, although exact drug etiology for SJS/TEN in all cases was not known in both studies. The details of these are mentioned in **Table 1**. In patients with SOC in the chronic phase with CM-related SJS/TEN, an association has been noted between HLA-A*02:06 in Japanese and Koreans, HLA-B*44:03 in Indian, Brazilian Caucasians, Thai, and Japanese populations, and HLA-C*07:01 in the Indian and Thai population (Ueta, 2015; Jongkhajornpong et al., 2018). Also, a significant genome-wide association between CM-related SJS/TEN and *IKZF1* SNPs (single nucleotide polymorphisms) were noted in the Japanese, Korean, Indian, and Thai populations with severe mucosal involvement (SMI), suggesting that IKZF1 might be a potential marker for susceptibility to CM-related SJS/TEN with SMI (Ueta et al., 2015; Chantaren et al., 2019). The genotypes of the associated SNP in the *IKZF1* gene reflected a quantitative difference in the ratio of transcripts of the gene produced by alternative splicing (Ueta et al., 2015).

However, preemptive genotyping before prescribing cold medications may not be feasible as these are commonly prescribed

TABLE 1 | Descriptive information of the studies on genetic markers for Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN) in the Asian Indian population.

S.no	Author	Country	Allele studied	Number of Indian patients with SJS/TEN with allele	Number of controls with allele	Drug	Reported OR, 95% CI, *p* value
1	Mehta et al., 2009	Gujarat, Northwest India	HLA-B*15:02	6/8	0/10 normal controls	CBZ	71.40, 3.0–1,698, 0.0014
2	Chang et al., 2011	Malaysia (multi-ethnic population with Indians, predominantly South Indian)	HLA-B*15:02	2/2	47/300 normal controls from Malaysian population	CBZ	NA
3	Aggarwal et al., 2014	Chandigarh, North India	HLA-B*15:02	2/9 (CBZ) 0/8 (Phenytoin)	50 tolerant controls (0/37 on CBZ, 0/13 on phenytoin)	CBZ, PHT	NA, NA, 0.035
4	Khor et al., 2014	Malaysia (multi-ethnic population with Indians, predominantly South Indian)	HLA-B*15:02	2/5	52 Indian CBZ-tolerant controls	CBZ	16.7, 1.7–163, 0.0349
5	Ueta et al., 2014	India	HLA-A*02:06 HLA-B*44:03	1/20 12/20	3/55 normal controls 6/55 normal controls	Cold medicine¶ (multi-ingredient cold medications and NSAIDs)	0.91, 0.09–9.06, 0.939; 10.88, 4.04–29.3, 1.87. E-07
6	Ramanujam et al., 2016	New Delhi, North India	HLA-B*15:02	1/2	25 LEV-tolerant controls	LEV	NA
7	Khor et al., 2017	Malaysia (multi-ethnic population with Indians, predominantly South Indian)	HLA-B*15:02 HLA-A*31:01	2/6 (HLA-B*15:02) 3/6 (HLA-A*31:01)	2/57 (HLA-B*15:02) 5/57 (HLA-A*31:01) All 57 Indian CBZ-tolerant controls	CBZ	13.8, 1.51–124.99, 0.04; 10.4, 1.64–65.79, 0.023
8	Srivastava et al., 2017	India	HLA-B*15:02	1/3	NA	Lamotrigine	NA
9	Kannabiran et al., 2017	India	HLA-A*33:03 HLA-B*44:03 HLA-C*07:01	37/80 (HLA-A*33:03) 50/80 (HLA-B*44:03) 47/80 (HLA-C*07:01)	10/50 (HLA-A*33:03) 6/50 (HLA-B*44:03) 9/50 (HLA-C*07:01) All 50 normal controls	Cold medicine‡	2.8, 1.4–5.5, 2.9.E-02; 10.1, 4.4–23.1, 1.3.E-09; 6.2, 3.0–12.7, 6.9.E-07
10	Devi, 2018	Kerala, South India	HLA-B*15:02	1/4 (CBZ), 0/8 (phenytoin)	11 tolerant controls (0/3 on CBZ, 0/18 on phenytoin)	CBZ, PHT	NA
11	Ihtisham et al., 2019	New Delhi, North India	HLA-B*57:01 HLA-DRB1*07:01	2/5 in CBZ-induced SJS/TEN cases (HLA-B*57:01)3/5 in CBZ-induced SJS/TEN cases (HLA-DRB1*07:01)	4/70 CBZ-tolerant positive for HLA-B*57:01; 12/70 CBZ-tolerant controls positive for HLA-DRB1*07:01	CBZ	11.00, 1.41–85.81, 0.05; 7.25, 1.09–48.18, 0.01

HLA, human leucocyte antigen; CBZ, carbamazepine; PHT, phenytoin; LEV, levetiracetam; NSAIDs, non-steroidal anti-inflammatory drugs; and NA, not available.

¶The exact drug etiology for SJS/TEN was not known in all patients; specific phenotype of patients with severe ocular complications in the chronic phase of SJS/TEN were studied.

‡The exact drug etiology for SJS/TEN were known in 28.8% (23/80) patients; specific phenotype of patients with severe ocular complications in the chronic phase of SJS/TEN were studied.

drugs and are available over-the-counter (Tangamornsuksan et al., 2020). Ascribing the cause of SJS/TEN to cold medications is problematic due to protopathic bias, where NSAIDs may be given to patients for the prodromal symptoms, which occur when SJS/TEN has already set in but is yet to evolve into a full-blown disease (Horwitz and Feinstein, 1980; Roujeau et al., 2018). Hence, the ALDEN (assessment of drug causality for epidermal necrolysis) algorithm comes into play here, where strict guidelines are followed to find out if a certain drug caused SJS/TEN so as not to create a situation, where drugs that might not have caused SJS/TEN are labeled so and have to be avoided (Sassolas et al., 2010). Strict definitions are required for labeling the day of disease-onset (Kelly et al., 1995). With AED's, there is credible evidence that they are known to cause SJS/TEN (Roujeau et al., 1995; Mockenhaupt et al., 2008). However, the same amount of evidence for cold-medications, such as salicylates, ibuprofen, and acetaminophen, causing SJS/TEN does not exist (Mockenhaupt et al., 2008; Lebrun-Vignes et al., 2018). Also, most studies that have found a genetic association between CM-related SJS/TEN and HLA type have been in the population of SJS/TEN patients in the chronic phase (Ueta, 2015), which predisposes these studies to a recall bias. Using the ALDEN algorithm in the chronic phase may not be accurate unless rigorous documentation is available. Thus, it may be difficult to conclude that NSAIDs were the primary reason for SJS/TEN, especially in patients

on multiple medications. Roujeau et al. (2018) suggested that it is possible that idiopathic SJS/TEN or SJS/TEN caused due to infections such as *Mycoplasma pneumoniae* could be labeled CM-related SJS/TEN if the ALDEN algorithm is not rigorously followed.

In a systematic review from India, NSAIDs were found to be responsible in 16% cases, cold-medications among these constituted 55% of total NSAIDs causing SJS/TEN (Patel et al., 2013). However, no drug causality algorithms were used to deduce this information. Also, self-medication with NSAIDs is common due to the availability of these over-the-counter (OTC; Doomra and Goyal, 2020). In a recent study from India, NSAIDs were found to be the second most common causative factor for SCAR (Thakkar et al., 2017). However, the information regarding causative drugs, especially NSAIDs in this study was not available in one-fourth cases due to use of OTC medications and absence of documentation. Hence, preemptive genotyping for these drugs may not be practical.

DRAWBACKS OF PREEMPTIVE GENOTYPING BASED ON HLA ASSOCIATIONS

One possible drawback of preemptive genotyping is highlighted by Chen et al. (2016) in a study, where they evaluated the cost-effectiveness of pharmacogenetic screening. They noted that HLA-B*15:02 screening policy in Hong Kong has not been cost-effective due to a shift in prescription from CBZ to alternate AED's, an increase in SJS/TEN caused by phenytoin intake post a policy to implement screening, poor adherence to the policy (Chen et al., 2014), an unwillingness of clinicians to wait for the screening results before prescribing alternative AED's causing an increase in expenditure by screening but this not being translated to immediate benefits of screening. The unwillingness to wait for the screening test's result was due to a long-turnaround time for the result and the need for an additional consultation to be scheduled. Clinicians preferred prescribing phenytoin as no genetic screening was required, this led to an increase in SJS/TEN caused by phenytoin, such that the overall burden of AED-induced SJS/TEN was unchanged (Chen et al., 2014). Chen et al. (2016) suggested that the cost-effectiveness of implementing this screening test may be improved by enhancing policy adherence by clinicians, making clinicians aware of SJS/TEN caused by other AED's, and by less expensive rapid point-of-care genotyping. Full genotyping may be expensive and specific allele typing may be more practical and cost-effective. Testing for specific HLA alleles, including HLA-B*15:02 should be made easily accessible and economical. At present, the expected cost of single HLA genotyping in India is approximately 80–100 USD with a turnaround time of 2–3 weeks, making this test expensive combined with a long-waiting time to decide if the drug can be prescribed.

Using an alternative, safer AED without the need to performing genotyping is another form of reducing costs (Locharernkul et al., 2011). However, this requires more research on safety profiles of different AED's. Although prevention of SJS/TEN will benefit a patient with high-risk of developing it, it is not clear whether the additional cost of screening will be covered by insurers, employers or the national health care systems (Locharernkul et al., 2011).

Also, HLA genotype may not be the only predictive factor for the development of SJS/TEN. Other than HLA genotype, factors such as initial drug dosing and renal function tests could also impact the risk of drug-induced SJS/TEN (Stamp et al., 2012; Ramasamy et al., 2013; Chung et al., 2015). For example, for allopurinol-induced SCAR, HLA-B*58:01 allele is not absolutely necessary or sufficient to explain the disease. The positive predictive value is estimated to be 2.7%, implying that other risk factors may be involved in the pathogenesis (Lonjou et al., 2008; Tassaneeyakul et al., 2009; Chung et al., 2015). Hence, in conjunction with HLA genotyping, further investigations are required to explain the role of HLA in predicting the development of SJS/TEN.

DIRECTIONS FOR FUTURE RESEARCH

Further research is required in finding the true incidence rates of SJS/TEN in India, preferably *via* a registry-based approach. The most common causative drugs that cause SJS/TEN in India need to be ascertained nation-wide. Prescribing patterns of these drugs need to be studied to be able to quantify the risk of SJS/TEN with the use of such medications. Physicians should be made aware of the pharmacogenomics of SJS/TEN and availability of preemptive genotyping. Physicians should also be made aware of CPIC guidelines for appropriate therapeutic usage of drugs that commonly cause SJS/TEN, when genotyping results are available.

Genotyping for specific HLA associations could be made more accessible, less expensive, with rapid results.

More studies need to be conducted in the normal population in various communities across the country in order to ascertain the prevalence of certain HLA alleles implicated in the development of SJS/TEN. Next, studies need to be conducted, preferably *via* a multi-centric approach in patients with SJS/TEN after exposure to a certain drug, to find if an HLA association exists. However, such studies first need to establish drug causality stringently. Once these factors are taken into consideration, certain policy recommendations can be instituted. Prevention of SJS/TEN may be possible by the integration of an effective pharmacovigilance system into routine health care.

CONCLUSIONS

Although SJS/TEN is considered as a rare disease, the burden of the disease is great with high degrees of morbidity and mortality with severe long-term sequelae in survivors affecting

multiple organ systems. These create a substantial economic burden for the patient as well as the caregivers. Further research on primary prevention of this dreaded disease is necessary.

AUTHOR CONTRIBUTIONS

SS: concept and design of study. SS and MK: literature search and interpretation of data. SS, MK, CK, PD, VS, and SB: drafting the article or revising it critically for important intellectual content and final approval of the version to be published. All authors contributed to the article and approved the submitted version.

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Meta-Analysis of ABCG2 and ABCB1 Polymorphisms With Sunitinib-Induced Toxicity and Efficacy in Renal Cell Carcinoma

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Background: ABCG2 and ABCB1 are genes related to the pharmacokinetics of sunitinib and have been associated with its toxicity and efficacy. However, the results have been controversial. This study aimed to evaluate the associations of ABCG2 and ABCB1 polymorphisms with sunitinib-induced toxicity and efficacy in renal cell carcinoma (RCC) by meta-analysis.

Methods: *PubMed, EMBASE, Cochrane Library*, and *Web of Science* were systematically searched for studies investigating the associations of the ABCG2 rs2231142 polymorphism with sunitinib-induced toxicity and the associations of the ABCB1 rs1128503 and ABCB1 rs2032582 polymorphisms with sunitinib-induced toxicity and clinical outcomes. The associations were evaluated by effect size (ES) with 95% confidence intervals (CIs).

Results: Eight and five studies were included in the toxicity and efficacy analysis, respectively, including a total of 1081 RCC patients. The ABCG2 rs2231142 A allele was associated with an increased risk of sunitinib-induced thrombocytopenia and hand-foot syndrome (HFS) in Asians (ES = 1.65, 95% CI = 1.15–2.36, $p = 0.006$; ES = 1.52, 95% CI = 1.02–2.27, $p = 0.041$). However, the ABCG2 rs2231142 polymorphism was not associated with sunitinib-induced hypertension or neutropenia (ES = 1.09, 95% CI = 0.69–1.73, $p = 0.701$; ES = 0.87, 95% CI = 0.57–1.31, $p = 0.501$). Compared with the C allele, the ABCB1 rs1128503 T allele was associated with a decreased risk of sunitinib-induced hypertension but worse progression-free survival (PFS) (ES = 0.44, 95% CI = 0.26–0.77, $p = 0.004$; ES = 1.36, 95% CI = 1.07–1.73, $p = 0.011$). There was no significant association between the T allele or C allele of ABCB1 rs1128503 and overall survival (OS) (ES = 0.82, 95% CI = 0.61–1.10, $p = 0.184$). The ABCB1 rs2032582 T allele was associated with worse PFS than the other alleles (ES = 1.46, 95% CI = 1.14–1.87, $p = 0.003$), while there was no significant association between the T allele or other alleles and sunitinib-induced hypertension, HFS, or OS (ES = 0.77, 95% CI = 0.46–1.29, $p = 0.326$; ES = 1.02, 95% CI = 0.65–1.62, $p = 0.919$; ES = 1.32, 95% CI = 0.85–2.05, $p = 0.215$).

Conclusion: The results indicate that the ABCG2 rs2231142 polymorphism may serve as a predictor of sunitinib-induced thrombocytopenia and HFS in Asians, while the ABCB1

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rs1128503 polymorphism may serve as a predictor of sunitinib-induced hypertension, and both the ABCB1 rs1128503 and rs2032582 polymorphisms may serve as predictors of PFS in RCC. These results suggest a possible application of individualized use of sunitinib according to the genetic background of patients.

Keywords: renal cell carcinoma, sunitinib, ABCG2, ABCB1, polymorphism, meta-analysis

INTRODUCTION

Renal cell carcinoma (RCC) is a common cancer with high malignancy, and 20–30% of RCC patients already suffer from metastatic lesions at the time of diagnosis (Basso et al., 2010; Sun et al., 2011). Sunitinib is a first-generation tyrosine kinase inhibitor (TKI) that was approved for the treatment of metastatic RCC (mRCC) in 2006 (Diekstra et al., 2016). In patients with mRCC, sunitinib has been associated with improvements in progression-free survival (PFS) and overall survival (OS) compared with interferon-alpha (Motzer et al., 2009), and it achieves significant response rates in both Western and Japanese patients (Motzer et al., 2006; Tomita et al., 2010). The Metastatic Renal Cell Carcinoma database Consortium International (IMDC) model is the most widely used prognostic models for the prognosis of mRCC in clinical practice and clinical trials (Rini et al., 2018). Currently, sunitinib remains the first-line standard of care for IMDC favorable-risk patients, and for IMDC intermediate- and poor-risk patients, immune checkpoint inhibition, sequencing, and combined systemic therapy have been reported to have an OS benefit (de Velasco et al., 2019; Loo et al., 2019; Stuhler et al., 2020). Increased exposure to sunitinib is associated with improved PFS and OS but also an increased risk for adverse events (Houk et al., 2009; Motzer et al., 2009). Sunitinib-associated toxicities include thrombocytopenia, neutropenia, leucopenia, hand-foot syndrome (HFS), hypertension, mucositis, and diarrhea (Kalantari, 2009; Escudier et al., 2014; Atkins et al., 2015). The therapeutic efficacy and toxicity of sunitinib are very heterogeneous and have been difficult to predict before treatment initiation. Single-nucleotide polymorphisms (SNPs) in genes encoding metabolism enzymes or transporters related to the pharmacokinetics (PK) and pharmacodynamics (PD) of sunitinib have been identified to be associated with the toxicity and efficacy of sunitinib in previous studies (Garcia-Donas et al., 2015; Rodriguez-Antona and Taron, 2015), especially SNPs in genes related to the PK pathways of sunitinib in patients with RCC (Junker et al., 2013; Fiszer and Krzyzosiak, 2014; Narjoz et al., 2015).

Sunitinib is a substrate of ATP-binding cassette member B1 (ABCB1) and another efflux transporter encoded by ATP-binding cassette member G2 (ABCG2) (Low et al., 2016). ABCG2 and ABCB1 are the PK-related genes of sunitinib (Watanabe et al., 2017). The most common functional SNP in ABCG2 was reported to be rs2231142 (421C/A), and those in ABCB1 were rs2032582 (2677G/TA), rs1128503 (1236T/C), and rs1045642 (3435C/T) (Kato et al., 2017; Watanabe et al., 2017; Reustle et al., 2018). ABCG2 rs2231142 was located at Q141K, ABCB1 rs1045642 at | 1154 | , ABCB1 rs1128503 at G412G, and ABCB1 rs2032582 at A893 S/T. The associations of ABCB1 and ABCG2 polymorphisms with sunitinib-induced toxicity and clinical outcomes in patients with RCC have been investigated. However, the associations were controversial. ABCG2 rs2231142 was reported to have no association with thrombocytopenia in Whites and Asians (van Erp et al., 2009; Kato et al., 2017; Zhang et al., 2018). However, in other studies, ABCG2 rs2231142 was associated with severe thrombocytopenia (Low et al., 2016), and patients carrying the ABCG2 rs2231142 AA genotype were more likely to develop thrombocytopenia, neutropenia, and HFS even after adjustment (Kim et al., 2013). Chu et al. reported that the ABCG2 rs2231142 A allele was correlated with a 3-fold decrease in the risk of neutropenia (Chu et al., 2015). Garcia-Donas et al. reported that ABCG2 rs2231142 was not associated with hypertension but seemed to confer protection against HFS, while ABCB1 rs1128503 and rs2032582 seemed to confer protection against hypertension (Garcia-Donas et al., 2011). The ABCB1 rs2032582 TT, AT, and GT genotypes were reported to be significantly correlated with grade 2 and grade 3 HFS (Zhang et al., 2018). However, de Velasco et al. reported that ABCB1 rs2032582 was not associated with sunitinib-induced toxicity (de Velasco et al., 2016). Beuselinck et al. reported that both the PFS and OS of patients with mRCC were significantly associated with SNP rs1128503 in ABCB1 but were not associated with ABCB1 rs2032582 (Beuselinck et al., 2013), while ABCB1 rs1128503 and rs2032582 were not associated with PFS or OS in patients with mRCC in another study (Garcia-Donas et al., 2011).

Identifying the effects of ABCG2 and ABCB1 polymorphisms on sunitinib-induced toxicity and efficacy in patients with RCC could help to optimize the therapeutic management strategy and maximize the clinical benefits of sunitinib. Here we used a meta-analysis to evaluate the associations of ABCG2 rs2231142, ABCB1 rs1128503, and ABCB1 rs2032582 polymorphisms with sunitinib-induced toxicity and those of ABCB1 rs1128503 and ABCB1 rs2032582 polymorphisms with PFS and OS in patients with RCC.

METHODS

Search Strategy and Selection Criteria

PubMed, EMBASE, Cochrane Library, and *Web of Science* were systematically searched using the keywords "sunitinib" and "polymorphisms" to identify eligible studies. Only articles in English were included. The last search was updated on 7 February 2020. We also retrieved eligible studies in the references of all relevant articles. Furthermore, we contact the authors to request missing information to strengthen the analysis.

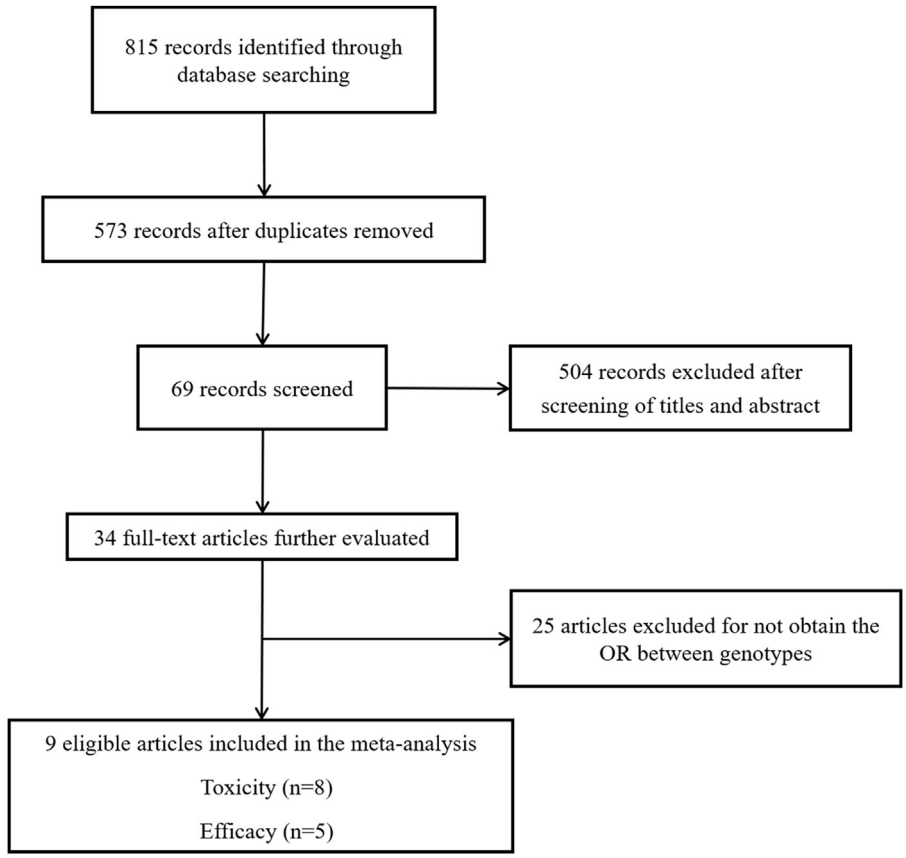

FIGURE 1 | Flow diagram of the study selection process.

The inclusion criteria for studies were as follows: 1) studies of RCC patients who received sunitinib treatment; 2) studies evaluating the associations of ABCG2 rs2231142 polymorphism with sunitinib-induced thrombocytopenia, HFS, and hypertension; 3) studies evaluating the associations of ABCB1 rs1128503 and rs2032582 polymorphisms with sunitinib-induced hypertension and HFS; 4) studies evaluating the associations of ABCB1 rs1128503 and rs2032582 polymorphisms with PFS and OS; and 5) studies with available odds ratios (ORs) between genotypes.

The exclusion criteria for studies were as follows: 1) letters, reviews, and case reports; 2) nonclinical studies; and 3) studies that were duplicate publications. In addition, we pooled complete data in the meta-analysis if multiple studies had overlapping data.

Data Extraction

Two authors independently selected the relevant articles and then extracted the following data from the articles: first author's name, publication year, study design, sample size, treatment regimen, age, sex, region/ethnicity, ABCG2 rs2231142, ABCB1 rs1128503 and ABCB1 rs2032582 phenotype, genotyping methods, evidence of Hardy-Weinberg equilibrium in cases, adverse events and clinical outcome in ABCG2 rs2231142, ABCB1 rs1128503 and ABCB1 rs2032582 phenotypes, and the ORs of ABCG2 rs2231142 A allele vs. C allele, ABCB1 rs1128503 T allele vs. C allele, and ABCB1 rs2032582 T allele vs. other alleles. If there was any controversy, it was resolved by discussion among the authors.

Statistical Analysis

STATA software (version 12.0; Stata Corp, College Station, TX, USA) was used to perform the statistical tests. The associations of ABCG2 rs2231142, ABCB1 rs1128503, and ABCB1 rs2032582 polymorphisms with sunitinib-induced toxicity, and ABCB1 rs1128503 and ABCB1 rs2032582 polymorphisms with PFS and OS were evaluated by effect size (ES) with 95% CIs (Cheng et al., 2020). The heterogeneity between studies was assessed by the chi-square-based Q-test and I^2 tests. Both no-heterogeneity criteria were required to be met ($p > 0.05$ AND $I^2 < 50\%$) to use the fixed-effects model; otherwise, the random-effects model was used to calculate the pooled ES. The Z test was used to investigate the significance of the ES, and $P_Z < 0.05$ was considered statistically significant. The stability of the results was evaluated by sensitivity analysis. The publication bias among studies was determined by Egger's and Begg's tests, and $P_E < 0.05$ was considered significant.

TABLE 1 | Characteristics of studies included in the meta-analysis.

Study	Year	Study design	Sample size	Treatment regimen	Age (year)	Male (%)	Region/ ethnicity	ABCG2 rs2231142 phenotype (CC/CA/AA)	ABCB1 rs1128503 (TT/CT/CC)	ABCB1 rs2032582 (GG/GT ± GA/TT+AT)	Genotyping method	Hardy-Weinberg equilibrium
Diekstra et al. (2015)	2015	Retrospective	330	Sunitinib 50, 37.5, or 25 mg/day	61 (55,69)	69	White 321, black 5, Asian 3, Latin American 1, Arab 3	-	-	-	KASPar SNP	Yes
Chu et al. (2015)	2015	Retrospective	97	Sunitinib 50 or 37.5 mg/day	58 (18–79)	77.3	Chinese 86, Malay 7, Indian 4	50/38/7	35/49/9	25/44/27	PCR	Yes
Garcia-Donas et al. (2011)	2011	Prospective	95	Sunitinib 50, 37.5, or 25 mg/day	65 (42–87, 56–73)	68	Caucasian	85/10/0	36/45/14	38/39/15	KASPar SNP genotyping system	Yes
Kim et al. (2013)	2013	Retrospective	65	Sunitinib 50 mg/day	59 (36–81)	78.5	Korean	-	-	-	TaqMan SNP genotyping assays	Yes
Low et al. (2016)	2016	Prospective	219	Sunitinib	63 (82–83)	73.5	Japanese	-	-	-	PCR	-
Numakura et al. (2017)	2017	Prospective	70	Sunitinib 50 or 37.5 mg/day	64 (28–85)	79	Japanese	CC 33, CA ± AA 31	CT ± TT 52, CC 12	GG 27, others 37	PCR-restriction fragment length polymorphism method	Yes
de velasco et al. (2016)	2016	Prospective	159	Sunitinib	61 (53–67)	72	Caucasian	-	-	50/71/32	iPlex gold platform	Yes
Zhang et al. (2018)	2018	Prospective	53	Sunitinib 50, 37.5, or 25 mg/day	52 (45–61)	75.47	Chinese	19/24/10	27/23/3	10/26/17	LifePro thermal cycler	Yes
Beuselinck et al. (2013)	2013	Retrospective	88	Sunitinib	59 (38–84)	68	Caucasian 83/ unknown 5	-	38/35/15	32/36/12	High-throughput SNP genotyping	-

"-"no description.

RESULTS

Study Selection

The study selection process is shown in **Figure 1**. We initially retrieved 815 articles from electronic databases, including *PubMed, EMBASE, Science Direct*, and *Web of Science*. We included 34 relevant studies after removing the duplicates and reviewing the titles, abstracts, and full texts. Then, 25 articles were excluded because they did not determine the ORs between genotypes. Ultimately, 8 articles published between 2011 and 2018 assessing the relationships of ABCG2 rs2231142, ABCB1 rs1128503, and ABCB1 rs2032582 polymorphisms with sunitinib-induced toxicity, and 5 articles published between 2011 and 2017 assessing the relationships of ABCB1 rs1128503 and ABCB1 rs2032582 polymorphisms with PFS and OS were included in the current meta-analysis (Garcia-Donas et al., 2011; Beuselinck et al., 2013; Kim et al., 2013; Diekstra et al., 2015; Chu et al., 2015; de Velasco et al., 2016; Low et al., 2016; Numakura et al., 2017; Zhang et al., 2018).

Characteristics of the Included Studies

The main characteristics of all eligible studies are shown in **Tables 1–3**. In total, 9 studies including 1081 patients were enrolled in the pooled analysis. The total sample size is the sum of the sample in each study except for Garcia-Donas et al. study, because patients in the study by Diekstra et al. were pooled from 5 studies including study of Garcia-Donas et al., and we only used data from Garcia-Donas et al. when those from Diekstra et al. did not indicate the specific toxicity or efficacy. The included studies are all observational. Five studies were prospective cohort studies, and four studies were retrospective cohort studies. Five studies (55.6%) reported on Asian individuals, and 4 (44.4%) studies reported mainly on Caucasian individuals. Sunitinib was given as monotherapy in the included studies. The variant allele A frequency of ABCG2 rs2231142 in Asians was significantly higher than that in Caucasians (34.1% vs. 5.3%), the variant allele C frequency of ABCB1 rs1128503 in the two different ethnic groups was similar (37.6% vs. 31.1%), and the variant AT genotype frequency of ABCB1 rs2032582 in Asians was a little higher than that in Caucasians (29.9% vs. 16.5%) in the included studies. The selected results contained the necessary information according to the STrengthening the REporting of Genetic Association Studies (STREGA) (Little et al., 2009).

Relationship Between the ABCG2 rs2231142 Polymorphism and Toxicity

Thinking the consistency of the overall results, we initially analyzed the associations of the ABCG2 polymorphisms with sunitinib-induced toxicities without regard to ethnic background. The meta-analysis results are shown in **Table 4**. There was no heterogeneity between studies assessing the relationships of the ABCG2 rs2231142 polymorphism with sunitinib-induced thrombocytopenia ($I^2 = 0$, $p > 0.05$), and the fixed-effects model was applied to the analysis. There was heterogeneity between studies assessing the relationships of ABCG2 rs2231142 polymorphism with sunitinib-induced hypertension,

TABLE 2 | Data for studies included in the meta-analysis of sunitinib-induced toxicities.

Study	Year	Thrombocytopenia (OR, 95% CI)	Hypertension (OR, 95% CI)			Hand-foot syndrome (OR, 95% CI)		Neutropenia (OR, 95% CI)
		ABCG2 rs2231142 (A allele vs. C allele)	ABCG2 rs2231142 (A allele vs. C allele)	ABCB1 rs1128503 (T allele vs. C allele)	ABCB1 rs2032582 (T allele vs. other alleles)	ABCG2 rs2231142 (A allele vs. C allele)	ABCB1 rs2032582 (T allele vs. other alleles)	ABCG2 rs2231142 (A allele vs. C allele)
Diekstra et al. (2015)	2015	-	0.03 (0.001, 0.85)	-	-	-	-	-
Chu et al. (2015)	2015	-	-	-	-	-	-	0.3 (0.1, 0.9)
Garcia-Donas et al. (2011)	2011	-	-	0.41 (0.20, 0.81)	0.42 (0.21, 0.84)	0.11 (0.01, 0.92)	1.00 (0.55, 1.82)	-
Kim et al. (2013)	2013	1.74 (0.81, 3.75)	-	-	-	6.76 (2.16, 21.13)	1.11 (0.37, 3.28)	1.89 (0.76, 4.75)
Low et al. (2016)	2016	1.856 (1.172, 2.939)	1.23 (0.713, 2.124)	-	-	1.24 (0.776, 1.98)	-	0.856 (0.512, 1.431)
Numakura et al. (2017)	2017	0.73 (0.26, 2.05)	0.66 (0.24, 1.80)	0.63 (0.18, 2.21)	5.37 (1.06, 9.52)	1.08 (0.36, 4.73)	3.17 (1.02, 14.61)	-
de velasco et al. (2016)	2016	-	-	-	0.57 (0.13, 2.46)	-	-	-
Zhang et al. (2018)	2018	2.3 (0.4, 12.0)	4.4 (0.7, 26.5)	0.4 (0.1, 1.4)	0.5 (0.1, 2.0)	1.4 (0.2, 7.9)	0.3 (0.1, 1.6)	-

"-"no description.

TABLE 3 | Data for studies included in the meta-analysis of sunitinib efficacy.

Study	Year	Progression-free survival (HR, 95% CI)		Overall survival (HR, 95% CI)	
		ABCB1 rs1128503 (T allele vs. C allele)	ABCB1 rs2032582 (T allele vs. other alleles)	ABCB1 rs1128503 (T allele vs. C allele)	ABCB1 rs2032582 (T allele vs. other alleles)
Diekstra et al. (2015)	2015	1.42 (1.07, 1.90)	1.36 (1.03, 1.81)	0.66 (0.46, 0.94)	-
Garcia-Donas et al. (2011)	2011	-	-	-	1.47 (0.85, 2.54)
Numakura et al. (2017)	2017	5.8 (0.87, 7.51)	2.03 (0.74, 2.57)	0.36 (0.31, 7.63)	0.33 (0.29, 3.74)
Chu et al. (2015)	2015	1.7 (0.9, 3.2)	1.5 (0.5, 4.7)	1.7 (1.0, 3.1)	2.0 (0.8, 5.0)
Beuselinck et al. (2013)	2013	0.464 (0.234, 0.918)	-	0.415 (0.193, 0.894)	-

"-"no description.

HFS, and neutropenia (I^2 = 61.4%, 68.3%, and 68.6%, respectively), and the random-effects model was applied to the analysis. Based on the results, we found that compared with the C allele, the ABCG2 rs2231142 A allele was significantly associated with an increased risk of sunitinib-induced thrombocytopenia in Asians (ES = 1.65, 95% CI = 1.15–2.36, p = 0.006; **Figure 2**), while there were no significant associations between the A allele and C allele in sunitinib-induced hypertension, HFS, or neutropenia (ES = 1.09, 95% CI = 0.69–1.73, p = 0.701; ES = 1.40, 95% CI = 0.94–2.08, p = 0.094; ES = 0.87, 95% CI = 0.57–1.31, p = 0.501; **Figure 2**). Since the patients included in the meta-analysis of thrombocytopenia and neutropenia were all Asian (**Table 2**), we only conducted a subgroup analysis of HFS and hypertension in Asians. Compared with the C allele, the ABCG2 rs2231142 A allele was significantly associated with an increased risk of sunitinib-induced HFS (ES = 1.52, 95% CI = 1.02–2.27, p = 0.041; **Figure 3**). There was also no significant associations between the A allele and C allele in sunitinib-induced hypertension in Asians (ES = 1.17, 95% CI = 0.74–1.86, p = 0.504; **Figure 3**).

Correlations of the ABCB1 rs1128503 Polymorphism With Toxicity and Clinical Outcomes

We only performed the meta-analysis of rs1128503 in ABCB1 in mixed ethnicity as the variant allele C frequency in Asians and Caucasians was similar. There was no heterogeneity between studies assessing the associations of the ABCB1 rs1128503 polymorphism with sunitinib-induced hypertension (I^2 = 0, p > 0.05), and the fixed-effects model was applied to the analysis. There was heterogeneity between studies assessing the relationship of ABCB1 rs1128503 polymorphism with sunitinib-induced PFS and OS (I^2 = 82.4%, 68.3%, p < 0.05), and the random-effects model was applied to the analysis. The results showed that compared with the C allele, the ABCB1 rs1128503 T allele was significantly associated with a decreased risk of sunitinib-induced hypertension and worse PFS (ES = 0.44, 95% CI = 0.26–0.77, p = 0.004; ES = 1.36, 95% CI = 1.07–1.73, p = 0.011; **Figure 4**). There was no significant association between the T allele and C allele in sunitinib-induced OS (ES = 0.82, 95% CI = 0.61–1.10, p = 0.184; **Figure 4**).

TABLE 4 | Summary of the meta-analysis and publication bias.

Allele	No. of studies	Heterogeneity P value	I^2 value (%) for heterogeneity test	Model	ES (95%CI)	P Value	Z	P Value for Egger's (Begg's) bias test
ABCG2 rs2231142 (A allele vs. C allele)								
Hand-foot syndrome	5	0.013	68.3	R	1.40 (0.94, 2.08)	0.094	1.67	0.737 (1.000)
Hand-foot syndrome (Asian)	4	0.055	60.4	R	1.52 (1.02, 2.27)	0.041	2.04	-
Thrombocytopenia (Asian)	4	0.421	0	F	1.65 (1.15, 2.36)	0.006	2.72	0674 (1.000)
Hypertension	4	0.051	61.4	R	1.09 (0.69, 1.73)	0.701	0.38	-
Hypertension (Asian)	3	0.191	39.7	F	1.17 (0.74, 1.86)	0.504	0.67	-
Neutropenia (Asian)	3	0.041	68.6	R	0.87 (0.57, 1.31)	0.501	0.67	-
ABCB1 rs1128503 (T allele vs. C allele)								
Hypertension	3	0.830	0	F	0.44 (0.26, 0.77)	0.004	2.87	-
PFS	4	0.001	82.4	R	1.36 (1.07, 1,73)	0.011	2.54	0.155 (0.296)
OS	4	0.024	68.3	R	0.82 (0.61, 1.10)	0.184	1.33	-
ABCB1 rs2032582 (T allele vs. other alleles)								
Hypertension	4	0.001	80.6	R	0.77 (0.46, 1.29)	0.326	0.98	-
Hand-foot syndrome	4	0.121	48.4	F	1.02 (0.65, 1.62)	0.919	0.10	-
PFS	3	0.516	0	F	1.46 (1.14, 1.87)	0.003	2.95	0.536 (1.000)
OS	3	0.065	63.3	R	1.32 (0.85, 2.05)	0.215	1.24	-

"-" not available.

Correlations of the ABCB1 rs2032582 Polymorphism With Toxicity and Clinical Outcomes

There was heterogeneity between studies assessing the associations of the ABCB1 rs2032582 polymorphism with sunitinib-induced hypertension and OS (I^2 = 80.6%, 63.3%), and the random-effects model was applied to the analysis. There was no heterogeneity between studies assessing the relationship of the ABCB1 rs2032582 polymorphism with sunitinib-induced HFS or PFS (I^2 = 48.4%, 0, $p > 0.05$), and the fixed-effects model was applied to the analysis. The results showed that compared with the other alleles, the ABCB1 rs2032582 T allele was significantly associated with worse PFS (ES = 1.46, 95% CI = 1.14–1.87, $p = 0.003$; **Figure 5**). In contrast, there was no significant association between the T allele and other alleles in sunitinib-induced hypertension, HFS, or OS (ES = 0.77, 95% CI = 0.46–1.29, $p = 0.326$; ES = 1.02, 95% CI = 0.65–1.62, $p = 0.919$; ES = 1.32, 95% CI = 0.85–2.05, $p = 0.215$; **Figure 5**).

Sensitivity Analysis and Publication Bias

The sensitivity analysis in the meta-analysis of the relationships of the ABCG2 rs2231142 polymorphism with sunitinib-induced thrombocytopenia and neutropenia showed that no single study qualitatively altered the pooled ES, however, the study by Diekstra et al. may qualitatively altered the result of hypertension and that by Garcia-Donas et al. may qualitatively altered the result of HFS (**Figure 2**). When we omitted the article, the meta-analysis results for HFS changed but not the hypertension results, and no single study qualitatively altered the pooled ES in either analysis, which provided evidence of the stability of the meta-analysis (**Figure 3**). There was no publication bias for thrombocytopenia and HFS ($p > 0.05$, **Table 4**).

The sensitivity analysis in the meta-analysis of the relationships of the ABCB1 rs1128503 polymorphism with sunitinib-induced hypertension showed that no single study qualitatively altered the pooled ES; however, the study by Beuselinck et al. may qualitatively altered the result of PFS and that by Diekstra et al. may qualitatively altered the result of OS (**Figure 4**). When we omitted the article, the meta-analysis results for PFS (ES = 1.58, 95% CI = 1.23–2.04, $p < 0.001$) and OS (ES = 1.27, 95% CI = 0.77–2.11, $p = 0.353$) were not changed, and no single study qualitatively altered the pooled ES in either analysis (**Figure 6**).

The sensitivity analysis in the meta-analysis of the relationships of the ABCB1 rs2032582 polymorphism with sunitinib-induced hypertension, HFS, PFS, and OS showed that no single study qualitatively altered the pooled ES (**Figure 5**). There was no publication bias for PFS in ABCB1 rs1128503, or ABCB1 rs2032582 polymorphisms ($p > 0.05$, **Table 4**).

DISCUSSION

In the current meta-analysis, we initially analyzed the associations of the ABCG2 rs2231142 polymorphism with sunitinib-induced thrombocytopenia, HFS, hypertension, and neutropenia; the associations of the ABCB1 rs1128503 polymorphism with sunitinib-induced hypertension, PFS, and OS; and the associations of ABCB1 rs2032582 polymorphisms with sunitinib-induced hypertension, HFS, PFS, and OS, without regard to ethnic background. However, we found heterogeneity between studies based on the results. We further considered the origin of the heterogeneity, and we found that the heterogeneity might derive from ethnicity as the variant allele A frequency of ABCG2 rs2231142 in Asians was significantly higher than that in Caucasians, then we conducted a subgroup analysis of hypertension and HFS in Asians. Next, we did the sensitivity analysis of all meta-analysis to evaluate their stability. The results showed that one article with population Caucasians altered the

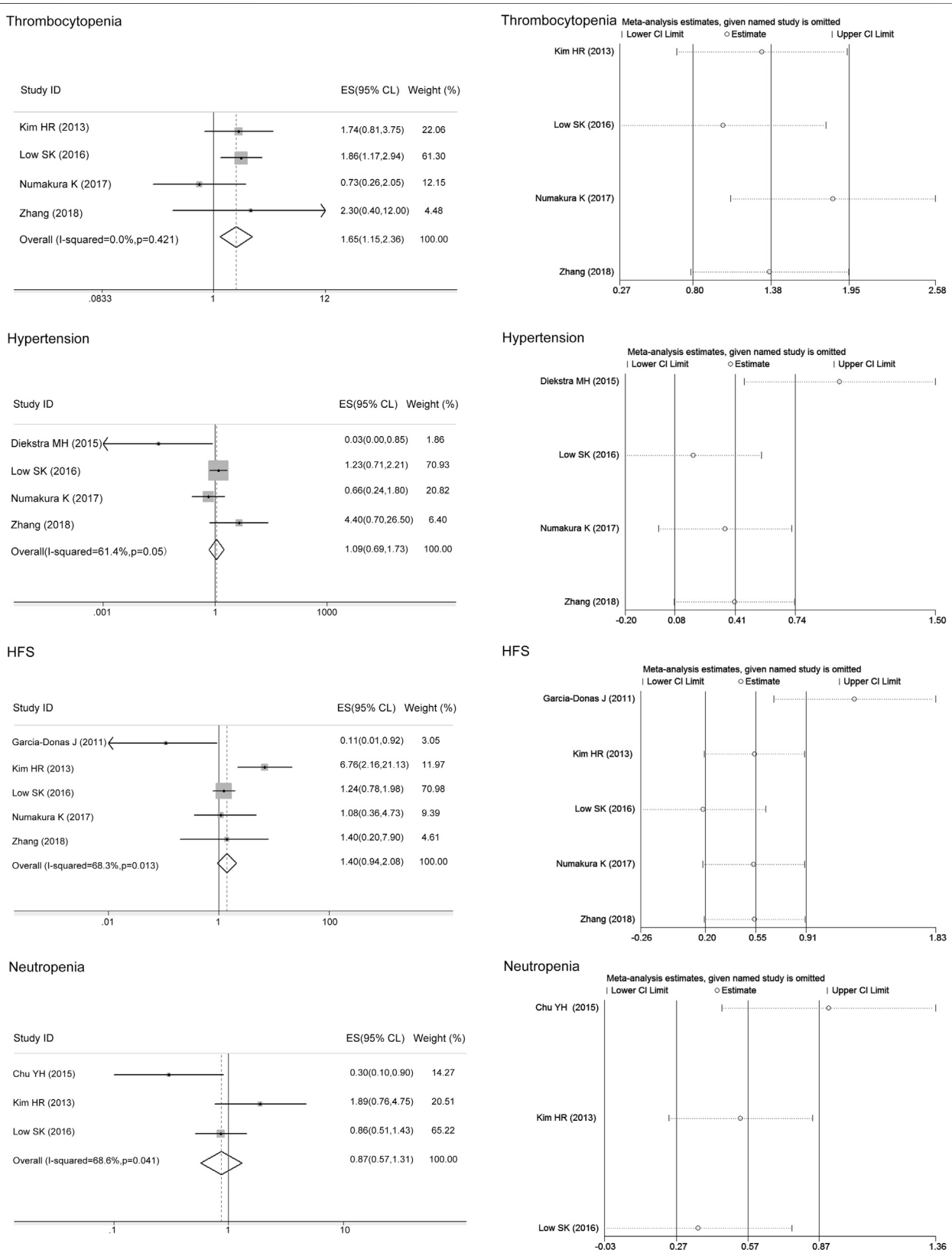

FIGURE 2 | Forest plots estimating the associations of ABCG2 rs2231142 polymorphism with sunitinib-induced toxicity and the corresponding sensitivity analysis (A allele vs. C allele). The ABCG2 rs2231142 A allele was significantly associated with an increased risk of thrombocytopenia in Asians compared with the C allele, while there were no significant associations between the A allele and C allele in hypertension, HFS, or neutropenia. The sensitivity analysis showed that no single study qualitatively altered the pooled ES of thrombocytopenia and neutropenia; however, the study by Diekstra et al. altered the hypertension results, and the study by Garcia-Donas et al. altered the HFS results.

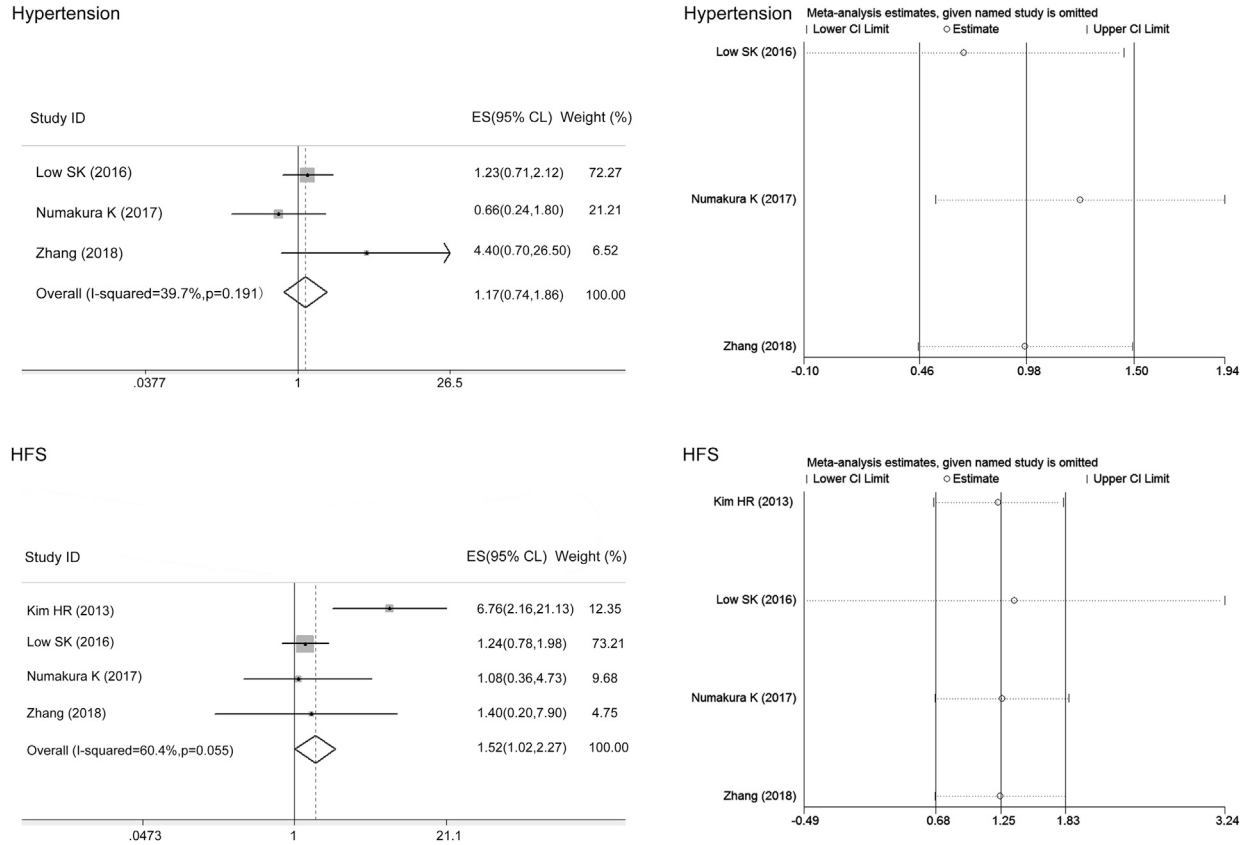

FIGURE 3 | Forest plots estimating the associations of the ABCG2 rs2231142 polymorphism with sunitinib-induced toxicities in Asians and the corresponding sensitivity analysis (A allele vs. C allele). Compared with the C allele, the ABCG2 rs2231142 A allele was significantly associated with an increased risk of HFS in Asians. There were no significant associations between the A allele and C allele in hypertension in this population. The sensitivity analysis showed that no single study qualitatively altered the pooled ES.

results of HFS in ABCG2, which indicated that the heterogeneity might derive from ethnicity. For ABCB1 rs1128503 and ABCB1 rs2032582 results, there were no article qualitatively altered the results. The sensitivity analysis results provided evidence of the stability of the meta-analysis.

The therapeutic efficacy of sunitinib is heterogeneous among different IMDC risk groups (Rini et al., 2018). In studies included in the meta-analysis, they did not mention the IMDC stratification, and we could not analyze the effect of IMDC on toxicity and clinical outcomes. The treatment regimen of sunitinib in the included articles was not completely consistent, and five studies had three starting dose (50 or 37.5 or ≤37.5, mg/day) (Garcia-Donas et al., 2011; Diekstra et al., 2015; Chu et al., 2015; Numakura et al., 2017; Zhang et al., 2018). In Chu et al. study, the starting dose (50 or 37.5 or ≤37.5, mg/day) was not associated with toxicities, including leukopenia, neutropenia, or diarrhea but was associated with PFS and OS (Chu et al., 2015), and we used the results of multivariate analysis in the meta-analysis, which showed no significant association. Zhang et al. study showed no significant association between toxicity of grade <3 or ≥3 and each dosage (25, 37.5, and 50 mg/d) (Zhang et al., 2018). The effect of dose on toxicities and clinical outcome were not mentioned in the other three studies (Garcia-Donas et al., 2011; Diekstra et al., 2015; Numakura et al., 2017). RCC is more common in men. However, the included studies did not investigate the differences between these genetic polymorphisms in different genders. In the included studies, Chu et al. reported that the gender was not associated with the leucopenia, neutropenia, diarrhea, PFS or OS (Chu et al., 2015). In Diekstra et al. study, gender seemed to show association with thrombocytopenia and toxicity grade >2 in univariate analysis, however, there was no association in the multivariate analysis (Diekstra et al., 2015). Zhang et al. showed that gender was associated with hypertension in univariate analysis, but the results of multivariate analysis showed no significant association (Zhang et al., 2018). In Low et al. study, gender was only associated with leucopenia (Low et al., 2016), which was not included in the meta-analysis. In summary, we used ORs of multivariate analysis in the pooled ES analysis, excluding other possible factors affecting toxicity and efficacy of sunitinib, such as gender, age, sunitinib dosage, laboratory indexes, and other related gene polymorphisms in each included study, which strengthened our conclusions.

Compared with non-Asians, Asians are more likely to develop sunitinib-induced adverse effects (Kim et al., 2011; Lee et al., 2014). The incidence of sunitinib-induced hematotoxicity in Japanese

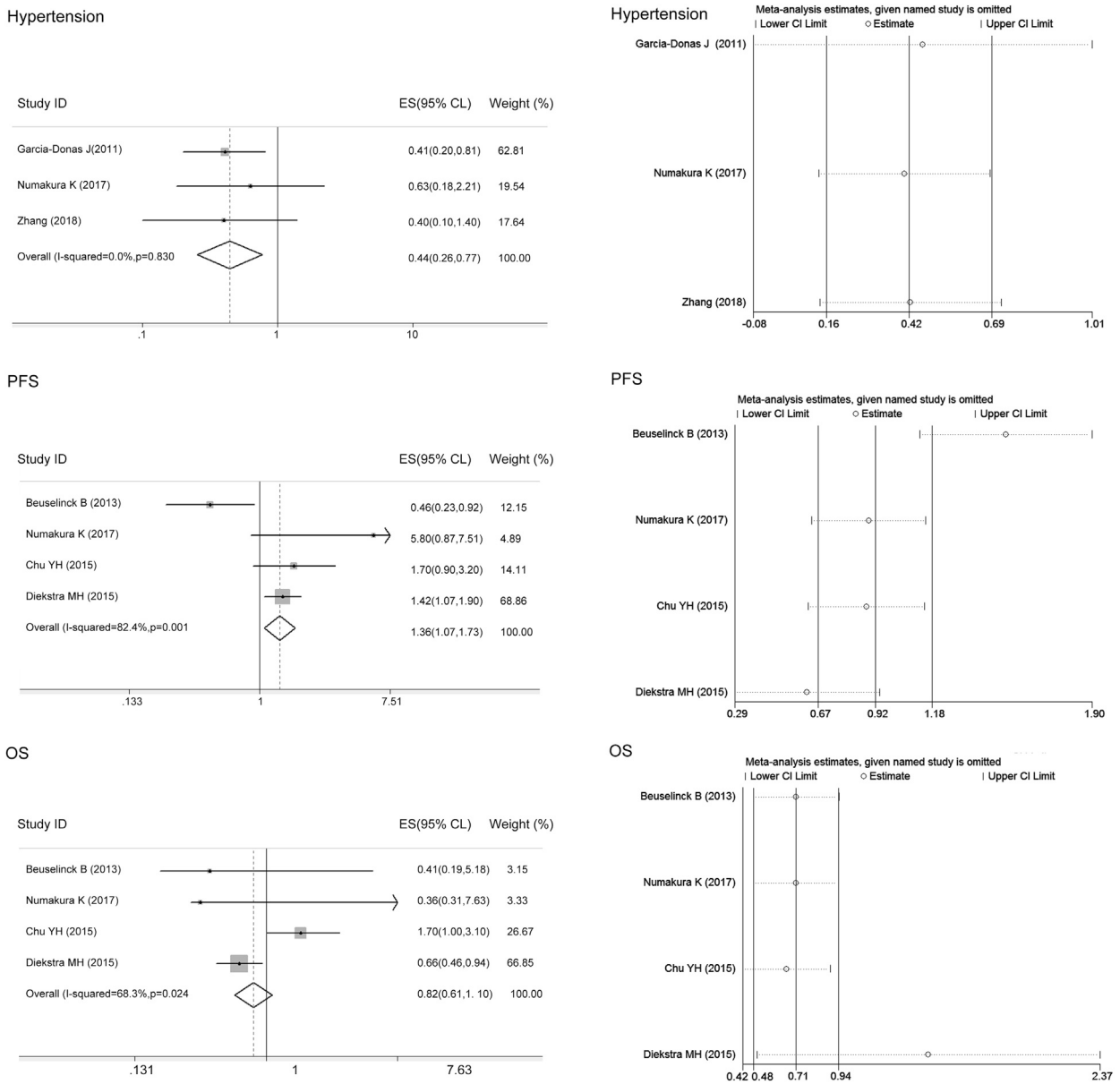

FIGURE 4 | Forest plots estimating the associations of the ABCB1 rs1128503 polymorphism with sunitinib-induced hypertension, progression-free survival (PFS), and overall survival (OS), and the corresponding sensitivity analysis (T allele vs. C allele). Compared with the C allele, the ABCB1 rs1128503 T allele was significantly associated with a decreased risk of hypertension and worse PFS. There was no significant association between the T allele and C allele in OS. The sensitivity analysis showed that no single study qualitatively altered the pooled ES of hypertension; however, the study by Beuselinck et al. altered the PFS results, and the study by Diekstra et al. altered the OS results.

patients with RCC is higher when compared to European populations (Low et al., 2016; Kato et al., 2017). The AA genotype of rs2231142 in ABCG2 has been associated with an increase in systemic exposure to sunitinib, possibly causing sunitinib-related adverse events. In the current meta-analysis, we found that compared with the C allele, the ABCG2 rs2231142 A allele was significantly associated with an increased risk of sunitinib-induced thrombocytopenia in Asians. However, we could not find associations of ABCG2 rs2231142 polymorphisms with hypertension and neutropenia in the meta-analysis.

Garcia-Donas et al. reported that the A allele of ABCG2 rs2231142 seemed to confer protection against HFS in Caucasians (Garcia-Donas et al., 2011), while in the other four studies carried out in Asians, an increased risk of development of HFS was found (Kim et al., 2013; Low et al., 2016; Numakura et al., 2017; Zhang et al., 2018). In our results, there was no significant association between the A allele and C allele in sunitinib-induced HFS. However, when we conducted a subgroup analysis in Asians, we found that compared with the C allele, the ABCG2 rs2231142 A allele was significantly associated with an increased risk of sunitinib-induced HFS. A possible reason for this

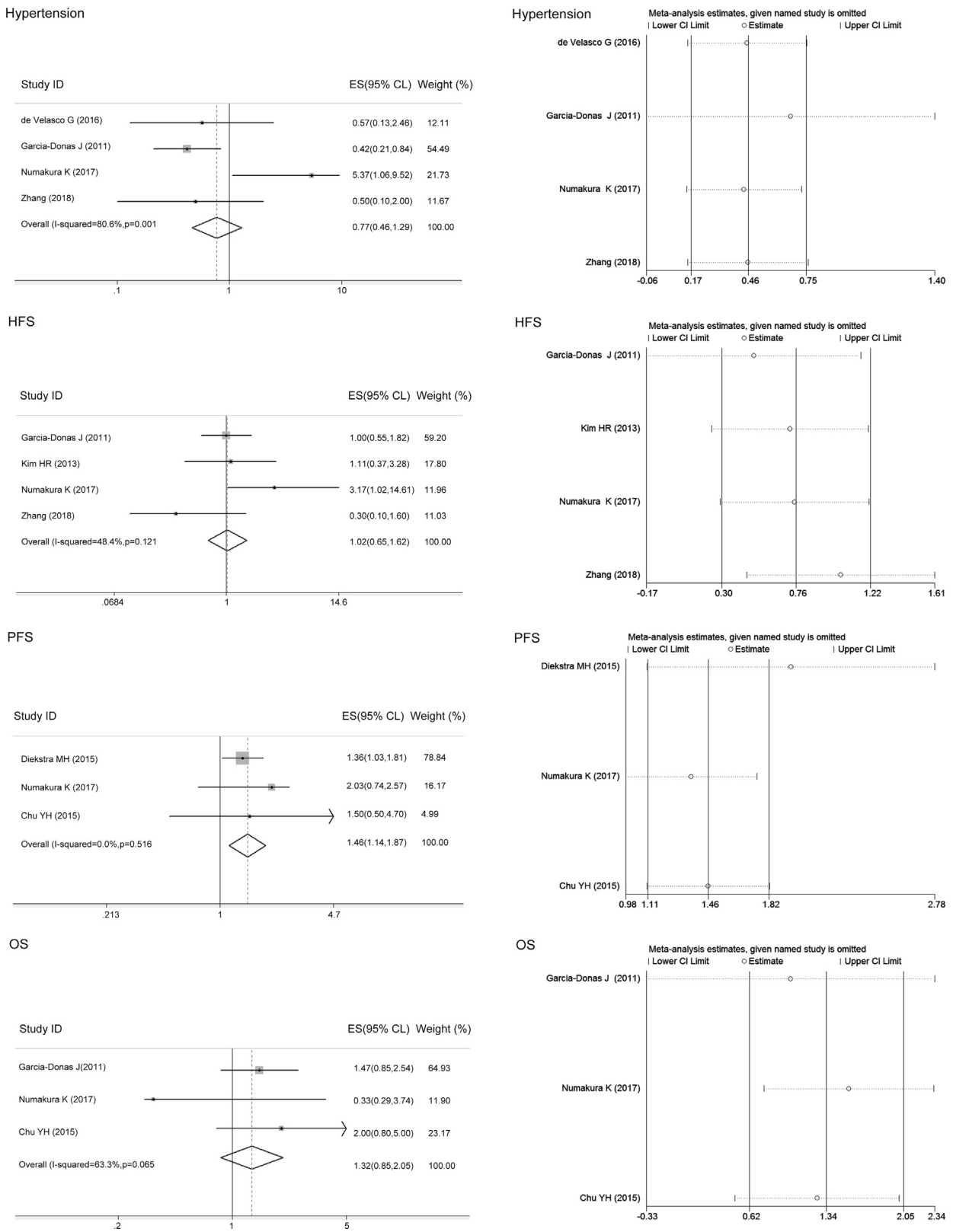

FIGURE 5 | Forest plots estimating the associations of the ABCB1 rs2032582 polymorphism with sunitinib-induced hypertension, hand-foot syndrome (HFS), progression-free survival (PFS), and overall survival (OS), and the corresponding sensitivity analysis (T allele vs. other alleles). Compared with the other alleles, the ABCB1 rs2032582 T allele was significantly associated with worse PFS. In contrast, there was no significant association between the T allele and other alleles in sunitinib-induced hypertension, HFS, or OS. The sensitivity analysis showed that no single study qualitatively altered the pooled ES.

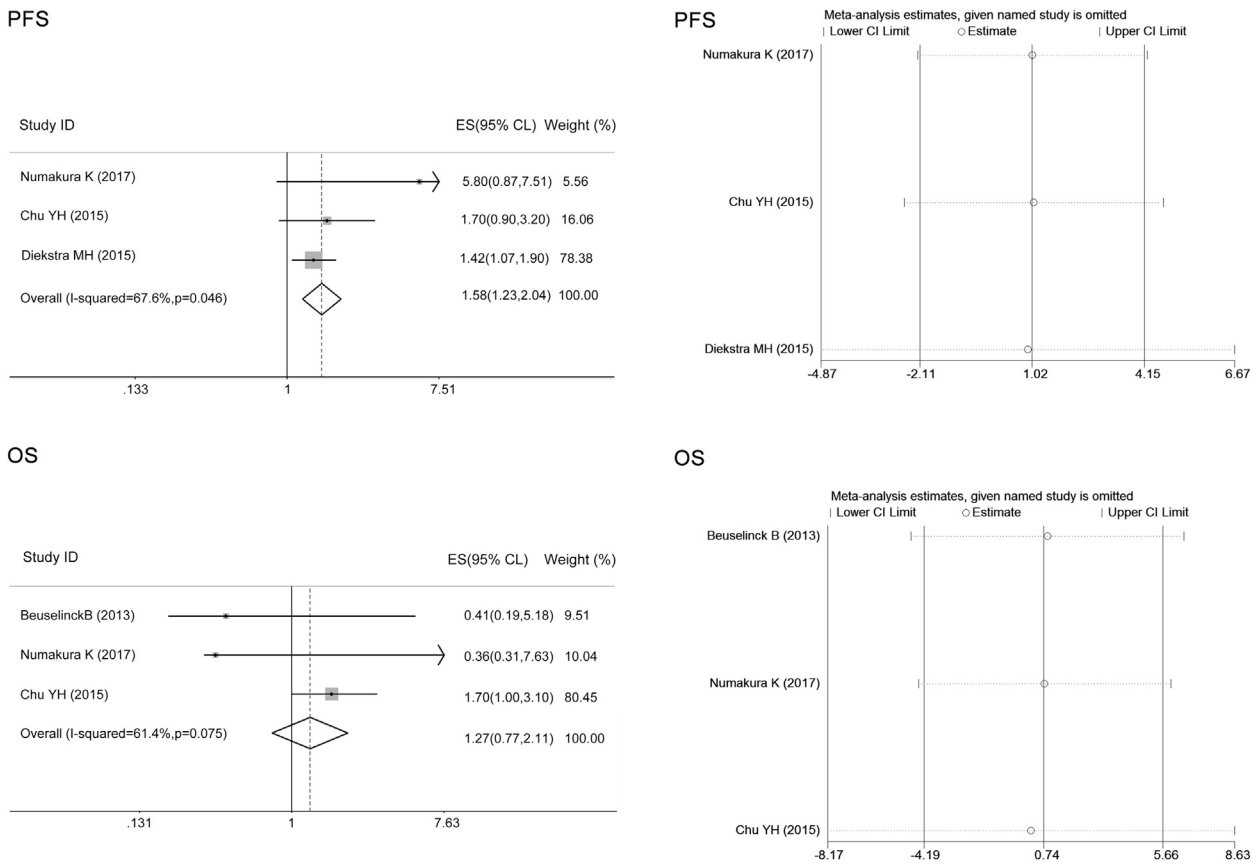

FIGURE 6 | Forest plots estimating the associations of ABCB1 rs1128503 polymorphism with sunitinib-induced progression-free survival (PFS) and overall survival (OS) when we omitted the article that altered the pooled ES (T allele vs. C allele). The ABCB1 rs1128503 polymorphism was associated with PFS but not OS, and no single study qualitatively altered the pooled ES in both analysis.

difference might be the small number of patients with homozygous alleles among Caucasians. The ABCG2 rs2231142 A allele is located within the ATP-binding cassette domain that regulates the ATP binding activity of ABCG2 protein, which reduces ATPase activity (Mizuarai et al., 2004), induces the reduction of transport activity, increases drug accumulation, decreases the efflux velocity of the drug (Imai et al., 2002), and thus increases the systemic exposure to sunitinib and is subsequently more likely to lead to the development of toxicity (Mizuno et al., 2012). It has been reported that the variant allele A of ABCG2 rs2231142 in Asians is approximately 30%, in Caucasians is approximately 10%, and in African Americans is approximately 5% (Diekstra et al., 2016). Diekstra et al. reported that the ABCG2 rs2231142 polymorphism was significantly associated with sunitinib-induced dose reduction after cycle 1, 2, or 3 (Diekstra et al., 2015). Although other previous studies have reported that the ABCG2 rs2231142 polymorphism is not significantly associated with toxicity-related dose reduction (Garcia-Donas et al., 2011; Kato et al., 2017; Zhang et al., 2018) or time to dose reduction in mRCC patients (Numakura et al., 2017), further studies should verify whether dose adjustment based on early onset thrombocytopenia and HFS in Asians prolongs sunitinib treatment.

Patients carrying the variant genotypes of rs1128503 and rs2032582 in ABCB1 showed an increased clearance of sunitinib and its active metabolite SU12662, consequently leading to lower exposure to sunitinib, reduced hypertension, and decreases in PFS and OS (Garcia-Donas et al., 2011; Beuselinck et al., 2014; Diekstra et al., 2014; Teo et al., 2015). In the current meta-analysis, we found that compared with the C allele, the ABCB1 rs1128503 T allele was significantly associated with a decreased risk of sunitinib-induced hypertension but worse PFS in Asians and Caucasians; and the ABCB1 rs2032582 T allele was significantly associated with worse PFS compared with other alleles, which was consistent with previous studies (Diekstra et al., 2015; Chu et al., 2015; Numakura et al., 2017). However, there was no significant association between the ABCB1 rs2032582 T and G alleles in sunitinib-induced hypertension, HFS, or OS. Four included studies also investigated the association of haplotype ABCB1 (rs1128503, rs2032582, rs1045642) with sunitinib induced toxicities and clinical outcome. In Kim et al. study, there were no significant differences among CGC, TTT, and TGC haplotype of ABCB1 (rs1128503, rs2032582, rs1045642) in thrombocytopenia, neutropenia, anemia, or HFS (Kim et al., 2013). Diekstra et al. reported that PFS and OS were improved in the presence of CGT in haplotype ABCB1 (rs1128503, rs2032582, rs1045642) (Diekstra et al., 2015), however, there was no significant

difference in median PFS between the present haplotype and absent haplotype in another study (Beuselinck et al., 2013). In Chu et al. study, ABCB1 rs1045642, 1236 rs1128503, rs2032582 TTT haplotype was correlated with a 10-fold (p = 0.03) decrease in the risk of neutropenia and inferior PFS and OS (Chu et al., 2015). We should also pay attention to patients carrying the opposite haplotype.

SNPs in sunitinib target candidate genes, including vascular endothelial growth factor receptors (VEGFRs) 1, 2, and 3; Fms-like tyrosine kinase 3 receptor (FLT3); and PK-related genes, including cytochrome P450 1A1 (CYP1A1), CYP3A5, NR1/2, and NR1/3, have also been reported to be associated with sunitinib-induced toxicity and efficacy in patients with mRCC (van Erp et al., 2009; Garcia-Donas et al., 2011; van der Veldt et al., 2011; Eechoute et al., 2012; Kim et al., 2012; Beuselinck et al., 2013; Beuselinck et al., 2014; Kato et al., 2017; Watanabe et al., 2017). SNPs rs2010963 and rs2070744 in VEGF were reported to be associated with increased chances for the occurrence and duration of hypertension (Eechoute et al., 2012; Kim et al., 2012). The FLT3 738 T/C polymorphism and the CYP3A5 A allele (CYP3A5*1) of rs776746 were reported to be associated with dose reduction due to sunitinib-induced toxicity (Garcia-Donas et al., 2011; Kato et al., 2017). Van Erp et al. observed an increased risk for leukopenia in CYP1A1 rs1048943 and FLT3 rs1933437 and an absence of CAG in the NR1/3 haplotype (rs2307424, rs2307418, rs4073054); CYP1A1 rs1048943 was associated with mucosal inflammation; and EGFR2 rs2305948 was associated with any toxicity > grade 2 (van Erp et al., 2009). Watanabe A et al. reported that the STAT3 polymorphism contributes to a risk factor for stomatitis (Watanabe et al., 2017). The rs9582036, rs9582036, and rs9554320 in VEGF-R1; rs2981582 and rs2305948 in VEGF-R2; rs307826 and rs307821 in VEGF-R3; and rs3025039 in VEGF-A were reported to be associated with PFS and OS in mRCC patients (Garcia-Donas et al., 2011; Kim et al., 2012; Beuselinck et al., 2013; Beuselinck et al., 2014). Compared with the disadvantageous genetic profile carriers, carriers of the genetic profile (at least an A allele in CYP3A5, a TCG copy in ABCB1, or a missing CAT copy in NR1/3) showed improved PFS and OS (van der Veldt et al., 2011). SNP rs2276707 in NR1/2 and SNPs rs2307424 and rs4073054 in NR1/3 were also reported to be associated with PFS and OS in patients with mRCC (Beuselinck et al., 2013).

We did not include rs1045642 in ABCB1 in the meta-analysis because we could not obtain the ORs between the genotypes or because the included article numbers were below three. ABCG2 polymorphisms have also been reported to be associated with other sunitinib-induced toxicity in mRCC patients, such as hypothyroidism (Werbrouck et al., 2018). The T alleles of ABCB1 rs1128503 and ABCB1 rs2032582 were reported to be correlated with decreases in the risk of neutropenia and diarrhea, respectively (Chu et al., 2015). We did not include all sunitinib-induced toxicities or SNPs associated with sunitinib-induced toxicity and efficacy for the above reason, or there was no controversy.

To our knowledge, the present study is the first meta-analysis to investigate the associations of ABCB1 and ABCG2 polymorphisms with sunitinib-induced toxicity and efficacy in patients with RCC. Based on our results, compared with the C allele, the ABCG2 rs2231142 allele A was significantly associated with increased risks of sunitinib-induced thrombocytopenia and HFS in Asians, while the T alleles of ABCB1 rs1128503 and ABCB1 rs2032582 were significantly associated with worse PFS than the other alleles. The results indicate that the ABCG2 rs2231142 polymorphism may serve as a predictor of sunitinib-induced thrombocytopenia and HFS in Asians, while the ABCB1 rs1128503 polymorphism may serve as a predictor of sunitinib-induced hypertension, and both the ABCB1 rs1128503 and rs2032582 polymorphisms may serve as predictors of PFS in RCC. Although our analysis showed no significant relationships of ABCB1 and ABCG2 polymorphisms with other sunitinib-induced severe toxicity or OS, we should also pay more attention to the use of sunitinib in Asian patients. The results may support the possible application of individualized use of sunitinib according to the genetic background of patients with RCC. Genotyping for ABCG2 rs2231142, ABCB1 rs1128503 and rs2032582 polymorphism could become a clinical routine practice to select the appropriate dose to decrease the risk of sunitinib-induced thrombocytopenia and HFS in Asians while ensuring efficacy. The population pharmacokinetic model based on the genotypes should be established to predict the occurrence of sunitinib-induced toxicities and the efficacy.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

LC and FS designed the study, performed the literature search and screening, performed the data analysis, and drafted the manuscript. ZC performed the literature search and screening and extracted the literature data. PY and ZL assisted in extracting the literature data. BW assisted in the figure design. All authors approved the final version of the manuscript.

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Association Between Variants in Calcineurin Inhibitor Pharmacokinetic and Pharmacodynamic Genes and Renal Dysfunction in Adult Heart Transplant Recipients

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Background: The goal of the study was to assess the relationship between single nucleotide variants (SNVs) in calcineurin inhibitor (CNI) pharmacokinetic and pharmacodynamic genes and renal dysfunction in adult heart transplant (HTx) recipients.

Methods: This retrospective analysis included $N = 192$ patients receiving a CNI at 1-year post-HTx. Using a candidate gene approach, 93 SNVs in eight pharmacokinetic and 35 pharmacodynamic genes were chosen for investigation. The primary outcome was renal dysfunction 1-year after HTx, defined as an estimated glomerular filtration rate (eGFR) <45 ml/min/1.73m^2.

Results: Renal dysfunction was present in 28.6% of patients 1-year after HTx. Two SNVs [transforming growth factor beta 1 (*TGFB1*) rs4803455 C > A and phospholipase C beta 1 (*PLCB1*) rs170549 G > A] were significantly associated with renal dysfunction after accounting for a false discovery rate (FDR) of 20%. In a multiple-SNV adjusted model, variant A allele carriers of *TGFB1* rs4803455 had lower odds of renal dysfunction compared to C/C homozygotes [odds ratio (OR) 0.28, 95% CI 0.12–0.62; $p = 0.002$], whereas *PLCB1* rs170549 variant A allele carriers had higher odds of the primary outcome vs. patients with the G/G genotype (OR 2.66, 95% CI 1.21–5.84, $p = 0.015$).

Conclusion: Our data suggest that genetic variation in *TGFB1* and *PLCB1* may contribute to the occurrence of renal dysfunction in HTx recipients receiving CNIs. Pharmacogenetic markers, such as *TGFB1* rs4803455 and *PLCB1* rs170549, could help identify patients at increased risk of CNI-associated renal dysfunction following HTx, potentially allowing clinicians to provide more precise and personalized care to this population.

Keywords: renal dysfunction, estimated glomerular filtration rate, heart transplant, pharmacogenetics, pharmacogenomics, calcineurin inhibitor, single nucleotide variant

INTRODUCTION

Renal dysfunction is a major challenge following heart transplantation, with over 50% of patients experiencing some degree of renal impairment at 1-year post-transplant (Hamour et al., 2009; Navarro-Manchon et al., 2010). Renal dysfunction is associated with significant morbidity and a 4-fold increase in the risk of mortality in heart transplant recipients (van Gelder et al., 1998; Ojo et al., 2003; Alba et al., 2016; Kolsrud et al., 2018). The development of renal dysfunction is multifactorial, with contributing factors such as older age at transplant, other comorbidities (e.g., hypertension and diabetes mellitus), and long-term use of nephrotoxic drugs such as calcineurin inhibitors (CNIs; Wilkinson and Cohen, 1999; Ojo, 2007).

Calcineurin inhibitors remain the cornerstone of maintenance immunosuppressant therapy following heart transplantation, with 98% of patients receiving a CNI, most commonly tacrolimus, at 1-year post-transplant (Khush et al., 2019). Although excellent at preventing rejection, CNIs are associated with the development of chronic renal dysfunction, the exact mechanism of which is unknown. Importantly, systemic CNI exposure is not predictive of renal impairment, as renal dysfunction often occurs despite CNI trough blood levels being within goal range (van Gelder et al., 1998; Wilkinson and Cohen, 1999).

The field of pharmacogenetics seeks to characterize the impact of genetic variation, including single nucleotide variants (SNVs), on drug disposition, response, and adverse effects (Roden et al., 2019). To date, only two pharmacogenetic studies have comprehensively investigated the association between SNVs and renal function in heart transplant recipients; however, CNI prescribing patterns in those studies do not reflect contemporary clinical practice, with only 21–36% of patients on tacrolimus therapy (Lachance et al., 2012; Asleh et al., 2018). Given the deleterious consequences of renal dysfunction after heart transplant and the contribution of CNIs to its development, there is a critical need to identify novel, patient-specific factors that increase the risk of this adverse effect. As such, the goal of this study was to evaluate the association between SNVs in key CNI pharmacokinetic and pharmacodynamic genes and renal dysfunction in heart transplant recipients taking CNIs at 1-year post-transplant.

MATERIALS AND METHODS

Study Population

The data for this retrospective cohort study were obtained from heart transplant patients who had participated in a parent pharmacogenomic study at the University of Colorado. The overarching goal of the parent study was to assess the association between SNVs and renal dysfunction in patients receiving CNIs in the first 5 years post-heart transplant. Patients were included in the parent study if they had received a heart-only transplant, were at least 18 years old at the time of transplant, and were prescribed a CNI post-transplant. Patients were excluded if they underwent multi-organ transplant or did not provide informed consent. In total, 253 adult heart transplant recipients were enrolled in the parent study at routine clinic visits, where a mouthwash or blood sample was collected for genetic analysis. Demographic and clinical data were retrospectively collected from participants' medical records at the following time points: pre-transplant, transplant discharge, 6 months post-transplant, and annually from 1 to 5-years post-transplant. The parent study was approved by the Colorado Multiple Institutional Review Board and all participants provided written, informed consent.

Here, we describe analyses for the 1-year post-transplant time point. Parent study participants were included in these analyses if they received a CNI at the 1-year post-transplant clinic visit and had at least one estimated glomerular filtration rate (eGFR) measurement available in their medical records in the 3 months prior to transplant and at 1-year after transplantation (±2 months). Participants were excluded if they did not receive a CNI at the 1-year post-transplant clinic visit or if eGFR measurements were unavailable. Those transplanted at hospitals other than the University of Colorado were included if relevant clinical data were available in their medical records. The number of participants from the parent study who met eligibility criteria for the 1-year analysis was $N = 192$.

Study Outcomes

The primary outcome of the study was renal dysfunction (yes/no), defined as an eGFR <45 ml/min/1.73 m^2 [i.e., grade 3B or worse per the Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines] calculated using the Modification of Diet in Renal Disease (MDRD) formula, at 1-year post-transplant (National Kidney Foundation, 2002; Zalawadiya et al., 2017). The single eGFR measurement closest to the 1-year transplant anniversary (±2 months) was used to assess renal dysfunction. The secondary outcomes were the continuous measurements of eGFR 1-year after transplantation and change in eGFR from pre-transplant to 1-year post-transplant. CNI exposure was defined as being prescribed either cyclosporine or tacrolimus at the 1-year post-transplant clinic visit.

Genotyping, Quality Control, and Imputation

Genomic DNA was isolated from either buccal cells in mouthwash samples or leukocytes in whole blood using a commercially available kit (QIAamp® DNA Mini Kit; Qiagen, Valencia, CA, United States). Genotyping was performed on a customized version of the Illumina® Expanded Multi-Ethnic Global Array (CU-MEGA^{EX}; Infinium, 2015). The CU-MEGA^{EX} contains over 2 million genetic variants, including over 20,000 drug metabolism and excretion markers, and incorporates content from Phase 3 of the 1,000 Genomes Project, the Consortium on Asthma among African-ancestry Populations in the Americas (CAAPA), and Population Architecture using Genomics and Epidemiology (PAGE; Matise et al., 2011; 1000 Genomes Project Consortium, 2015; Mathias et al., 2016).

Genotype quality control was performed using PLINK v1.90 (Chang et al., 2015). Patients with array-wide call rates <90% were excluded from the analysis. Next, we selected a set of high-quality SNVs to determine heterozygosity, relatedness, and

principal components (PCs). High-quality SNVs were defined as having Hardy-Weinberg equilibrium values of $p > 1 \times 10^{-6}$, call rates >95%, and minor allele frequencies >1%. Patients with excessively high heterozygosity rates (i.e., >3 SD above the mean) and one sibling from a pair of related samples (defined as a pairwise identity by descent >0.185) were excluded from the analysis. Using the scree test, the top four PCs were selected for inclusion in adjusted models based on the "elbow" of the scree plot (**Supplementary Figure S1**). Variants not present on the CU-MEGA^{EX} were imputed using the Michigan Imputation server (Das et al., 2016). Imputation was performed using Minimac4, with the Haplotype Reference Consortium version r1.1 2016 serving as the reference panel (McCarthy et al., 2016). SNVs with imputation values of $r^2 > 0.6$, minor allele frequencies >5%, and Hardy-Weinberg equilibrium values of $p > 1 \times 10^{-6}$ in Americans of European ancestry were considered for analysis.

Following quality control and imputation procedures, we employed a candidate gene approach, whereby we selected SNVs in CNI pharmacokinetic and pharmacodynamic genes. Candidate genes were chosen based on the following criteria: (1) genes involved in CNI clinical pharmacology and renal pathophysiology; (2) genes previously associated with renal function in transplant populations; and (3) significant predictors of non-transplant chronic kidney disease in genome wide association studies (Baan et al., 2000; Lacha et al., 2001; van de Wetering et al., 2006; Kottgen et al., 2008; Smith et al., 2008; Jacobson et al., 2012; Lachance et al., 2012). Within these genes, we chose SNVs based on functional or clinical significance, annotation in pharmacogenomic databases,[1] and/or HapMap tagging variants. In total, 93 SNVs were evaluated – 22 SNVs in eight CNI pharmacokinetic genes and 71 SNVs in 35 CNI pharmacodynamic genes (**Supplementary Table S1**).

Statistical Analyses

Descriptive data are presented as mean ± SD or *n* (%). The Shapiro-Wilk test was used to assess for normality; non-normally distributed data were log-transformed prior to analysis and back-transformed for presentation. Relationships between demographic and clinical variables [i.e., age at transplant, sex, pre-transplant eGFR, pre-transplant renal dysfunction (eGFR <45 ml/min/1.73 m^2; yes/no), heart failure etiology [ischemic cardiomyopathy (CM) vs. other], pre-transplant left ventricular assist device (LVAD; yes/no), pre-transplant hypertension (yes/no), pre-transplant diabetes (yes/no), body mass index (BMI) 1-year post-transplant, transplant era, type of CNI used 1-year post-transplant (i.e., cyclosporine vs. tacrolimus), and angiotensin-converting enzyme (ACE) inhibitor or angiotensin II receptor blocker (ARB) prescribed 1-year post-transplant (yes/no)] and the primary and secondary outcomes were assessed using Pearson's correlation coefficients, with significant covariates ($p < 0.05$) included in the relevant adjusted analyses. Patients were divided into one of three transplant eras, as defined by the most recent International Society for Heart and Lung Transplantation annual report, based on their date of transplant (era 1, 1989–2001; era 2, 2002–2009; era 3, 2010–2016; Khush et al., 2019). To account for potential population stratification, the top four PCs were included in all adjusted analyses performed in the entire cohort. SNVs were analyzed as wild-type (WT) homozygotes vs. variant carriers using a dominant model.

For the primary outcome, univariate analyses were performed using Fisher's exact tests to identify SNVs associated with renal dysfunction (yes/no). SNVs that were significant after accounting for a false discovery rate (FDR) of 20% were included in a multiple-SNV logistic regression model, adjusting for pertinent covariates and the top four PCs. As exploratory analyses, SNVs suggestively associated with the primary outcome in the univariate analyses [unadjusted $p < 0.05$, but not significant after accounting for multiple comparisons (FDR of 20%)], were investigated in single-SNV logistic regression models, adjusting for relevant covariates and the top four PCs. Univariate analyses to assess the relationship between SNVs and each secondary outcome were performed using *t*-tests. Single-SNV analyses were carried out using linear models and included SNVs suggestively associated [i.e., unadjusted $p < 0.05$ but not significant following adjustment for multiple comparisons (FDR of 20%)] with the secondary outcomes plus relevant covariates and the top four PCs.

For all primary and secondary outcomes, SNV analyses including relevant covariates were also performed in non-Hispanic Americans of European ancestry to assess whether significant and suggestive SNVs remained associated with each outcome in this subgroup. Statistical analyses were performed using R version 3.5.2 (R Foundation for Statistical Computing, Vienna Austria).

RESULTS

Patient Characteristics

Of the 253 participants enrolled in the parent study, 192 participants met the inclusion and exclusion criteria for this 1-year post-transplant analysis. The most common reason participants from the parent study were excluded was the absence of an eGFR measurement prior to transplant ($n = 44$). Characteristics of the 192 participants are shown in **Table 1** and described as follows. Most participants were transplanted between May 1989 and August 2016 at the University of Colorado (96.4%). The cohort was primarily of European ancestry (79.2%) and male (77.1%), with a mean ± SD age at transplant of 49 ± 12 years. Tacrolimus (53.1%) was the most frequent CNI used 1-year post-transplant. Pre-transplant hypertension and pre-transplant renal dysfunction were present in 33.3 and 20.3% of patients, respectively. Less than one-third of participants (27.6%) had an LVAD prior to transplant. Approximately two-thirds of patients (63.5%) were prescribed an ACE inhibitor or an ARB 1-year post-transplant.

Renal Dysfunction at 1-Year Post-transplant

Renal dysfunction was present in 28.6% of patients at 1-year post-transplant. Demographic and clinical variables associated with renal dysfunction 1-year post-transplant were the presence

[1]www.pharmgkb.org

TABLE 1 | Participant demographic and clinical characteristics.

Characteristic	Entire cohort (*N* = 192)	No renal dysfunction at 1-year post-transplant (*N* = 137)	Renal dysfunction[a] at 1-year post-transplant (*N* = 55)	*p*
Sex (male)	148 (77.1%)	110 (80.3%)	38 (69.1%)	0.128
Age at transplant (years)	49 ± 12	47 ± 13	55 ± 9	<0.001
Race				0.222
European American	152 (79.2%)	106 (77.4%)	46 (83.6%)	
African American	17 (8.9%)	15 (10.9%)	2 (3.6%)	
Asian	7 (3.6%)	6 (4.4%)	1 (1.8%)	
Other[b]	16 (8.3%)	10 (7.3%)	6 (10.9%)	
Ethnicity				1.000
Non-hispanic	172 (89.6%)	123 (89.8%)	49 (89.1%)	
Hispanic	20 (10.4%)	14 (10.2%)	6 (10.9%)	
Reason for transplant				0.957
Nonischemic CM	91 (47.4%)	67 (48.9%)	24 (43.6%)	
Ischemic CM	64 (33.3%)	44 (32.1%)	20 (36.4%)	
Valvular CM	9 (4.7%)	6 (4.4%)	3 (5.5%)	
Mixed etiology	8 (4.2%)	6 (4.4%)	2 (3.6%)	
Congenital CM	8 (4.2%)	5 (3.6%)	3 (5.5%)	
Other	12 (6.3%)	9 (6.6%)	3 (5.5%)	
Pre-transplant hypertension	64 (33.3%)	47 (34.3%)	17 (30.9%)	0.736
Pre-transplant diabetes	25 (13.0%)	16 (11.7%)	9 (16.4%)	0.477
Pre-transplant eGFR (ml/min/1.73 m^2)	65 ± 27	71 ± 28	51 ± 16	<0.001
Pre-transplant renal dysfunction[a]	39 (20.3%)	21 (15.3%)	18 (32.7%)	0.010
Pre-transplant LVAD	53 (27.6%)	39 (28.5%)	14 (25.5%)	0.724
Body mass index (kg/m^2)[c]	27.0 ± 4.8	26.5 ± 4.6	28.1 ± 5.3	0.056
eGFR 1-year post-transplant (ml/min/1.73 m^2)	55 ± 20	63 ± 19	37 ± 6	<0.001
Change in eGFR (ml/min/1.73 m^2)[d]	−10 ± 21	−8 ± 23	−14 ± 15	0.037
Calcineurin inhibitor[c]				0.004
Tacrolimus	102 (53.1%)	82 (59.9%)	20 (36.4%)	
Cyclosporine	90 (46.9%)	55 (40.1%)	35 (63.6%)	
Tacrolimus total daily dose (mg/day)[c]	6.3 ± 4.8	6.5 ± 5.1	5.3 ± 3.2	0.291
Tacrolimus trough level (ng/ml)[c]	11.4 ± 3.8	11.7 ± 3.8	9.8 ± 3.6	0.019
Cyclosporine total daily dose (mg/day)[c]	278 ± 96	284 ± 92	269 ± 102	0.339
Cyclosporine trough level (ng/ml)[c]	238 ± 101	242 ± 109	232 ± 89	0.630
Antiproliferative agent[c]				0.011
Mycophenolate	153 (79.7%)	115 (83.9%)	38 (69.1%)	
Azathioprine	28 (14.6%)	14 (10.2%)	14 (25.5%)	
None	11 (5.7%)	8 (5.8%)	3 (5.5%)	
ACE inhibitor/ARB[c]	122 (63.5%)	88 (64.2%)	34 (61.8%)	0.744
Transplant era				0.031
Era 1: 1989–2001	60 (31.3%)	35 (25.5%)	25 (45.5%)	
Era 2: 2002–2009	58 (30.2%)	45 (32.8%)	13 (23.6%)	
Era 3: 2010–2016	74 (38.5%)	57 (41.6%)	17 (30.9%)	

Data are presented as mean ± SD or N (%). Percentages may not equal 100% due to rounding. ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker; CM, cardiomyopathy; eGFR, estimated glomerular filtration rate; and LVAD, left ventricular assist device.

[a]eGFR <45 ml/min/1.73 m^2 calculated using the Modification of Diet in Renal Disease (MDRD) formula.

[b]Includes n = 15 Hispanics and n = 1 American Indian.

[c]At 1-year clinic visit.

[d]Change in eGFR from pre-transplant to 1-year post-transplant.

of pre-transplant renal dysfunction (p = 0.007), lower pre-transplant eGFR (p < 0.001), older age at transplant (p < 0.001), cyclosporine use at 1-year post-transplant (p = 0.003), and receiving a transplant in an earlier era (p = 0.021). Pre-transplant renal dysfunction (yes/no) was selected for inclusion in covariate-adjusted models rather than the continuous measurement of eGFR due to the dichotomous nature of the primary outcome (Oetjens et al., 2014). Transplant era remained significantly associated with renal dysfunction at the 1-year time point after adjusting for type of CNI prescribed (p = 0.035). Tacrolimus trough blood levels were significantly lower in patients with vs. without renal dysfunction at 1-year post-transplant (9.8 ± 3.6 ng/ml vs. 11.7 ± 3.8 ng/ml; p = 0.019), likely reflecting clinical intervention in response to decreased eGFR. Cyclosporine trough blood levels, tacrolimus total daily dose, and cyclosporine total daily dose did not differ significantly between the two groups at 1-year post-transplant.

Two SNVs [transforming growth factor beta 1 (*TGFB1*) rs4803455 C > A and phospholipase C beta 1 (*PLCB1*) rs170549 G > A] were significantly associated with renal dysfunction 1-year post-transplant in the univariate analyses after adjusting for an FDR of 20% (unadjusted p = 0.004 for both SNVs). Renal dysfunction was present in 43% of participants with the *TGFB1* rs4803455 C/C genotype vs. 22% of variant A

carriers, and 20% of participants with the *PLCB1* rs170549 G/G genotype vs. 39% of variant A carriers. *TGFB1* rs4803455 and *PLCB1* rs170549 remained significantly associated with the primary outcome in a multiple-SNV covariate-adjusted logistic regression model (**Table 2**). Specifically, *TGFB1* rs4803455 variant A carriers had lower odds of renal dysfunction when compared to participants with the C/C genotype [odds ratio (OR) 0.28; 95% CI 0.12–0.62; $p = 0.002$]. In contrast, *PLCB1* rs170549 variant A allele carriers had higher odds of renal dysfunction than G/G homozygotes (OR 2.66; 95% CI 1.21–5.84; $p = 0.015$). Additionally, both SNVs were significantly associated with pre-transplant renal dysfunction, with this morbidity occurring in 30% of participants with the *TGFB1* rs4803455 C/C genotype vs. 16% of variant A carriers ($p = 0.018$), and 12% of participants with the *PLCB1* rs170549 G/G genotype vs. 30% of variant A carriers ($p = 0.002$). When limiting the multiple-SNV covariate-adjusted analysis to non-Hispanic Americans of European ancestry, both *TGFB1* rs4803455 C > A (OR 0.39; 95% CI 0.17–0.90; $p = 0.027$) and *PLCB1* rs170549 G > A (OR 2.52; 95% CI 1.10–5.77; $p = 0.029$) remained significantly associated with the outcome (**Supplementary Table S2**).

Six SNVs in the univariate analyses were suggestively associated with renal dysfunction 1-year post-transplant but were not significant after accounting for an FDR of 20% (**Table 3**). As an exploratory analysis, each SNV was evaluated in a single-SNV logistic regression model, adjusting for pre-transplant renal dysfunction, age at transplant, type of CNI prescribed at 1-year post-transplant, transplant era, and the top four PCs. Five SNVs [calcium voltage-gated channel subunit alpha1 D (*CACNA1D*) rs893365 C > T, protein phosphatase 3 catalytic subunit gamma (*PPP3CC*) rs10108011 A > G, *PPP3CC* rs2461494 A > G, protein kinase AMP-activated non-catalytic subunit gamma 2 (*PRKAG2*) rs7805747 G > A, and nuclear receptor subfamily 3 group C member 2 (*NR3C2*) rs1490453 G > A] remained suggestively associated with the primary outcome in the single-SNV covariate-adjusted models. The odds of renal dysfunction were higher in variant carriers of *PPP3CC* rs10108011, *PPP3CC* rs2461494, and *PRKAG2* rs7805747, whereas the odds of renal dysfunction were lower in variant carriers of *CACNA1D* rs893365 and *NR3C2* rs1490453. When these five SNVs were evaluated in non-Hispanic Americans of European ancestry, only *CACNA1D* rs893365 and *PRKAG2* rs7805747 continued to be associated with lower and higher odds of renal dysfunction 1-year post-transplant, respectively, after adjusting for covariates (**Supplementary Table S3**).

eGFR 1-Year Post-transplant

A histogram of the eGFR measurements closest to the 1-year transplant anniversary is shown in **Figure 1**. The mean ± SD time from the 1-year anniversary to the eGFR measurement was 10 ± 9 days. The mean eGFR 1-year post-transplant was 55 ± 20 ml/min/1.73 m^2. Demographic and clinical variables associated with lower eGFR 1-year post-transplant were female sex ($p = 0.018$), presence of pre-transplant renal dysfunction ($p < 0.001$), lower pre-transplant eGFR ($p < 0.001$), older age at transplant ($p < 0.001$), cyclosporine use at 1-year post-transplant ($p = 0.001$), and higher BMI at 1-year post-transplant ($p = 0.002$). The measurement of pre-transplant eGFR was included as a covariate in adjusted analyses, as the outcome (i.e., eGFR 1-year post-transplant) was a continuous variable.

TABLE 2 | Multiple-SNV adjusted model for SNVs significantly associated with renal dysfunction[a] 1-year post-transplant.

Gene name	rs number	Chromosome	Alleles[b]	MAF (%)	Reference group	Adjusted[c] odds ratio (95% CI)	Adjusted[c] value of *p*
TGFB1	rs4803455	19	C > A	45	C/C	0.28 (0.12–0.62)	0.002
PLCB1	rs170549	20	G > A	28	G/G	2.66 (1.21–5.84)	0.015

MAF, minor allele frequency; PLCB1, phospholipase C beta 1; SNV, single nucleotide variant; and TGFB1, transforming growth factor beta 1.
[a]Defined as an eGFR <45 ml/min/1.73m².
[b]Major > minor alleles, respectively.
[c]Model adjusting for pre-transplant renal dysfunction, age at transplant, cyclosporine use at 1-year post-transplant, transplant era, and the top four principal components (PCs).

TABLE 3 | Unadjusted and adjusted single-SNV analyses for SNVs suggestively associated with renal dysfunction[a] at 1-year post-transplant.

Gene name	rs number	Chromosome	Alleles[b]	MAF (%)	Reference group	Unadjusted odds ratio (95% CI)	Unadjusted value of *p*	Adjusted[c] odds ratio (95% CI)	Adjusted[c] value of *p*
CACNA1D	rs893365	3	C > T	49	CC	0.44 (0.22–0.89)	0.025	0.28 (0.12–0.69)	0.005
PPP3CC	rs10108011	8	A > G	42	AA	2.30 (1.13–4.68)	0.021	3.30 (1.39–7.82)	0.007
PPP3CC	rs2461494	8	A > G	20	AA	2.19 (1.16–4.15)	0.021	2.62 (1.24–5.54)	0.012
PRKAG2	rs7805747	7	G > A	24	GG	2.11 (1.12–3.98)	0.024	2.13 (1.03–4.39)	0.041
NR3C2	rs1490453	4	G > A	18	GG	0.47 (0.23–0.97)	0.043	0.44 (0.20–0.99)	0.047
PLCB1	rs227129	20	G > A	28	GG	1.98 (1.05–3.75)	0.039	1.89 (0.92–3.89)	0.083

CACNA1D, calcium voltage-gated channel subunit alpha1 D; MAF, minor allele frequency; NR3C2, nuclear receptor subfamily 3 group C member 2; PLCB1, phospholipase C beta 1; PPP3CC, protein phosphatase 3 catalytic subunit gamma; PRKAG2, protein kinase AMP-activated non-catalytic subunit gamma 2; and SNV, single nucleotide variant.
[a]Defined as an eGFR <45 ml/min/1.73m².
[b]Major > minor alleles, respectively.
[c]Models adjusted for pre-transplant renal dysfunction, age at transplant, cyclosporine use at 1-year post-transplant, transplant era, and the top four PCs.

Five SNVs were suggestively associated with eGFR 1-year post-transplant ($p < 0.05$; **Table 4**), but they did not withstand correction for multiple testing (FDR of 20%). As an exploratory analysis, each SNV was evaluated in a single-SNV adjusted linear regression model, adjusting for the demographic and clinical variables above. Two SNVs in the protein phosphatase 3 regulatory subunit B alpha (*PPP3R1*) gene, rs875 A > G and rs12465425 T > G, remained suggestively associated with the outcome in single-SNV adjusted models, with variant G allele carriers having higher eGFR 1-year post-transplant compared to WT homozygotes for both SNVs. Neither of these two *PPP3R1* SNVs were suggestively associated with eGFR 1-year post-transplant in the single-SNV adjusted models when limiting the analysis to non-Hispanic Americans of European ancestry (**Supplementary Table S4**).

Change in eGFR

The mean change in eGFR from pre-transplant to 1-year post-transplant was −10 ± 21 ml/min/1.73 m^2 (**Figure 2**). Demographic and clinical variables associated with a greater decrease in eGFR and included in the multivariable models were presence of a LVAD prior to transplant ($p < 0.001$), absence of pre-transplant diabetes ($p = 0.033$), younger age at transplant ($p < 0.001$), and not being prescribed an ACE inhibitor or ARB 1-year post-transplant ($p = 0.013$). No SNVs were significantly associated with change in eGFR

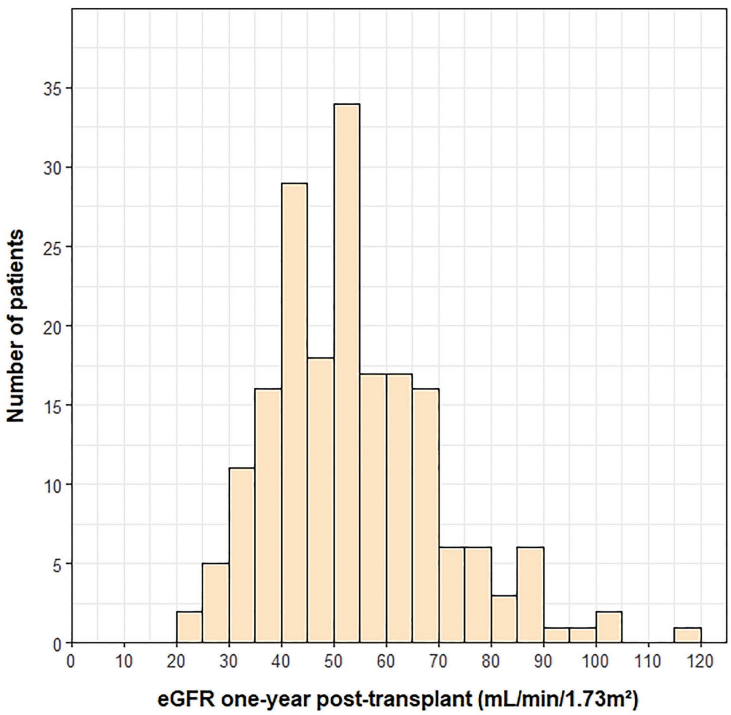

FIGURE 1 | Histogram of eGFR measurements at 1-year post-transplant. One outlier (eGFR = 210) was not included on the histogram.

TABLE 4 | Unadjusted and adjusted single-SNV analyses for SNVs suggestively associated with eGFR[a] at 1-year post-transplant.

Gene name	rs number	Chromosome	Alleles[b]	MAF (%)	Unadjusted value of *p*	Adjusted eGFR[c] (95% CI) WT homozygotes	Adjusted eGFR[c] (95% CI) variant carriers	Adjusted[c] value of *p*
PPP3R1[d]	rs875	2	A > G	32	0.017	49 (46–51)	54 (51–57)	0.009
PPP3R1[d]	rs12465425	2	T > G	32	0.013	49 (47–52)	54 (51–57)	0.010
PPP3CC	rs10108011	8	A > G	42	0.016	54 (50–57)	50 (48–53)	0.142
PRKAG2	rs7805747	7	G > A	24	0.012	52 (50–55)	50 (47–53)	0.219
PLCB1	rs170549	20	G > A	28	0.025	51 (49–55)	51 (48–54)	0.808

eGFR, estimated glomerular filtration rate; MAF, minor allele frequency; PLCB1, phospholipase C beta 1; PPP3CC, protein phosphatase 3 catalytic subunit gamma; PPP3R1, protein phosphatase 3 regulatory subunit B alpha; PRKAG2, protein kinase AMP-activated non-catalytic subunit gamma 2; SNV, single nucleotide variant; and WT, wild-type.

[a]*Calculated using the MDRD formula.*

[b]*Major > minor alleles, respectively.*

[c]*Models adjusted for sex, pre-transplant eGFR, age at transplant, cyclosporine use at 1-year post-transplant, body mass index (BMI) at 1-year post-transplant, and the top four PCs.*

[d]*PPP3R1 SNVs are correlated ($r^2 = 0.91$).*

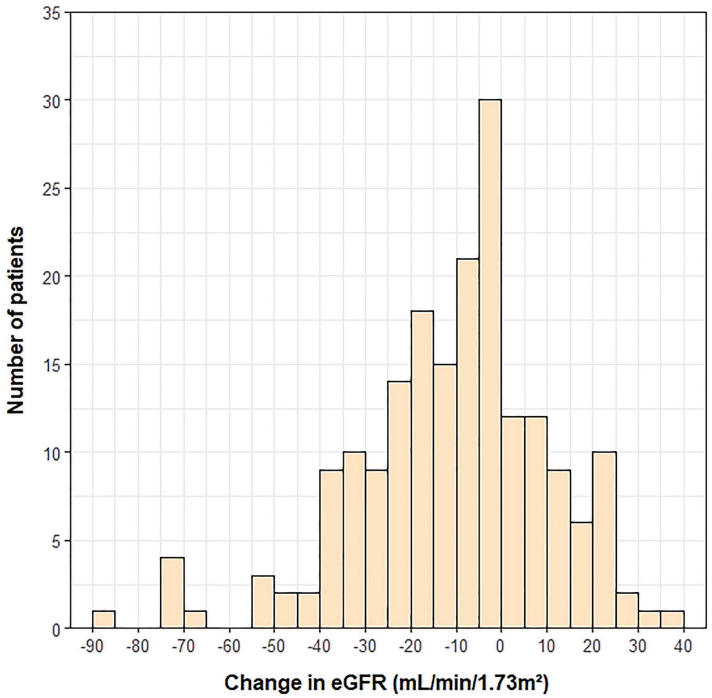

FIGURE 2 | Histogram depicting the change in eGFR from pre-transplant to 1-year post-transplant.

TABLE 5 | Unadjusted and adjusted single-SNV analyses for SNVs suggestively associated with change in eGFR[a] from pre-transplant to 1-year post-transplant.

Gene name	rs number	Chromosome	Alleles[b]	MAF (%)	Unadjusted value of *p*	Adjusted change in eGFR[c] (95% CI) WT homozygotes	Adjusted change in eGFR[c] (95% CI) variant carriers	Adjusted[c] value of *p*
COL3A1[d]	rs1800255	2	G > A	23	0.008	−10 (−15 to −4)	−16 (−21 to −10)	0.041
COL3A1[d]	rs1878201	2	A > G	24	0.010	−9 (−15 to −4)	−15 (−21 to −10)	0.042
CACNA1D	rs893365	3	C > T	49	0.022	−17 (−24 to −9)	−11 (−16 to −6)	0.117
NR1I2	rs2056530	3	C > T	26	0.029	−11 (−17 to −6)	−13 (−19 to −8)	0.542

CACNA1D, calcium voltage-gated channel subunit alpha1 D; COL3A1, collagen type III alpha 1 chain; eGFR, estimated glomerular filtration rate; MAF, minor allele frequency; NR1I2, nuclear receptor subfamily 1 group I member 2; SNV, single nucleotide variant; and WT, wild-type.

[a]*Calculated using the MDRD formula.*

[b]*Major > minor alleles, respectively.*

[c]*Models adjusted for presence of a LVAD pre-transplant, pre-transplant diabetes, age at transplant, ACE inhibitor or ARB prescribed 1-year post-transplant, and the top four PCs.*

[d]*COL3A1 SNVs are correlated ($r^2 = 0.85$).*

using an FDR of 20%. Four SNVs were suggestively associated with the outcome in univariate analyses ($p < 0.05$; **Table 5**) and were included in exploratory single-SNV multivariable models, adjusting for the covariates listed above and the top four PCs. Two SNVs (rs1800255 G > A and rs1878201 A > G) in the collagen type III alpha 1 chain (*COL3A1*) gene remained suggestively associated with change in eGFR from pre-transplant to 1-year post-transplant in the single-SNV adjusted models. Variant carriers had greater decline in eGFR in the 1st-year post-transplant vs. WT homozygotes for both SNVs. *COL3A1* rs1800255 and rs1878201 were no longer suggestively associated with the outcome in single-SNV adjusted analyses in non-Hispanic Americans of European ancestry (**Supplementary Table S5**).

DISCUSSION

In this study, we utilized a candidate gene approach to examine the association between 93 SNVs in CNI pharmacokinetic and pharmacodynamic genes and renal impairment following heart transplantation. Our study, in which the majority of patients were prescribed tacrolimus at the 1-year time point, is one of the largest and most comprehensive to date in this population. Two SNVs, *TGFB1* rs4803455 and *PLCB1* rs170549, were associated with renal dysfunction 1-year post-transplant after accounting for an FDR of 20%. TGFB-1, encoded by *TGFB1*, is a cytokine involved in the development of fibrosis, a key histological feature of chronic CNI exposure (Randhawa et al., 1993; Border and Noble, 1994). The expression

of this important protein is modulated by the cell signaling enzyme, PLCB1 (Slattery et al., 2005).

We found variant A allele carriers of rs4803455 C > A, located in intron 2 of *TGFB1*, had lower odds of renal dysfunction compared to C/C homozygotes. TGFB-1 has been associated with increased fibrosis in multiple tissues, including the kidneys (Border and Noble, 1994). Importantly, two of the hallmarks of chronic renal dysfunction after transplantation are interstitial fibrosis and tubular atrophy (Myers et al., 1984; Randhawa et al., 1993). Intrarenal TGFB-1 expression has been shown to increase following administration of tacrolimus and cyclosporine (Khanna et al., 2002; Roos-van Groningen et al., 2006). Lachance et al. previously reported an association between *TGFB1* rs4803455 and eGFR in heart transplant recipients, of whom 21% were receiving tacrolimus and the average time post-transplant was 4.3 years (Lachance et al., 2012). However, that study did not describe which allele conveyed worse renal function, nor did it provide an estimate of the strength of the association. In non-transplant populations, variant A allele carriers of rs4803455 have been reported to have reduced risk of myopia, asthma, the formation of carotid plaque, breast cancer, and endometrial cancer (Zha et al., 2009; Wu et al., 2010; Deng et al., 2011; Boone et al., 2013; Yang et al., 2016). To date, rs4803455 has not been directly shown to influence *TGFB1* gene expression or protein function. Due to its location in intron 2, further investigation is warranted to identify if a functional variant in linkage disequilibrium with rs4803455 is driving the observed associations.

Other studies have reported an association between the *TGFB1* missense variant, Leu10Pro (L10P), and renal function in heart transplant recipients (Baan et al., 2000; Lacha et al., 2001; van de Wetering et al., 2006). Codon 10 proline carriers had a higher risk of renal dysfunction in patients taking CNIs compared to leucine/leucine patients in two studies, whereas Lacha et al. reported worse progression of renal dysfunction in leucine carriers (Baan et al., 2000; Lacha et al., 2001; van de Wetering et al., 2006). We found no association between L10P and renal function outcomes at 1-year post-transplant in our cohort. Our results agree with Klauke et al. (2008) and Lachance et al. (2012) both of whom found no association between L10P and renal function in heart transplant recipients. Possible reasons for the discrepant results between studies include the variable post-transplant time points analyzed and differing definitions of renal dysfunction. Nonetheless, our results, in combination with previous findings, suggest that SNVs in *TGFB1* may play a role in the development of renal dysfunction following heart transplantation.

We also found that carriers of the intronic *PLCB1* rs170549 variant A allele had higher odds of renal dysfunction when compared to individuals with the G/G genotype. PLCB1 catalyzes the formation of diacylglycerol and is expressed in multiple tissues, including vascular smooth muscle cells (Schelling et al., 1997; Hisatsune et al., 2005). In turn, diacylglycerol activates protein kinase C, which regulates a variety of cellular targets *via* phosphorylation (Nishizuka, 1988). Protein kinase C has been shown to modulate cyclosporine-induced up-regulation of TGFB-1 and inhibition of endothelial nitric oxide synthase by tacrolimus (Slattery et al., 2005; Chiasson et al., 2011). Furthermore, *PLCB1* rs170549 has previously been associated with eGFR in adult heart transplant recipients, although the study did not describe which allele was associated with worse renal function, nor the magnitude of the association (Lachance et al., 2012). Given that rs170549 is located in an intron, it may be in linkage disequilibrium with another functional variant in *PLCB1*, which could influence its protein structure and/or expression. Thus, additional studies are needed to elucidate the mechanism by which *PLCB1* rs170549, or a functional SNV linked with this intronic variant, influences the development of renal dysfunction in heart transplant recipients receiving CNIs.

In exploratory analyses, we found carriers of the *CACNA1D* rs893365 variant T allele had lower odds of renal dysfunction 1-year post-transplant. *CACNA1D* encodes a subunit of the calcium channel, $Ca_v1.3$, which plays an important role in the production and secretion of aldosterone from the adrenal cortex (Yang et al., 2020). Aldosterone increases sodium and fluid retention which, in turn, results in higher blood pressure as part of renin-angiotensin-aldosterone system (RAS) activation. RAS activation leads to vasoconstriction of renal afferent and efferent arterioles, changes which are also observed following CNI treatment (Kaye et al., 1993; Textor et al., 1995; Mennuni et al., 2014). Another intronic variant in *CACNA1D*, rs9810888, has previously been associated with the presence of hypertension in non-transplanted adults (Lu et al., 2015). As such, genetic variation in *CACNA1D* may increase the odds of developing hypertension, a condition that contributes to the occurrence of renal dysfunction after heart transplantation and which is exacerbated by CNI use (Lindenfeld et al., 2004). We also found *PRKAG2* rs7805747 variant A carriers had higher odds of renal impairment 1-year after transplant than G/G homozygotes. *PRKAG2* encodes the gamma-2 subunit of AMP-activated protein kinase, an enzyme that regulates metabolism during cellular stress (Hardie et al., 1998). Furthermore, AMP-activated protein kinase may protect against the development of tubulointerstitial renal fibrosis, a common histological finding in patients treated with CNIs, through the inhibition TGFB-1 (Naesens et al., 2009; Lee et al., 2013). *In silico* data suggest *PRKAG2* rs7805747 could affect the binding of transcription factors, such as hepatocyte nuclear factor 4 alpha, to *PRKAG2* (Wang et al., 2018). Additionally, the *PRKAG2* rs7805747 variant A allele has been associated with higher odds of renal dysfunction in genome-wide association studies in non-transplant populations (Kottgen et al., 2010; Pattaro et al., 2016). Given the exploratory nature of our analyses, the lack of *in vitro* data describing the functional significance of *CACNA1D* rs893365 and *PRKAG2* rs7805747, and the intronic location of both variants, further studies are necessary to determine how these SNVs, or linked functional variants, impact renal function in heart transplant recipients prescribed CNIs.

There are limitations to our study that deserve to be acknowledged. First, there was a broad range of dates of transplant in our cohort (1989–2016). Patient care during

this period has changed, and some of these changes might have influenced renal dysfunction (e.g., type of CNI used, CNI goal ranges). Second, it is likely that survival bias is present, with some patients with renal dysfunction dying prior to study enrollment. Furthermore, we did not include patients with severe renal impairment whose CNI was discontinued in favor of an mTOR inhibitor (i.e., sirolimus or everolimus), which may also bias our results. Third, multiple suggestive SNVs from the whole cohort were not associated with the primary and secondary outcomes when the analysis was limited to non-Hispanic Americans of European ancestry. While this may be due, in part, to a reduction in power, we cannot exclude the possibility that population stratification may have impacted these findings. Fourth, the functional effects of many of the significant and suggestive SNVs have not been reported in the literature. Future studies should focus on replicating these findings and determining the mechanistic underpinnings of their effects. Fifth, the two SNVs significantly associated with renal dysfunction 1-year post-transplant in our cohort were also associated with pre-transplant renal dysfunction. While these SNVs continued to be associated with the primary outcome after accounting for pre-transplant renal impairment in multi-SNV adjusted models, it is possible that the effects of these variants on renal function post-transplant may exist independent of CNI exposure. Finally, we used a single eGFR measurement in our primary and secondary outcomes. Although this is a common approach to assess renal function post-transplant, it may not capture other acute processes that may have occurred around that time point (e.g., acute kidney injury, dehydration; Sanchez-Lazaro et al., 2015; Asleh et al., 2018).

In conclusion, we provide additional evidence that SNVs in the pro-fibrotic *TGFB1* and cell signaling *PLCB1* genes may play a role in the development of renal dysfunction in adult heart transplant recipients taking CNIs. Novel genetic markers, such as *TGFB1* rs4803455 and *PLCB1* rs170549, may serve as tools that clinicians can utilize to assess a patient's risk of CNI-associated renal dysfunction following heart transplantation, thereby improving clinical care and advancing precision medicine in this population.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Colorado Multiple Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CA conceptualized the study idea. KO, CA, and KD designed the study and collected and analyzed the data. LS and NR aided in data analysis. AA, JL, and RP were involved in patient recruitment and provided clinical input. KO and CA wrote the manuscript. All authors contributed to the article 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/fgene.2021.658983/full#supplementary-material

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Clozapine Metabolism is Associated With Absolute Neutrophil Count in Individuals With Treatment-Resistant Schizophrenia

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Up to one-third of those with schizophrenia fail to respond to standard antipsychotics and are considered to have treatment-resistant schizophrenia, a condition for which clozapine is the only evidence-based medication. While up to 60% of treated individuals obtain therapeutic benefits from clozapine, it is currently underprescribed worldwide, partly because of concerns related to its broad adverse effect profile. In particular, the potential effects of clozapine on the immune system have gained relevance after a recent study showed that drug plasma concentrations were inversely correlated with neutrophil counts in individuals routinely undergoing treatment. Seeking to investigate this relationship in more detail, we extracted metabolic, immune, and genetic data from a UK cohort of long-term clozapine users linked to a clozapine monitoring service, CLOZUK2 (N = 208). Whilst a correlation analysis was compatible with the original results, a multiple linear regression accounting for dose and other confounding factors additionally allowed us to estimate the decrease in absolute neutrophil counts to approximately 141 cells/mm^3 for every 0.1 mg/L increase in clozapine concentration. However, this association was attenuated after controlling for the metabolic ratio between clozapine and its main metabolite, norclozapine, which was itself negatively associated with neutrophil concentrations. Further analyses revealed that these relationships are likely moderated by genetic factors, as three pharmacogenomic SNPs previously associated to norclozapine plasma concentrations and the metabolic ratio (rs61750900, rs2011425 and rs1126545) were shown to be independently associated with a variation in neutrophil counts of about 400 cells/mm^3 per effect allele. Such results are compatible with an effect of norclozapine, but not necessarily clozapine, on immune cell counts, and highlight the need for further investigations into the potential role of genetic determinants of clozapine pharmacokinetics in the occurrence of adverse effects during treatment.

Keywords: clozapine, norclozapine, metabolic ratio, genetics, treatment-resistant schizophrenia

INTRODUCTION

Schizophrenia is typically a severe, chronic neuropsychiatric disorder affecting an estimated 0.7% of the global population (McGrath et al., 2008). Although antipsychotic medications are generally effective, 20–30% of patients are classified as having 'Treatment-Resistant' Schizophrenia (TRS), defined as a lack of symptomatic relief following trials of at least two antipsychotics at an adequate dose for a reasonable time (Howes et al., 2017). For these individuals, treatment options are often limited to a single medication; clozapine, the first atypical antipsychotic drug developed, and the only drug currently available with proven efficacy in TRS. Clozapine has also been shown to have robust effects on preventing suicide (Meltzer, 2011) in this patient subgroup. However, although an estimated two-thirds of patients with treatment-resistant symptoms would respond beneficially to clozapine (Meltzer, 2011), it is still widely under-prescribed, in part due to the risk of serious adverse effects (De Fazio et al., 2015). One of these, affecting 0.38–0.8% of patients, is agranulocytosis, a potentially fatal condition in which there is a significant decrease in granulocytes, the most abundant form of which are neutrophils (Alvir et al., 1993). To minimize the risk of agranulocytosis, regular haematological monitoring is required for safe and effective treatment with clozapine. Clozapine-induced agranulocytosis is most likely to develop within the first 18 weeks of treatment (Atkin et al., 1996), during which time patients' white blood cell levels are measured weekly and treatment stopped if agranulocytosis is detected or monitoring shows a sustained but milder decrease of white blood cells (neutropenia). This need for intensive physical health monitoring is an additional burden for the use of clozapine, and has been indicated by service users as a reason for discontinuing treatment (Legge et al., 2016).

In contrast to hematological monitoring, measures of clozapine concentrations in plasma do not seem to be strong indicators of agranulocytosis onset (Hasegawa et al., 1994), which to this day is a manageable but unpredictable risk of this treatment (de Leon et al., 2020). However, potential associations between clozapine dose and ANC in those taking the drug remain poorly understood. Several mechanisms have been proposed in favor of the hypothesis that clozapine might have a subtle dose-dependent effect on ANC (Vaquero-Baez et al., 2019), which could, in turn, lead to neutropenia. One such mechanism is the production of a reactive nitrenium ion following the bioactivation of either clozapine or one of its primary metabolites (Liu and Uetrecht, 1995). In turn, this short-lived but highly reactive intermediate may act as a hapten leading to the destruction of neutrophils via an immune response, or it may disrupt neutrophil function by binding to crucial cellular proteins (Pirmohamed and Park, 1997). This effect could potentially be enhanced if an infection occurs, since inflammatory processes are known to increase clozapine plasma concentrations (de Leon et al., 2020; Siskind et al., 2020). Complicating the matter further is the fact that there is substantial variation of plasma clozapine concentration between individuals who have been prescribed the same dose; with modifying factors including age, weight, smoking and likely genomic variation affecting enzymes involved in clozapine metabolism (Olsson et al., 2015). It is important to better understand the link between clozapine doses, clozapine metabolism and neutrophil counts for several reasons. First, because it would allow for the evaluation of the effectiveness of interventions aimed at lessening the potential detrimental impacts of clozapine on the immune system, such as those proposed in response to the COVID-19 pandemic (Pandarakalam, 2020; Siskind et al., 2020). Additionally, in line with recent research on underrepresented populations (Legge et al., 2019), improved comprehension of the biology behind potential adverse effects could help with the refinement of current dosing and titration protocols, therefore increasing the number of TRS patients who can be safely prescribed the medication and benefit from its therapeutic effects.

There have been a small number of studies investigating the relationship between plasma concentrations of clozapine metabolites and blood dyscrasias, but results are so far inconclusive. In a study of 5 patients with confirmed agranulocytosis, plasma clozapine and norclozapine levels were below toxic ranges and not significantly different to 59 patients on clozapine who did not develop agranulocytosis (Hasegawa et al., 1994). Another study with a cohort of 37 schizophrenia patients taking clozapine reported no significant correlations between neutrophil count and serum clozapine and norclozapine levels (Oyewumi et al., 2002). However, a more recent observational study of 129 patients did report a significant positive correlation between norclozapine serum concentrations and ANC, with the same relationship being observed with the clozapine/norclozapine ratio (Smith et al., 2017). Finally, and most recently, a study of 41 patients found a negative association between plasma clozapine concentration and neutrophil count, reporting an R^2 = 0.447 from multiple regression analyses (Vaquero-Baez et al., 2019). While all of these studies were arguably small for a cross-sectional design, the effect sizes reported in the latter could be conventionally considered as "large" (Cohen, 1992), which make them interesting candidates for further investigation. Motivated to test these findings on a larger cohort, we used similar statistical methods on the UK-based CLOZUK2 study of people with TRS (Pardiñas et al., 2018). We also extended the regression approach to incorporate genetic data, specifically genetic variants that have been recently found to be associated with clozapine metabolism (Pardiñas et al., 2019).

METHODS

Samples, Neutrophil Count and Clozapine Concentration Data

ANC, clozapine concentration and genetic data were obtained as part of the CLOZUK2 study. Full details of recruitment and quality control of the samples are provided in Pardiñas et al. (2018). Curation of ANC data and assessment of genetic ancestry are described in Legge et al. (2019), while curation of the

TABLE 1 | Pharmacogenomic SNPs used in the regression analyses, and their association to clozapine metabolite concentrations in the GWAS from Pardiñas et al. (2019).

Phenotype/SNP	Minor Allele	Beta	s.e.	MAF[a]
Clozapine				
rs2472297	T	–0.089	0.013	27.94
Norclozapine				
rs61750900	T	–0.149	0.018	9.9
rs2011425	G	–0.112	0.019	8.65
Metabolic ratio				
rs61750900	T	0.212	0.012	9.9
rs1126545	T	0.078	0.01	14.22

[a]*Based on the European Subset of CLOZUK2 (see Pardiñas et al., 2019 for details).*

longitudinal clozapine concentration data is described in Pardiñas et al. (2019).

Inclusion Criteria

The dataset was restricted to individuals of European ancestry who had a plasma clozapine measurement (mg/L) within 21 days of an ANC (cells/mm^3) measurement. For any individual who had more than one clozapine plasma concentration sample within that window, the measurement closest to the ANC was selected. The clozapine/norclozapine ratio ("metabolic ratio") was calculated for each individual, and anyone with a ratio greater than 3 and less than 0.5 was excluded (n = 19), in line with current recommendations to detect confounding factors such as drug interactions, as well as treatment non-adherence (Ellison and Dufresne, 2015). Finally, any individual prescribed clozapine for the treatment of psychosis in Parkinson's disease was also excluded (n = 1), leaving a final sample of 208 individuals.

Statistical Analysis of Metabolite Data

As a direct replication of Vaquero-Baez et al. (2019), Spearman correlations were computed to assess the bivariate relationships between plasma clozapine concentration, plasma norclozapine concentration, time on clozapine treatment, daily clozapine dose and ANC. Expanding on those analyses, multivariate linear regression models were used to assess the association between plasma clozapine concentration (mg/L) and ANC (cells/mm^3) accounting for eight additional covariates: norclozapine plasma concentration (mg/L), daily clozapine dose (mg), time on clozapine treatment (days), time between clozapine concentration measurement and ANC measurement (days), time between clozapine dose and blood sampling (hours), sex (male/female), age (years) and age^2 (in order to account for a possible non-linear effect of age on ANC; Prabhakar et al., 2009). Following on from this baseline model, the metabolic ratio and four pharmacogenomic variants (further details below) were added to refine our assessment of the relationship between clozapine and ANC and to identify potential mediators. All statistical analyses were conducted using R v4.0.2.

Pharmacogenomic Analyses

Four SNPs were selected for inclusion in these models, based on work conducted by Pardiñas et al. (2019), in which GWAS of clozapine levels, norclozapine levels, and clozapine/norclozapine metabolic ratio were conducted to identify pharmacogenomic variants affecting the metabolism of clozapine. Details of these SNPs can be seen in **Table 1**. Another SNP reported to be associated with clozapine concentration (rs28379954; Smith et al., 2020) was also considered for analysis, but was not included due to poor imputation quality in CLOZUK2, which led to missing data in 78/208 individuals in our sample.

RESULTS

Descriptive statistics for the final sample can be seen in **Table 2**. This cohort represents long-term clozapine users, who at the time of sampling had ANC levels not indicative of neutropenia (ANC ≥1,500 cells/mm^3) by clozapine monitoring guidelines (Nielsen et al., 2016).

Correlation Analysis

Mirroring the approach of Vaquero-Baez et al. (2019), Spearman's correlation statistics are reported in **Table 3**. None of the variables examined was significantly associated with ANC in this bivariate analysis.

Regression Procedure

Three regression models were considered: *Model 1* includes clozapine plasma concentration and the eight covariates described above. *Model 2* introduces the metabolic ratio as an additional covariate, following previous clozapine therapeutic drug monitoring studies (Rostami-Hodjegan et al., 2004; Couchman et al., 2013). *Model 3* additionally incorporates four genetic variants associated with clozapine metabolism (**Table 1**).

TABLE 2 | Covariates used in the correlation and regression analyses, and their distribution in the CLOZUK2 sample described in this study.

Variable	Average ± SD	
	Male, *N* = 147	**Female, *N* = 61**
Clozapine (mg/L)	0.47 (0.28)	0.51 (0.32)
Norclozapine (mg/L)	0.27 (0.17)	0.29 (0.17)
Daily dose (mg/Day)	357 (136)	322.2 (134)
Age (Years)	40.3 (13.2)	42.7 (14.2)
Time on treatment (Years)	3.26 (0.8)	3.21 (1.1)
Absolute neutrophil count (1,000 Cells/mm^3)	3.1 (1.2)	3.1 (1.2)

TABLE 3 | Spearman's rank correlations of several variables with ANC, reproducing the approach of Vaquero-Baez et al. (2019).

Outcome	Lowest ANC	
	Correlation coefficient (rho)	*p*
CLZ	−0.125	0.062
DMC	−0.033	0.629
Time on clozapine	−0.073	0.289
Daily dose	−0.033	0.629

Effect size estimates of all covariates included in our ANC regression models are shown in **Table 4**.

Model 1

Both clozapine and norclozapine level were significantly associated with ANC; clozapine level was negatively associated ($\beta = -1.41$, $p = 0.009$) and a positive association was found for norclozapine ($\beta = 1.77$, $p = 0.049$). For each 0.1 mg/L increase in plasma clozapine concentration, there was an associated decrease in ANC of 141 cells/mm^3, and for each 0.1 mg/L increase in norclozapine concentration, there was an increase in ANC of 177 cells/mm^3.

Model 2

With the addition of metabolic ratio in the model, the effect sizes of clozapine and norclozapine shifted toward zero, becoming nonsignificant likely because of collinearity with this covariate. Thus, their initial association appeared to be explained by their ratio, which was negatively associated with ANC ($\beta = -0.69$, $p = 0.021$). For every unit increase in the clozapine/norclozapine ratio we estimated an associated decrease of 690 cells/mm^3 in ANC.

Model 3

Of the four SNPs included in the analysis, 3 were significantly associated with ANC; rs61750900_T ($\beta = -0.41$, $p = 0.048$), rs2011425_G ($\beta = 0.45$, $p = 0.026$) and rs1126545_T ($\beta = 0.33$, $p = 0.039$). Each minor allele was associated with a decrease in ANC of 410 cells/mm^3 and increases of 450 cells/mm^3 and 330 cells/mm^3, respectively. In this model, the metabolic ratio remained significantly associated with a decrease in ANC ($\beta = -0.54$, $p = 0.035$).

DISCUSSION

Clozapine and Neutrophil Counts

Our results demonstrate a relationship between clozapine concentration and ANC, which appeared to be mediated through the metabolic ratio of clozapine and its main metabolite norclozapine. As the ratio of clozapine/norclozapine increased, ANC decreased, and this relationship appeared to be largely independent of clozapine dose. High metabolic ratios in the normal range (i.e. not suggestive of non-adherence; Ellison and Dufresne, 2015) could either be indicative of slow metabolism of clozapine or relatively rapid clearance of norclozapine, and have been previously associated to better cardiometabolic and cognitive outcomes (Costa-Dookhan et al., 2020). Regression results also highlighted a relationship with ANC of pharmacogenomic variants associated with the clozapine/norclozapine metabolic ratio, as well as norclozapine concentration. Interestingly, two of these variants (rs61750900 and rs2011425) are missense polymorphisms in UGT genes, which glucuronidize norclozapine and thereby facilitate its excretion, supporting the role of this metabolite in altering ANC (Erickson-Ridout et al., 2012). The third significantly associated SNP (rs1126545) is a missense variant located within CYP2C18. This gene, while not part of the current consensus clozapine metabolic pathway (Thorn et al., 2018), has also been shown to be able to participate in its bioactivation *in vitro* (Dragovic et al., 2013). The fourth SNP (rs2472297), not associated significantly with ANC in any of our analyses, is an intergenic variant located between two cytochrome P450 genes, CYP1A1 and CYP1A2. This marker, also commonly found in coffee consumption and caffeine metabolism GWAS, has been tentatively associated to the regulation of CYP1A2 activity (Pardiñas et al., 2019), which drives the primary

TABLE 4 | Results of the three regression analyses with ANC as outcome.

Variable	Model 1		Model 2		Model 3	
	Estimates	*p*	Estimates	*p*	Estimates	*p*
Clozapine concentration	**−1.410**	**0.009**	0.540	0.585	0.060	0.950
Norclozapine concentration	**1.770**	**0.049**	−1.450	0.385	−0.570	0.738
Daily dose	0.000	0.887	0.000	0.931	0.000	0.948
Gender [male]	0.030	0.886	0.040	0.829	0.080	0.626
Days between clozapine/ANC measurements	−0.010	0.113	−0.010	0.124	−0.010	0.105
Age	**0.120**	**0.001**	**0.110**	**0.001**	**0.120**	**0.001**
Age squared	**−0.001**	**0.004**	**−0.001**	**0.004**	**−0.001**	**0.004**
Time on clozapine	**−0.001**	**0.036**	**−0.150**	**0.044**	**−0.001**	**0.020**
Time between dose and sample	−0.050	0.105	0.060	0.080	−0.050	0.122
Ratio			**−0.690**	**0.021**	**−0.540**	**0.035**
rs2472297_T (clozapine)					−0.130	0.324
rs61750900_T (norclozapine + ratio)					**−0.410**	**0.048**
rs2011425_G (norclozapine)					**0.450**	**0.026**
rs1126545_T (ratio)					**0.330**	**0.039**

Bold highlight indicates statistically significant effect sizes ($p < 0.05$).

reaction transforming clozapine into norclozapine (Prior and Baker, 2003). While the exact biological effect that any these variants might have on neutrophil count itself cannot be inferred from our results, our analysis does highlight a number of potential avenues for further research.

Replication of Previous Results

Although our main findings replicate those of Vaquero-Baez et al. (2019), the effect sizes we observed were substantially smaller. Besides the effects of winner's curse (Kraft, 2008), and the use of a larger cohort (208 vs. 41), there are a number of reasons why this may be the case. Firstly, the original study was conducted in Mexico, and the individuals recruited are likely of different ancestry (Ruiz-Linares et al., 2014) to our cohort that was recruited in the UK and made up only of those of European ancestry as inferred from genetic analyses. It cannot be ruled out that the work of Vaquero-Baez et al. (2019) has uncovered a population- or ancestry-specific effect, although no literature exists at this time to support this. An increased prevalence of neutropenia has been found in individuals with schizophrenia of African ethnicity (Kelly et al., 2007), and "benign" (constitutional) neutropenia rates vary widely based on genetic ancestry (Haddy et al., 1999; Legge et al., 2019), but no specific risk has been found in Mexican, Latino or Native American people to date.

Another noteworthy difference is that the daily clozapine dose prescribed is substantially different between the two cohorts, as inferred from a comparison of the study descriptive statistics. In our study average clozapine doses were 348 mg/day for males and 313 mg/day for females, while Vaquero-Baez et al. (2019) reported average doses of 223 mg/day for males and 105 mg/day for females (*t*-test $p_{male} = 1.31 \times 10^{-10}$; $p_{female} = 1.43 \times 10^{-6}$). There was also a significant difference in the average time that the cohorts had been on treatment with clozapine, upwards of three years in our study with 3.26 years for males and 3.21 years for females, and less than one year in Vaquero-Baez et al. (2019) with 10 months for males and 6.5 months for females (*t*-test $p_{male} = 5.9 \times 10^{-3}$; $p_{female} = 6.18 \times 10^{-16}$). This might have contributed to the smaller effect size we observed between clozapine metabolites and ANC, as our sample represents individuals who are long-term clozapine users and therefore likely excludes the approximately 10% of people who, after a year of treatment, might go on to exhibit neutropenia or other immune-related adverse effects (Myles et al., 2018). However, the main associations we found across our successive tests suggest that clozapine might have sustained effects on ANC even in individuals without obvious hematological adverse effects. This is consistent with the rationale for the current practice of continued hematological monitoring of people being prescribed clozapine, and supports that additional measures to lower their risk of infections might indeed be warranted even in long term clozapine users (Siskind et al., 2020), for example ensuring their timely access to the annual influenza vaccine (Pandarakalam, 2020).

Limitations

There are a few limitations in this work that have to be noted. Although several covariates were used in the regression analyses, no data were available for the CLOZUK2 cohort for several factors that are known to affect clozapine metabolism, including concomitant medications (Singh et al., 2015), the use of tobacco (Smith and Mican, 2014) and the consumption of coffee (Raaska et al., 2004). Additionally, the cross-sectional nature of the ANC data available means there is no information regarding neutrophil trajectories throughout the individuals' time on treatment. Replicating this analysis in a longitudinal dataset would allow for better estimations of the magnitude of the detected associations and whether they vary along particular time or titration windows. This would additionally enable clarifying, through more sophisticated causal modeling, the exact relationship between clozapine metabolic ratio and ANC.

CONCLUSION

Increased blood clozapine concentration is associated with decreased ANC in people with treatment-resistant schizophrenia who are long-term recipients of clozapine, even if they have not developed agranulocytosis or neutropenia during their treatment. Further investigation suggests this relationship is mediated by the clozapine/norclozapine metabolic ratio, with higher values (increased levels of clozapine proportional to norclozapine) associated with lower ANC. Furthermore, these effects can be partially explained by common genetic variants, some of which have a functional impact on enzymes involved in norclozapine glucuronidation (rs61750900 and rs2011425). Investigating these effects in a longitudinal cohort could shed further light on the relationship between clozapine and neutrophils, potentially offering biological insights. A fuller mechanistic understanding of the linkage between clozapine and ANC might allow improving the wellbeing of individuals taking clozapine by supporting targeted interventions such as prioritizing seasonal vaccinations. Additional research might also lead to the elucidation of appropriate trials to assess the incorporation of genetic variants into clozapine monitoring and/or titration protocols, thus tapping into currently unrecognized but potentially valuable sources of information to improve clozapine safety at all stages of treatment.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: To comply with the ethical and regulatory framework of the CLOZUK project, access to individual-level data requires a collaboration agreement with Cardiff University. Requests to access these datasets should be directed to Prof. James T. R. Walters (WaltersJT@cardiff.ac.uk).

ETHICS STATEMENT

The CLOZUK project was reviewed and approved by the UK Multicentre Research Ethics Committee (ref. 10/WSE02/15). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

SL, JW, and AP conceived and designed the study. SL, MN, AK, JJ, and MH contributed and/or curated data. IW performed all statistical analyses under the supervision of AP. IW, JW, and AP interpreted results. IW, SL, LM, MO, MO'D, JW, and AP participated in the primary drafting of the manuscript. All authors had the opportunity to review and comment on the manuscript, and all approved the final manuscript.

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Robust Performance of Potentially Functional SNPs in Machine Learning Models for the Prediction of Atorvastatin-Induced Myalgia

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Background: Statins can cause muscle symptoms resulting in poor adherence to therapy and increased cardiovascular risk. We hypothesize that combinations of potentially functional SNPs (pfSNPs), rather than individual SNPs, better predict myalgia in patients on atorvastatin. This study assesses the value of potentially functional single nucleotide polymorphisms (pfSNPs) and employs six machine learning algorithms to identify the combination of SNPs that best predict myalgia.

Methods: Whole genome sequencing of 183 Chinese, Malay and Indian patients from Singapore was conducted to identify genetic variants associated with atorvastatin induced myalgia. To adjust for confounding factors, demographic and clinical characteristics were also examined for their association with myalgia. The top factor, sex, was then used as a covariate in the whole genome association analyses. Variants that were highly associated with myalgia from this and previous studies were extracted, assessed for potential functionality (pfSNPs) and incorporated into six machine learning models. Predictive performance of a combination of different models and inputs were compared using the average cross validation area under ROC curve (AUC). The minimum combination of SNPs to achieve maximum sensitivity and specificity as determined by AUC, that predict atorvastatin-induced myalgia in most, if not all the six machine learning models was determined.

Results: Through whole genome association analyses using sex as a covariate, a larger proportion of pfSNPs compared to non-pf SNPs were found to be highly associated with myalgia. Although none of the individual SNPs achieved genome wide significance in univariate analyses, machine learning models identified a combination of 15 SNPs that predict myalgia with good predictive performance (AUC >0.9). SNPs within genes identified in this study significantly outperformed SNPs within genes previously

reported to be associated with myalgia. pfSNPs were found to be more robust in predicting myalgia, outperforming non-pf SNPs in the majority of machine learning models tested. **Conclusion:** Combinations of pfSNPs that were consistently identified by different machine learning models to have high predictive performance have good potential to be clinically useful for predicting atorvastatin-induced myalgia once validated against an independent cohort of patients.

Keywords: statin, myalgia, whole genome sequencing, machine learning, pharmacogenomics

INTRODUCTION

Cardiovascular disease is a leading cause of death worldwide (World Health Organization – Cardiovascular Disease, 2020). High blood cholesterol levels increase the risk of cardiovascular disease, making lipid-lowering medications such as statins important for the therapeutic management of this risk factor (SEARCH Collaborative Group et al., 2010; Cholesterol Treatment Trialists et al., 2012; Silverman et al., 2016). Statins, or 3-hydroxy-3-methylglutaryl CoA reductase inhibitors, are generally well tolerated. However up to 25% of individuals have reported some degree of statin-associated muscle symptoms (SAMS) (Bruckert et al., 2005; Cohen et al., 2012). These side effects range from myalgia (with or without elevations in serum creatine kinase) to severe rhabdomyolysis (Alfirevic et al., 2014). Although severe forms of muscle toxicity such as myopathy and rhabdomyolysis are rare, the most common event leading to discontinuation of statins are muscle symptoms, in particular those without significant elevation in creatine kinase (McGinnis et al., 2007; Wei et al., 2013; Stroes et al., 2015). As treatment of hypercholesterolaemia is life-long, poor adherence to prescribed statin therapy increases the risk of cardiovascular events (Chowdhury et al., 2013; Saxon and Eckel, 2016). It is therefore important to be able to identify patients with muscle symptoms of pharmacological origin so that they can receive appropriate management. These patients could also receive alternative non-statin therapies such as the more expensive PSCK9 inhibitors or ezetimibe (Bakar et al., 2018).

Previous pharmacogenomic studies have reported genetic variations that are associated with SAMS, most notably the rs4149056 polymorphism in the *SLCO1B1* gene. (Link et al., 2008; Wilke et al., 2012). This polymorphism has been included in CPIC guidelines for simvastatin therapy. While the pharmacokinetic basis of rs4149056 and simvastatin-induced myopathy has been established in several clinical studies (Pasanen et al., 2006; Voora et al., 2009; Carr et al., 2013), it is unclear whether this variant is also associated with SAMS in patients on lower doses of statin, milder myalgia or from different populations (Donnelly et al., 2011.; Hubacek et al., 2015; Sai et al., 2016; Zhong et al., 2018). For instance, Donnelly et al. (2011) reported an association of *SLCO1B1* variants with mild myalgia in patients receiving high doses of statin, but Huback et al. (2015) reported that *SLCO1B1* polymorphisms were not associated with risk of myalgia in a Czech population. Furthermore, this association is strongest for simvastatin, and there are conflicting reports for atorvastatin treatment which is the most widely prescribed high-potency statin (Voora et al., 2009; Carr et al., 2013; Brunham et al., 2018). Atorvastatin, simvastatin and other statins differ in the ring that is attached to their active moieties as well as in the form that they are administered in (Turner and Pirmohamed, 2019; Ward et al., 2019). These statins therefore have different pharmacokinetic characteristics and involve different genes and SNPs for their metabolism and transport.

In addition to SNPs in the *SLCO1B1* gene, SNPs in several other pathways including statin metabolism [e.g., cytochrome P450 (CYP) genes (Frudakis et al., 2007; Shek et al., 2017) and glycine amidinotransferase (GATM) (Mangravite et al., 2013)], statin transport [e.g., ATP binding cassette (ABC) transporters (Zhang et al., 2019)], and immune response (e.g human leukocyte antigen (HLA) (Sai et al., 2016) and leukocyte immunoglobulin-like receptor (LILR) (Siddiqui et al., 2017)] have also been implicated in SAMS (reviewed in Ward et al., 2019; Turner and Pirmohamed, 2019). For some of these SNPs, further studies have shown that the associations do not replicate (e.g., the *GATM* variant) (Floyd et al., 2014; Luzum et al., 2015). Clinical factors such as age, sex, ethnicity, daily dose, body mass index, drug-drug interactions, comorbidities, duration of statin use and use of concomitant medications have also been implicated with SAMS (SEARCH Collaborative Group et al., 2010; Cohen et al., 2012; Tournadre, 2020), although the association of these covariates again varies with each study.

Hence, this study aims to examine the role of genetic and clinical factors for predicting atorvastatin-induced myalgia in the Singapore population, which comprises mainly of individuals of Chinese, Malay and Indian descent. Genetic polymorphisms associated with myalgia were obtained by whole genome sequencing (WGS). Unlike exome or targeted sequencing technologies previously used in the discovery of statin associated myopathy variants (Ruano et al., 2007; Ruano et al., 2011; Bakar et al., 2018; Floyd et al., 2019), WGS allows for the detection of polymorphisms in both coding and non-coding regions. Furthermore, our group has found that non-coding regions contain a larger proportion of potentially functional SNPs compared to coding regions (Bachtiar et al., 2019a), which makes WGS a more suitable platform compared to other technologies. Floyd et al. (2019) reported that there was no evidence linking rare coding variants to adverse statin reactions, and given our small sample size, we have decided to focus on common variants in this study.

The potentially functional SNPs (Wang et al., 2011) uncovered from this study, as well as from other known genes in the atorvastatin pathway, were used for predicting myalgia using a

variety of machine learning approaches. Machine learning has previously been used to predict drug response or dosage in fields such as cancer, psychiatry and cardiovascular disease (Liu et al., 2015; Huang et al., 2018; Athreya et al., 2019). To our knowledge, it has not been applied to predict the risk of statin-induced myalgia based on pharmacogenomic data. Insights gained from this study can therefore help to reveal important clinical and genetic risk factors that are predictive of atorvastatin-induced myalgia, as well as demonstrate the utility of machine learning approaches in pharmacogenomics.

MATERIALS AND METHODS

Study Cohort

This study examined 183 subjects on atorvastatin therapy from the Surveillance and Pharmacogenomics Initiative for Adverse Drug Reactions (SAPhIRE) project. Written informed consent was obtained from all participants and the study protocol was approved by the National Healthcare Group Domain Specific Review Board (NHG DSRB). For patients who reported muscle pain, severity of symptoms was scored based on two criteria, regional distribution pattern and temporal pattern. The scoring for regional distribution is as follows - "non-specific, intermittent" was given a score of 1, "symmetric hip flexors/thigh aches" was given a score of 3 while "symmetric calf aches" and "symmetric upper proximal aches" were given a score of 2. For temporal pattern, "onset < 4 weeks" was given a score of 3, "4–12 weeks" was given a score of 2 and ">12 weeks" was given a score of 1. The scores for the two patterns were added with scores ranging from 0 (no muscle pain) to 6, and patients who responded with a score of 0–2 were defined as the statin tolerant group while those with a score of 4–6 were defined as the myalgia group. All 30 patients in the myalgia group were selected for further analysis, while 153 out of 946 patients were randomly selected from the atorvastatin tolerant group to form the controls. From this study cohort, 48% were self-reported Chinese, 31% Indian and 21% Malay, and patients had a mean age of 57.4 (95% CI: 55.9–59.0) years, although one patient did not have age data. Patients were treated with atorvastatin for 10–5,046 days with a daily dose ranging from 5 to 80 mg. Demographics (including age, sex, height and weight), comorbidities and medications of all patients were recorded. Each patient provided a venous blood sample which was transferred into EDTA tubes and stored at −80°C for genetic analyses.

Whole Genome Sequencing

Genomic DNA was extracted and purified from whole blood using the Omega Bio-Tek E. Z.N.A. Blood DNA mini kit (Norcross, GA, United States). DNA concentration was measured using Qubit® DNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, United States). Fragment distribution of DNA library was measured using the DNA Nano 6000 Assay Kit of Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, United States). A total amount of 1.0 μg DNA per sample was used as input material for the DNA sample preparations. Sequencing libraries were generated using NEBNext® DNA Library Prep Kit following manufacturer's recommendations and indices were added to each sample. The genomic DNA is randomly fragmented to a size of 350 bp by shearing, then DNA fragments were end polished, A-tailed, and ligated with the NEBNext adapter for Illumina sequencing, and further PCR enriched by P5 and indexed P7 oligos. The PCR products were purified (AMPure XP system) and resultant libraries were analyzed for size distribution by Agilent 2100 Bioanalyzer and quantified using real-time PCR. Sequencing was performed on the Illumina platform (HiSeq X) using a paired-end read length of 150 bp. Data files have been uploaded to the European Nucleotide Archive with accession number PRJEB40922.

Sequence Alignment and Data Processing

Read pairs with adapter contamination, more than 10% bases uncertainty or >50% low quality bases in either read were first discarded. Burrows-Wheeler Aligner (BWA) was utilized to map the paired-end reads to the human reference genome b37 (ftp.broadinstitute.org/bundle/b37/human_g1k_v37_decoy.fasta.gz) and duplicate reads marked using Picard (http://picard.sourceforge.net) (Li and Durbin, 2009). The BAM files were further processed following the GATK Best Practices Workflow (https://www.broadinstitute.org/gatk/guide/best-practices). Single-sample genotypes were called using GATK HaplotypeCaller (McKenna et al., 2010) followed by hard filtering with the following options: QualByDepth > 2.0, FisherStrand < 60.0, MappingQuality > 40, MappingQualityRankSumTest > − 12.5, ReadPosRankSumTest > − 8.0 and StrandOddsRatio < 3.0. Variants were annotated using ANNOVAR according to the hg19 reference genome (Wang et al., 2010). Downstream analyses were only performed on biallelic SNPs that passed all quality filters above, had less than 10% of genotype missingness, deviation from Hardy-Weinberg equilibrium $p > 0.001$ and minor allele frequency >10%. Genotypic data from myalgia patients, controls and 1,000 Genomes was used in a principal component analysis (PCA) using PLINK 1.9 (Chang et al., 2015) to identify racial stratification in our dataset, and figures were plotted in R.

Univariate and Single Variant Analysis

Statistical analyses for all clinical parameters (expressed as mean, 95% CI) were performed using R 3.6.1. Fisher's exact tests were used for categorical variables and t-tests for continuous variables. Unlike the chi-squared test, Fisher's exact test does not require the expected frequencies of cases and controls to be large, and was the more suitable test given the small sample size in this study. To determine the association of genetic polymorphisms with myalgia, binary logistic regression was performed on the 4,554,532 SNPs with known rs numbers using PLINK 1.9. Additive, dominant and recessive models for genotypes were separately tested. Sex was included as a covariate as it was found to be significantly associated with myalgia, and the first two principal components (PCs) were used to correct for population substructure. SNPs obtained from this single variant analysis were ranked according to the lowest *p*-value out of the three genotypic models tested.

For a 0.1 minor allele frequency cutoff, assuming a reported prevalence rate of 0.2 (prevalence has been reported to be up to 25%) and a case control ratio of 1:5, to detect an odds ratio of 5 with $p < 5 \times 10^{-8}$ and 80% statistical power, a sample size of 34 cases for the additive model and 35 cases for the dominant model is required. However, the prevalence rate of myalgia in this study may not be 0.2 as there were only 30 patients with myalgia out of the 976 patients on atorvastatin therapy whose clinical data was available. Assuming a prevalence rate of 30/976 = 0.03, 66 cases would be required to detect the above effects.

Selection of Potentially Functional SNPs

Potential functionalities of SNPs found in this study were evaluated using the pfSNP resource developed by our laboratory (Wang et al., 2011). pfSNPs include SNPs that reside within regions under natural selection forces, as well as those predicted to alter the expression, structure, function, or activity of the associated gene. For coding SNPs, functionality was determined based on whether the SNP resides within protein modification sites such as phosphorylation sites, within important protein domains/functional regions, or are predicted to affect exonic splice enhancer/silencer sites or nonsense-mediated decay. Furthermore, within the coding region, synonymous mutations were assessed for significant codon usage bias as this could potentially influence the speed of the translation process (Kimchi-Sarfaty et al., 2007), while predicted deleteriousness was used for selecting non-synonymous pfSNPs (Bachtiar et al., 2019b). In addition to the pfSNP resource, expression quantitative trait loci (eQTLs) from the GTEx database (gtexportal.org) (Carithers et al., 2015), the eqtlGen consortium (eqtlgen.org) (Võsa et al., 2018) and the Jansen study (eqtl.onderzoek.io) (Jansen et al., 2017) were also used to identify potentially functional SNPs (pfSNPs). Cumulative counts of potentially functional (pf) SNPs were compared with non-pf SNPs for the top 100 SNPs most associated with myalgia based on univariate association *p*-values.

Selection of Candidate SNPs for Prediction

Three separate groups of SNPs were used as inputs into the machine learning models. These were: 1) SNPs that were most highly associated with myalgia from our results, 2) SNPs residing in 128 genes in the atorvastatin pathway from the drug databases Drugbank, CHEMBL, CTD and PharmGKB as previously obtained by our group (**Supplementary Table S1**) (Bachtiar et al., 2019b), and 3) SNPs in nine genes reported to be associated with atorvastatin-induced myalgia from the literature (**Supplementary Table S2**) (Ruano et al., 2007; Ruano et al., 2011; Brunham et al., 2018). SNPs in these three groups were ranked by their *p*-value of association with myalgia from our univariate analysis, and the top 50 overall, pf and non-pf SNPs from these three groups were extracted and separately used for training the models. For genes found to be associated with myalgia from the literature, only 20 non-pf SNPs were found. SNPs with missing values as well as those with greater than 80% correlation with a more significant SNP were removed. Non-pf SNPs that had greater than 80% correlation with pfSNPs were also removed from the non-pf group.

Predictions Using Machine Learning

Six classifiers were selected for predicting myalgia. These include regression based methods such as logistic regression and elastic nets; tree based methods such as random forests and boosted trees and other popular machine learning approaches such as neural networks and support vector machines. As there is currently no consensus as to which approach is best for genomic data, these six models were selected as a broad representation of popular machine learning models used for prediction. SNPs that performed well on most or all models represent SNPs that are able to predict myalgia to a high degree of confidence. As the different models use different approaches for learning and prediction, consistent results from the majority of models would increase our confidence about the validity of the results. All predictions were made using the R caret package in conjunction with the glm, glmnet, rf, gbm, nnet and svmRadial packages for training the individual models (Kuhn, 2008). Default caret training settings were used and sex was included as a predictor in all models. Predictive performance using the top 5–50 (in intervals of 5) overall, pf and non-pf SNPs from all three groups were separately obtained using the average 5-round 5-fold cross validation area under ROC curve (AUC) as the performance score. The unpaired t-test with Bonferroni correction ($n = 3$) was used to determine if there was a significant difference in mean AUC values of models using pfSNPs, non-pf SNPs and all SNPs. All six models were also trained without SNP data using 1) only sex as a predictor and 2) all clinical characteristics as predictors for determining the baseline model.

RESULTS

Demographic and Clinical Characteristics

There were 88 Chinese, 57 Indians and 38 Malays in the dataset and patients ranged in age from 25 to 81 years (mean = 57.4, CI = 55.9–59.0). The ethnic distribution in the study cohort is generally reflective of the Singapore population, although there was a lower percentage of Chinese and a higher percentage of Indians in the study cohort. This can be attributed to the higher prevalence of coronary heart disease in Singapore Indians requiring statin pharmacotherapy resulting in a higher proportion of Indians among statin users (Hughes et al., 1990; Ounpuu and Yusuf, 2003). All patients were treated with atorvastatin and the demographic and clinical characteristics of patients according to myalgia status is shown in **Table 1**. Of these characteristics, only sex was found to be significant ($p < 0.05$), with females more likely to have statin induced myopathy than males (**Table 1**). None of the comorbidities and drug treatments were found to be significantly associated with myalgia.

Population Stratification

PCA analyses showed that Chinese patients from our dataset clustered more closely with 1,000 Genomes East Asian populations, and Indian patients from our dataset clustered more closely with 1,000 Genomes South Asian populations (**Supplementary Figure S1A**). Chinese, Malay and Indian

TABLE 1 | Clinical/demographic characteristics of myalgia (cases) and non-myalgia (controls) subjects.

Characteristic	Descriptor	Group		*p*
		Myalgia *n* = 30	**Non myalgia *n* = 153**	
Age (yrs)[a]		56.7 (52.6–60.8)	57.6 (55.9–59.2)	0.71
BMI (kg/m^2)		27.3 (25.5–29.1)	26.3 (25.6–27.0)	0.29
Statin dose (mg)		36.0 (28.7–43.3)	38.4 (35.4–41.5)	0.54
Days on statin		702 (344–1,060)	805 (657–953)	0.59
Reported ethnicity	Chinese	14 (46.7%)	74 (48.4%)	0.44
	Indian	12 (40.0%)	45 (29.4%)	
	Malay	4 (13.3%)	34 (22.2%)	
Sex	Male	22 (73.3%)	136 (88.9%)	0.038
	Female	8 (26.7%)	17 (11.1%)	
Alcohol consumption	No	27 (90.0%)	141 (92.2%)	0.72
	Yes	3 (10.0%)	12 (7.8%)	
Smoking	No	25 (83.3%)	119 (77.8%)	0.63
	Yes	5 (16.7%)	34 (22.2%)	
Myocardial infarction	No	6 (20%)	12 (7.8%)	0.085
	Yes	24 (80%)	141 (92.2%)	
Renal problems	No	23 (76.7%)	132 (86.3%)	0.18
	Yes	7 (23.3%)	21 (13.7%)	
Liver problems	No	28 (93.3%)	146 (95.4%)	0.64
	Yes	2 (6.7%)	7 (4.6%)	
Hypertension	No	13 (43.3%)	57 (37.3%)	0.54
	Yes	17 (56.7%)	96 (62.7%)	
Diabetes mellitus	No	20 (66.7%)	82 (53.6%)	0.23
	Yes	10 (33.3%)	71 (46.4%)	
Hypercholesterolemia	No	11 (36.7%)	44 (28.8%)	0.39
	Yes	19 (63.3%)	109 (71.2%)	
Blood thinner	No	5 (16.7%)	15 (9.8%)	0.33
	Yes	25 (83.3%)	138 (90.2%)	
Glucose lowering	No	21 (70%)	78 (51%)	0.071
	Yes	9 (30%)	75 (49%)	
Cholesterol lowering	No	28 (93.3%)	133 (86.9%)	0.54
	Yes	2 (6.7%)	20 (13.1%)	
Heart protective	No	19 (63.3%)	74 (48.4%)	0.16
	Yes	11 (36.7%)	79 (51.6%)	
Blood pressure lowering	No	23 (76.7%)	91 (59.5%)	0.099
	Yes	7 (23.3%)	62 (40.5%)	

[a]*One sample in the non-myalgia group was missing age data.*

patients from our dataset were also fairly well separated when projected on to the first two principal components (**Supplementary Figure S1B**), although there was some overlap between Chinese and Malay patients due to genetic admixture between the two ethnicities (Deng et al., 2015).

Single Variant Analyses

4,554,532 SNPs with known rs numbers passed quality control in our dataset, with the majority of variants residing in intergenic and intronic regions (**Supplementary Figure S2**). To identify single SNP variants that might be associated with statin induced myalgia, logistic regression adjusting for the first two principal components and sex was performed. Most of the SNPs that were highly associated with myalgia were located outside exons and untranslated (UTR) regions (**Figure 1A**), highlighting an important limitation of exome based platforms. A *p*-value of 5×10^{-8} is commonly used to determine significance in genome wide studies, based on an assumption of 1,000,000 independent tests and patterns of linkage disequilibrium in individuals of European descent (Fadista et al., 2016). Although none of the variants in our analyses met this *p*-value threshold, 15 suggestive SNPs ($p < 1 \times 10^{-5}$) were found, with genes *RHOBTB1* on chromosome 10 and *SUSD1* on chromosome 9 containing the most number of suggestive SNPs, all of which were potentially functional (**Figure 1B**; **Table 2**). The top SNP for *RHOBTB1*, rs10821852, is an intronic SNP with an odds ratio (OR) of 5.66 (95% CI: 2.70–11.8, *p*: 4.23×10^{-6}, assuming an additive genotypic model) while the top SNP for *SUSD1*, rs10981237 is an intronic SNP with an OR of 21.67 (95% CI: 5.68–82.8, *p*: 6.81×10^{-6}, assuming a recessive genotypic model) (**Table 2**).

Distribution of Potentially Functional SNPs

Of the 4,554,532 SNPs with known rs numbers, approximately 60% (2,774,804) were potentially functional. The cumulative number of pfSNPs was consistently higher than that of non-pf SNPs in the top 100 SNPs most associated with myalgia (**Figure 1C**).

Good Predictive Performance Using 15 SNPs

Predictive performance was greatest when using SNPs that were highly associated with myalgia from this study (highest AUC: 1,

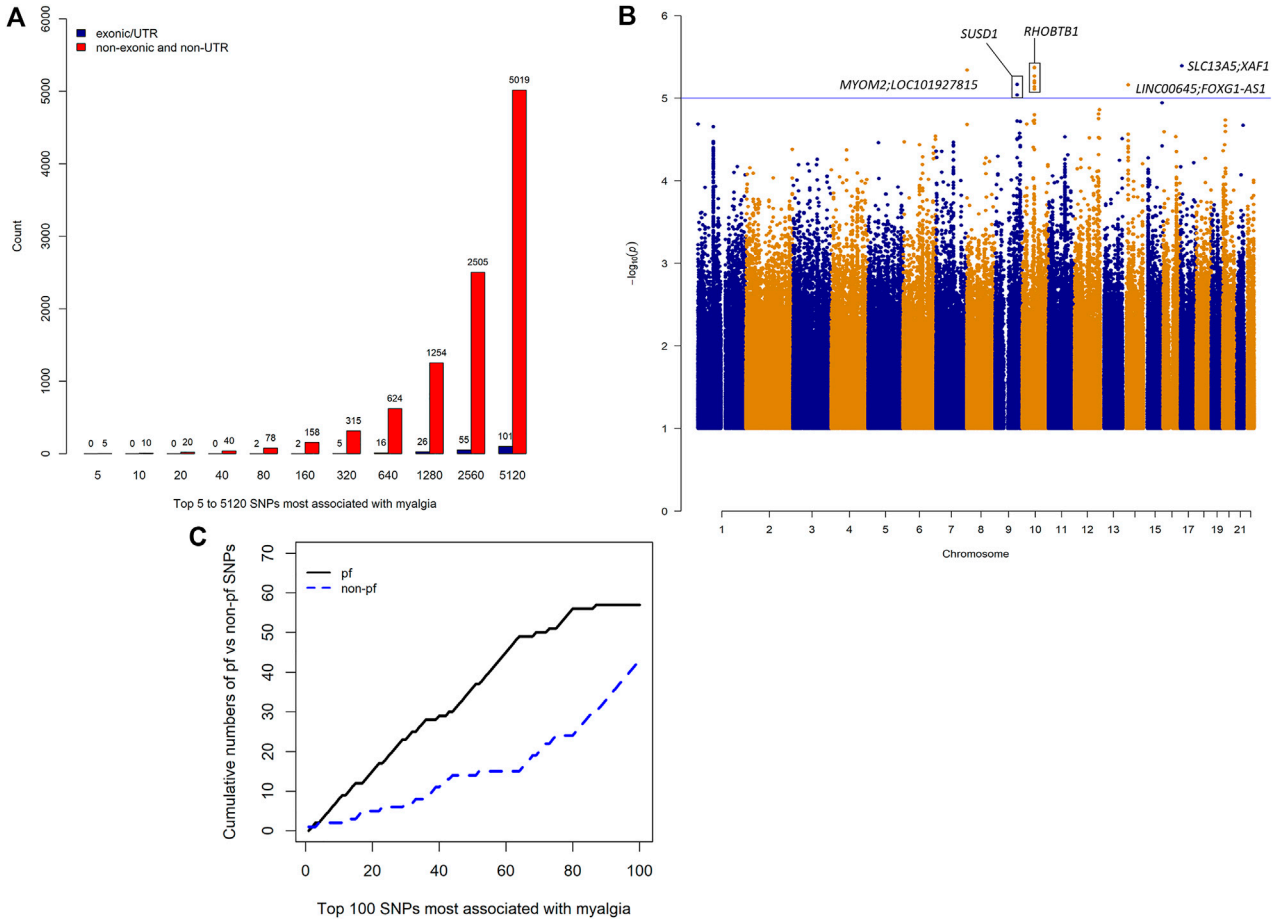

FIGURE 1 | Whole genome sequencing results. **(A)** Number of exonic/UTR variants vs non-exonic/non-UTR variants in the top 5 to 5,120 SNPs most associated with myalgia. **(B)** Manhattan plot of association of SNPs with atorvastatin-induced myalgia. The line indicates a *p*-value threshold of 1×10^{-5}, which can be considered to be a suggestive threshold of genome wide significance for small sample sizes. **(C)** Cumulative numbers of potentially functional (pf) and non-pf SNPs in the top 100 SNPs most associated with myalgia.

TABLE 2 | Top single variant associations with atorvastatin-induced myalgia ($p < 1 \times 10^{-5}$).

rsID	Chr	BP	OR (CI)	*p*	Model	Location	Gene	Intergenic distances	pfSNP
rs8082182	17	6,622,978	17.87 (5.244–60.91)	4.05*E* − 06	REC	Intergenic	*SLC13A5*; *XAF1*	dist = 6,238; dist = 36,178	NA
rs10821852	10	62,660,939	5.658 (2.704–11.84)	4.23*E* − 06	ADD	Intronic	*RHOBTB1*		eQTL
rs12263661	10	62,661,320	6.321 (2.88–13.87)	4.27*E* − 06	ADD	Intronic	*RHOBTB1*		eQTL
rs7011427	8	2,152,675	8.345 (3.369–20.67)	4.54*E* − 06	REC	Intergenic	*MYOM2*; *LOC101927815*	dist = 59,295; dist = 234,544	NA
rs750593	10	62,662,314	6.097 (2.798–13.28)	5.39*E* − 06	ADD	Intronic	*RHOBTB1*		eQTL
rs10821851	10	62,660,911	5.145 (2.529–10.47)	6.16*E* − 06	ADD	Intronic	*RHOBTB1*		eQTL
rs4437981	10	62,662,503	6.013 (2.758–13.11)	6.45*E* − 06	ADD	Intronic	*RHOBTB1*		eQTL
rs4575214	10	62,662,468	6.013 (2.758–13.11)	6.45*E* − 06	ADD	Intronic	*RHOBTB1*		eQTL
rs2893868	10	62,661,125	5.998 (2.753–13.07)	6.50*E* − 06	ADD	Intronic	*RHOBTB1*		eQTL
rs10981237	9	1,14,817,524	21.67 (5.675–82.76)	6.81*E* − 06	REC	Intronic	*SUSD1*		eQTL
rs16916623	9	114,821,568	21.67 (5.675–82.76)	6.81*E* − 06	REC	Intronic	*SUSD1*		eQTL
rs8011850	14	29,117,256	9.648 (3.593–25.91)	6.87*E* − 06	ADD	Intergenic	*LINC00645*; *FOXG1-AS1*	dist = 1,008,414; dist = 77,192	NA
rs2893869	10	6,2,661,961	5.787 (2.688–12.46)	7.25*E* − 06	ADD	Intronic	*RHOBTB1*		eQTL
rs10821853	10	62,661,057	5.286 (2.548–10.97)	7.74*E* − 06	ADD	Intronic	*RHOBTB1*		eQTL
rs55744607	9	114,815,563	17.78 (4.986–63.37)	9.11*E* − 06	REC	Intronic	*SUSD1*		eQTL

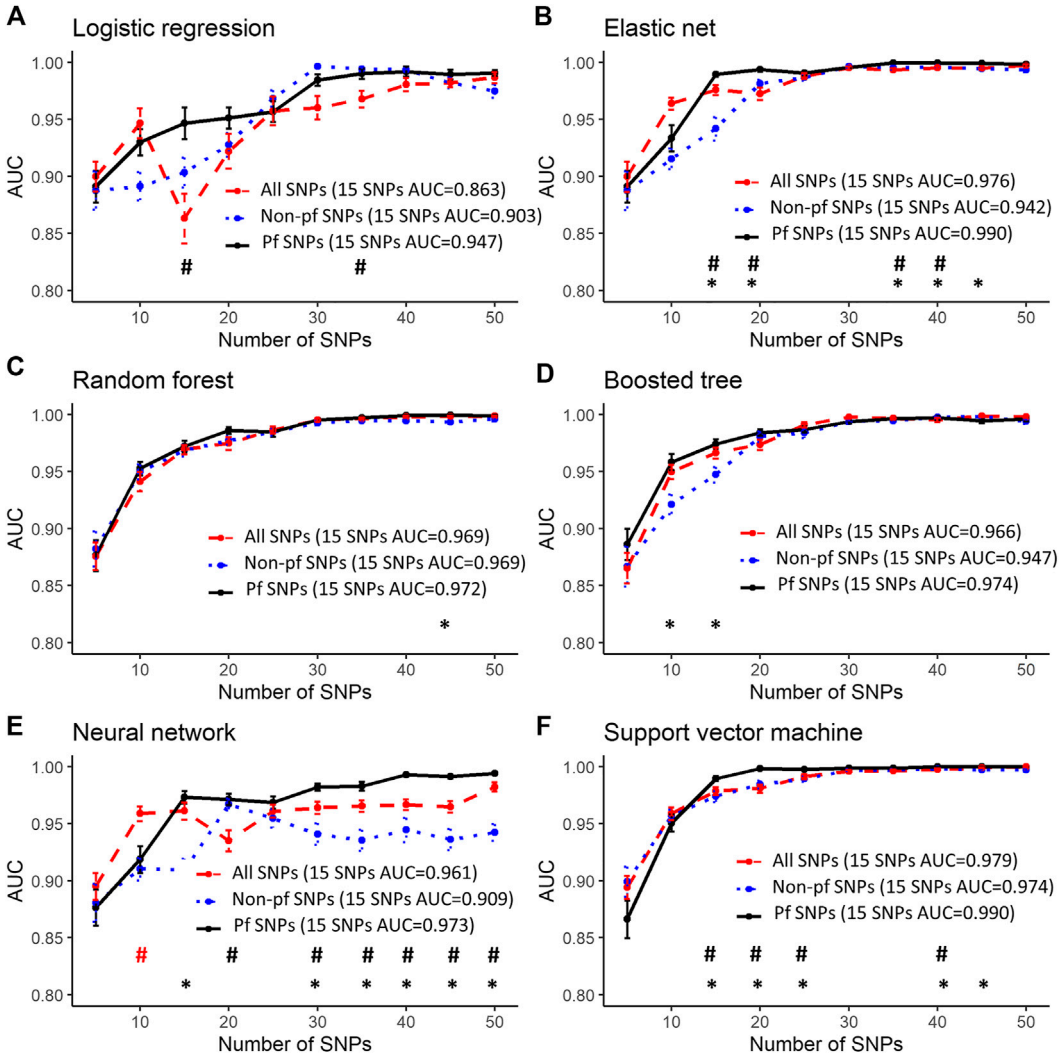

FIGURE 2 | Predictive performance using the top 5 to 50 SNPs most associated with myalgia from our dataset. Error bars denote the standard error of the mean. #'s indicate statistical significance when comparing between all SNPs and pfSNPs while *'s indicate statistical significance when comparing between non-pf SNPs and pfSNPs. Statistical significance when comparing between all SNPs and non-pfSNPs is not shown. The colors represent the input set with the higher AUC (red—all SNPs, blue—non-pf SNPs and black—pfSNPs). Bonferroni corrected unpaired *t*-test *p*-values ($p < 0.05$) were used for determining statistical significance.

Figure 2) followed by SNPs in atorvastatin pathway genes (highest AUC: 0.936, **Figure 3**) and SNPs in myalgia associated genes from previous studies (highest AUC: 0.794, **Figure 4**). For all models and inputs, close to maximal AUCs were generally achieved when 15 SNPs were used, after which there was either minimal increase in predictive performance, or a decrease in AUC values (**Figures 2–4**). However, for SNPs in myalgia associated genes from previous studies, mean AUC values did not increase with increasing number of SNPs, suggesting that most of these SNPs were not predictive (**Figure 4**). Out of the top five pfSNPs in this group, four were within the *ABCG2* gene while one was at the *HTR3B* locus. In terms of the best performing machine learning model when 15 SNPs were used as inputs, the best model was support vector machine (AUC: 0.990) for SNPs found from this study (**Figure 2**), random forest (AUC: 0.89) for atorvastatin pathway SNPs (**Figure 3**) and boosted tree (AUC:0.790) for SNPs in genes from previous studies (**Figure 4**).

Robust Performance of Potentially Functional SNPs in Predicting Myalgia

The best performing models described above for a 15 SNP input were obtained when using only pfSNPs, and not when using all SNPs or non-pf SNPs. Furthermore, when comparing pfSNPs to combined SNPs, in four of the machine learning models (logistic regression, elastic net, neural network, support vector machine), pfSNPs outperformed combined SNPs when 15 or more SNPs were used (**Figures 2A, B, E, F**, # indicates Bonferroni corrected $p < 0.05$). The predictive performance of pfSNPs was only significantly lower than the combined SNPs when a small number of 10 SNPs was used in the neural network model

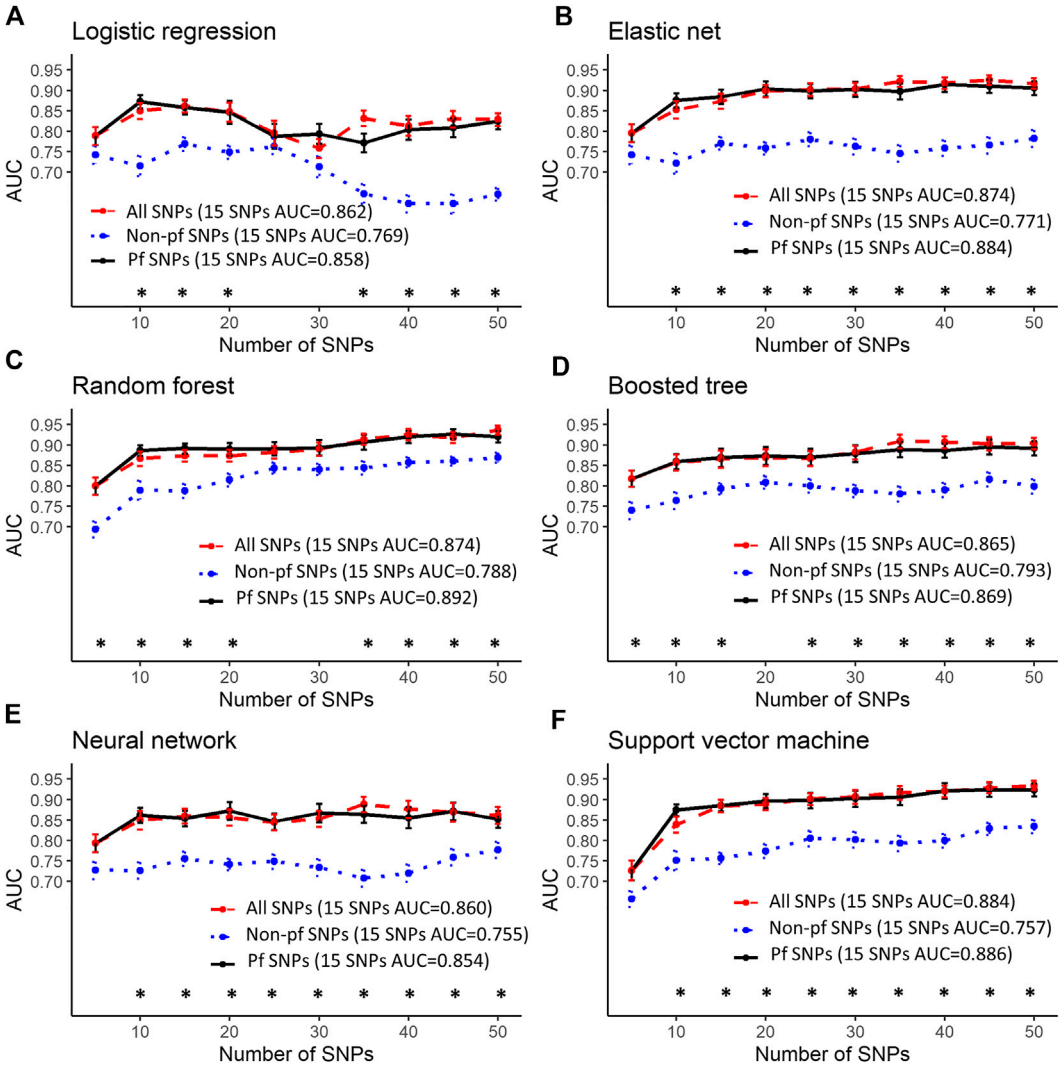

FIGURE 3 | Predictive performance using the top 5–50 SNPs in atorvastatin pathway genes. Error bars denote the standard error of the mean. #'s indicate statistical significance when comparing between all SNPs and pfSNPs while *'s indicate statistical significance when comparing between non-pf SNPs and pfSNPs. Statistical significance when comparing between all SNPs and non-pfSNPs is not shown. The colors represent the input set with the higher AUC (red—all SNPs, blue—non-pf SNPs and black—pfSNPs). Bonferroni corrected unpaired *t*-test *p*-values ($p < 0.05$) were used for determining statistical significance.

(**Figure 2E**, # indicates Bonferroni corrected $p < 0.05$). However, the AUC achieved using this 10 combined SNPs was 0.959 which was lower than when 15 pfSNPs were used in the same neural network model (AUC: 0.973). In the remaining models (**Figures 2C,D**) as well as for atorvastatin SNPs (**Figure 3**) and literature review SNPs (**Figure 4**), there was no significant difference between using pfSNPs and using combined SNPs. When comparing pfSNPs to non-pf SNPs, pfSNPs outperformed non-pf SNPs in almost all models and input sets (**Figures 2–4**, * indicates Bonferroni corrected $p < 0.05$). Additionally, the baseline performance of models only incorporating sex as a predictor (best AUC: 0.58) or using all clinical variables (best AUC: 0.57) (**Supplementary Table S3**) was significantly poorer than models incorporating both pfSNPs and sex (**Figures 2–4**).

DISCUSSION

In this study, we hypothesize that rather than individual SNPs, a combination of several potentially functional SNPs (pfSNPs) can better predict myalgia in patients on atorvastatin. Among the demographic and clinical characteristics examined, only sex was significantly ($p < 0.05$) associated with myalgia, with females having a higher risk. This is concordant with reports from previous studies (Link et al., 2008; Bakar et al., 2018; Tournadre, 2020). Through whole genome association analyses with sex as a covariate, we first demonstrated that among the top 100 SNPs that were most associated with myalgia, the cumulative number of pfSNPs was consistently higher than that of non-pf SNPs (**Figure 1C**) highlighting the importance of pfSNPs. To identify the combination of pfSNPs/non-pfSNPs that can predict

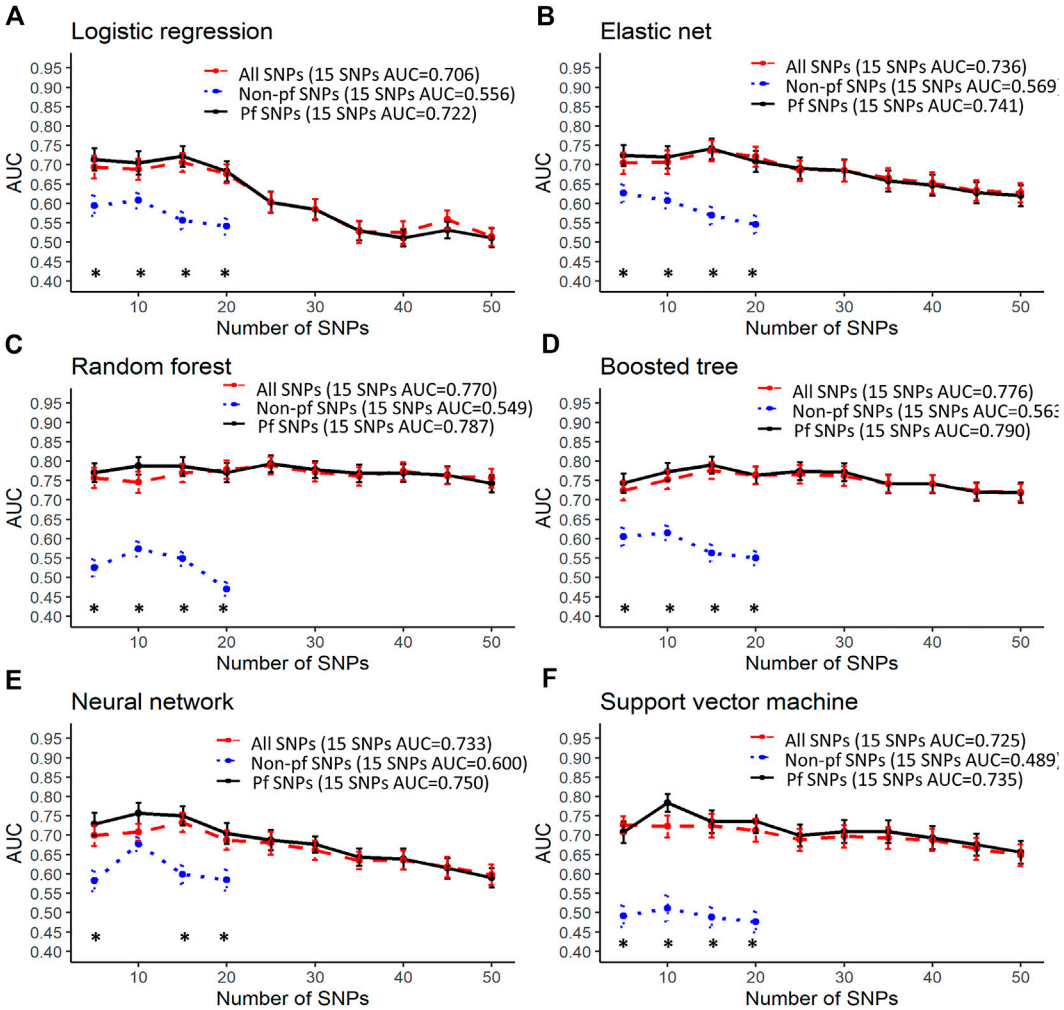

FIGURE 4 | Predictive performance using the top 5–50 SNPs in genes found to be associated with myalgia from previous studies. Error bars denote the standard error of the mean. #'s indicate statistical significance when comparing between all SNPs and pfSNPs while *'s indicate statistical significance when comparing between non-pf SNPs and pfSNPs. Statistical significance when comparing between all SNPs and non-pfSNPs is not shown. The colors represent the input set with the higher AUC (red—all SNPs, blue—non-pf SNPs and black—pfSNPs). Bonferroni corrected unpaired *t*-test *p*-values ($p < 0.05$) were used for determining statistical significance. Only 20 non-pf SNPs were found in genes associated with myalgia from the literature.

atorvastatin-induced myalgia, six different, but commonly used machine learning models were employed to identify the minimum number of pfSNPs/non-pfSNPs necessary to achieve optimal sensitivity and specificity, determined through the area under ROC curve (AUC), in most, if not all the six models. pfSNPs consistently outperforms non-pfSNPs in predicting myalgia. To our knowledge, this is the first study examining pfSNPs and utilizing machine learning models in the prediction of myalgia.

From the whole genome sequencing results, potentially functional SNPs in *RHOBTB1* and *SUSD1* were found to be highly associated with atorvastatin-induced myalgia. *RHOBTB1* is a member of the Rho GTPase family of signaling proteins with high levels of expression in the stomach, skeletal muscle, placenta, kidney and testis (Ramos et al., 2002). *RHOBTB1* is a tumor suppressor gene involved in head and neck cancer and is also involved in protecting against hypertension by improving vasodilator function (Xiao et al., 2017; Mukohda et al., 2019). Knockdown of *RHOBTB1* was also found to promote cardiomyocyte proliferation (Xiao et al., 2017). Given its high expression in skeletal muscle, as well as its role in cardiomyocyte proliferation and preventing vascular smooth muscle dysfunction, it is possible that this gene is also involved in preventing myalgia. Not much is known about *SUSD1*, which encodes for the sushi domain-containing protein 1 precursor. The sushi domain has been found in a number of proteins and is a motif for protein-protein interactions (Wei et al., 2001). SNPs in *SUSD1* has been previously associated with venous thromboembolism (Tang et al., 2013) and neurocognitive disabilities (Nilsson et al., 2017).

Most of the machine learning models gave similar AUC values making it difficult to draw definitive conclusions as to which

model performs best. However, models including only clinical factors (**Supplementary Table S3**) were found to have a poorer performance than models incorporating sex and genetic factors (**Figures 2–4**), demonstrating the higher predictive potential of SNPs compared to clinical factors. When 15 or more SNPs were used, elastic net, neural network and support vector machine models with potentially functional SNPs as inputs had significantly better mean AUCs compared to the same models incorporating non-pf SNPs and total SNPs as seen in **Figure 2**. Furthermore, the overall best models at the 15 SNP level for each of the three datasets (associated SNPs from this study, SNPs in artovastatin pathway genes and SNPs in genes found from the literature) all utilized pfSNPs as inputs (**Figures 2–4**). Taken together, these results suggest that SNP functionality is an important factor to consider for improving predictive performance. The importance of SNP functionality was also underscored by the fact that the raw count of pfSNPs was higher than non-pf SNPs in the top 100 variants most associated with myalgia.

Interestingly, we found that SNPs in genes previously reported to be associated with myalgia had the poorest predictive performance of the three groups. Furthermore, predictive AUCs in this group did not increase as the number of SNPs used was increased. Genes in this group include the serotonin receptor genes *HTR3B* and *HTR7* (Ruano et al., 2007), efflux transporter *ABCG2*, uptake transporter *SLCO1B1*, cytochrome P450 genes *CYP3A4* and *CYP2D6*, and other candidate genes such as *COQ2*, *ATP2B1*, *DMPK* (Ruano et al., 2011). Our results suggest that only a few SNPs in this group had predictive value, with *ABCG2* and *HTR3B* being the strongest candidate genes. It is also interesting to note that the rs4149056 variant in the *SLCO1B1* gene had a relatively high uncorrected *p*-value of 0.1 in our study. Furthermore, the minor allele frequencies of this variant were higher in controls than in cases for Singaporeans of Chinese, Malay and Indian ethnicities (**Supplementary Table S4**). These findings suggest that the rs4149056 variant may not have the same effect for milder myalgia, in non-European populations, or due to the type of statin used. These reasons were also alluded to in the review by Turner and Pirmohamed (2019) when discussing the role of *SLCO1B1* in statin-related myotoxicity.

There are however some limitations to this study. The relatively small number of samples, with only 30 patients reporting definitive myalgia, limits the discovery *p*-value to only a suggestive threshold, and could be a possible reason why *SLCO1B1* was not detected to be significant. Nevertheless, smaller sample sizes are not unusual in pharmacogenomic association studies due to the large effect sizes of pharmacogenomic variants, unlike complex disease association analyses (Maranville and Cox, 2016). Furthermore, in this study, being unable to achieve genome wide significance for single SNPs is not pertinent as the univariate *p*-values were merely used for the ranking of SNPs to facilitate the identification of a combination of multiple potentially functional SNPs that best predict atorvastatin-induced myalgia using six different machine learning algorithms. The combination of pfSNPs that were found by most, if not all, of the six different machine learning models to show high sensitivity and specificity in predicting myalgia highlights the robustness of our strategy. A second caveat is that predictive performance of the machine learning models, while achieving good cross validation AUCs, should ideally be validated against an independent test set. Nonetheless, cross validation is a useful indicator of the generalizability of the model and by utilizing the lowest number of SNPs with good AUCs, which we found to be in the 15 SNP range, we hope to minimize overfitting. We aim to validate these SNPs in an independent test set in a future study. The results of this study, while limited by the small sample size, represent a proof of concept of the potential of both machine learning methods and potentially functional polymorphisms in the prediction of drug response.

In conclusion, machine learning models with potentially functional SNPs were found to have good and robust properties for predicting atorvastatin-induced myalgia. However, SNPs in candidate genes previously reported to be associated with myalgia did not show good predictive properties, at least in this Singapore population. Combinations of pfSNPs that were consistently identified by different machine learning models to have high predictive performance have good potential to be clinically useful for predicting atorvastatin-induced myalgia once validated against an independent cohort of patients.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB40922.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the National Healthcare Group Domain Specific Review Board (NHG DSRB). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BO: data processing, statistical analysis, machine learning modeling, data interpretation, and writing of manuscript. RR and YJ: data processing and statistical analysis. AY: machine learning modeling and writing of manuscript. YK and SY: data acquisition and data processing. JHSL: whole genome sequencing and data processing. SC: data interpretation and cirtical review of manuscript. JT and JJL: Patient recruitment, data collection, study plan and critical review of manuscript throughout the editorial process. CL and CD: study plan, data acquisition, data interpretation, critical review of the

manuscript throughout the editorial process, and approval of the final manuscript draft submitted for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.605764/full#supplementary-material

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frontiers in Pharmacology

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Analysis of Genetic and Non-genetic Predictors of Levodopa Induced Dyskinesia in Parkinson's Disease

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Background: Levodopa (L-Dopa), representing the therapeutic gold standard for the treatment of Parkinson disease (PD), is associated with side effects like L-Dopa induced dyskinesia (LID). Although several non-genetic and genetic factors have been investigated for association with LID risk, contrasting results were reported and its genetic basis remain largely unexplored.

Methods: In an Italian PD cohort (N = 460), we first performed stepwise multivariable Cox Proportional Hazard regressions modeling LID risk as a function of gender, PD familiarity, clinical subtype, weight, age-at-onset (AAO) and years-of-disease (YOD), L-Dopa dosage, severity scores, and scales assessing motor (UPDRS-III), cognitive (MoCA), and non-motor symptoms (NMS). Then we enriched the resulting model testing two variants—rs356219 and D4S3481—increasing the expression of the *SNCA* gene, previously suggested as a potential mechanism of LID onset. To account for more complex (non-linear) relations of these variables with LID risk, we built a survival random forest (SRF) algorithm including all the covariates mentioned above.

Results: Among tested variables (N = 460 case-complete, 211 LID events; total follow-up 31,361 person-months, median 61 months), disease duration showed significant association ($p < 0.005$), with 6 (3–8)% decrease of LID risk per additional YOD. Other nominally significant associations were observed for gender—with women showing a 39 (5–82)% higher risk of LID—and AAO, with 2 (0.3–3)% decrease of risk for each year increase of PD onset. The SRF algorithm confirmed YOD as the most prominent feature influencing LID risk, with a variable importance of about 8% in the model. In genetic models, no statistically significant effects on incident LID risk was observed.

Conclusions: This evidence supports a protective effect of late PD onset and gender (men) against LID risk and suggests a new independent protective factor, YOD. Moreover, it underlines the importance of personalized therapeutic protocols for PD patients in the future.

Keywords: SNCA, α-synuclein, levodopa induced dyskinesia, Parkinson disease, rs356219, D4S3481

INTRODUCTION

Dyskinesia is characterized by involuntary dystonic and/or choreic movements of the trunk, limbs, and face (Finlay et al., 2014). One of the main risk factors predisposing to the onset of dyskinesia is Levodopa (L-Dopa) dosage, which currently represents the therapeutic gold standard for Parkinson's disease (PD) (Coelho and Ferreira, 2014), which led to define a specific subtype of motor complications called L-Dopa Induced Dyskinesia (LID). Different risk factors have been associated with the onset of LID in PD patients, both modifiable and non-modifiable (Prashanth et al., 2011). Non-modifiable risk factors include age, gender, PD age at onset (AAO), clinical subtype, and genetic factors, while modifiable factors include L-Dopa dosage, duration of treatment, and body weight (Arabia et al., 2002; Sharma et al., 2006; Warren et al., 2013). Women generally show a greater incidence of dyskinesia than men (Zappia et al., 2005; Warren et al., 2013) and tend to develop dyskinesia earlier in relation to time of L-Dopa administration (Hassin-Baer et al., 2011). This may be due to a higher bio-availability of L-Dopa in women, which may be in turn be due to their usually lower body weight (Coelho and Ferreira, 2014). However, a higher LID risk for women has not been confirmed in other studies (Coelho and Ferreira, 2014; Campêlo and Silva, 2017). Similarly, early PD onset was associated with an increased risk of LID: a 5-years follow-up study of PD patients showed a prevalence of LID up to 50% at age 40–59, and of 16% after 70 years (Kumar et al., 2005), while another study found that the rates of dyskinesia in PD patients after 5 years of L-Dopa treatment were 70, 42, 33, and 24% for onsets at 40–49, 50–59, 60–69, and 70–79 years, respectively (Ku and Glass, 2010). Similarly, patients with AAO <40 years had a higher incidence of LID than those with AAO ≥50 years (Kostic et al., 1991). In line with this evidence, patients with longer disease duration—which is partly dependent on AAO—are more likely to develop LID (Ahlskog and Muenter, 2001). Of note, age has been detected as a risk factor in a single cross-sectional study, which reported a positive correlation between patients' age and time-to-onset of motor complications, indicating that older patients develop dyskinesia later than younger patients (Schrag and Quinn, 2000). Similarly, disease duration has been also associated with prevalent risk of LID and motor fluctuations in a PD patients cohort from Ghana (Cilia et al., 2014). Among clinical subtypes of PD, the tremor-dominant subgroup has shown lower risk of dyskinesia compared to the bradykinetic and mixed manifestations subgroups (Zhang et al., 2013). PD stage has also been associated with LID risk, with patients in the early stage of the disease—with Hoehn and Yahr (HY) score of 1—showing a larger median time to LID from the beginning of L-Dopa treatment, compared to patients in a later HY stage (Kostic et al., 2002). Likewise, dyskinesia has been reported to be less frequent and less severe in late-stage PD patients (see Coelho and Ferreira, 2012 (Coelho and Ferreira, 2014)). A recent analysis of Chinese PD patients revealed a positive association of prevalent LID risk with high HY scores and low Unified Parkinson's disease Rating Scale part III (UPDRS-III) under active L-Dopa treatment, which suggested progression of the disease and severity of motor symptoms as risk factors, in addition to early AAO, long disease duration, gender (women being more affected), and high L-Dopa equivalent dose (Zhou et al., 2019). Of note, the emergence of dyskinesia had no association with the initiation time of L-Dopa (Zhou et al., 2019). On the contrary, a community-based study found that the overall dose of L-Dopa was the most important predictor of motor fluctuations in PD patients, with dose and treatment having the strongest impact on LID prevalence (Schrag et al., 1998). These studies suggest that L-Dopa dosage may be more important than the duration of treatment. In other words, the higher the dose, the greater the risk of dyskinesia (Zhang et al., 2013).

Overall, PD patients show a remarkable heterogeneity in their response to L-Dopa and this likely suggests that there is a certain genetic predisposition toward the development of LID (Kalinderi et al., 2019). A handful of studies investigated so far variants in the genes encoding dopamine receptors (*DRD1*, *DRD2*, and *DRD3*), brain-derived neurotropic factor (*BDNF*) and leucine-rich repeat kinase 2 (*LRKK2*) (reviewed in (Kalinderi et al., 2019)), which often led to contrasting or inconclusive findings, warranting further genetic studies on LID. Another known PD-causative gene, *SNCA* (4q22.1, coding for α-synuclein) has been robustly implicated and investigated in PD etiology and progression (Stanic et al., 2016), but has so far been mostly neglected with regard to motor complications connected to the treatment of the disease, except for few studies. One of these identified a heterozygous autosomal dominant point mutation in the *SNCA* gene (c.158C > A; p. A53E in transcripts NM_000345.3, NM_001146054.1, NC_000004.11), in two Finnish related PD patients characterized by severe bradykinesia, very slight tremor and early onset of LID (Martikainen et al., 2015). In C. elegans models overexpressing human α-synuclein, chronic exposure to L-Dopa led to hyperactivity without meaningful increase in motor activity, this being proposed as a simple animal model of LID (Gupta et al., 2016). In spite of this interesting hypothesis, so far only two studies attempted to investigate the association between variants increasing *SNCA* expression levels and LID risk. Corrado et al. tested the influence of the 263 base pairs (bp) allele of the D4S3481 microsatellite marker on the incident risk of LID, in a longitudinal cohort of Italian PD patients (Corrado et al., 2018). They reported no significant differences between 263 allele carriers vs non carriers. More recently, Kim et al. (2020) tested an independent variant associated with the increase of *SNCA* expression levels—rs356219—in a longitudinal cohort of PD patients, reporting associations with motor fluctuations, but not with incident LID (Kim et al., 2020). Of note, these genetic studies were carried out in relatively small samples and are in contrast with preliminary evidence implicating α-synuclein levels in dyskinesia etiology (see above). This, along with the relatively comprehensive knowledge gained so far on *SNCA*, compared to other candidate genes, prompted us to focus on *SNCA* to further investigate potential genetic influences on LID onset (see below).

To sum up, research on risk and protective factors for LID onset present two main open issues. One is the partially contrasting results reported for many potential predictors by

the different epidemiological studies, which warrant further investigations on the effect of these risk factors in cohorts of PD patients, possibly in a multivariable setting and across different ethnicities and ancestries, as well as within non-linear models. The other one is represented by the contrast among genetic/molecular studies involving *SNCA* (see above), which warrant further investigation of its influence on LID onset, especially of those variants leading to overexpression of the *SNCA* gene, to verify the hypothesis suggested by Gupta et al. (Gupta et al., 2016), or alternatively replicate the lack of associations detected for D4S3481 (Corrado et al., 2018) and rs356219 (Kim et al., 2020). Here, we investigated these two aspects, in a well characterized PD cohort from Central-Southern Italy (Gialluisi et al., 2019). First, we performed a multivariable survival analysis to analyze potential risk/protective effects of a number of non-genetic factors influencing LID risk. Then, we investigated in a similar setting potential effects of two variants previously implicated in PD etiology (Campêlo and Silva, 2017) and increasing the expression levels of *SNCA* gene, namely rs356219 (Fuchs et al., 2008; Pihlstrøm et al., 2018; Luo et al., 2019) and D4S3481 (Chiba-Falek et al., 2005; Fuchs et al., 2008; Trotta et al., 2012). Finally, we explored more complex relationships deploying exploratory supervised machine learning algorithms to detect the most influential variables on incident LID risk. We gained interesting insights in the epidemiology of LID and provide a notable contribution to knowledge in the field.

MATERIALS AND METHODS

PD Patients Cohort and Outcome Definition

472 PD patients (288 males) from Central and Southern Italy were recruited at IRCCS Neuromed, Italy (Gialluisi et al., 2019). All the cases were diagnosed with PD according to published diagnostic criteria (Postuma et al., 2015). The project was approved by the institutional Ethical Committee and written informed consent was obtained from all the participating subjects. All analyses were carried out in R (see URLs).

The main outcome of survival analyses (absence/presence of dyskinesia) was assessed by a trained neurologist. The time-to-event was defined as the number of months between the beginning of L-Dopa treatment (based on consultation of the hospital database or self-report by the patients through drug prescription) and the onset of LID, or alternatively the end of follow-up time (right-censoring on December 31st 2018).

Analysis of Incident LID Risk vs Non-genetic Factors

Basic assumptions of Cox Proportional Hazard (PH) regressions—proportionality of hazards, absence of outlier observations and linearity of effects—where checked for all the variables analyzed (see below), through Df-beta, Martingale and Schoenfeld residuals, where applicable.

To investigate the relation of non-genetic factors with the incident risk of LID, we applied a multivariable Cox PH regression—through the *cox. zph*() function of the *survival* package (see URLs) (Chung et al., 2014)—modeling LID onset as a function of gender, PD familiarity (familial/sporadic), clinical subtype (rigid-bradykinetic/tremorigenic/mixed), weight, PD AAO and years of disease (YOD), daily L-Dopa dosage (mg/day), HY scores, and scales assessing motor (UPDRS-III), cognitive (Montreal Cognitive Assessment, MoCA), and non-motor symptoms (NMS). Missing observations were imputed in all those patients with at least 50% of the measures available (N = 460), through a k-nearest neighbor (knn) algorithm implemented by the *kNN*() function (k = 10) of the *VIM* package (Zhang, 2015). Indeed, imputation is advised in presence of a patchy missing data pattern for variables with <50% missing values. This way, sample size was maximized to N = 460 (**Table 1**), which underwent statistical analyses.

In Cox PH models, we implemented a multivariable stepwise regression approach through the *stepAIC*() function of the *MASS* package (see URLs), which kept within each model only those covariates determining a gain in the tradeoff between the goodness-of-fit of a given model and its parsimony (i.e., the number of parameters included) (Akaike, 2011). In other words, in this analysis only those covariates which significantly contributed to an increase in the total variance explained by the model—in spite of the addition of a parameter to the regression—were kept, allowing to "clean" the model for potential bias introduced by other collinear covariates. For this analysis we set a significance threshold $\alpha = 4.5 \times 10^{-3}$, applying a Bonferroni correction for eleven different covariates tested. Moreover, full Cox regression models including all the covariates were run to compare results with stepwise regressions.

Analysis of Incident LID Risk vs Candidate SNCA Variants

After investigating non-genetic variables, we analyzed the effect on LID onset of two *SNCA* genetic variants increasing the expression levels of the gene, namely rs356219 (Fuchs et al., 2008; Pihlstrøm et al., 2018; Luo et al., 2019) and D4S3481 (Chiba-Falek et al., 2005; Fuchs et al., 2008; Trotta et al., 2012) (see below for details on genotyping). To this end, we built multivariable Cox PH regression models adjusted for the non-genetic variables retained in the stepwise regression above. This analysis was driven by the hypothesis that these markers could affect LID onset, as previously suggested for other *SNCA* variants (see Introduction section). Specifically, we tested these associations under the assumptions of additive effect for the SNP rs356219, as previously tested by Kim et al. (Kim et al., 2020), and of a dominant effect for the microsatellite marker D4S3481, where carriers of the 263 bp allele (see below) were tested against non-carriers, as in Corrado et al. (Corrado et al., 2018). A Bonferroni correction for two independent genetic variants tested was applied, resulting in $\alpha = 0.025$.

Genotyping of the Candidate SNCA Variants

rs356219

rs356219 (hg19 coordinates chr4:90637601; A/G; allelic frequencies ~49/51%)–lying in the 3′ untranslated region

TABLE 1 | Description of the neuromed PD cohort used for analysis.

Set	Total	Fpd	Spd
N	460	196	264
Age (mean ± SD)	66.57 ± 8.84	66.31 ± 9.10	66.77 ± 8.65
AAO (mean ± SD)	58.17 ± 10.05	57.82 ± 10.55	58.42 ± 9.67
Disease duration (mean ± SD)	8.39 ± 6.16	8.51 ± 6.67	8.30 ± 5.77
Sex ratio (M/F)	282/178	124/72	158/106
Dyskinesia (D/ND)	211/249	98/98	113/151
PD clinical subtype (RB/t/mixed)	316/71/73	141/24/31	175/47/42
Weight (mean ± SD)	74.22 ± 10.92	73.47 ± 10.93	74.78 ± 10.89
BMI (mean ± SD)	26.90 ± 3.36	26.71 ± 3.46	27.05 ± 3.28
UPDRS (mean ± SD)	21.77 ± 10.44	21.09 ± 11.03	22.25 ± 9.98
MoCA (mean ± SD)	23.23 ± 4.72	23.19 ± 4.50	23.27 ± 5.03
HY (mean ± SD)	1.94 ± 0.87	1.92 ± 0.92	1.94 ± 0.83
NMS (mean ± SD)	56.39 ± 34.65	57.56 ± 34.95	55.52 ± 34.47

Here we report a description of PD cohort. AAO, age at onset; BMI, body mass index; D/ND, dyskinetic/non dyskinetic; HY, hoehn e yahr; M/F, male/female; MoCA, montreal cognitive assessment; NMS, non motor symptoms; RB/T, rigid-bradykinetic/tremorigen; SD, standard deviation; UPDRS, unified parkinson's disease rating scale.

(3′UTR) of the *SNCA* gene (4q22.1) and previously associated with its circulating levels of expression (Chiba-Falek, 2001; Chiba-Falek et al., 2005; Fuchs et al., 2008; Cronin et al., 2009; McCarthy et al., 2011; Trotta et al., 2012; Cardo et al., 2014; Campêlo and Silva, 2017)–was genotyped using TaqMan® custom assays (Bio-Rad, United States), according to the manufacturer's protocol, and analyzed in a Bio-Rad® CFX96™ Real Time PCR detection system. About 10–50 ng of DNA were amplified with 5 μL of 2X TaqMan universal PCR master mix, 0.5 μL of 20X primer and TaqMan probe dye mix. Cycling conditions were 3 min at 95°C, followed by 40 cycles of 15 s at 95°C and 30 s at 60°C. Genotyping was performed on 470 PD cases for which DNA samples were available at the time of genetic analyses.

We performed a general quality control (QC) of genotyped samples, in PLINK v1.9 (Cardo et al., 2014). These showed a call rate >98% (17 samples with missing genotype) and were in Hardy Weinberg Equilibrium (HWE, $p = 0.62$), suggesting a good quality of genotyping.

D4S3481 (Rep1)

D4S3481 (also known as Rep1) was analyzed in 469 PD patients of the Neuromed cohort, as described in (Maraganore et al., 1996) and in following studies (Corrado et al., 2018). Briefly, the region was amplified through polymerase Chain Reaction (PCR) from genomic DNA, using the following primer pairs: Fam5′-CCTGGCATATTTGATTGCAA-3′ and 5′-GACTGGCCCAAG ATTAACCA-3′. PCR reactions (25 μL final volume) containing 2 mmol/L MgCl2, 0.5 μM of each primer, 200 μM dNTPs, 1 unit of Taq polymerase (Life Technologies) and approximately 20 ng of genomic DNA. Thermal cycling was performed with an initial denaturation of 180 s at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at 61°C, 45 s at 72°C, and a terminal extension of 10 min at 72°C. PCR products were resolved by capillary electrophoresis on an ABI-3130XL DNA Analyzer (Applied Biosystem, Foster City, CA, United States), using GeenScan-500 ROX (Applied Biosystem) as molecular weight marker. Allelic sizes were assessed using the GeneMapper® Software Version 4.0 SNPlex™ (Applied Biosystem, Foster City, CA, United States). This method allows to determine the length of dinucleotide repeats at the investigated locus, and typically results in number of repeats ranging between 255 and 263. Since we detected only one sample carrying the 255 allele, and two samples with the 257 allele, and these alleles are usually neglected due to their low frequency (Corrado et al., 2018), we removed them before the analyses, as done elsewhere (Maraganore et al., 1996). This variant showed genotyping call rate >97% (29 samples with missing genotype) and was in HWE ($p = 0.28$).

Machine Learning Analyses

To validate observations made in Cox PH models and explore more complex relationships of the investigated predictors with LID risk, we built survival random forest (SRF) algorithms using the above mentioned covariates as input features and dyskinesia events and time-to-dyskinesia as a label. SRF represent a class of supervised machine learning algorithms used to predict incident outcomes in a longitudinal setting and are potentially more powerful than classical statistical models since they model also non-linear and interactive functions in the prediction. In particular, two models were deployed, both including and excluding *SNCA* variants as features. SRF were built using the *rfsrc*() function of the randomForestSRC package in R (see URLs). After hyperparameter tuning, model training and testing, we performed a variable importance analysis (*vimp*() function) to establish the most important features (or variables) influencing the prediction of incident LID events. Details on the construction of the SRF are reported in Supplementary Methods.

RESULTS

Survival Models With Non-genetic Predictors

The multivariable Cox PH regression analyzing the association of non-genetic factors with the incident risk of LIDs was performed in 460 PD cases for which all phenotypic, clinical and pharmacological information was available after imputation,

TABLE 2 | Results of the stepwise multivariable Cox PH regression modeling LID onset vs non-genetic factors.

Exposure	HR	CI (lower)	CI (upper)	z	p
Gender (women)	1.39	1.05	1.82	2.33	0.02
Familiarity	1.24	0.95	1.63	1.55	0.12
AAO	0.98	0.97	1.00	−2.33	0.02
Hoehn and yahr	0.89	0.77	1.04	−1.43	0.15
YOD	0.94	0.92	0.97	−3.80	1.4×10^{-4}

Here we report only exposures which were retained in stepwise multivariable models. AAO, age at onset; CI (lower/upper), inferior and superior limits of the 95% Confidence Interval of HRs; HR, hazard ratio; p, p-value; YOD, years of disease; z, z-score. Significant associations surviving Bonferroni correction ($p < 4.5 \times 10^{-3}$) are highlighted in bold.

with a total of 211 LID events. These subjects were followed for a total of 31,361 person-months (median follow-up time 61 months). The AIC-based stepwise approach retained five variables in the model, namely gender, PD familiarity, age-at-onset (AAO), staging (H&Y) and duration (YOD), whose associations with the incident risk of LID are reported in **Table 2**. In particular, YOD showed significant associations surviving correction for multiple testing, with a longer duration of disease being protective against LID onset (HR [CI] per additional year of disease = 0.94 [0.92–0.97], $p = 1.4 \times 10^{-4}$). Nominally significant associations ($p = 0.02$) were observed for AAO (HR [CI] = 0.98 [0.97–1.00] per year increase) and gender (HR [CI] = 1.39 [1.05–1.82] for women compared to men), although they did not survive Bonferroni correction (see **Table 2**).

Exploratory Survival Models With Genetic Predictors

SNCA genetic variants were analyzed in a final sample size of N = 456 for rs356219 and N = 455 for D4S3481, with a total of 208 LID events for rs356219 and 210 LID events for D4S3481. Total follow-up time was 31,153 person-months for rs356219 and 31,140 for D4S3481, while median time was 61 months for rs356219 and 60.5 for D4S3481. We observed no statistically significant genetic effects of these variants on incident LID risk (**Figures 1A,B**), although D4S3481-263 allele carriers showed a nominally significant protective association with incident LID (**Supplementary Table S2**). This prompted us to compare follow-up times of the Rep1 263 bp allele carriers vs non-carriers through a rank sum test, which revealed a significant difference between the two groups (W = 4.18, $p = 0.04$), with 263 bp allele carriers showing a higher follow-up time (**Supplementary Figure S1A**), in line with the protective effect observed in the survival analysis (**Supplementary Table S2**). No differences were observed in the follow-up time of rs356219 genotype classes (**Supplementary Figure S1B**).

Survival Random Forest

A SRF model predicting LID onset as a function of non-genetic predictors showed a sensitivity of 39% and a specificity of 76% in the test set. A variable importance analysis (**Figure 2A**) revealed that the most influential features in predicting incident LID risk were YOD (standardized feature importance 7.8%), NMS (5.4%)

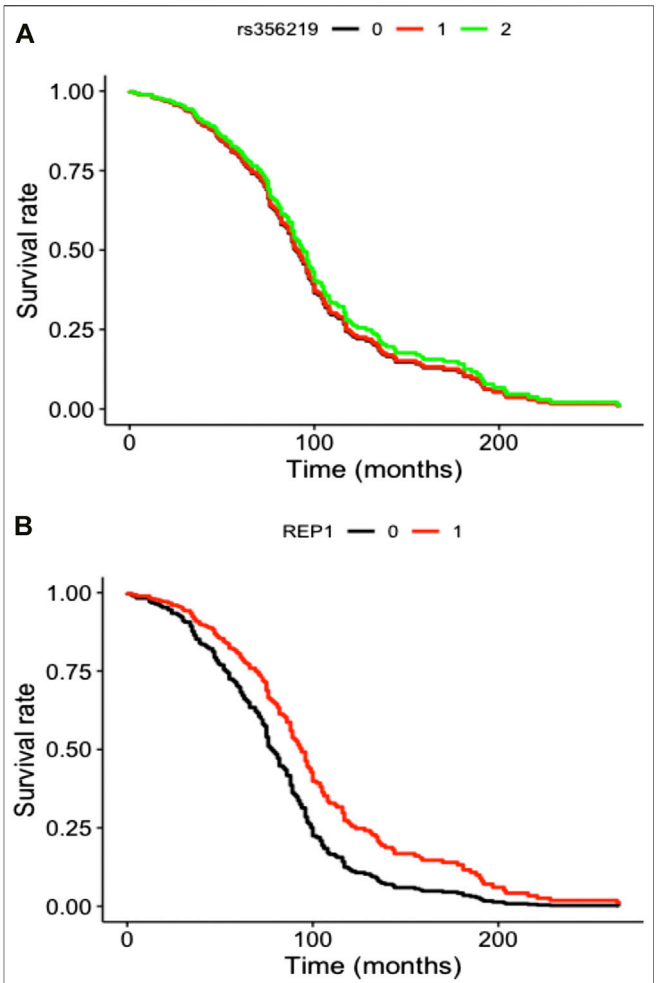

FIGURE 1 | Cox curves of genetic model tested modeling incident LID risk vs **(A)** additive genetic model of rs356219 and vs **(B)** pseudo-recessive genetic model of D4S8134. Genotype classes were defined as **(A)** AA (black), AG (red) and GG (green) genotypes for rs356219 and **(B)** 263 bp allele carriers (black) vs all other genotypes (red) for D4S8134.

and L-Dopa dosage (4.5%). In the genetic SRF (including also SNCA variants) performance was slightly worse (sensitivity: 27%, specificity: 73%), but the feature importance rank was substantially stable (YOD: 5.7%, NMS: 3.9%, L-Dopa dosage: 3.7%), with SNCA variants showing a negligible contribution (**Figure 2B**).

DISCUSSION

In the present study, we analyzed the influence of both non-genetic and genetic factors on the incident risk of LID in a cohort of Italian PD patients through a multifaceted approach implying the use of both classical statistics and supervised machine learning methods, which revealed two main findings.

First, a multivariable analysis of non-genetic exposures previously implicated in LID onset showed a significant association with years of disease (YOD), which in our study

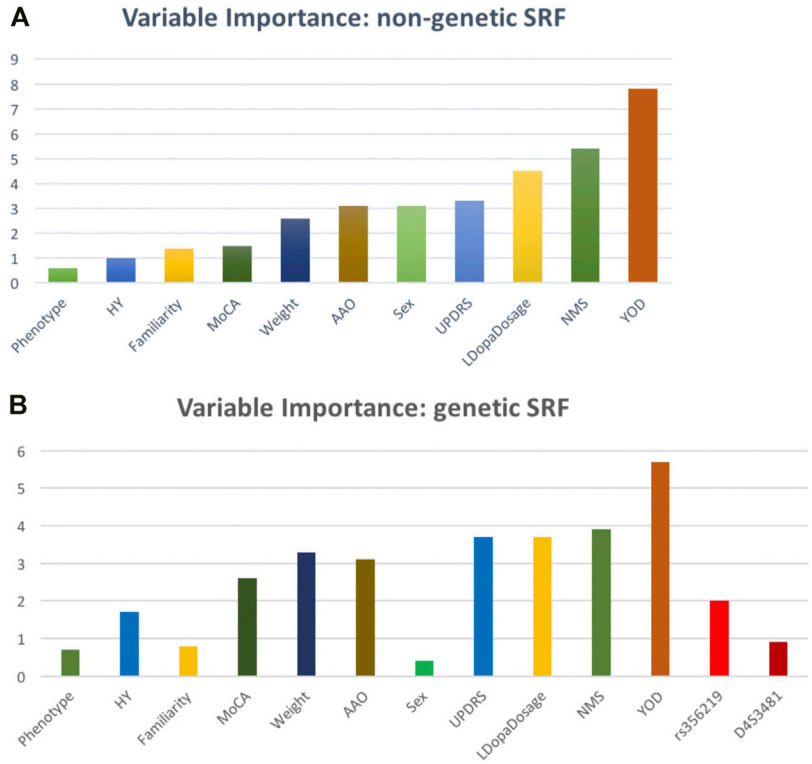

FIGURE 2 | Relative variable importance (%) of the LID risk predictors tested within the survival random forest **(A)** excluding and **(B)** including genetic (*SNCA*) features.

conferred a protective effect. This is in contrast with previous studies reporting that patients with longer disease duration—which is only partly dependent on age at onset (AAO)—are more likely to develop LID (Ahlskog and Muenter, 2001; Cilia et al., 2014; Coelho and Ferreira, 2014; Tran et al., 2018; Zhou et al., 2019). This discrepancy may be explained by the multivariable setting of our exploratory analysis, where also AAO showed a significant association with incident LID risk, in the expected direction (see below for further discussion). Second, the same multivariable models revealed a nominally significant association of incident LID risk with gender, in line with previous studies reporting a higher risk of LID for women compared to men (Zappia et al., 2005; Warren et al., 2013). However, this association was attenuated after constraining the model to adjust for weight (see full Cox PH model). This suggests that a generally lower weight of women and the resulting higher bioavailability of levodopa may play a role in this association (Coelho and Ferreira, 2014). We also observed a protective effect of late PD onset against LID risk. These findings corroborate previous observations reporting negative associations of LID risk with AAO (Kumar et al., 2005; Ku and Glass, 2010; Warren et al., 2013; Hashim et al., 2014). Importantly, these associations were observed in a multivariable setting and were all mutually independent, although only that with duration of disease survived a conservative correction for multiple testing and persisted mutual adjustments of all the other covariates in the full model.

Of note, we observed no evidence for a linear association between L-Dopa daily intake and incident LID risk, in spite of previous literature reporting it as one of the most important risk factors for dyskinesia (Warren et al., 2013; Coelho and Ferreira, 2014; Eusebi et al., 2018). This may be due to the fact that different studies analyzed differently exposure to L-Dopa intake. Indeed, some reported association with L-Dopa dosage at the beginning of the pharmacological treatment (Warren et al., 2013), while others took the latest prescription as dosage of reference (Bjornestad et al., 2016), and other works analyzed L-Dopa Equivalent Daily Dosage (LEDD) (Tomlinson et al., 2010; Zhou et al., 2019). Interestingly, our survival random forest model reported L-Dopa dosage among the most influential features, suggesting that it may contribute to LID risk in a more complex, non-linear fashion. While the topic is still controversial, previous pioneering studies in the field have previously reported a concordant evidence with our observation on L-Dopa. In a large African cohort of PD patients, Cilia and colleagues observed that motor fluctuations and dyskinesias were not associated with the duration of L-Dopa therapy, but rather with higher levodopa daily dose, beyond longer disease duration (Pandey and Srivanitchapoom, 2017). Others have reported the prevalence of LID to increase with disease and treatment duration, and that it usually takes approximately 3–5 years after administering L-Dopa for developing dyskinesia (Bjornestad et al., 2016). Our findings well fit the hypothesis that L-Dopa substantially increases the risk of LID, although this influence might dissipate in the long term (Coelho and Ferreira, 2014). Overall, these lines of evidence support the idea that

the practice to delay L-Dopa therapy to postpone the occurrence of LID may not be justified for all PD patients. In the future, a personalized approach to L-Dopa therapy based on the characteristics of the patients may be more suited to minimize risk-to-benefit ratio. While we are still far from this goal and large and well characterized PD datasets are warranted to this purpose, this study represents a first exploratory attempt in this perspective.

When we examined candidate *SNCA* genetic variants, we observed only a nominally significant protective influence of 263 bp allele of D4S3481 against incident LID risk, compared to non-carriers in a dominant model, while no evidence of association for rs356219 was detected. Similarly, SRF models including also *SNCA* genetic variants revealed a negligible contribution to the prediction of incident LID risk. This evidence is in line with the lack of longitudinal associations with LID previously reported by Corrado et al. for D4S3481 (Corrado et al., 2018) and by Kim et al. for rs356219 (Kim et al., 2020). At present, it remains difficult to say whether this is due to the total lack of influence of these two variants—or possibly of the *SNCA* gene as a whole—on the occurrence of LID, since both have been under-investigated in this regard. Overall, further genetic studies on these and other *SNCA* variants are warranted in larger samples, to clarify the relation of this gene with LID onset and risk, which has been fairly neglected so far.

Strengths and Limitations

In addition to the lack of detailed information on L-Dopa treatment duration for some PD patients, additional limitations which may have limited power include the relatively low sample size of the study, which was however counterbalanced by the use of advanced machine-learning based imputation techniques to maximize N. Computing L-Dopa equivalent daily dose (LEDD) for each participant may help to have a slightly more precise and comparable information to sum dopamine dosages coming from different sources (e.g., carbidopa). Although different approaches have been suggested to compute LEDD, no agreement has been reached on a gold standard procedure and different studies report different L-Dopa dosage exposures (Bjornestad et al., 2016; Zhou et al., 2019). Finally, the assessment of dyskinesia made by qualified neurologists only reported the absence/presence of motor complications, hence missing information on the time spent with or without LID in the different stages of the disease, as well as on the severity of motor complications. We are now planning a recall of the cohort to assess in detail these aspects.

In spite of these limitations, this still represents one of the largest genetic studies on LID and one of the most richly characterized as for non-genetic exposures. Indeed, to our knowledge no attempt has been made so far to predict LID onset through supervised machine learning techniques, which show a notable power compared to classical statistical models since they account also for more complex relationships of the investigated predictors and may pave the way to personalized medicine approaches.

Overall, we provide a contribution to knowledge on the epidemiology of LID, which will help to better understand both environmental and genetic influences of this phenomenon. This represents an important translational milestone in developing future personalized strategies for the treatment and management of PD patients in the future.

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 the IRCCS Neuromed. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AG conceived the study and designed statistical analyses. AG and AT performed statistical analyses. AT provided theoretical background and reviewed available literature. TE was responsible for molecular genetics analyses and cohort management. NM, EO, AT, TE and NP carried out phenotypic assessment. AT and AG wrote the manuscript, with contributions from all the co-authors. All the authors participated to discussion and interpretation of the results.

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frontiers in Pharmacology

REVIEW
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Role of Pharmacogenetics in Adverse Drug Reactions: An Update towards Personalized Medicine

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Adverse drug reactions (ADRs) are an important and frequent cause of morbidity and mortality. ADR can be related to a variety of drugs, including anticonvulsants, anaesthetics, antibiotics, antiretroviral, anticancer, and antiarrhythmics, and can involve every organ or apparatus. The causes of ADRs are still poorly understood due to their clinical heterogeneity and complexity. In this scenario, genetic predisposition toward ADRs is an emerging issue, not only in anticancer chemotherapy, but also in many other fields of medicine, including hemolytic anemia due to glucose-6-phosphate dehydrogenase (G6PD) deficiency, aplastic anemia, porphyria, malignant hyperthermia, epidermal tissue necrosis (Lyell's Syndrome and Stevens-Johnson Syndrome), epilepsy, thyroid diseases, diabetes, Long QT and Brugada Syndromes. The role of genetic mutations in the ADRs pathogenesis has been shown either for dose-dependent or for dose-independent reactions. In this review, we present an update of the genetic background of ADRs, with phenotypic manifestations involving blood, muscles, heart, thyroid, liver, and skin disorders. This review aims to illustrate the growing usefulness of genetics both to prevent ADRs and to optimize the safe therapeutic use of many common drugs. In this prospective, ADRs could become an untoward "stress test," leading to new diagnosis of genetic-determined diseases. Thus, the wider use of pharmacogenetic testing in the work-up of ADRs will lead to new clinical diagnosis of previously unsuspected diseases and to improved safety and efficacy of therapies. Improving the genotype-phenotype correlation through new lab techniques and implementation of artificial intelligence in the future may lead to personalized medicine, able to predict ADR and consequently to choose the appropriate compound and dosage for each patient.

Keywords: adverse drug reaction, long QT syndrome, brugada syndrome, seizure, diabetes, proarrhythmia, genetic test, personalized medicine

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Abbreviations: AIP, acute intermittent porphyria; ADR, adverse drug reaction; BrS, Brugada syndrome; CYP, Cytochrome P450; DILI, drug induced liver injury; DNA, Deoxyribonucleic acid; GABAA, γ-aminobutyric acid type A receptors; G6PD, glucose-6-phosphate dehydrogenase deficiency; HLA, human leukocyte antigen; HCV, hepatitis C virus; LQTS, long QT syndrome; MH, malignant hypertermia; MHC, major histocompatibility complex; MODY, maturity onset diabetes of the young; mtDNA, mytocondrial deoxyribonucleic acid; mRNA, messenger ribonucleic acid; miRNA, micro ribonucleic acid; NAD, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide hydrogenated; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NGS, next generation sequencing; NSAID, nonsteroidal anti-inflammatory drugs; PCT, porphyria cutanea tarda; RNA, ribonucleic Acid; SJS, Stevens-Johnson syndrome; TdP, torsade-de pointes; TEN, toxic epidermal necrolysis.

INTRODUCTION

Adverse drug reactions (ADRs) remain an important and frequent cause of worldwide morbidity and mortality (Bouvy et al., 2015). Despite the dramatic technical improvements in molecular diagnoses, the causes of ADRs are still poorly understood. ADRs can be due to a huge variety of drugs and can involve every organ or apparatus, and several ADRs have recently been associated with specific genetic mutations. In this scenario, genetic predisposition toward ADRs is an emerging issue, not only in anticancer chemotherapy, but also in many other fields of contemporary medicine, including glucose-6-phosphate dehydrogenase (G6PD) deficiency, malignant hyperthermia, epidermal tissue necrosis (Lyell's Syndrome and Stevens-Johnson Syndrome), epilepsy, thyroid diseases, porphyria, aplastic anemia, Long QT Syndrome, and Brugada Syndrome (Swen et al., 2011; García-González et al., 2016; Weitzel et al., 2017). This awareness led to a well-differentiated subspecialty of clinical genetics called "pharmacogenetics," defined as "the study of how variations in a few genes affect the response to medications" (Wilke et al., 2007; Daly 2017), aimed to prevent ADRs and to optimize therapies, considering the individual variations in either pharmacokinetics or pharmacodynamics. Both pharmacokinetics, which is the complex interplay of absorption, distribution, metabolism and excretion, and pharmacodynamics, which is the physiologic consequence of the interaction between drug metabolites and receptors, are genetically encoded (Martinez-Matilla et al., 2019). The family of genes called CYP encodes the vast majority of enzymes regulating both pharmacokinetics and pharmacodynamics (Dresser et al., 2000).

In our view, the main role of pharmacogenetics is to translate genetic information into everyday medical practice, trying to lower the impact of ADRs, both for patients and for the healthcare system. It is beyond the scope of this review to discuss the full array of pharmacogenetic variants, whereas its aim is to present an update on the genetic background of ADRs, and to provide some red flags useful in everyday clinical practice, showing how genetics can be helpful for the prevention and the treatment of patients experiencing ADRs. Wider use of genetic testing and implementation of artificial intelligence techniques in the future may favor personalized medicine (Leopold and joseph, 2018), able to predict ADR, and consequently to choose the appropriate and safer drug and dosage for each patient (Kalinin and Higgins, 2018; Schaik et al., 2020).

Impact of Adverse Drug Reactions in Clinical Practice

In 2020, a systematic review of observational studies emphasized that, despite the negative clinical outcomes and high costs of ADRs, the lack of consistent definitions and good data collection methods limited the possibility to provide an accurate and comprehensive picture of the problem (Zhu et al., 2020). Nonetheless, according to this review, the cost per patient of preventable ADRs was measurable in thousands of dollars, associated with a considerably increased length of hospital stays (Sultana et al., 2013), with a cost estimate of over 30 billion dollars just in the United States (Formica et al., 2018).

However, the damage is not only economic, but consists of plenty of human lives lost. For instance, according to a meta-analysis published in 1998, more than 100,000 patients die yearly in the US because of ADRs (Lazarou et al., 1998). The most harmful ADRs involve the treatment of inflammatory, gastrointestinal, renal, and blood coagulation disorders. This is the reason why nonsteroidal anti-inflammatory drugs (NSAID), diuretics, anticoagulants, and antiplatelets have been recognized over time to be the major culprits, with prescribing errors being major contributors to the problem.

Definitions of Adverse Drug Reactions

To understand how ADRs may affect the clinical practice, it is first necessary to define them. According to the World Health Organization (WHO) definition established in 1972, ADR can be defined as "a response to a drug which is noxious and unintended, and which occurs at doses normally used in man for the prophylaxis, diagnosis, or therapy of disease, or for the modifications of physiological function" (International Drug Monitoring: The Role of National Centres, 1972). According to the European Medicines Agency (EMA), "ADR is any untoward medical occurrence in a patient or clinical trial subject administered a medicinal product, and which does not necessarily have a causal relationship with this treatment" (Baldo, Francescon, and Fornasier 2018). However, in 2010 the European Union stated that the ADR definition should also encompass medication errors and off-label uses, and that there must be at least a reasonable possibility of causal relationship between drug and reaction (DIRECTIVE 2010/84/EU OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of December 15, 2010). According to the Federal Drug Administration (FDA), ADR is "Any noxious, unintended and undesired effect of a drug, which occurs at doses used in humans for prophylaxis, diagnosis or therapy. This excludes therapeutic failures, intentional and accidental poisoning and drug abuse" (Wu et al., 2019). Besides, "adverse event means any untoward medical occurrence associated with the use of a drug in humans, whether or not considered drug related" (Sakaeda et al., 2013). Noteworthy, ADRs are caused by multiple mechanisms, and indeed we now distinguish two main types of adverse drug reactions (Edwards and Aronson 2000; Wilke et al., 2007) with possible implications for the therapeutic management:

- ***Dose-related reactions*** (***Type A reactions***) caused by an overdosing of the compound relative to individuals and resulting in either toxic effects or side effects. This type of ADR is predictable and related to the pharmacodynamics and pharmacokinetics of the drug, and it is usually associated with milder effects and thus with lower mortality. Both pharmacokinetic and pharmacodynamics properties are related to enzyme proteins, and thus may depend on genetics. Therefore, a recent field within genetics has been called "pharmacogenetics," studying both variants and mutations related to drug metabolism, dealing with treatment and prevention of many ADRs. The most relevant

clinical application of Pharmacogenetics is in anticancer chemotherapy (Lu et al., 2015), but this field is beyond the aims of this review.

- ***Non-dose related or idiosynchrasic reactions*** (***Type B reactions***), less common than dose-related reactions, generally considered unpredictable, although screening tests can indeed predict some of these reactions (Edwards and Aronson 2000). They can be caused by different mechanisms, often related to genetic conditions. Possible examples are malignant hyperthermia, porphyria, glucose 6 phosphate dehydrogenase deficiency, or drug-induced liver injury. The causes of non-dose related ADRs are not yet fully understood. ADRs can be caused by altered pharmacokinetics, due to altered absorption or metabolism, but also by altered pharmacodynamic properties, such as the alteration of the target receptor, or its pathway, or binding to unwanted receptors. Also, for non-dose related ADRs, both pharmacokinetic and pharmacodynamic properties are related to enzyme proteins, thus depending on genetics. However, the most likely mechanism for idiosyncratic reactions is an immunological cause (Uetrecht and Naisbitt 2013), for example related to mastocytes activity.

Role of Genetic Codification in Adverse Drug Reactions

Several observations suggest that ADRs may run in families, although generally a Mendelian pattern of inheritance cannot be identified. Even in cases where genetic mutations are identified, these are only indicative of susceptibility to ADRs but cannot predict them with certainty (Wilke et al., 2007). Moreover, ADRs may happen in patients harbouring variants of uncertain significance (VUS), rather than pathogenic mutations or full syndromic clinical pictures (Pirmohamed, 2010). We provide here a synthetic glossary on the terms used in this review, and a more comprehensive compendium of pharmacogenetics nomenclature can be found in a recent article (Kalman et al., 2016). The nomenclature for genes in the text in provided in accordance with HUGO guidelines https://www.genenames.org/about/guidelines.

Genetic information is stored in complex molecules, such as genomic DNA, mitochondrial DNA (mtDNA), and various kinds of RNAs (Brenner, 2001). This information is organized in the genetic code, consisting of sixty-four nucleotides triplets, three stop signals, several regulatory sequences and regions interacting with both mtDNA and full RNA sets (Wang et al., 2009). All information stored in the genetic code specifies twenty canonical and two additional aminoacids (Ambrogelly et al., 2007), forming a huge variety of proteins. Proteins are the key-of-life, allowing countless activities in the cells, interacting with the environment to control and balance all aspects of cell life. Thus, the study of the genetic basis of ADR has to consider the complex interplay between the genetic information and environmental factors in a given disease condition (Afsar et al., 2019). Finally, it must be considered that the huge variability of genetic information is transmitted from one generation to the other, not always unaltered, inserting a further degree of variability, with possible great clinical relevance (Cismaru et al., 2020).

"Gene" is a DNA sequence occupying a precise position (called "locus") in the genomic DNA itself. Every individual has two copies of non-sexual genetic information defined as "allele," that can be considered alternative forms of a gene occurring at the same locus, each autosomic allele being inherited from either parent (García-González et al., 2016). The genetic sequence of every allele can be different from an individual to another. An allele is defined as "variant" when found with a frequency below 1% in the general population, excluding inbred coupling (Lo et al., 2003). In clinical settings, when a variant is associated to at least one specific pathologic phenotype, it is usually referred as a "mutation". Instead, when a variant has a frequency above 1% in the general population, it is defined "Single Nucleotide Polymorphism (SNP)" (Karki et al., 2015). SNPs are the most common contributors to variation in genetic code: copy number variants, insertions, deletions and duplications are the possible mechanisms. Not all SNPs have the same influence toward the risk of ADRs, however SNPs affecting regulatory RNAs have been recently described to have an important role in drug effects (Sadee et al., 2011). In pharmacogenetics, both relevant variants and SNPs are marked with the symbol "*" (star). A "haplotype" is the order by which either single variants or SNPs are positioned across a gene (Sangkuhl et al., 2020). The relevance of haplotypes in pharmacogenetics is a direct consequence of the interactions between coding and non-coding genetic information.

Genes can encode one or more messenger RNA (mRNA), each mRNA allowing the synthesis of a protein or having a regulatory role (Portin and Wilkins 2017). The discovery of the huge amount of non-coding RNAs challenged the traditional definition of genes, demonstrating both the overlapping of many biological functions and the clinical relevance of genetic regulatory information (Gerstein et al., 2007). A recent review focused on the role of microRNAs (miRNAs), 18–22 nucleotide RNAs modulating the expression of multiple protein-encoding genes at the post-transcriptional level (Latini et al., 2019). MiRNAs modulate the pharmacological response, regulating numerous genes involved in the pharmacokinetics and pharmacodynamics of drugs, and differences in the levels of circulating miRNAs or genetic variants in the sequences of the miRNA genes can contribute to inter-individual variability in drug response, both in terms of toxicity and efficacy. For their stability in body fluids and the easy availability and accurate quantification, miRNAs could be ideal biomarkers of individual response to drugs (Latini et al., 2019).

Role of Metabolism in Adverse Drug Reactions

Genetic mutations affecting both pharmacokinetics and pharmacodynamics can confer a predisposition toward ADR (Mehrotra et al., 2007). Probably, the prevalent mechanism affecting a drug behavior inside the body is related to the renal and hepatic metabolism (Doogue and Polasek 2011; Talal et al., 2017). If these two systems are impaired, the direct consequence will be a defective clearance of all compounds

relying upon renal and hepatic metabolism. Besides, other disease conditions, including congestive heart failure, diabetes, peripheral vascular, pulmonary, rheumatologic, and malignant diseases are associated with increased ADR (Zhang et al., 2009).

Furthermore, other important modulators affecting the predisposition to ADRs are the immunologic factors. There are four main types of immunological reactions to drugs, namely the immediate hypersensitivity IgE-mediated, IgG-mediated, serum sickness, and delayed type cell-mediated (Dispenza, 2019). Also, immunological and genetic factors can interact in the pathogenesis of ADRs, through specific alleles in HLA-genes (Ferner and Aronson, 2019). Covering the role of modulators is beyond the scope of this review. Noteworthy, the modulating factors involved in drug metabolism may interplay not only with a disease specific mutation, but also with other environmental factors, such as age and pollutants.

Environmental, Age and Gender Factors in Adverse Drug Reactions

The effect of both environmental and age and gender factors in drug metabolism is a well-described phenomenon. Several studies describe the relationship between age and drug metabolism (O'Mahony and Woodhouse, 1994; Benedetti et al., 2007). Pediatric age (Gregornik et al., 2021) and, even more so, geriatric age are at higher risk for ADRs (Seripa et al., 2015). Geriatric patients at higher risk for ADRs as they take several drugs, with possible mutual metabolic interferences. Moreover aging can lead to impaired biotransformation of several drugs, especially for decreased activity of many cytochrome enzymes (Wauthier et al., 2007).

The gender factor in ADRs is often underestimated, even if gender differences in drug metabolism may be a cofactor of ADRs. Indeed, men and women have a different chromosome, translating into diverse hormonal, metabolic, structural and immunological states. In general, women are at higher risk for ADRs, in particular when treated with thyroid hormones, TNF-alpha inhibitors and analeptics (Tran et al., 1998). Also, the risk of pro-arrhythmic effect of several cardiac and non-cardiac drugs is higher in women (Roden, 2016).

Besides age and gender, other environmental factors may affect ADRs, particularly cigarette smoking and pollutants, which can modify CYP1A1 and CYP1A2 expression (Wardlaw et al., 1998). This might alter the pharmacokinetics of many drugs, such as haloperidol, propranolol and flecainide. Viral infections can likewise change the behavior of a drug and induce an adverse drug effect, namely with ampicillin rash in infectious mononucleosis, Reye's syndrome in patients with Influenza B and Varicella Zoster viruses and multiple drugs with HIV positive patients due to their altered metabolism (Levy, 1997).

Adverse Drug Reactions in Different Disease Conditions

Genetics has been known to affect drug metabolism since the discovery of glucose-6-phosphate dehydrogenase (G6PD) deficiency. This condition is also known as "favism" because affected patients can experience hemolytic anemia after a meal with fava beans. Over time, researchers have found associations between hemolytic anemia and the consumption of other foods (like beans or peas), or the use of certain drugs (Beutler, 2008). Other classical conditions related to ADRs are malignant hyperthermia, Lyell syndrome, Stevens-Johnson Syndrome, porphyria, Fanconi Syndrome, Long QT Syndrome, and Brugada Syndrome (Swen et al., 2011; García-González et al., 2016; Weitzel et al., 2017). For most of them, a genetic background has been defined, especially following the technical improvements of genetic testing due to Next Generation Sequencing (NGS) (Hwang et al., 2017; Yohe and Thyagarajan, 2017). This new knowledge brought two important pieces of clinical information: different kinds of ADRs can share the same genetic background (Hwang et al., 2017), and even common genetic polymorphisms might play a relevant role in the aetiology of ADRs, especially in elderly patients (Just et al., 2017).

Drug Induced Haemolytic and Aplastic Anaemias

The most relevant drug-induced anaemias are haemolytic, sideroblastic or aplastic anaemia. The most common syndrome causing drug-induced haemolytic anaemia is the glucose-6-phosphate dehydrogenase (G6PD) deficiency (Pamba et al., 2012; Luzzatto et al., 2016; Belfield and Tichy, 2018).

Glucose-6-Phosphate Dehydrogenase Deficiency

In humans, G6PD deficiency is the most common enzyme deficiency due to an X-linked mutation associated with reduced G6PD enzymatic activity (Beutler 2008; Belfield and Tichy, 2018). This enzyme is involved in the pentose phosphate pathway, through which NADPH is produced. NADPH is required to protect human cells from oxidative stress, and when its concentration decreases, many tissues suffer from its impaired function. This is more evident in tissues with frequent cell divisions, like red bone marrow, in which erythrocytes mature and become functional. G6PD deficiency affects almost half a billion people all over the world. Due to the position of G6PD in the X chromosome, males can display two possible genotypes: wild type (meaning "non carrier") or affected carrier. In females, instead, there are three possible genotypes: homozygous (affected), hemizygous (either affected or non-affected) and wild type (unaffected). The enzymatic deficit can be diagnosed by the detection of enzymatic activity with different methods. Noteworthy, most individuals with G6PD deficiency of either sex do not show a specific clinical picture throughout their life. However, exposure to either drugs or infection is sufficient to experience oxidative stress, responsible for the NADPH depletion, eliciting an acute haemolytic crisis. In these situations, symptoms can include jaundice, fatigue, back or abdominal pain, and haemoglobinuria (Beutler, 2008). Thus, G6PD deficiency should be included in differential diagnosis in case of patients affected by acute haemolysis after exposure

TABLE 1 | Drugs to be Avoided in Patients with Glucose-6-Phosphate Dehydrogenase Deficiency.

- **General medications**
  - anacin
  - empirin
  - excedrin
  - pepto bismol
  - acetylsalicilates
  - aspirin
  - bufferin
  - ecotrin
- **Antimalarials**
  - chloroquine
  - mefloquine
  - pamaquine
  - primaquine
  - quinidine
  - quinine
- **Sulfonamides and sulfones**
  - dapsone
  - furosemide
  - sulfacetamide
  - sulfamethoxazole
  - sulfanilamide
  - sulfasalazine
  - sulfisoxazole
  - nitrofurans
  - nitrofurantoin
- **Quinolones and fluoroquinolones**
  - ciprofloxacin
  - levofloxacin
  - moxifloxacin
  - norfloxacin
- **Other medications**
  - acetylphenylhydrazine
  - beta-naphthol
  - chloramphenicol
  - dimercaprol
  - fava beans
  - glyburide
  - menthol
  - penicillamine
  - phenazopyridine
  - phenylhydrazine
  - probenecid
  - rasburicase
  - tolbutamide

to known oxidative drugs. A list of medications that should be avoided in patients with G6PD deficiency is provided in **Table 1** complete list is available at www.g6pd.org. It is also possible to perform a molecular analysis to detect known mutations of the *G6PD* gene. This gene is characterized to be highly polymorphic in the general population, lacking mutational hotspots and with common missense mutations, regardless of ethnicity (Luzzatto et al., 2016). The two more frequent mutations are V68M and N126D, accounting for about half of the cases (Pamba et al., 2012). Other mutations can be found with a particularly high frequency in specific ethnic groups, such as in the Arab world (Doss Priya et al., 2016). Since the discovery of G6PD deficiency, many other syndromes and genetic variants have been associated with adverse drug reactions by linkage analysis and genome sequencing. Besides the G6PD deficiency, which will be further detailed among haemolytic anaemias, there are some other well-known genetic syndromes which are correlated to adverse drug reactions, with the most known being malignant hyperthermia.

Aplastic Anaemia

An example of drug-induced aplastic anemia can be found in patients with Fanconi Anemia (Mehta and Tolar, 1993; Auerbach, 2009). This is a genetic syndrome, usually autosomal recessive for B*RCA2*, *BRIP1*, *FANCA*, *FANCC*, *FANCD2*, *FANCE*, *FANF*, *FANCG*, *FANC*I genes, while autosomal dominant for *RAD51* or even X-linked for *FANCB* genes (Mehta and Tolar, 1993). The Fanconi anemia pathway comprises 19 gene products (FANCA to FANCT) (Ceccaldi et al., 2016). In most patients, typical congenital physical features, like short stature and skin pigmentation, besides bone marrow failure, characterize Fanconi Syndrome. This condition is the most common cause of adult-onset aplastic anemia, typically preceded by chemotherapy or chemo radiation, frequent therapies in those patients, due to their tendency to develop solid malignancies. Noteworthy, even the gene carrier status for pathogenic mutations in some Fanconi anemia genes (*FANCD2*, *FANCA*, *FANCB*, *FANCC*) has been associated with higher risk of bone marrow failure under replicative cell stress conditions, such as chemotherapy (Durrani et al., 2019).

Germline inactivation of any one of the Fanconi anemia genes causes Fanconi anaemia, resulting in sensitivity to interstrand-crosslinks (ICLs). ICLs are DNA lesions that inhibit essential processes, such as replication and transcription, and they must be repaired or bypassed for the cell to survive (Ceccaldi et al., 2016). Thus, ICLs predisposes patients to bone marrow failure and development of cancer.

In summary, individuals developing aplastic anaemia following chemo or radiotherapy should be suspected for Fanconi Syndrome and investigated for a possible carrier status, and once identified should undergo a careful oncologic and hematologic work-up, and avoid the most damaging chemo and radiotherapy, to preserve their bone marrow as much as possible (Mehta and Tolar, 1993).

Porphyrias

Haemoglobin and myoglobin proteins are essential for life, both including a non-protein structure called "heme group" (Phillips 2019). The complex metabolic process to produce heme groups is linked to a specific enzyme pathway inside liver cells (Szlendak et al., 2016). Specific genes encode for each enzyme involved in the heme group pathway: so far, eight enzymes have been identified (Puy et al., 2010). Thus, there are eight known porphyria phenotypes, each caused by a specific genetic mutation: thus, porphyria is not a single disease, but a group of genetically different disorders, sharing some clinical manifestations. These disorders may be due either to altered levels or to altered activity of the enzyme encoded by a certain gene. The involved genes are *PBGD*, *ALAS*, *ALAD, UROS*, *UROD*, *CPOX*, *PPOX*, and *FECH* (Yasuda et al., 2019). It is relevant that all porphyria genes map on autosomes, while the only gene mapping on the X chromosome is *ALAS*, associated with a

rare form of drug-related sideroblastic anemia (Furuyama and Sassa, 2000). This association is so characteristic that the onset of a sideroblastic anemia after a drug treatment is the main suspicion element for the erythropoietic porphyria (Phillips, 2019).

When an enzyme responsible for the heme group's synthesis is deficient or defective, its specific substrate and all the other precursors (normally modified by that enzyme) can accumulate in the bone marrow, liver, skin, or other tissues, causing toxic effects. These precursors accumulate in the patient's blood and are eliminated in the urine, bile, or stools (Balwani et al., 2017). This explain why the clinical pictures of all forms of porphyria can be characterized by hematologic manifestations (anaemia), neurologic disorders (sleep disorders, muscle weakness, seizures, chronic back, limbs, and abdominal pain), gastrointestinal disorders (constipation, diarrhoea, vomiting), and skin involvement (skin and mucosal ulcerations). Those signs and symptoms can be triggered not only by fever, viral infections (like C hepatitis), but also by certain drugs, especially anaesthetics and anticonvulsants. **Table 2** provides a list of drugs associated with porphyria.

The most common forms of porphyria are acute intermittent porphyria (AIP) and porphyria cutanea tarda (PCT). Both forms can be diagnosed incidentally, often after puberty, since hormones are known triggers of accumulation of heme-group precursors in target organs (Haberman et al., 1975).

The pharmacogenetic relevance of porphyria is high, since several different drugs can trigger the clinical manifestations. The two most common forms of porphyria, AIP and PCT, have an identified genetic background: about 25% of PCT and 100% of AIP are inherited as autosomal dominant conditions. Thus, it is sufficient to harbor one mutated copy in either the *PBGD* or *UROD* gene to manifest the disease. Altogether, all forms of porphyria afflict fewer than 200,000 people in the United States, while in Europe the most common porphyria (PCT) has a prevalence of 1: 10,000 individuals, the other forms being much less common. The treatment of porphyria is beyond the scope of this study and is illustrated in detail elsewhere (Dawe, 2017).

The mechanisms of adverse drug reactions associated with porphyria are complex, likely due to competition between drug metabolizing enzymes and to enzyme dysfunctions inside liver cells (Bickers, 1982). Some forms of porphyria, especially AIP, show a specific association with anaesthetics like Articaine, Bupivacaine, Lidocaine, Mepivacaine, Prilocaine, and Ropivacaine (Nagarajappa et al., 2019). Moreover, porphyria can be diagnosed following the onset of skin lesions, like in Stevens Johnson syndrome, often exacerbated by sunlight exposure (i.e., photosensitivity). This commonly happens in patients harbouring single copy mutations in the *PBGD* gene. In other situations, an ADR due to psychotropic drugs like Valproic Acid (Noval Menéndez et al., 2012) can be the first sign. Noteworthy, a high incidence of psychiatric symptoms has been described in patients subsequently receiving a clinical diagnosis of porphyria (Santos et al., 2017). In summary, different variants of porphyria are metabolic disorders genetically determined, still difficult to diagnose. Their presence must be suspected in case of unexplained chronic abdominal pain (Valle Feijóo et al., 2015) or of adverse drug reactions against either anaesthetics or mood-modulating drugs. The relevance of genetic testing is growing, but at present there are no specific guidelines, unless a pathogenic gene mutation has already been found (Whatley and Badminton, 2013). Owing to many diverse clinical pictures, it is not possible, according to current knowledge, to perform genetic testing in porphyria as a first line diagnostic procedure (Stölzel et al., 2019). The only case in which genetic testing is strongly recommended is when a pathogenic mutation has already been found in the family (Whatley and Badminton, 2013). The pre-symptomatic genetic testing in those cases can be helpful to minimize the risk of porphyria acute attacks, in some cases very harmful.

Malignant Hyperthermia

Malignant hyperthermia can be considered a pharmacogenetic disorder, which is most known for its sudden adverse reactions to certain drugs. Malignant hyperthermia is basically a metabolic muscle disease caused by heterozygous mutations in three genes to the best of current knowledge (Miller et al., 2018). The metabolic defect genetically encoded in this condition, regardless of the mutated gene, provokes a very fast and uncontrolled calcium increase from skeletal muscle cells (Riazi et al., 2018). It is common that a patient does not know they are affected by malignant hyperthermia until the administration of the triggering drugs (Wappler, 2010). **Table 3** provides a list of drugs associated with malignant hyperthermia. The most harmful drugs for a patient affected by malignant hyperthermia are either volatile anaesthetics or succinylcholine (Horstick et al., 2013). Most of the individuals known to be affected by this syndrome are carriers of single copy mutations in the *RYR1* gene, located on chromosome 19q13.1, although rare mutations have also been found in the genes *CACNA1S* and *STAC3* (Horstick et al., 2013; Riazi et al., 2018). The incidence of this syndrome ranges between 1:10,000 and 1:250, 000 and the individuals affected develop a life-threatening adverse drug reaction upon administration of volatile anaesthetics such as halothane, sevoflurane, desflurane, and isoflurane. The signs of malignant hyperthermia include hyperthermia, tachycardia, tachypnoea, increased carbon dioxide production and oxygen consumption, acidosis, hyperkalaemia, muscle rigidity, and rhabdomyolysis, all related to a hypermetabolic response (Rosenberg et al., 2015). This kind of response is the main reason for the increasing body temperature that characterizes the clinical picture of such patients. In these cases, it is sufficient to avoid administration of the above-mentioned drugs, opting instead for intravenous anaesthetics. However, if the adverse reaction is already ongoing, a specific treatment consists of intravenous administration of Dantrolene, together with interruption of the causing drug and supportive therapy (Schneiderbanger et al., 2014). The knowledge of gene mutation causing malignant hyperthermia is crucial to avoid a potentially fatal anesthesia complication. Indeed, for over 30 years the diagnosis of malignant hyperthermia relied on *in vitro* functional tests, with a low negative predictive value (Loke and MacLennan, 1998). In suspected malignant hyperthermia (MH), the functional studies can have

TABLE 2 | Drugs associated with porphyrias.

• **Anaesthetics**
articaine
bupivacaine
lidocaine
mepivacaine
prilocaine
ropivacaine
• **Anticonvulsants**
carbamazepine
phenytoine
phenobarbitone
primidone
ethosuximide
tiagabine
felbamate
valproate
oxcarbazine

TABLE 3 | Drugs associated with malignant hyperthermia.

• **Depolarizing muscle relaxants**
succinylcholine (suxamethonium)
• **Inhaled general anaesthetics**
chloroform (trichloromethane, methyl trichloride)
desflurane
enflurane
halothane
Isoflurane
methoxyflurane
sevoflurane
Trichloroethylene
Xenon

inconclusive results. This is due to the high frequency of polymorphic variants in RYR1 gene, the most commonly mutated gene in MH patients. Vice versa, genetic testing can provide clinically useful genotype–phenotype correlations (Zhou et al., 2007). These can be used in case of inconclusive results of a functional test. Thus, the introduction of genetic testing raised the negative predictive value up to 95% (Girard et al., 2004). Therefore, recently malignant hyperthermia became a rare condition, especially for family members of a proband, in whom a pathogenic mutation has already been identified. Notwithstanding, functional tests are still considered the gold standard for a definite diagnosis of malignant hyperthermia, either when a variant of uncertain significance is found, or when genetic testing results are negative (Yang et al., 2019).

Drug Induced Cutaneous Reactions

Human skin is very often affected by adverse drug reactions, with highly variable clinical pictures (Chung et al., 2016; Lerch et al., 2018). The observation of a genetic background in patients affected by cutaneous ADRs was somewhat an unexpected finding (Gerogianni et al., 2018). Infact, the two major phenotypes of cutaneous ADRs are the **Lyell Syndrome**, also known as toxic epidermal necrolysis (TEN), and the **Stevens-Johnson Syndrome (SJS)** (Lerch et al., 2018; Oakley and Krishnamurthy, 2020). Variable muco-cutaneous lesions, displaying epidermal detachment and painful ulcerations in mouth, eyes, and genitalia, characterize both conditions. Such reactions are thought to be caused by an immunological response (Miliszewski et al., 2016). Both conditions share an onset characterized by a flu-like syndrome with fatigue and fever, besides either a neutrophilia or eosinophilia (Chung et al., 2016; Miliszewski et al., 2016). Furthermore, during the clinical course of both syndromes, immune deficiency can be diagnosed, often being the cause of death due to septic shock (Lerch et al., 2018). Currently, Lyell and Stevens-Johnson syndromes are considered as different expressions of the same clinical entity, sharing a similar genetic background, but mainly distinct by the grade of epidermal involvement. Specifically, if the area of detached skin is less than 10%, it is defined as SJS, while if it is more than 30%, it is defined as TEN, and if between 10 and 30% it will be considered an overlap syndrome (Banik et al., 2020). Of note, the drug dosage does not correlate to the severity of skin lesions, nor to their extension.

Genetic studies in Asian populations showed a correlation between HLA-B*1,502 and the two phenotypes (Chung and Hung, 2010), both correlating with ADRs following administration of carbamazepine (Fan et al., 2017; Wang et al., 2017). However, this correlation was not found in Caucasian populations, probably due to the lower prevalence of HLA-B*1,502 in those regions: therefore, US FDA now recommends testing for HLA-B*1,502 in Asian individuals before carbamazepine administration. Other HLA variants were found to be related to SJS/TEN are HLA-B*5,801 in Han Chinese patients and HLA-B*5,701 and HLA-A*3,101 in patients of European descent (Fan et al., 2017). Also, non-HLA-related genes have been linked to SJS/TEN such as *CYP2C9*3*, *GSTM1* and *TRAF3IP2* (Lerch et al., 2018). From a clinical standpoint, many drugs can cause such life-threatening adverse drug reactions, however the most common belong to aromatic anticonvulsants, such as carbamazepine (Wang et al., 2017). Other potentially harmful drugs are sulfonamides, allopurinol, and acetaminophen (Miller et al., 2018; Miliszewski et al., 2016; Ban et al., 2016). **Table 4** provides a list of drugs potentially associated with TEN. These drugs are thought to bind to cellular peptide and type 1 Major Histocompatibility Complex (MHC), to create an immunogenic compound, resulting in a strong immune response (Miliszewski et al., 2016), leading first to nonspecific signs such as fever, malaise, and upper respiratory tract symptoms. Over the next few days, the patient develops a blistering rash with erosions, more localized in SJS, and more generalized in TEN (Kohanim et al., 2016; Miliszewski et al., 2016). Unfortunately, there is no specific therapy for this type of reaction. The patient should undergo supportive therapy to reduce the immune response, and lesion cleaning by antiseptics and antibiotics to reduce the risk of infection (Miliszewski et al., 2016). The strong association between specific HLA proteins stimulated further research to identify other possible risk or protective HLA alleles (Fan et al., 2017; Wang et al., 2017). A recent article published by a Japanese group also described single nucleotide polymorphisms in the *IKZF1* gene as a possible predisposing factor toward ocular involvement

in SJS patients (Kohanim et al., 2016; Ueta, 2020). Further studies are required to confirm these results.

Drug Induced Epilepsy

The diagnosis of epilepsy is complex due to the variety of possible phenotypes and causes, including possible adverse drug reactions, involving either Central and/or Peripheral Nervous system, together with other organs (Godhwani and Bahna 2016; Božina et al., 2019). Indeed, drug-induced seizures are a common and dangerous consequence of several drugs, with more than 9% of all cases of status epilepticus being caused by drug or poison administration, usually antidepressants, stimulants, and antihistamines (Chen et al., 2016; Fricke-Galindo et al., 2018). Possible genetic predispositions toward drug-induced seizures is scarcely known, but since 2019, some experimental studies have shed new light on this hot topic. First, Wong and co-workers (Wong et al., 2019) studied the benefits of Donepezil administration in a murine model of Dravet syndrome. This condition is a common form of childhood onset epilepsy, caused by heterozygous mutations in the *SCN1A* gene. Their group also studied the role of HuperzineA (Hup-A), an acetylcholinesterase inhibitor, in the prevention of drug-induced seizures in *SCN1A* mutant mice, demonstrating the role of both muscarinic and γ-Aminobutyric acid type A (GABAA) receptors in Hup A-mediated seizure protection (Wong et al., 2016). The translation of these studies to the human species might be useful to prevent the side effects of anticonvulsant drugs in Dravet syndrome patients.

Drug Induced Thyroid Diseases

Many drugs can cause thyroid dysfunction, either through a misbalance of the TSH-T3-T4 axis, or through an autoimmune mechanism. A common example of the latter is the autoimmune thyroid disease consequent to interferon-alpha treatment in patients with chronic Hepatitis C Virus (HCV) infection, also called interferon-induced thyroiditis (Hasham et al., 2013). Supporting a probable genetic predisposition of this condition is the linkage between variants in the genes *SP100*, *SP110*, *SP140*, *HLA*, and *TAP1* (Hasham et al., 2013). Also, polymorphisms affecting both UGT1A9 (UGT1A9-rs3832043) and nuclear receptor PXR (NR1I2-rs3814055, NR1I2-rs2472677, and NR1I2-rs10934498), possibly resulting in downregulation of liver metabolizing enzymes of Sorafenib (i.e., CYP and UGT), were associated with both plasma overexposure and severe thyroid toxicities upon Sorafenib intake in papillary thyroid cancer (Ba et al., 2020).

As most thyroiditis, also ADR-induced thyroiditis may start with an increase in thyroid hormones, then followed by a depression, usually permanent. For this reason, this reaction must be identified and treated promptly, or the patient will have to receive replacement thyroid hormones for their lifetime.

One of the most frequent ADR-induced thyroiditis is due to Amiodarone, a common antiarrhythmic drug (Ahmed et al., 2011). In the case of Amiodarone-related thyroid disorders, the mechanisms underlying AMIO thyroid toxicity have been elusive, and no link has been found yet between genetic variants and its thyroid disorders, both hypothyroidism and thyrotoxicosis (Jabrocka-Hybel et al., 2015). Noteworthy, some patients developing thyroid-related amiodarone adverse reactions are already affected by diverse thyroid disorders. Then, it is possible that variants impairing iodine metabolism may also modify the effects of iodine in amiodarone on the thyroid. Recently, it was suggested that Amiodarone treatment could induce endoplasmic reticulum (ER) stress in human thyroid cells, with possible implications of this effect in Amiodarone-induced destructive thyroiditis (Lombardi et al., 2015).

Drug Induced Liver Diseases

Drug induced liver injury (DILI) is the leading cause of acute liver failure in Western Countries. Many studies have investigated the possible genetic predisposition in these events, mainly as to HLA-related genes. DILI is well documented in patients assuming amoxicillin and clavulanic acid, and specifically it has been observed that patients with increased susceptibility to this reaction carry HLA-DRB1*15:01, HLA-DQB1*06:02, HLA-A*02:01, and HLA-B*18:01 variants. However also other HLA variants have been found to be correlated with DILI, caused by Flucloxacillin, Minocycline, Lumiracoxib, Lapatinib, Ximelagatran, and Ticlopidine (Clare et al., 2017). Noteworthy, a genetic predisposition for non-alcoholic fatty liver disease (NAFLD) and Non-Alcoholic Steatohepatitis (NASH) has been observed, with the main player of these conditions being the variant I148M in the *PNPLA3* gene. Moreover, variants in the *TM6SF2, MBOAT7*, and *GCKR* genes have been found to be correlated with a moderate risk of NAFLD (Eslam et al., 2018). Therefore, in affected patients, the administration of substantial doses of multiple drugs mainly metabolized by the liver should be avoided, since hepatotoxic drugs may accelerate the NAFLD process.

Diabetes and Adverse Drug Reactions

Many patients diagnosed with either type 1 or 2 diabetes are in reality affected by maturity onset diabetes of the young, also known as MODY (Valkovicova et al., 2019). This is a condition caused by a genetic mutation in any of the genes *ABCC8*, *APPL1*, *BLK*, *CEL*, *GCK*, *HNF1A*, *HNF1B*, *HNF4A*, *INS*, *KCNJ11*, *KLF11*, *NEUROD1*, *PAX4* or *PDX1*. The MODY phenotypes can be due to a variety of different genetic mechanisms, partially still not well understood but with possible implications for the therapeutic management. Regardless of genetic aetiology, a diabetic patient harboring such mutations might experience treatment failures with a frequency much higher than diabetics patients without those mutations (Chakera et al., 2015). Some forms are manageable just with diet, while others respond well to insulin and others to oral antidiabetic drugs (e.g., sulfonylureas and GLP-1 antagonists). In general, it is clinically relevant to assess the genetic origin of diabetes, as an erroneous therapy could lead to treatment failures, such as hyperglycaemic hyperosmolar syndrome or, inversely, severe hypoglycaemia (Bain, 2009).

Proarrhythmic Drug Effects

Many different cardiac and non-cardiac drugs (mainly antiarrhythmic drugs) and non-cardiac drugs (including antihistaminic, antipsychotic, anti-depressant, antibiotics,

TABLE 4 | Drugs associated with epidermal necrolysis (Stevens–Johnson and Lyell syndromes)

• **Sulfamidics**
Cotrimoxazole
Sulfasalazine
• **Penicillins**
Amoxicillin
Ampicillin
• **Quinolones**
Ciprofloxacine
Norfloxacine
Levofloxacine
Moxifloxacine
• **Anticonvulsants**
Lamotrigine
Valproic acid
Phenobarbital
Phenytoine
• **Miscellaneous**
Allopurinol
Piroxicam
Abacavir

antimalarial, gastrointestinal, and anaesthetic agents) have been described to favour the onset of fatal arrhythmias, defined as pro-arrhythmic effect (Roden and Anderson, 2006). Pro-arrhythmias may be due either to the prolongation of ventricular repolarization, defined as acquired long QT syndrome (LQTS), or to the induction of a Brugada pattern, unmasking Brugada Syndrome (BrS) (Roden and Anderson, 2006; Locati et al., 2017; Locati et al., 2019).

Drug-Induced QT Prolongation and Proarrhythmias

Many different cardiac drugs (*mainly antiarrhythmic drugs*) and non-cardiac drugs (*including antihistaminic, antipsychotic, anti-depressant, antibiotics, antimalarial, gastrointestinal, and anesthetic agents*) have been implicated in the prolongation of the ventricular repolarization and pro-arrhythmias (Locati et al., 2019; Roden, 2016) (**Table 5**). Virtually all QT prolonging drugs act by blocking the potassium channels, mainly the rapid component of the delayed rectifier potassium channel (I_{kr}) encoded by the human Ether-à-go-go-Related Gene (*hERG*) (Antzelevitch and Burashnikov, 2011; Roden, 2016; Goversen et al., 2019; Locati et al., 2019; Roden, 2016). The complete and updated list of specific drugs that prolong the QT interval is available at www.qtdrugs.org. Some of these drugs have either been restricted or withdrawn from the market due to the increased incidence of torsade-de pointes (TdP), a particular polymorphic ventricular tachycardia typically associated with congenital or drug-induced prolonged QT interval.

Reduced Repolarization Reserve and Proarrhythmias

Approximately 5–20% of patients with drug-induced TdP have mutations in genes causing LQTS. These patients have normal to borderline QTc interval at baseline but can develop QT prolongation and TdP, when exposed to I_{kr} blocking drugs (Roden, 2016). The risk of pro-arrhythmic effect is higher in women, in patients with structural heart disease, heart failure and electrolyte disturbance, due to use of diuretics or to renal or gastrointestinal diseases (Roden, 2016). The vulnerability to a pro-arrhythmic effect of a given drug is bound to the concept of "repolarization reserve" (Roden and Anderson, 2006). According to this theory, the loss of one component (such as rapidly delayed rectified potassium currents, I_{Kr}) ordinarily will not lead to failure of repolarization (i.e., marked QT prolongation). However, individuals with subclinical lesions in other components of the system e.g., slowly delayed rectified potassium currents (I_{Ks}) or calcium currents, may display little or no QT changes until I_{Kr} block is superimposed. Among antiarrhythmic drugs, ***Class IA agents*** (Quinidine, Procainamide and Disopyramide), blocking both Na^+ and K^+ channels (mainly I_{kr}) can induce TdP either at therapeutic or subtherapeutic doses and can be precipitated by concomitant hypokalemia or hypomagnesaemia (Locati and Bagliani, 1999; **Figure 1**). On the other hand, ***Class III agents*** (Dofetilide, Ibutilide, Sotalol, Amiodarone), potent I_{Kr} blockers, prolong QT interval in a dose-dependent manner. Pro-arrhythmic effects may be favoured by concomitant factors, such as female sex, electrolyte imbalance, bradycardia, atrial fibrillation, or reduced left ventricular function, and the concomitant use of other QT prolonging drugs (Roden 2016; Locati and Bagliani, 1999; Frommeyer and Eckardt, 2016; Hreiche et al., 2008). In addition, several ***non-cardiac drugs*** (including antihistaminic, antipsychotic, anti-depressant, antibiotics, antimalarial, gastrointestinal, and anaesthetic agents) have been described to alter ventricular repolarization and favour arrhythmogenesis, by blocking the potassium channels, mainly the rapid component of the delayed rectifier potassium channel (I_{kr}).

Sex Differences in Proarrhythmias

Women develop proarrhythmia with cardiac and non-cardiac dugs, and particularly with antiarrhythmic drugs, more often than men (Linde et al., 2018; Roden, 2016). Women have higher resting heart rates (HRs) and longer rate-corrected QT (QTc) intervals than men do, and sex differences in the electrical properties of cardiomyocytes and the myocardial conduction system have been extensively described (Curtis and Narasimha, 2012). Sex-based differences in clinical arrhythmias could be explained by changes in ion channel function, action potential (AP) morphology, autonomic tone, modulated by the effects of sex hormones and by genetics factors. Differences in the activity and density of ion channels, particularly K+ channels, in female cardiomyocytes may increase vulnerability to QT prolongation in women (Curtis and Narasimha, 2012). Several sex-based differences in function, structure, quantity, and currents of cardiomyocyte ion channels, including sodium (Na+), potassium (K+), and calcium (Ca2+) channels and their components, have been described. Sex hormones may affect Na + channel function and the transmural distribution of Na + channel current (INa), and particularly testosterone reduces the transmural dispersion in amplitude of Ina in males, while higher transmural dispersion of INa increases the risk of ventricular arrhythmias in females. Furthermore, a reduced expression of a

TABLE 5 | Cardiac and non-cardiac drugs associated with QT interval prolongation[a].

Cardiac medications
- antiarrhythmic drugs

Class ia (Quinidine, procainamide, Disopyramide)
Class III (dofetilide, ibutilide, sotalol, amiodarone)

Non-cardiac medications
- antihistamines (*Terfenadine*[b], *Astemizole*[b])
- neuroleptic (*Haloperidol*, *Droperidol*, *Thioridazine*, *Chlorpromazine*)
- atypical antipsychotics (*Sertindole*[b], *Ziprasidone*, *Risperidone*, *Zimeldine*, *Citalopram*)
- antidepressants (*Amitriptyline*, *Imipramine*, *Maprotiline*, *Doxepin*, *Fluoxetin*)
- opiate agonists (*Methadone*, *Levomethadyl*)
- anesthetic agents (*Sevoflurane*, *Isoflurane*)
- antibiotics
- quinolones (*Sparfloxacin*[b], *Levofloxacin*, *Moxifloxacin*, *Grepafloxacin*[b])
- macrolides (*Erythromycin*, *Clarithromycin*, *Azitromycine*)
- antimalarials (*Quinine*, *Halofantrine*)
- immunosuppressants (*Hydroxychloroquine*, *Methotrexate*)
- antiprotozoal (*Pentamidine*)
- antifungal (*Azole group*)
- anti-motility agents (*Cisapride*[b], *Domperidone*)
- other (*Arsenic trioxide*, *Bepridil*, *Probucol*)

[a]*Complete and updated list of drugs can be obtained fromwww.qtdrugs.org or https://crediblemeds.org/*
[b]*Withdrawn from market or discontinued.*

variety of delayed rectifier K+ currents (IK) subunits was described in women compared with that in men (Curtis and Narasimha, 2012). Consequently, women have a higher risk than men for TdP in association with QT prolongation with class I and III antiarrhythmics (such as d,l-sotalol, amiodarone, dofetilide, and ibutilide), as well as with several non-cardiac drugs (see **Table 5**; Roden, 2016).

Factors Favouring Proarrhythmic Effects of Drugs

The pro-arrhythmic effect of a drug can be potentiated by the simultaneous use of multiple QT prolonging drugs, e.g., antibiotics, and antidepressants, or immunosuppressants (Roden, 2016). Drug-to-drug interactions have also been described, as several of these drugs are metabolized by the cytochrome P450 enzymes, CYP3A4 or CYP2D6 (Vos and Paulussen, 2004). Patients with liver dysfunction or co-administration of other drugs or food that inhibit the CYP3A4 or CYP2D6 can result in higher drug levels, and favor pro-arrhythmic effects (Roden, 2016; Zanger and Schwab, 2013; Tornio and Backman, 2018). Recently, during the COVID-19 pandemic, in order to provide an urgent remedy, multiple drugs by off-label use were administered to the patients, with a scarce knowledge of potential safety implications. Consequently, QT prolongation and cardiac arrhythmias have been described, particularly following the administration of hydroxychloroquine, an immunosuppressant derived from chloroquine, alone or in combination with antibiotics, such as azithromycin (Bernardini et al., 2020).

Also, impaired renal function may induce higher drugs and catabolite levels, favoring pro-arrhythmias (Reiffel and Appel, 2001). Furthermore, inherited salt-wasting renal disorders, such as Gitelman and Bartter syndromes, provoking hypokalaemia or hypomagnesemia, may favour pro-arrhythmias upon exposure to additional clinical stressors of repolarization, such as QT prolonging cardiac and non-cardiac drugs (Pachulski et al., 2005; Tsukakoshi et al., 2018).

In summary, gene–drug interactions may also exist at different levels, favouring pro-arrhythmic effects of drugs (**Figure 2**):

- Rare ion-channel mutations that increase the risk of QT prolongation by drugs
- Common genetic variants that potentiate the QT prolonging effect of drugs
- Variation within drug metabolizing and transporting proteins that influence drug pharmacokinetics
- Simultaneous use of multiple QT prolonging drugs.

Congenital Long QT Syndrome and Proarrhythmic Drug Effects

Among the cardiac syndromes known to be associated with adverse reactions to certain drugs, by far the most known is the **Long QT syndrome (LQTS)**. This syndrome is rather common, with an estimated prevalence from 1:2,000 to 1: 20,000 (Schwartz et al., 1995; Schwartz et al., 2009). LQTS is characterized by a prolonged duration of ventricular repolarization, exposing affected individuals to a higher risk of fatal ventricular arrhythmias, such as torsade de pointes and ventricular fibrillation. Every patient diagnosed with this condition should receive the list of drugs further lengthening the QT interval, which must be avoided to prevent the occurrence of malignant arrhythmias (Fazio et al., 2013). A continuously updated list of QT prolonging drugs are available at the website https://crediblemeds.org/. Of the 17 genes reported as causative for LQTS, nine genes (*AKAP9*, *ANK2*, *CAV3*, *KCNE1*, *KCNE2*, *KCNJ2*, *KCNJ5*, *SCN4B*, *SNTA1*) were classified as having limited or disputed evidence as LQTS-causative genes. Only three genes (*KCNQ1*, *KCNH2*, *SCN5A*) were curated as definitive genes for typical LQTS. Another four genes (*CALM1*, *CALM2*, *CALM3*, *TRDN*) were found to have strong or definitive evidence for

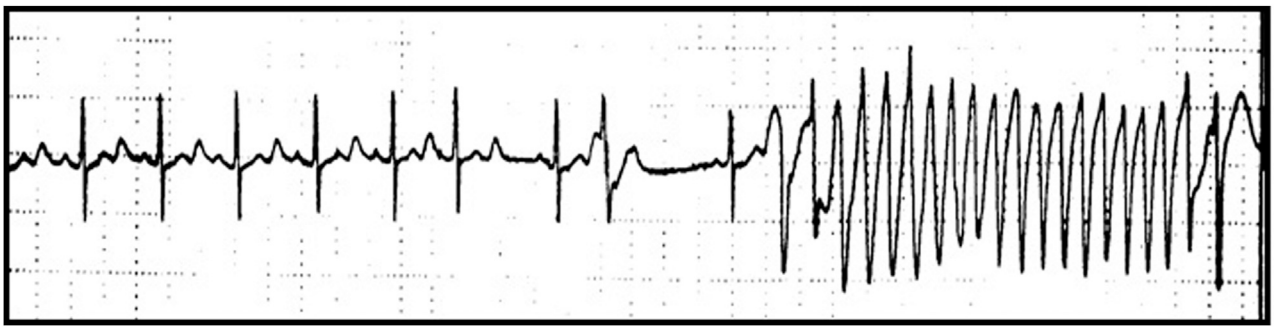

FIGURE 1 | QT prolongation and Torsade-de-pointes (TdP) following Quinidine therapy. Marked QT prolongation (corrected QT, QTc 520 ms) and Torsade-de-pointes (TdP) following Quinidine therapy (dosage quinidine polygalacturonate 275 mg b. i.d, equivalent to quinidine sulfate 200 mg b. id.) recorded during Holter monitoring in a female patient (age 62 years) with history of paroxysmal atrial fibrillation.

causality in LQTS with atypical features, including neonatal atrioventricular block, while the remaining gene (*CACNA1C*) had moderate-level evidence for causing LQTS (Adler et al., 2020; Zareba et al., 1998; Kutyifa et al., 2018). The three most common genotypes (LQT1, LQT2, and LQT3), accounting for most of LQTS cases, tend to have genotype-specific syncope triggers for cardiac events, characteristic T-wave morphologies, QT interval, and age and gender correlated features of high risk (Goldberg et al., 1991; Mathias et al., 2013; Giudicessi et al., 2018). Genotype specific age and gender differences have also been described (Zareba et al., 2003) Subjects with LQT1 and LQT2 tend to have several "warning" syncopal episodes before a sudden death, while in LQT3 the first presentation is commonly sudden death. Beta-blockers are the first-line therapy in LQT1 and LQT2 patients, while they have less efficacy in LQT3. Of note, in all genotypes, the strongest predictors for high risk are previous cardiac arrest or syncope and a QTc interval above 500 msec recorded at any time during follow-up (Locati, 2006).

- **LQT1** accounts for 40–55% of cases of the LQTS and is caused by mutations in the *KVLQT1* (also called *KCNQ1*) gene, encoding Iks. LQT1 patients typically show a prolonged QT interval and a broad-based T-wave on the resting ECG, and children and adolescents, predominantly male, are at highest risk, especially during exercise and particularly swimming.
- **LQT2** accounts for 35–45% of cases of congenital LQTS and is caused by a variety of mutations in the *hERG* (also known as *KCNH2*) potassium channel gene, encoding Ikr. The mutations may involve either the pore or the non-pore region of the hERG channel. Pore mutations carry high-risk of cardiac events and may affect young patients, whereas non-pore mutations often lead to TdP only in the presence of hypokalemia. In adult women with LQT2, particularly the post-partum period is potentially at highest risk for events. In LQT2, ventricular arrhythmias are typically observed during sleep, or during emotional or auditory stimulations. In addition to causing LQT2, the unique structural features of the tetrameric hERG/Kv11.1 channel make it particularly susceptible to blockade by an array of pharmacologic agents resulting in acquired or drug-induced LQTS (Roden, 2016; Frommeyer and Eckardt, 2016; Giudicessi et al., 2018).
- **LQT3** is caused by mutations in the sodium channel gene (*SCN5A*), and it is characterized by events occurring at rest or during sleep. LQT3 is due to perturbation of Na^+-channel inactivation that can prolong the cellular action potential and alter cellular excitability, and it has the higher frequency of fatal cardiac events. In LQT3 patients, gene carriers are often bradycardic with very prolonged QT interval and late onset T waves on resting ECG, and typical T wave alternance can be observed before the occurrence of TdP, and it appears to be less responsive to beta-blockers.

Drug-Induced Brugada Pattern and Brugada Syndrome

Another syndrome strictly correlated to drug-induced arrhythmias is **Brugada Syndrome (BrS).** BrS is characterized by a prolongation of ventricular depolarization mainly localized on the epicardial right ventricle outflow tract, typically manifested on the surface ECG by the elevation of the J point with a coved morphology defined as the Brugada pattern (Antzelevitch and Patocskai, 2016; Pappone and Santinelli, 2019; Priori et al., 2015). BrS is associated with a high risk of cardiac arrest and sudden death due to ventricular tachycardia and ventricular fibrillation. Typically, the Brugada pattern can be spontaneous or induced by sodium blocking drugs, like Ajmaline or Flecainide (Pappone and Santinelli, 2019; Turker et al., 2017; **Figure 3**). Thus, every patient diagnosed with BrS should avoid the use of sodium-blocker antiarrhythmics, and of several other drugs, including psychotropics, anaesthetics, and recreational drugs, such as alcohol and cocaine (Pappone and Santinelli, 2019; **Table 6**). The updated list of drugs associated with BrS is provided at http://www.brugadadrugs.org/. BrS is commonly considered a channelopathy, with an autosomal-dominant pattern of inheritance. The first gene variation associated with BrS was in the *SCN5A* gene, encoding the alpha-subunit of the

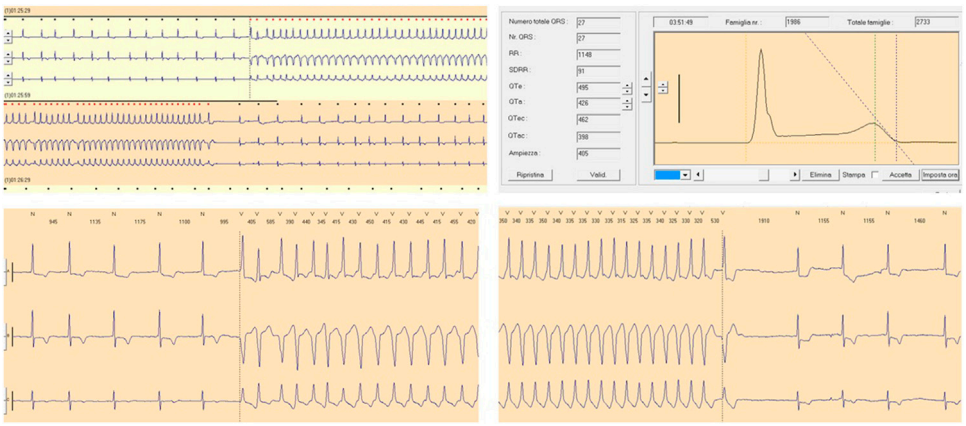

FIGURE 2 | QT prolongation and ventricular tachycardia following Paroxetine therapy. Moderate QT prolongation (corrected QT, QTc 462 ms) and ventricular tachycardia following Paroxetine therapy (dosage 20 mg per day) recorded during Holter monitoring in a female patient (age 70 years) with a history of depression.

cardiac sodium channel, Nav1.5, leading to a reduction of sodium currents (Ina) (Antzelevitch and Patocskai, 2016). Since then, more than 450 pathogenic variants have been identified in 24 genes encoding sodium, potassium, and calcium channels or associated proteins (*ABCC9, CACNA1C, CACNA2D1, CACNB2, FGF12, GPD1L, HCN4, HEY2, KCND2, KCND3, KCNE3, KCNE5, KCNH2, KCNJ8, PKP2, RANGRF, SCN10A, SCN1B, SCN2B, SCN3B, SCN5A, SEMA3A, SLMAP, and TRPM4*). Approximately 20–25% of BrS patients are genetically diagnosed with pathogenic variations in *SCN5A*, also accounting for the most severe clinical forms (Ciconte et al., 2020). However, known BrS susceptibility genes can only explain 30–35% of the clinically diagnosed cases, indicating that 65–70% of BrS patients remain genetically unsolved (Priori et al., 2015). Similarly as in drug-induced LQTS, latent forms of BrS can be induced or unmasked by a wide variety of drugs and pathological conditions. Of note, BrS is frequently associated with atrial fibrillation, whose therapeutic agents such class IC drugs (Flecainide and Propafenone) can unmask cases of latent or undiagnosed BrS (Pappone et al., 2009; Turker et al., 2017). Regarding the possible risk associated with anaesthetics, an important recent study conducted on 36 patients showed the safety of Propofol among high-risk patients clinically affected by BrS (Ciconte et al., 2018).

DISCUSSION AND CONCLUSIONS

Adverse drug reactions (ADRs) are often under evaluated and misdiagnosed, despite being an important cause of both morbidity and mortality all over the world. The technical improvement of genetic testing in the last years enabled the identification of multiple genes involved in ADRs. Thus, identifying the genetic risk factors particularly for idiosynchrasic ADRs could significantly decrease the morbidity and the mortality, and reduce healthcare costs (Wilke et al., 2007). Unfortunately, today the number of drugs for which it is possible to perform pharmacogenetic testing is still limited. The main clinical fields in which pharmacogenetic testing is already available are hemolitc anaemias, malignant hyperthermia, porphyrias, severe skin disorders, Brugada and long QT syndromes. Given the prominent role of genetics in pharmacokinetics and pharmacodynamics, an increasing use of pharmacogentic testing is likely to occur in the near future. Of note, today in case ADRs, the diagnostic work-up tends to consider environmental and genetic factors as mutually excluding, rather than interplaying. However, in reality ADRs could be viewed as a sign, a sort of "red flag," of a possible genetic disorder. Those signs could be nonspecific (as Epidermal Necrolysis), or more related to specific genetic background (as QT interval prolongation and/or Brugada pattern). The clinical usefulness of "red flags" is double: first, raising the suspicion of an ADR, and second prompting a genetic counseling, particularly in case of familial recurrence. Thus, ADRs should become a new untoward "stress test," which should raise the suspicion of a patient being carrier of a specific genetic background. In this context, the demonstration of a clinically relevant genetic background can optimize the safety of many common drugs, including anticonvulsants, anaesthetics, antibiotics, antiretroviral, and antiarrhythmics. The wider use of pharmacogenetic testing in the work-up of ADRs will have at least four consequences: 1) New clinical diagnosis of previously unsuspected diseases; 2) Improvement in the safety and efficacy of the therapeutic management, particularly in paediatric and elderly patients; 3) Repositioning of old drugs for new clinical indications, particularly in rare diseases; 4) Development of new drugs designed for certain specific genetic backgrounds. In this area, improving the genotype-phenotype correlation through new lab techniques and implementation of artificial intelligence in the future may lead to personalized medicine, able to predict ADR and consequently to choose the appropriate compound and dosage for each patient (Kalinin and Haggins, 2018) and the repositioning of old drugs for rare diseases (Scherman and Fetro, 2020).

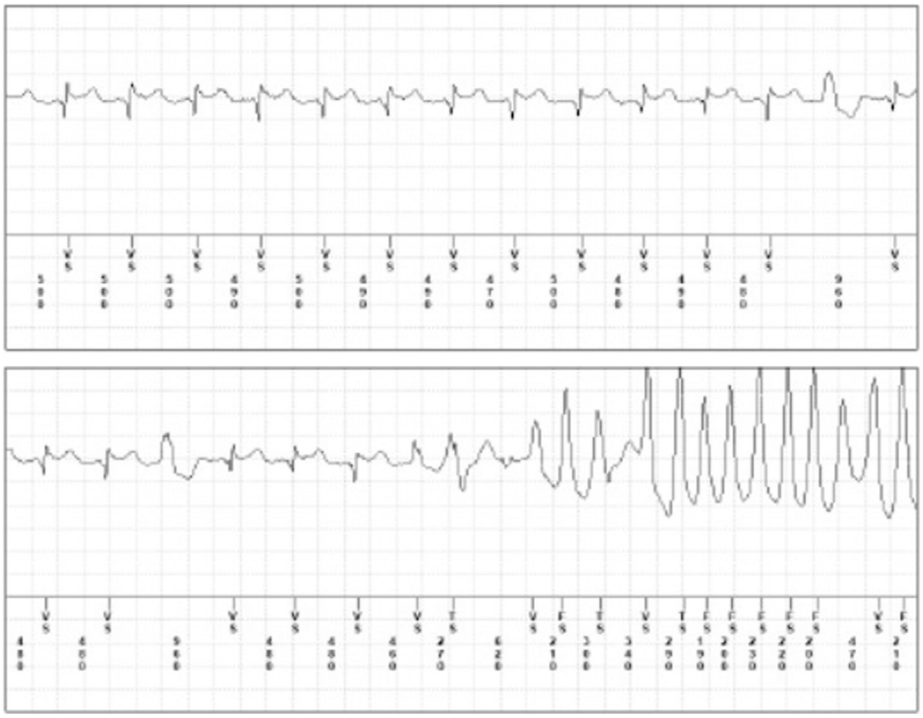

FIGURE 3 | Brugada Pattern and ventricular tachycardia following flecainide therapy. Brugada Pattern and ventricular tachycardia following flecainide therapy (dosage 100 mg b. i.d) recorder by implantable loop recorder in a male patient (age 48 years) with a history of paroxysmal atrial fibrillation.

TABLE 6 | Cardiac and non-cardiac drugs associated with Brugada Syndrome.

Drugs able to unmask Brugada type 1 pattern, and to be avoided in patients with diagnosis of Brugada Syndrome.

- Antiarrhythmic drugs*: Ajmaline, allapinin, ethacizine, flecainide, pilsicainide, procainamide, propafenone
- Psychotropic drugs: Amitriptyline, clomipramine, desipramine, lithium, loxapine, nortriptyline, oxcarbazepine, trifluoperazine anesthetics/analgesics*: bupivacaine, procaine, propofol
- Other substances: Acetylcholine, alcohol (toxicity), cannabis, cocaine, ergonovine

*For furthers advices please visit www.brugadadrugs.org/emergencies

Drugs preferably avoided in patients with diagnosis of Brugada Syndrome.

- Antiarrhythmic drugs: Amiodarone, cibenzoline, disopyramide, lidocaine**, propranolol, verapamil, vernakalant
- Psychotropic drugs: Bupropion, carbamazepine, clothiapine, cyamemazine, dosulepine, doxepin, fluoxetine, fluvoxamine, imipramine, lamotrigine, maprotiline, paroxetine, perphenazine, phenytoin, thioridazine
- Anesthetics/analgesics: Ketamine, tramadol other substances: Dimenhydrinate, diphenhydramine, edrophonium, indapamide, metoclopramide, terfenadine/ Fexofenadine

**lidocaine use for local anesthesia (e.g., by dentists) does seem to be safe if the amount administered is low and if it is combined with adrenaline (epinephrine) which results in a local effect only

Further recommendations

- Recreational drugs (*alcohol, cocaine*) are also potentially dangerous in susceptible patients
- In case of fever, electrocardiographic monitoring is appropriate in combination with lowering of body temperature (e.g., by paracetamol/acetaminophen)
- Possible active drugs may be present in medications containing a combination of drugs
- The presence or absence of a specific drug on this list do not preclude a certain harmful or safe use of that specific drug in this patient respectively
- For most recent recommendations (and disclaimer) on drugs to be avoided by brugada syndrome patients, please visit http://www.brugadadrugs.org

AUTHOR CONTRIBUTIONS

Conceptualization: EM and ETL; data curation: All authors; formal analysis: All authors; funding acquisition: CP; investigation: All authors; resources: CP; supervision: EM, ETL, and CP; visualization: EM, ETL; writing–original draft preparation: EM and ETL; All authors have reviewed and provided corrections, and have agreed to be co-authors on the submitted version of the paper.

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Association Between Genetic Polymorphisms of Metabolic Enzymes and Azathioprine-Induced Myelosuppression in 1,419 Chinese Patients: A Retrospective Study

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The aim of this study was to investigate the correlation between genetic polymorphisms of azathioprine-metabolizing enzymes and adverse reactions of myelosuppression. To this end, a retrospective analysis was performed on 1,419 Chinese patients involving 40 different diseases and 3 genes: *ITPA* (94C>A), *TPMT*3* (T>C), and *NUDT15* (415C>T). Strict inclusion and exclusion criteria were established to collect the relative cases, and the correlation between azathioprine and myelosuppression was evaluated by adverse drug reaction criteria. The mutation rates of the three genes were 29.32, 3.73, and 21.92% and grades I to IV myelosuppression occurred in 54 (9.28%) of the 582 patients who took azathioprine. The highest proportion of myelosuppression was observed in 5 of the 6 (83.33%) patients carrying the *NUDT15* (415C>T) TT genotype and 12 of the 102 (11.76%) patients carrying the *NUDT15* (415C>T) CT genotype. Only the *NUDT15* (415C>T) polymorphism was found to be associated with the adverse effects of azathioprine-induced myelosuppression (odds ratio [OR], 51.818; 95% CI, 5.280–508.556; $p = 0.001$), which suggested that the *NUDT15* (415C>T) polymorphism could be an influencing factor of azathioprine-induced myelosuppression in the Chinese population. Epistatic interactions between *ITPA* (94C>A) and *NUDT15* (415C>T) affect the occurrence of myelosuppression. Thus, it is recommended that the genotype of *NUDT15* (415C>T) and *ITPA* (94C>A) be checked before administration, and azathioprine should be avoided in patients carrying a homozygous *NUDT15* (415C>T) mutation. This study is the first to investigate the association between genetic polymorphisms of these three azathioprine-metabolizing enzymes and myelosuppression in a large number of cases with a diverse range of diseases.

Keywords: azathioprine, ITPA, TPMT, NUDT15, myelosuppression, adverse drug reaction

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INTRODUCTION

Azathioprine (AZA) is a classic immunosuppressant that is widely used for post-transplant rejection, severe rheumatoid arthritis, systemic lupus erythematosus, pemphigus (Joly et al., 2020), inflammatory bowel disease (Ran et al., 2020), dermatomyositis, and other diseases (Mack et al., 2020). It is also recommended for the treatment of immune checkpoint inhibitor-related renal and musculoskeletal adverse events (Thompson et al., 2020). However, AZA can also cause drug adverse reactions (ADRs), including myelosuppression, hepatotoxicity, gastrointestinal reactions (nausea, vomiting, and diarrhea), and alopecia (Food and Drug Administration, 2018). Among them, myelosuppression is particularly harmful and could result in leukopenia, thrombocytopenia, pancytopenia, and even some life-threatening conditions (Panda et al., 2018).

AZA is metabolized into the active 6-thioguanine nucleotides (6-TNGs) by a series of enzymes *in vivo* (Yang et al., 2014; Moon and Loftus, 2016; Kishibe et al., 2018; Wang et al., 2018). AZA is first metabolized to 6-mercaptopurine (6-MP) by glutathione S-transferase (GST), and then converted into 6-thioinosine monophosphate (6-TIMP) with the help of hypoxanthine-guanine phosphoribosyl transferase (HGPRT). Subsequently, 6-TIMP is dehydrogenized into 6-thioxanthosine monophosphate (6-TXMP) by inosine monophosphate dehydrogenase (IMPDH), and then further metabolized to 6-TNGs by guanosine monophosphate synthetase (GMPS), which finally integrates into DNA and RNA molecules to exert cytotoxic and immunosuppressive effects. Moreover, 6-TGTP also binds to Rac1, and inactivates it by regulating the Vav-Rac1 signaling pathway in T lymphocytes; this results in the inhibition of Rac1 target genes, such as nuclear factor kappa beta (NF-κβ), finally leading to the increased apoptosis of activated T lymphocytes (Tiede et al., 2003; Poppe et al., 2006) (**Supplementary Figure S1**).

To reduce the risk of ADRs resulting from the use of AZA, researchers have attempted to establish AZA-metabolizing enzymes to predict the occurrence of myelosuppression and liver toxicity and adjust the dosage according to the genotype. The Clinical Pharmacogenetics Implementation Consortium (CPIC) first published guidelines for adjusting the dose of AZA based on the thiopurine S-methyltransferase (*TPMT*) polymorphism in 2011 (Relling et al., 2011), which were later updated in 2013 and 2018 (Relling et al., 2013; Relling et al., 2019). Currently, the guidelines recommend that patients with a normal *TPMT* metabolizer can use the standard recommended dose, those with intermediate metabolizers are recommended to use 30–80% of the normal dose, and those with poor metabolizers with nonmalignant conditions are not recommended to use AZA. Patients with poor *TPMT* metabolizers with malignancy are recommended to reduce the daily dose by 10-fold and to receive the dose thrice weekly instead of daily. Although some previous studies have investigated the association of AZA-induced ADRs with *TPMT*, inosine triphosphate pyrophosphatase (*ITPA*), nucleoside diphosphate-liked moiety X motif 15 (*NUDT15*), *GST,* multidrug resistance protein 4 (*MRP4*), *HGPRT*, *IMPDH*, and xanthine oxidase (*XO*), the results were varying due to ethnic differences in gene distribution (Krishnamurthy et al., 2008; Kudo et al., 2009; Yang et al., 2014; Burgis, 2016; Choi et al., 2019; Yang et al., 2019). Moreover, the dosage recommended in the CPIC guidelines is inaccurate in that it cannot be individualized among individuals of different races and regions. Among these genes, the most well-studied are *ITPA*, *TPMT*, and *NUDT15*. Some studies have reported that mutations in *ITPA* have no association with AZA-induced myelosuppression (Al-Judaibi et al., 2016; Steponaitiene et al., 2016). Other studies have indicated that the incidence of hepatotoxicity increases with a high *TPMT* enzyme activity, and that there is a high risk of myelosuppression with a low *TPMT* enzyme activity, due to its homozygous mutation. The Food and Drug Administration (FDA) recommends that the *TPMT* genotype of patients should be determined before using AZA (FDA). However, studies have shown that the frequency of *TPMT* gene mutations in the Asian population is only approximately 1.5–3%, thereby showing a high specificity but a low sensitivity. However, Asians have a low tolerance to AZA and a high incidence of leukopenia, which makes it necessary to explore predictive genes suitable for the Asian population specifically. In recent years, some studies have shown that *NUDT15* might be highly correlated with AZA-induced myelosuppression in Asians (Chao et al., 2017; Wang et al., 2018; Banerjee et al., 2020; Kang et al., 2020), and the CPIP guideline also recommends that the *NUDT15* genotype should be determined prior to the administration of AZA (2018) (Relling et al., 2019).

AZA is widely used in clinical settings, and genetic testing is essential for patients who need to take this drug for an extended duration. The abovementioned genes, *ITPA*, *TPMT*, and *NUDT15*, are currently being tested at the West China Hospital of Sichuan University, and the dose of AZA is being adjusted by doctors in accordance with the results of genetic tests to avoid adverse reactions. However, it has been found clinically that some patients with no mutations in these genes suffered myelosuppression, while others with homozygous mutations did not. To provide a reference for the analysis of genetic test results and accurate medication, this study was performed to explore the correlation between the polymorphism of these three genes and AZA-induced myelosuppression. As large-volume analytical studies, especially those involving diverse diseases, remain rare, this study is particularly important given that we examined a large number of cases with various diseases.

MATERIALS AND METHODS

Patients

All included cases were collected from outpatient, emergency, and inpatient data of the West China Hospital of Sichuan University.

Inclusion and Exclusion Criteria

Related data of patients who underwent genetic testing of AZA-metabolizing enzyme genes from January 2016 to January 2019 in

our hospital were extracted from the database. After the removal of duplicates, the patient information, including age, sex, clinical department, diagnosis, white blood cell count (WBC), and AZA daily dose, was compiled using the hospital information system. To determine myelosuppression, patients taking AZA who had complete routine blood examination results were included, while those who did not receive AZA or had incomplete WBC records were excluded.

Myelosuppression Criteria

According to the Common Terminology Criteria for Adverse Events (CTCAEs) version 5.0 published by the United States Department of Health and Human Services and the hospital leukocyte count index standard, myelosuppression was defined as a WBC count $<3.5 \times 10^9$/l; a WBC count of $3–3.5 \times 10^9$/l was defined as grade I, $2–3 \times 10^9$/l as grade II, $1–2 \times 10^9$/l as grade III, and $<1 \times 10^9$/l as grade IV. Adverse drug reaction correlation evaluation criteria of the National Medical Products Administration of China were used to evaluate AZA and myelosuppression correlation, and the Naranjo score was also used when the judgment results were controversial (National Health Commission PRC, 2011; Naranjo et al., 1981). The result "possible" was considered to be an adverse reaction of myelosuppression, and the results were judged by two clinical pharmacists after a double cross-check.

Statistical Analysis

Microsoft Office Excel 2010 was used to input data, and SPSS 25.0 (IBM Corp., Armonk, NY, United States) was used for statistical analysis. Continuous variables are presented as mean ± SD. The independent t-test or Mann–Whitney U test was used to investigate the difference between two unrelated groups, and the one-way analysis of variance was used for comparison between multiple groups. Categorical variables were compared by the chi-square test or Fisher's test, and the Bonferroni correction was used for pairwise comparison between groups. The related factors of myelosuppression were analyzed by logistic regression analysis. Chi-square goodness-of-fit was used to confirm the agreement of the *ITPA* (94C>A), *TPMT*3* (T>C), and *NUDT15* (415C>T) genotype frequencies with the expected frequencies (Hardy–Weinberg equilibrium). The multifactor dimensionality reduction (MDR) method was used to examine gene–gene interactions, and MDR Permutation Testing software (version 1.0 Beta 2) was used for replacement testing. *P*-values < 0.05 were considered statistically significant.

RESULTS

Patient Characteristics

A total of 1,419 available cases were covered in this study, including 742 (52.29%) inpatients and 677 (47.71%) outpatients/emergency patients (**Figure 1**). Among them, there were more female patients (65.19%), and the average age was 45.96 ± 14.41 years. Of the total cases, males comprised 494 (34.81%), with an average age of 42.74 ± 16.32 years. Nineteen departments were involved in the study, and the study population included 40 diseases, including pemphigus, inflammatory bowel disease, and autoimmune hepatitis (**Supplementary Figure S2**), among which, pemphigus was the most common (347, 24.45%).

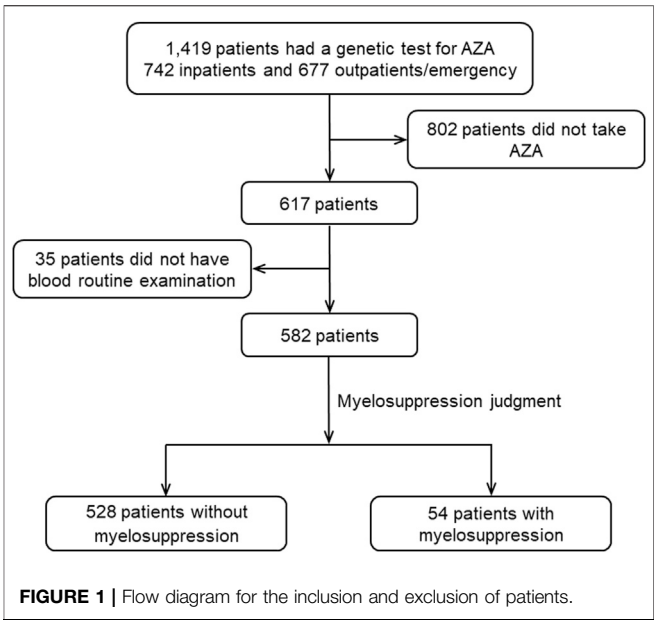

FIGURE 1 | Flow diagram for the inclusion and exclusion of patients.

Genetic Mutation

Among the 1,419 patients, 1,279 (90.13%) had *ITPA* (94C>A) (rs1127354), *TPMT*3* (T>C) (rs1142345), and *NUDT15* (415C>T) (rs116855232), while 140 (9.87%) had *TPMT*3* (T>C) and *NUDT15* (415C>T). The genotypes of all patients are shown in **Supplementary Table S1**. The *ITPA* (94C>A), *TPMT*3* (T>C), and *NUDT15* (415C>T) genotype distributions were in Hardy–Weinberg equilibrium (p = 0.959, 0.811, and 0.406, respectively). The mutation rate of *TPMT*3* (T>C) was the lowest (3.73%), similar to the previous reports in Asian populations (Kumagai et al., 2001; Chen et al., 2014; van et al., 2014). The respective proportions of wild type, heterozygous mutation, and homozygous mutation of the three genes were statistically significant ($p = 1.374 \times 10^{-68}$), and pairwise comparison between different genotypes by chi-square test also showed statistical significance. The data are shown in **Table 1**.

Overall Incidence of Myelosuppression

A total of 617 (43.48%) patients had AZA administration records, 582 (94.33%) of whom had complete routine blood examination results. Myelosuppression occurred in 54 (9.28%) patients, and the incidence of myelosuppression with grades from I to IV was 37.04% (20/54), 42.59% (23/54), 11.11% (6/54), and 9.26% (5/54), respectively.

Incidence of Myelosuppression According to Genotype

ITPA (94C>A) Genotype

A total of 516 patients carrying the *ITPA* (94C>A) genotype were included, among whom, 48 (9.30%) suffered from

TABLE 1 | Mutations of *ITPA*, *TPMT*3*, and *NUDT15*.

Genotype	*ITPA* (94C>A)	*TPMT*3* (T>C)	*NUDT15* (415C>T)	*P*-value[a]		
Wild type	904 (70.68%)	1,366 (96.27%)	1,108 (78.08%)	1.551×10^{-72}	0.059×10^{-3}	1.868×10^{-46}
Heterozygote	340 (26.58%)	51 (3.59%)	283 (19.95%)			
Homozygote	35 (2.74%)	2 (0.14%)	28 (1.97%)			
Total	1,279	1,419	1,419			

Data are n (%).

[a] *The differences in mutations were statistically significant between ITPA (94C>A) and TPMT*3 (T>C) ($p = 1.551 \times 10^{-72}$), and between ITPA (94C>A) and NUDT15 (415C>T) ($p = 0.059 \times 10^{-3}$), and between TPMT*3 (T>C) and NUDT15 (415C>T) ($p = 1.868 \times 10^{-46}$).*

myelosuppression (grades I-IV). Patients carrying the *ITPA* (94C>A) AA genotype had the highest risk of myelosuppression, with an incidence of 25.00%, although this was not statistically significant. The mean daily dose (MDD) of AZA was significantly different among patients with different genotypes ($p = 0.002$). Multiple comparison results showed that only the AZA doses between the AC and CC genotype were significantly different ($p = 0.323 \times 10^{-3}$) (**Supplementary Table S2**).

*TPMT*3* (T>C) Genotype

A total of 582 cases with the *TPMT*3* (T>C) genotype were included, among whom, 54 (9.28%) had ADRs of myelosuppression (grades I-IV). Similarly, the incidence of myelosuppression varied according to genotype; patients carrying the *TPMT*3* (T>C) TC heterozygous mutation had the highest rate (20.00%), but there were no significant differences among the three groups. There were also no significant differences among the MDD of AZA among the different genotypes (**Supplementary Table S2**).

NUDT15 (415C>T) Genotype

The number of patients carrying *NUDT15* (415C>T) gene was 582, 54 (9.28%) of whom had ADRs of myelosuppression (grades I-IV). The incidence of myelosuppression varied by genotype; patients carrying the *NUDT15* (415C>T) TT homozygote mutant had the highest rate (83.33%; 3 [60.00%] with grade IV), and the difference among the three genotypes was significant ($p = 0.008 \times 10^{-3}$). After the Bonferroni correction, there was a statistically significant difference in the myelosuppression rate between patients with the TT genotype and the other two genotypes (CT and CC) (TT, CC: $p = 7.707 \times 10^{-11}$ and TT, CT: $p = 0.003 \times 10^{-3}$), and there was no statistically significant difference between the CT and CC genotypes ($p = 0.194$). The WBC count was significantly different among the three genotypes ($p = 0.002$), but the results of multiple comparisons showed that only the TT and CC genotypes were significantly different ($p = 0.002$). The difference in the MDD of AZA among different genotypes was also significant ($p = 0.010$), but the results of multiple comparisons showed that it was only statistically significant between the CT and CC genotypes ($p = 0.003$) (**Supplementary Table S2**).

Myelosuppression According to Genotype Combinations

Our results show that all of the patients who carried the *NUDT15* (415C>T) TT homozygote mutation, regardless the genotypes of *TPMT*3* (T>C) and *ITPA* (94C>A), had a higher incidence of myelosuppression, and mostly at grade IV. Moreover, 20 of 313 (6.39%) patients carried the wild-type versions of these three genes, one of whom had grade IV myelosuppression. One patient carried a homozygote mutation of *TPMT*3* (T>C), with wild-type *NUDT15* (415C>T) and *ITPA* (94C>A), and did not suffer myelosuppression (**Table 2**).

Factors Associated With Myelosuppression

Based on the occurrence of myelosuppression, all of the 582 patients who received AZA treatment and had routine blood examinations were divided into two groups: group A suffered myelosuppression and group B did not. The results showed that there were more females in both groups, but there was no significant difference in the ratio of males to females ($p = 0.240$), ages ($p = 0.866$), nor the MDD of AZA ($p = 0.410$) between the two groups of patients. The number of patients carrying different genotypes of *ITPA* (94C>A) and *TPMT*3* (T>C) was also not significantly different between the two groups, although the number of patients carrying *NUDT15* (415C>T) mutations was significantly different ($p = 0.008 \times 10^{-3}$) (**Table 3**).

Binary logistic regression analysis of myelosuppression-related factors showed that the risk of myelosuppression was significantly higher in patients with an *NUDT15* (415C > T) TT genotype than in those with the wild type (odds ratio [OR], 51.818; 95% CI, 5.280–508.556; $p = 0.001$). Beyond this, no significant difference in the incidence of myelosuppression was found among the patients with other genotypes of these three genes compared with their corresponding wild type. Other factors, including age ($p = 0.722$), sex ($p = 0.075$), and dose ($p = 0.490$), had no significant association with the incidence of myelosuppression (**Table 4**).

Gene–Gene Interactions

The gene–gene interaction results are shown in **Table 5**. The *NUDT15* (415C>T) locus had the highest testing balanced accuracy among the 3 SNPs. The optimal interaction models include *NUDT15* (415C>T) and *ITPA* (94C>A) with a maximum cross-validation (CV) consistency of 10 out of 10 and a maximum testing balanced accuracy of 0.5921 ($p < 0.05$ on the basis of 1000-fold permutation testing).

DISCUSSION

The retrospective case analysis was performed on a large number of relative cases involving various diseases. Current reports are

TABLE 2 | Incidence of myelosuppression in patients with different genotype combinations.

ITPA (94C>A)	*TPMT*3* (T>C)	*NUDT15* (415C>T)	Myelosuppression	Grade (n)
CA	TT	TT	1/1 (100%)	I (1)
-	TC	TT	1/1 (100%)	IV (1)
CC	TT	TT	3/4 (75.00%)	IV (2), I (1)
CA	TC	CC	2/5 (40.00%)	II (2)
AA	TT	CC	2/7 (28.57%)	II (1), III (1)
-	TT	CT	2/14 (14.29%)	I (1), III (1)
CC	TT	CT	8/59 (13.56%)	I (2), II (5), III (1)
CA	TT	CC	9/87 (10.34%)	I (6), II (2), III (1)
CC	TC	CC	1/10 (10.00%)	III (1)
CA	TT	CT	2/26 (7.69%)	II (1), IV (1)
CC	TT	CC	20/313 (6.39%)	I (8), II (10), III (1), IV (1)
-	TT	CC	3/49 (6.12%)	I (1), II (2)
AA	TT	CT	0/1	–
CC	TC	CT	0/2	–
-	TC	CC	0/2	–
CC	CC	CC	0/1	–

TABLE 3 | Comparison of differences between groups with and without myelosuppression.

	Group A (*n* = 54)	Group B (*n* = 528)	*P*-value
Gender: male/female	15/39	189/339	0.240
Age	43.50 ± 14.27	43.15 ± 14.42	0.866
ITPA (94C>A) CC/CA/AA	32/14/2	357/105/6	0.155
*TPMT*3* (T>C) TT/TC/CC	50/4/0	511/16/1	0.188
NUDT15 (415C>T) CC/CT/TT	37/12/5	437/90/1	0.008×10^{-3}
MDD of AZA (mg)	72.21 ± 35.44	68.90 ± 27.27	0.410

Data are n (%) or mean ± SD.

TABLE 4 | Regression analysis of factors associated with myelosuppression.

Variable	*P*-value	OR	95% CI
Gender (referent: male)	0.722	0.996	0.974–1.018
Age	0.075	1.940	0.934–4.028
MDD of azathioprine (mg)	0.490	1.004	0.993–1.015
ITPA (94C>A) CA (referent: CC)	0.197	1.585	0.787–3.191
ITPA (94C>A) AA (referent: CC)	0.061	4.945	0.928–26.358
*TPMT*3* (T>C) TC (referent: TT)	0.185	2.420	0.655–8.946
*TPMT*3* (T>C) CC (referent: TT)	1.000	0.000	0.000–
NUDT15 (415C>T) CT (referent: CC)	0.216	1.624	0.754–3.497
NUDT15 (415C>T) TT (referent: CC)	0.001	51.818	5.280–508.556

OR: odds ratio, CI: confidence interval.

mostly limited to a certain disease or a type [such as inflammatory bowel disease (Al-Judaibi et al., 2016; Wang et al., 2018; Walker et al., 2019; Kang et al., 2020), autoimmune disease (Fei et al., 2018; Fan et al., 2019), and acute lymphoblastic leukemia (Yang et al., 2015; Zhu et al., 2018)], and have generally included a small number of study subjects, with a focus on only one or two genes. In this study, 40 diseases were included, which covered all of the indications for AZA, and the correlation between genetic polymorphisms of AZA-metabolizing enzymes and AZA-induced myelosuppression were compared under different pathological states.

The most common single-nucleotide polymorphism (SNP) loci of *ITPA* in the population are 94C>A and IVS2 + 21A>C, and the mutation of *ITPA* (94C>A) leads to a high risk of ADRs (Arenas et al., 2007). Mutations at this locus affect protein expression by reducing the expression of the full length transcript, decreasing the catalytic activity and stability, and altering mRNA splicing events such as missplicing of exons 2 and 3. Finally, these mutations result in a poor expression of an unstable, catalytically compromised protein, and affect the activity of ITPA (Burgis, 2016). The reported frequency of the *ITPA* (94C>A) A allele is higher in Asians (11–19%) than in Caucasian, Hispanics, and Africans (1–7%) (Maeda et al., 2005; Hawwa et al., 2008; Okada et al., 2009). In this study, the mutation rate of *ITPA* (94C > A) was 29.32%, and the frequency of carrying the *ITPA* (94C>A) A allele was 16.02%, which was consistent with that reported in the current Asian population. Odahara et al. reported that the mutation rate of this gene was 39.6% in 48 Japanese inflammatory bowel disease (IBD) patients, and that the incidence of leukopenia in patients carrying this mutation was 36.8% (Odahara et al., 2015). However, the study indicated that leukopenia cannot be clearly attributed to the *ITPA* (94C>A) mutation as there may be other influencing factors. Moreover, Wroblova et al. reported a 13.8% mutation rate of the *ITPA* (94C > A) gene in 188 IBD patients, but no confirmed association was found between its polymorphism and myelosuppression toxicity (Wroblova et al., 2012). These studies also indicated that *ITPA* genetic polymorphisms may be associated with influenza-like symptoms, arthralgia, and pancreatitis (Zabala-Fernandez et al., 2011; Wroblova et al., 2012). However, the sample sizes of

TABLE 5 | Epistatic interactions between the variants of azathioprine metabolism influencing myelosuppression.

Model	Training balance accuracy	Testing balance accuracy	Cross-validation Consistency	χ^2 (*P*)	OR (95%CI)
NUDT15 (415C>T)	0.5732	0.4969	8/10	6.58 (0.0103)	2.21 (1.19–4.09)
NUDT15 (415C>T) and *ITPA* (94C>A)	0.6157	0.5921	10/10	12.20 (0.0005)	2.66 (1.51–4.69)
NUDT15 (415C>T), *ITPA* (94C>A), and *TPMT*3* (T>C)	0.6199	0.5507	10/10	12.47 (0.0004)	2.69 (1.53–4.74)

OR: odds ratio, CI: confidence interval.

previous studies have been limited, and relatively new studies are lacking. The number of patients included in our study is large, and the result is representative of the Chinese population. In our study, the incidence of myelosuppression in patients with different *ITPA* (94C>A) genotypes, from high to low, were homozygous mutation, heterozygous mutation, and wild type. Nevertheless, there was no significant difference in the incidence of myelosuppression among these three genotypes. Moreover, a correlation factor analysis suggested that compared to the wild type, homozygous, and heterozygous mutations in patients carried a high risk of myelosuppression, although this was not significant. Therefore, there was no significant correlation between the *ITPA* (94C>A) gene polymorphism and myelosuppression.

Collie-Duguid et al. reported that the rate of *TPMT* gene mutation was 10.1% (20/199) in Caucasians, 2.0% (2/99) in Southwest Asians, and 4.7% (9/192) in Chinese (Collie-Duguid et al., 1999). Common mutation loci in *TPMT* include *TPMT**2, *TPMT**3A, *TPMT**3B, and *TPMT**3C. *TPMT**3A is the dominant locus in Caucasians, and *TPMT**3C is the most common locus in Southeast Asian, African, and African–American populations. *TPMT* has ten exons, eight of which encode the 28-kDa protein. Nucleotide transversion (G238C) at one locus of *TPMT**2 leads to the substitution of a rigid proline for a more flexible alanine residue at codon 80 (Krynetski et al., 1995). This mutation causes changes in the tertiary structure of the *TPMT* protein, which reduces the stability and catalytic ability of the protein. *TPMT**3A contains two single nucleotide transversions, G460A in exon 7 and A719G in exon 10, which leads to amino acid substitutions at codon 154 (Ala > Thr) and codon 240 (Tyr > Cys) (Sahasranaman et al., 2008). *TPMT**3B and *TPMT**3C both have only one mutation locus, G460A in exon 7 and A719G in exon 10, respectively (Zelinkova et al., 2006). These variants destabilize the *TPMT* protein, and reduce its binding affinity to 6-MP (Naushad et al., 2021). In our study, the mutation rate of *TPMT*3* (T>C) was the lowest, at 3.73%, which was close to the previously reported mutation rate of 2.90% (15/522) in the Japanese population (Kumagai et al., 2001), and 3.17% (4/126) and 4.60% (4/87) in the Chinese population (Chen et al., 2014; Fei et al., 2018). Moreover, in these two studies (Chen et al., 2014; Fei et al., 2018), all of the mutant genotypes of *TPMT* were heterozygous and no homozygous mutation was found. The higher number of participants in our study could better reflect the mutation rate of this gene in the Chinese population (low). Chen et al. suggested that the *TPMT* gene polymorphism in Chinese SLE patients had a low sensitivity to predict leukopenia, resulting in a limited clinical value; therefore, they recommended that the AZA dose should be adjusted by monitoring the enzyme activity of *TPMT* (Chen et al., 2014). Two other studies on Chinese patients with autoimmune diseases demonstrated that the polymorphism of *TPMT* was not clearly associated with AZA-induced leukopenia (Fei et al., 2018; Fan et al., 2019). Although a meta-analysis of 14 published studies, involving 2276 patients with IBD, showed an association between the *TPMT* polymorphism and AZA-induced myelosuppression in Caucasians ($p < 0.00001$; pooled OR, 6.97; 95% CI, 3.89–12.47), no significant correlation was found in Asians ($p = 0.12$) (Liu et al., 2015). In our study, only two patients carried the *TPMT*3* (T>C) CC genotype, one of whom had an AZA treatment history but no myelosuppression. The incidence of myelosuppression in patients with the *TPMT*3* (T>C) TC genotype was significantly higher than that in patients with the wild genotype, but the difference was not statistically significant. The correlation factor analysis showed that patients with *TPMT*3* (T>C) TC had a higher risk of myelosuppression (OR, 2.420; 95% CI, 0.655–8.946) those with the wild type, but the difference was not significant ($p = 0.185$). Therefore, there was no correlation between the polymorphism of *TPMT*3* (T>C) and myelosuppression. Some studies in Western countries demonstrated a correlation between the *TPMT* gene polymorphism and the ADR of blood toxicity (Zabala-Fernandez et al., 2011; Al-Judaibi et al., 2016; Steponaitiene et al., 2016); however, for the Asian population, especially Chinese, there was no significant association between the *TPMT* gene polymorphism and myelosuppression.

There are four common mutation loci of *NUDT15*, including rs116855232, rs554405994, rs186364861, and rs147390019 (Moriyama et al., 2016), the most common of which is rs116855232 (415C>T, protein sequence p.Arg139Cys). Studies have reported that the *NUDT15* (415C>T) mutation does not affect enzymatic activity but does adversely affect protein stability (Valerie et al., 2016). This may be due to the loss of supportive intramolecular bonds, leading to a rapid degradation of proteasomes in cells. Other reports noted that *NUDT15* variants have no impact on the binding of "dGTP" to the *NUDT* protein. The *NUDT15* (415C>T) mutation increases aggregation "hot spots" and induces unfavorable torsion in the protein (Naushad et al., 2021). The frequency of mutation for this locus (15–30%) is higher in East Asian populations, including Japanese (Kakuta et al., 2018; Tanaka et al., 2018), Chinese (Chao et al., 2017; Fei et al., 2018), Koreans (Kim et al., 2017; Yi et al., 2018), and Indians (Banerjee et al., 2020), while the mutation rate is low in Caucasians (Yang et al., 2015; Walker et al., 2019) (European: 0.5–0.8% and Hispanic: 7.7%). Some studies have

investigated the mutation rate of *NUDT15* (415C>T) in the Chinese population, but the majority have had small sample sizes. Fan et al. reported an *NUDT15* (415C>T) mutation rate of 17.45% (26/149) in Chinese patients with autoimmune hepatitis, among whom, only 2 patients (1.34%) had homozygous mutations (Fan et al., 2019). Fei et al. studied 87 Chinese patients with autoimmune diseases, and found an *NUDT15* (415C>T) mutation rate of 32.18%, with only one patient (1.15%) carrying a homozygous mutation (Fei et al., 2018). In the current study, the *NUDT15* (415C>T) mutation rate was 21.92%, and 28 (1.97%) patients had an *NUDT15* (415C>T) homozygous mutation; these results are higher than those reported by Fan et al. but lower than those of Fei et al. In addition, Kakuta and colleagues found a 25.27% mutation rate of this gene among 1,282 Japanese patients (Kakuta et al., 2018), which was close to the 21.92% observed in our study. Therefore, it is conceivable that our data truly reflect the mutation rate of *NUDT15* in the Chinese population. Current studies suggest that polymorphism of *NUDT15* is significantly associated with leukopenia or myelosuppression (Moriyama et al., 2016; Chao et al., 2017; Kim et al., 2017; Fei et al., 2018; Kakuta et al., 2018; Wang et al., 2018; Fan et al., 2019; Kang et al., 2020). Moreover, the risk of adverse reactions has been found to be much higher in people carrying homozygous mutations than in those with the wild genotype. Our results showed that the rate of AZA-induced myelosuppression in patients carrying the *NUDT15* (415C>T) TT genotype was as high as 83.33%, and the incidence of grade IV myelosuppression was 60.00%, while patients with a heterozygous mutation and wild type had rates of 11.76 and 7.81%, respectively. Moreover, the incidence of myelosuppression was significantly different among patients with homozygous mutations, heterozygous mutations, and wild type ($p = 0.008 \times 10^{-3}$). Given that there was no significant difference in the MDD of AZA among these patient groups, the interference of dose difference on the incidence of myelosuppression could be eliminated. The correlation factor analysis showed that compared to the wild type, people carrying the homozygous mutant genotype had an extremely high risk of myelosuppression (OR, 51.818; 95% CI, 5.280–508.556; $p = 0.001$). The mutation frequency of *NUDT15* (415C>T) was 21.92%, which was significantly higher than the 3.73% of *TPMT*3* (T>C) ($p = 1.868 \times 10^{-46}$). Additionally, the analysis of factors associated with myelosuppression showed that the polymorphism of *NUDT15* (415C>T) was significantly associated with myelosuppression; thus, the *NUDT15* (415C>T) polymorphism is a promising predictor of AZA-induced myelosuppression in the Chinese population. According to the results, it is recommended to test the genotype of *NUDT15* (415C>T) before taking AZA, and AZA should be avoided in patients with a homozygous mutant genotype.

In the current study, the overall incidence of myelosuppression was 9.28%, which was close to the 8.05% (12/149) and 8.7% (81/935) described previously in Chinese and Indian populations (Fan et al., 2019; Banerjee et al., 2020), but lower than 18.17–23.81% reported in the other studies mentioned above (Kim et al., 2017; Fei et al., 2018; Kakuta et al., 2018; Yang et al., 2019). This difference may be due to the inherent limitations of the retrospective study and incomplete information on medication and examination which may lead to the absence of myelosuppression records for some patients. In addition, within the 582 patients with medication records, 83 of patients received an adjusted dose of AZA according to the gene test results to the safe range. The analysis of myelosuppression-related factors showed that sex, age, the MDD of AZA, and polymorphisms of *ITPA* (94C>A) and *TPMT*3* (T>C) had no significant association with myelosuppression, and that only the polymorphism of *NUDT15* (415C>T) was an influencing factor. The analysis of different combinations of genotypes indicate that patients with the *NUDT15* (415C>T) T allele were prone to suffer from myelosuppression and those who carried the *NUDT15* (415C>T) TT genotype faced an even high risk. The results of gene–gene interactions showed that *NUDT15* (415C>T) had the highest testing balanced accuracy, which also proved that this gene locus had a strong correlation with myelosuppression. There might be an interaction between *ITPA* (94C>A) and *NUDT15* (415C>T) loci, which together affected the occurrence of myelosuppression induced by azathioprine. At present, the epistatic interactions among the above three gene loci had not been reported. This study was the first to analyze the gene–gene interactions among *ITPA*, *NUDT15*, and *TPMT*. We also found that in patients whose three genes were the wild type, 20 (6.39%) of them suffered from myelosuppression, and one case was grade IV. This finding suggests that these genes are not sufficient to predict myelosuppression in all patients, and there may be other relative metabolic enzyme genes that remain to be explored in future studies (Inman et al., 2018).

This study has some limitations. First, the retrospective nature of the study meant that the information was incomplete in some cases, and some medication records and test results were missing; in particular, one patient with the *TPMT*3* (T>C) CC genotype had no medication records, which resulted in the exclusion of this population. In addition, as the genotype detection of *ITPA* (94C>A) was only initiated in our hospital in the last few years, there were 140 cases in whom only *TPMT*3* (T>C) and *NUDT15* (415C>T) were detected, with no information on the *ITPA* (94C>A) genotype. The degree of AZA-induced myelosuppression could only be evaluated by the information provided in the cases, which cannot be used to reconstruct the actual situation at the time, making it difficult to truly evaluate the severe grades of myelosuppression.

CONCLUSION

Our findings suggest that the polymorphism of *NUDT15* (415C>T) is a significantly relative factor in the context of AZA-induced myelosuppression, and epistatic interactions between *ITPA* (94C>A) and *NUDT15* (415C>T) affect the occurrence of myelosuppression. Therefore, it is recommended to test these two genes prior to administration of AZA. In people carrying a homozygous mutation of *NUDT15* (415C>T), the risk of myelosuppression is very high, and therefore AZA should be avoided during their treatment. However, in our hospital, the cost of the detection of these three metabolic enzyme genes is

628 times that of one tablet of AZA (100 mg). Thus, the detection of *ITPA* (94C>A) and *TPMT*3* (T>C) is not necessarily recommended for economic reasons but only to test the genotype of *NUDT15* (415C>T) for patients who have difficulty in paying medical expenses. Moreover, there may be other AZA-metabolizing enzyme genes that could better predict the incidence of AZA-induced myelosuppression, and further investigations are needed.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

This study was performed according to the recommendations of the ethical guidelines and approved by the Biomedical Ethics Committee of West China Hospital of Sichuan University (Nos. 2020973 and 2021402). All patients were exempt from providing informed consent.

AUTHOR CONTRIBUTIONS

Z-YC, Y-HZ, L-YZ, and Z-YH wrote the manuscript; X-JL and Z-YH designed the research; Z-YC, Y-HZ, ZQ, and Y-WP performed the research; Z-YC, Y-HZ, W-QS, and Z-YH analyzed the data; BW, YY, N-NC, RZ, M-YW, Z-HS, X-JL, and TX contributed new reagents/analytical tools.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.672769/full#supplementary-material

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frontiers in Genetics

SYSTEMATIC REVIEW
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The Genetics of Adverse Drug Outcomes in Type 2 Diabetes: A Systematic Review

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Background: Adverse drug reactions (ADR) are a major clinical problem accounting for significant hospital admission rates, morbidity, mortality, and health care costs. One-third of people with diabetes experience at least one ADR. However, there is notable interindividual heterogeneity resulting in patient harm and unnecessary medical costs. Genomics is at the forefront of research to understand interindividual variability, and there are many genotype-drug response associations in diabetes with inconsistent findings. Here, we conducted a systematic review to comprehensively examine and synthesize the effect of genetic polymorphisms on the incidence of ADRs of oral glucose-lowering drugs in people with type 2 diabetes.

Methods: A literature search was made to identify articles that included specific results of research on genetic polymorphism and adverse effects associated with oral glucose-lowering drugs. The electronic search was carried out on 3rd October 2020, through Cochrane Library, PubMed, and Web of Science using keywords and MeSH terms.

Result: Eighteen articles consisting of 10, 383 subjects were included in this review. Carriers of reduced-function alleles of organic cation transporter 1 (OCT 1, encoded by *SLC22A1*) or reduced expression alleles of plasma membrane monoamine transporter (PMAT, encoded by *SLC29A4*) or serotonin transporter (SERT, encoded by *SLC6A4*) were associated with increased incidence of metformin-related gastrointestinal (GI) adverse effects. These effects were shown to exacerbate by concomitant treatment with gut transporter inhibiting drugs. The CYP2C9 alleles, **2* (rs1799853C>T) and **3* (rs1057910A>C) that are predictive of low enzyme activity were more common in subjects who experienced hypoglycemia after treatment with sulfonylureas. However, there was no significant association between sulfonylurea-related hypoglycemia and genetic variants in the ATP-binding cassette transporter sub-family C member 8 (*ABCC8*)/Potassium Inwardly Rectifying Channel Subfamily J Member 11 (*KCNJ11*). Compared to the wild type, the low enzyme activity C allele at CYP2C8**3* (rs1057910A>C) was associated with less weight gain whereas the C allele at rs6123045 in the *NFATC2* gene was significantly associated with edema from rosiglitazone treatment.

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Conclusion: In spite of limited studies investigating genetics and ADR in diabetes, some convincing results are emerging. Genetic variants in genes encoding drug transporters and metabolizing enzymes are implicated in metformin-related GI adverse effects, and sulfonylurea-induced hypoglycemia, respectively. Further studies to investigate newer antidiabetic drugs such as DPP-4i, GLP-1RA, and SGLT2i are warranted. In addition, pharmacogenetic studies that account for race and ethnic differences are required.

Keywords: pharmacogenomics, type 2 diabetes, adverse drug outcomes, oral glucose-lowering drugs, gastrointestinal side effects, hypoglycemia, weight gain

INTRODUCTION

Diabetes mellitus refers to a group of metabolic disorders characterized by hyperglycemia resulting from insufficient production and/or ineffective response of cells to insulin. It is one of the major contributors to morbidity and mortality globally, and its prevalence continues to rise. By 2019, an estimated 463 million adults aged 20–79 years were living with diabetes which accounts 9.3% of the global population in this age group (International Diabetes Federation, 2019). This figure is expected to surge to 578 million (10.2%) by 2030 and to 700 million (10.9%) by 2045, most of this in the form of type 2 diabetes (T2D) (International Diabetes Federation, 2019). Poorly controlled diabetes progressively leads to chronic microvascular, macrovascular and neuropathic complications which manifest as renal failure, blindness, lower limb amputation, and accelerated vascular disease.

Several drugs are available for the management of T2D, referred to as glucose lowering agents. These include: biguanides (metformin), insulin secretagogues (sulfonylureas, meglitinides), thiazolidinediones, alpha glucosidase inhibitors (acarbose), incretin mimetics (GLP-1RAs, DPP-4is), amylin antagonists, sodium-glucose co-transporter-2 inhibitors (SGLT-2i), and insulin. These classes of drugs are either prescribed as monotherapy or given in combination.

The management of T2D is guided by national and international evidence-based guidelines (Buse et al., 2020), but there is noticeable inter-individual variability in treatment response as defined by glycemic reduction and adverse drug reactions. This variation is the net effect of several environmental and clinical factors including age, sex, adherence, concomitant therapy, drug interactions, and disease severity. In addition to these, a patient's genotype can affect interindividual differences in drug response.

Adverse drug reactions are major clinical and public health problems world-wide. In the UK, around 6.5% of hospital admissions are due to adverse drug reactions (Pirmohamed et al., 2004), and that almost 15% of patients experience an ADR during their admission (Davies et al., 2009, 2010). The projected annual cost of such admissions to the NHS was £466m (Davies et al., 2009). Glucose lowering agents are one of the drugs of great concern for ADRs (Ducoffe et al., 2016). They have a well-described set of ADRs that are detrimental to individuals' health and quality of life (**Table 1**). In the US, from 2010 to 2013, there were 600,991 ADRs associated with glucose-lowering agents with an average hospital marginal cost of $4, 312 resulting in an annual hospital cost of $2.59 billion (Spector et al., 2017). Optimizing drug therapy through the avoidance of ADRs will dramatically improve patient health while generating millions of dollars by saving unnecessary medical costs.

However, there is inter-individual variability in the type and severity of ADRs experienced by individuals taking glucose-lowering drugs. While clinical and environmental factors influence this, genomic factors are also important. Here, we aim to undertake a systematic review of pharmacogenomic studies of ADRs related to oral glucose-lowering drugs.

METHODS

This systematic review is reported according to the Preferred Reporting Items for the Systematic Reviews and Meta-analysis Protocols (PRISMA-P) 2015 Checklist (Shamseer et al., 2015).

Type of Participants

Participants included in eligible studies must be diagnosed with T2D and treated with oral glucose-lowering drugs.

Type of Exposure

We included studies in which participants genotype were investigated in relation to ADRs of oral glucose-lowering agents.

Outcomes

The primary outcome was the incidence of any of the adverse effects of glucose-lowering drugs (**Table 1**). For metformin, GI adverse effects were considered - hypoglycemia and weight gain for sulfonylureas and weight gain and edema for thiazolidinediones.

Eligibility Criteria

Inclusion Criteria

Studies assessing the effect of genetic variations on the incidence of adverse effects of oral glucose-lowering drugs in people with T2D, published up to 3rd October 2020 in English language without any geographical restriction were included in this review.

Exclusion Criteria

We did not consider news, qualitative studies, case reports, reviews, editorials, and comments; and all non-published studies and published in non-English languages. Studies in which relevant data on genetic polymorphisms and/or ADRs associated

TABLE 1 | List of adverse drug reactions related to glucose-lowering drugs in type 2 diabetes.

Drug	Side effects	Comments
Metformin	GI side effects (10%−25%): nausea, indigestion, abdominal cramp or bloating, or combination of these (Bailey and Turner, 1996; Goodman, 2017). Decrease in vitamin B12 concentration (5 to 10%) (Bell, 2010; de Jager et al., 2010; Aroda et al., 2016; Donnelly et al., 2020) Lactic acidosis (Misbin, 2004)	Symptoms are usually mild, transient, and reversible after dose reduction. When severe requires drug switch in about 5% of the population. Patient may develop anemia and/or peripheral neuropathy (Bell, 2010). Rare but serious
Thiazolidinediones	Increased risk of overweight, congestion, heart failure, fractures, bladder cancer (pioglitazone) and myocardial infarction (rosiglitazone) (Jearath et al., 2016).	An average of 2 to 4 kg weight gain in the first year of treatment (Yki-Järvinen, 2004; Winkelmayer et al., 2008). Higher risk of adverse cardiovascular events with rosiglitazone (Winkelmayer et al., 2008; Juurlink et al., 2009; Graham et al., 2010).
Sulfonylurea and meglitinides	Hypoglycemia including coma (Schopman et al., 2014). Weight gain of 1–3 kg (Meneilly, 2011; Guardado-Mendoza et al., 2013)	
GLP-1 analogs	Nausea and vomiting, injection site reactions	Could be mediated through neural activation of specific CNS neurons due to peripheral dosing of peptide (Madsbad et al., 2011).
DPP-4 inhibitors	Headache, nasopharyngitis, and urinary tract infections (sitagliptin) (Katzung et al., 2019).	Have no effects on body weight or risk of hypoglycemia (Salvo et al., 2016).
SGLT2 inhibitors	Genital and urinary tract infections. IV volume depression and hypotension can result from osmotic diuresis (Katzung et al., 2019).	

with oral glucose-lowering agents is lacking or impossible to extract were also excluded.

Search Strategy for Identifying Relevant Studies

A literature search was made to identify articles that included specific results of research on genetic polymorphisms and adverse effects associated with oral glucose-lowering agents. The electronic search was carried out on 3rd October 2020, through Cochrane Library, PubMed, and Web of Science using keywords and MeSH terms with no restriction for time of publication. The search strategy conducted in MEDLINE via PubMed, Cochrane, and Web of Science is shown in **Supplementary Material**. We also manually searched reference lists from relevant studies and contact experts in the field in order to identify additional eligible studies.

Selection of Studies for Inclusion in the Review

Two review authors (AMB and AYD) independently identified articles and then screened their titles and abstracts for eligibility. Thereafter, full texts of articles considered to be eligible were retrieved. Furthermore, the review authors independently assessed the eligibility for inclusion in the review based on the inclusion and exclusion criteria. Disagreements between the two authors was resolved by consensus. A PRISMA flow diagram (Shamseer et al., 2015) was employed to document the process of literature selection and the reasons for exclusion of articles were stated.

Data Extraction and Management

Two review authors (AMB and AYD) independently extracted data from the articles reviewed. A data extraction form for this purpose was designed. Data was collected on the first author's name, year of publication, geographical location (country where the study was performed), sample size, population characteristics, relevant genetic polymorphism, primary outcome measurements (incidence of adverse events after treatment with oral glucose-lowering agents). Any disagreements between the two review authors were resolved through discussion and consensus.

Study characteristics and the effect estimates of genetic polymorphisms on the incidence of adverse effects of oral glucose-lowering agents was presented in full, in tabular form. We summarized findings based on the type of oral glucose-lowering agent.

RESULT

Study Selection

Of the 362 studies identified, 66 were included for review of the full text. Of these studies, 18 studies met the inclusion criteria (**Figure 1**).

Characteristics of Included Studies

Eighteen articles comprised of 10, 383 subjects were included in this review (**Table 2**). Among these, two studies were multinational (Bailey et al., 2010; Dawed et al., 2019). While 12 studies were conducted in Europe (Holstein et al., 2005, 2011, 2012; Ragia et al., 2009, 2012, 2014; Tarasova et al., 2012; Dujic et al., 2015, 2016a,b, 2018; Dawed et al., 2016), three were in Asia

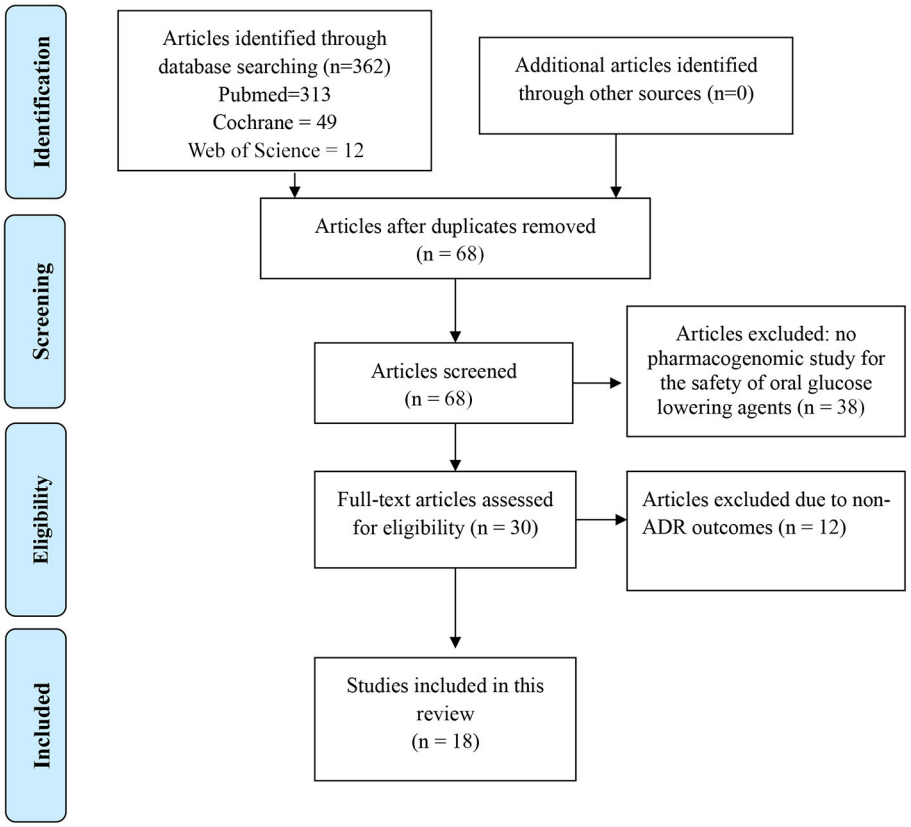

FIGURE 1 | PRISMA flow diagram for search results October 30, 2020.

(Kahn et al., 2006; Sato et al., 2010; Gökalp et al., 2011) and one was in the US (Ruaño et al., 2009).

Five of these studies comprising of 5,438 subjects were conducted to investigate association between genetic polymorphisms and metformin-related GI adverse effects (Tarasova et al., 2012; Dujic et al., 2015, 2016a,b; Dawed et al., 2019). Association between genetic polymorphisms and the risks of sulfonylurea-induced hypoglycemia was evaluated in nine studies comprising of 1,944 subjects (Holstein et al., 2005, 2011, 2012; Ragia et al., 2009, 2012, 2014; Sato et al., 2010; Gökalp et al., 2011; Dujic et al., 2018). Among the sulfonylureas, glimepiride was the most investigated (nine studies) (Holstein et al., 2005, 2012; Ragia et al., 2009, 2012, 2014; Sato et al., 2010; Gökalp et al., 2011; Dujic et al., 2018) followed by gliclazide (Ragia et al., 2009, 2012, 2014; Gökalp et al., 2011; Dujic et al., 2018) (five studies) and glibenclamide (Holstein et al., 2005, 2011, 2012; Sato et al., 2010; Dujic et al., 2018) (five studies). Four studies consisting of 3, 001 subjects evaluated the genetics of thiazolidinediones-induced edema and weight gain (Kang et al., 2006; Ruaño et al., 2009; Bailey et al., 2010; Dawed et al., 2016).

Metformin

Metformin is the first-line therapy for T2D. Around 30% of metformin treated subjects experience gastrointestinal (GI) side effects manifested as nausea, indigestion, abdominal cramps, bloating, diarrhea, or combination of these (**Table 1**) (Garber et al., 1997; Hirst J. A. et al., 2012). Metformin is an organic cation, and carrier proteins mediate its oral absorption, hepatic uptake, and renal elimination. Several solute carrier transporters, expressed in the membranes of the enterocytes, could be involved in the absorption of metformin from the intestinal lumen, including organic cation transporter (OCT) 1, plasma membrane monoamine transporter (PMAT), carnitine/cation transporter 1, OCT3 (encoded by *SLC22A3*), and serotonin reuptake transporter (SERT) (Han et al., 2015). Genetic variants in genes encoding these transporters have been reported in five articles (**Table 3**) (Tarasova et al., 2012; Dujic et al., 2015, 2016a,b; Dawed et al., 2019).

Association between genetic variants in *SLC22A1* (a gene encoding OCT1), and GI intolerance related to metformin therapy have been reported in three studies (Tarasova et al., 2012; Dujic et al., 2015, 2016a). A study conducted using the GoDARTS cohort (Hébert et al., 2018), in 251 metformin-intolerant and 1,915 metformin-tolerant individuals showed that the presence of two or more reduced-function alleles at R61C, C88R, G401S, M420del, or G465R increased the odds of GI side effects of metformin by more than 2-fold (Dujic et al., 2015). This effect was over 4-fold with concomitant use of OCT1-inhibiting drugs. The findings were replicated in another prospective observational cohort study from Bosnia and Herzegovina that included 92

TABLE 2 | Description of included studies.

References	Country	Study drug	Study period	Parent study	Population	Gene	Comparators	*N*
Dawed et al. (2019)	Multinational (Europe)	Metformin		IMI DIRECT	White Europeans aged between 18 and 90 years	*SLC29A4*	Metformin-intolerant Metformin-tolerant	286 1,128
Dujic et al. (2015)	Scotland, UK	Metformin	1 January 1994–1 June 2011.	GoDARTS	White Europeans	SLC22A1	metformin-intolerant Metformin- tolerant	251 1,915
Dujic et al. (2016b)	Scotland, UK	Metformin	1 January 1994–1 June 2011.	GoDARTS	White Europeans	*SLC6A4* *SLC22A1*	Metformin-tolerant Metformin-intolerant	1,356 164
Dujic et al. (2016a)	Bosnia and Herzegovina	Metformin			T2D diagnosis after the age of 35 years	*SLC22A1*	Metformin-tolerant Metformin-intolerant	49 43
Tarasova et al. (2012)	Latvia	Metformin	from 2003 to 2010	LGDB	Subjects with T2D older than 18 years	*SLC22A1, SLC22A2, SLC47A1*	Metformin-tolerant Metformin-intolerant	193 53
Gökalp et al. (2011)	Turkey	Glimepiride Gliclazide Glipizide	2003 and 2005	None	Subjects with T2D treated with SU for at least 3 months.	*CYP2C9* *CYP2C19* *CYP2C8*	Without hypoglycemia With hypoglycemia	93 15
Dujic et al. (2018)	Scotland	Glibenclamide Gliclazide Glimepiride Glipizide	1994–2010	GoDARTS	Subjects with T2D who were incident users of SU	*POR* *CYP2C9*	Without hypoglycemia With hypoglycemia	311 69
Holstein et al. (2011)	Germany	Glimepiride Glibenclamide Gliquidone.	1 January 2000 and 31 December 2009	None	Subjects with T2D treated with SU	*CYP2C9*	Without severe hypoglycemia With severe hypoglycemia	101 102
Holstein et al. (2005)	Germany	Glibenclamide Glimepiride	January 2000 and 31 December 2003	None	Subjects with T2D treated with SU	*CYP2C9*	Without severe hypoglycemia With severe hypoglycemia	337 20
Holstein et al. (2012)	Germany	Glimepiride Glibenclamide Gliquidone	January 2000–31 December 2010	None	Subjects with T2D admitted to the emergency department	*ABCC8*	Without hypoglycemia With hypoglycemia	100 111

(Continued)

TABLE 2 | Continued

References	Country	Study drug	Study period	Parent study	Population	Gene	Comparators	*N*
Sato et al. (2010)	Japan	Glimepiride Glibenclamide	January 2005 and October 2009	None	Subjects with T2D treated with Sus	*ABCC8*	Without hypoglycemia With hypoglycemia	32 125
Ragia et al. (2012)	Greece	Glimepiride Gliclazide	February 2007–September 2008	None	Subjects with T2D treated with SU	*KCNJ11*	Without hypoglycemia With hypoglycemia	84 92
Ragia et al. (2014)	Greece	Glimepiride Gliclazide	February 2007–September 2008	None	Subjects with T2D treated with SU	*CYP2C9*	Without hypoglycemia With hypoglycemia	84 92
Ragia et al. (2009)	Greece	Glimepiride Gliclazide	February 2007–September 2008	None	Subjects with T2D treated with SU	*CYP2C9*	Without hypoglycemia With hypoglycemia	84 92
Ruaño et al. (2009)	USA	Rosiglitazone Pioglitazone	Between February and June 2007	None	Subjects with T2D treated with rosiglitazone or pioglitazone for ≥4 months	**For BMI:** *ADORA1, PKM2, ADIPOR2, UCP2, APOH, IRS1, LIPA, RARB, and CHRM3* **For Edema:** *NPY, GYS1, CCL2, OLR1, GHRH, ADRB1, ACACB, SCARB2, HRH3 and ACE*		87
Kang et al. (2006)	South Korea	Rosiglitazone		None	Subjects with T2D aged between 35 and 80 years	*PLIN*		160
Dawed et al. (2016)	Scotland, UK	Rosiglitazone Pioglitazone			Subjects with T2D treated with TZD	*CYP2C8*		833
Bailey et al. (2010)	Multinational (21 countries)	Rosiglitazone Placebo		DREAM	Subjects with T2D ≥30 years of age	*NFATC2*	Rosiglitazone Placebo	965 956

TABLE 3 | Association between metformin and selected SNPs for the incidence of GI adverse outcomes.

References	Outcome measure	Drug (*n*)			Gene	SNP/genotype	Conclusion
		Drug	Tolerant	Intolerant			
Dawed et al. (2019)	Incidence of GI adverse effects	Metformin	1,128	286	*SLC29A4*	rs3889348G>A	The G allele at rs3889348 (*SLC29A4*) was associated with higher odds of gastrointestinal intolerance (OR 1.34 [1.09–1.65], $P = 0.005$).
Dujic et al. (2015)	Incidence of GI adverse effects	Metformin	1,915	251	*SLC22A1*	R61C C88R G401S M420del G465R	Compared to carriers of one or no deficient allele, carriers of two reduced-function OCT1 alleles had higher odds of intolerance (OR 2.41 [1.48-3.93], $P < 0.001$).
Dujic et al. (2016b)	Incidence of GI adverse effects	Metformin	1,356	164	*SLC6A4*	L*L* L*S* S*S*	Each S* alleles was associated with higher odds of metformin intolerance (OR = 1.28 [1.01–1.63], $P = 0.04$). Multiplicative interaction between *SLC6A4* and *SLC22A1* ($P = 0.003$)
					SLC22A1	R61C C88R G401S M420del G465R	
Dujic et al. (2016a)	Incidence of GI adverse effects	Metformin	49	43	*SLC22A1*	R61C M420del	Each OCT1 reduced-function allele was associated with higher odds of GI side effects (OR = 2.31 [1.07–5.01], $P = 0.034$).
Tarasova et al. (2012)	Incidence of GI side effects	Metformin	193	53	*SLC22A1* *SLC22A2* *SLC47A1*	rs12208357A>G rs34059508T>C rs628031A>G rs72552763D>I rs36056065I>D rs316019T>G rs2289669A>G	Each A allele at rs628031 was associated with lower odds of intolerance (OR = 0.39 [0.19–0.82], $P = 0.01$) Each 8 bp insertion at rs36056065 was associated with lower odds of intolerance (OR = 0.41 [0.23–0.72], $P = 0.01$)

newly diagnosed subjects in the first 6 months of metformin treatment (Dujic et al., 2016a). Likewise, Tarasova et al. showed significant associations between a SNP (rs628031) and an 8 bp insertion (rs36056065) in the *SLC22A1*, with GI side effects of metformin (Tarasova et al., 2012).

Dawed et al. reported association between rs3889348, a variant that alters intestinal expression of the *SLC9A4* (a gene encoding PMAT), with metformin related GI intolerance in 286 severe intolerant and 1,128 tolerant subjects. The G allele that reduces expression of *SLC29A4* was associated with 34% higher odds of intolerance. Concomitant administration of metformin transporter inhibiting drugs exacerbate GI intolerance by more than 3-folds (Dawed et al., 2019).

Considering involvement of serotonin reuptake transporter (SERT) in metformin intestinal absorption, Dujic et al. investigated association between the low-expressing S* allele derived from a composite SERT (*SLC6A4*)-5-HTTLPR/rs25531 genotypes and metformin intolerance. In this study, each S* allele was associated with 30% higher odds of intolerance. Interestingly, a multiplicative interaction between OCT1 and SERT genotypes was observed (Dujic et al., 2016b). Carriers of reduced function alleles in OCT1 at the background of the wild type SERT (L*L*) genotype had greater odds of intolerance (OR 9.25 [3.18–27.0]) compared to carriers of the S* allele.

Sulfonylureas

Sulfonylureas were the first oral glucose-lowering therapy introduced into clinical practice and along with metformin, are the most prescribed drugs for the management of T2D (Inzucchi et al., 2015). SU are transported into the liver by OATP1B1 (encoded by *SLCO1B1*) and metabolized mainly by the polymorphic CYP2C9 enzyme and to a lesser extent by CYP2C19 enzyme (Becker et al., 2008). CYP2C9*2 (R144C, rs1799853) and CYP2C9*3 (I359L, rs1057910) are the two most common variants that have been associated with poor metabolism of SU (Semiz et al., 2010). Sulfonylureas induce glucose-independent

TABLE 4 | Association between sulfonylureas and selected SNPs for the incidence of hypoglycemia.

References	Outcome measure	Sulfonylurea drug (%)			Gene	Genotypes	Conclusion
		Drug	Controls	Cases			
Gökalp et al. (2011)	Incidence of mild hypoglycemia	Glimepiride Gliclazide Glipizide	44 (47%) 41 (44%) 8 (9%)	6 (40%) 5 (33%) 4 (27%)	*CYP2C9*	CYP2C9**2* CYP2C9**3*	In the gliclazide group a significant association between *CYP2C9* genotypes and hypoglycemic attacks were observed ($P = 0.035$).
Dujic et al. (2018)	Incidence of SU-induced hypoglycemia	Glibenclamide Gliclazide Glimepiride Glipizide	5 (1.6%) 254(81.7%) 3 (1.0%) 49 (15.8%)	2 (2.9%) 53 (76.8%) 4 (5.8%) 10 (14.5%)	*POR* *CYP2C9*	POR**28* CYP2C9**2* CYP2C9**3*	The number of *CYP2C9* deficient alleles increased the odds of hypoglycemia nearly 3-fold (OR, 2.81; 95% CI, 1.30-6.09; $P = 0.009$) only at the POR*1/*1 genotype background Statistically significant interaction between *POR* and *CYP2C9* genotypes ($P = 0.007$).
Holstein et al. (2011)	Severe hypoglycemia	Glimepiride Glibenclamide Gliquidone	81 (80.2%) 18 (17.8%) 2 (2%)	76 (74.5%) 25 (24,5%) 1 (1.0%)	*CYP2C9*	CYP2C9**1* CYP2C9**2* CYP2C9 **3*	There was no overrepresentation of the CYP2C9 **2/*2*, **2/*3*, and **3/*3* variants in the SH group (2%) compared with the control group (5%).
Holstein et al. (2005)	Severe hypoglycemia	Glimepiride Glibenclamide	337	20	*CYP2C9*	CYP2C9**1* CYP2C9**2* CYP2C9 **3*	The *CYP2C9* genotypes **3/*3* and **2/*3* that are predictive of low enzyme activity were more common in the hypoglycemic group than in the comparison groups (10 vs. <2%, OR 5.2; 95% CI, 1.01, 27).
Holstein J. D. et al. (2012)	Severe hypoglycemia	Glimepiride Glibenclamide Gliquidone	80 18 2	82 28 1	*ABCC8*	Ser1369Ala	Ser1369Ala variant in *ABCC8* does not affect the response to sulfonylurea treatment and so, is not a major player in the etiology of severe hypoglycemia.
Sato et al. (2010)	Severe hypoglycemia	Glimepiride Glibenclamide	32	125	*ABCC8*	Ser1369Ala	No significant differences in the distribution of the Ser1369Ala genotype between patients with or without severe hypoglycemia ($p = 0.26$)
Ragia et al. (2012)	Mild hypoglycemia	Glimepiride Gliclazide	4 10	80 12	*KCNJ11*	E23K	*KCNJ11* E23K polymorphism did not affect hypoglycemia risk.
Ragia et al. (2014)	Mild hypoglycemia	Glimepiride Gliclazide	74 10	80 12	*CYP2C9* *POR*	CYP2C9**1* CYP2C9**2* CYP2C9**3* POR**1* POR**28*	POR**28* allele was not associated with severe hypoglycemia. CYP2C9 **2* allele increased the risk of hypoglycemia by more than 3 times (OR: 3.218, $p = 0.031$). POR**28* allele is masking the association of CYP2C9**2* allele with severe hypoglycemia.
Ragia et al. (2009)	Mild hypoglycemia	Glimepiride Gliclazide	74 10	80 12	*CYP2C9*	CYP2C9*1 CYP2C9*2 CYP2C9*3	The presence of CYP2C9**3* allele puts subjects with T2D at higher risk of hypoglycemia when receiving the SU

insulin release from the pancreatic β-cells by binding to the ATP-sensitive potassium (KATP) channels, SUR1 and Kir6.2, that are encoded by the *ABCC8* and *KCNJ11* genes, respectively.

Hypoglycemia is the most common adverse effect of SU. In a systematic review consisting of 22 randomized controlled trials, 10.1 and 5.9% of SU treated subjects experienced hypoglycemia as defined by blood glucose levels of ≤3.1 or ≤2.8 mmol/L, respectively (Schopman et al., 2014). Severe hypoglycemia with SU therapy is less common, with reported incidence of 0.8%. SU treatment also results in weight gain of 1–3 kg (Schopman et al.,

TABLE 5 | Association between thiazolidinediones and selected SNPs for the incidence of weight gain and edema.

References	Outcome measure	Drug	Sample size	Gene	SNP/genotype	Conclusion
Dawed et al. (2016)	Weight gain	Rosiglitazone Pioglitazone	519 273	*CYP2C8* *SLCO1B1*	CYP2C8*3 (rs10509681) 521T>C (rs4149056)	The CYP2C8*3 variant was associated with less weight gain ($P = 0.02$).
Bailey et al. (2010)	Edema	Rosiglitazone	965	GWAS	GWAS	rs6123045 an intronic SNP in the *NFATC2* was significantly associated with edema (OR 1.89 [95% CI 1.47–2.42]; $P = 5.32 \times 10^{-7}$)
Ruaño et al. (2009)	BMI	Rosiglitazone Pioglitazone	87	*ADORA1* *PKM2* *ADIPOR2* *UCP2* *APOH*	rs903361 rs2856929 rs7975375 rs660339 rs8178847	*ADORA1*-rs903361 was significantly associated with weight gain $P < 0.0003$)
Kang et al. (2006)	Weight gain	Rosiglitazone	160	*PLIN*	6209T>C 11482G>A 13041A>G 14995A>T	The A allele at 11482G>A was associated with less weight gain (GG, 1.33 ± 1.59 kg; GA, 0.85 ± 1.89 kg; and AA, 0.03 ± 1.46 kg; $P = 0.010$)

2014). Risk of hypoglycemia and weight gain may vary with age, gender, renal function, disease progression, drug exposure, and genetic constitution.

In this systematic review, we have included studies that investigated association between genetic variants in genes, *CYP2C9* and *ABCC8/KCNJ11*, that encode CYP2C9 and SUR1/Kir6.2 with risk of hypoglycemia (Holstein et al., 2005, 2011, 2012; Ragia et al., 2009, 2012, 2014; Gökalp et al., 2011) (**Table 4**). An association between reduced function CYP2C9**2* and CYP2C9**3* alleles with higher risk of SU related hypoglycemia was reported confirming earlier functional and pharmacokinetic data (Ragia et al., 2009; Gökalp et al., 2011). However, another study could not confirm the findings (Holstein et al., 2011). In the later study subjects within the control arm that carry slow metabolizing alleles were found to be treated with significantly lower doses than carriers of the wild type, whereas in the group with severe hypoglycemia, the dose was the same for all genotype groups. Another small study suggested a possible interaction between P450 oxidoreductase (*POR*) and *CYP2C9* genotypes (Ragia et al., 2014), where POR**28* allele could mask the effect of CYP2C9**2* allele on sulfonylurea-induced hypoglycemia. Indeed, a bigger study from the GoDARTS cohort confirmed this and therefore it is worth considering *CYP2C9* and *POR* genotypes jointly in studies involving the pharmacogenetics of SU (Dujic et al., 2018).

Three other studies investigated association between two strongly linked non-synonymous polymorphisms, S1369A (rs757110) and E23K (rs5219), in the *ABCC8* and *KCNJ11* genes, respectively with hypoglycemia (Sato et al., 2010; Holstein et al., 2012; Ragia et al., 2012). None of these studies showed statistically significant association between SU treatment and risk of hypoglycemia suggesting these polymorphisms may not play a major role in the etiology of hypoglycemia.

Thiazolidinediones

Thiazolidinediones are insulin sensitizers that act by increasing the transactivation activity of Peroxisome Proliferators Activated Receptors (PPARs). The clinically used TZDs, rosiglitazone and pioglitazone, suffer from serious side effects. Concerns about the cardiovascular safety of rosiglitazone due to fluid retention led suspension in the European market and several restrictions in the US (Woodcock et al., 2010; Shukla and Kalra, 2011). Unlike rosiglitazone, pioglitazone did not show any risk of cardiovascular side effects. However, concerns were raised on the apparent risk of bladder cancer with pioglitazone and hence it is not recommended in people with active or prior history of bladder cancer (Shukla and Kalra, 2011). TZDs are associated with an average of 2–4 kg weight gain in the first year of management (Yki-Järvinen, 2004). In addition, these agents result in peripheral edema in 4–6% (Graham et al., 2010).

This systematic review identified four articles that assessed association between genetic variants in candidate genes and weight gain and/or oedema after treatment with TZDs (Kang et al., 2006; Ruaño et al., 2009; Bailey et al., 2010; Dawed et al., 2016) (**Table 5**). A *post-hoc* analysis from the DREAM (Diabetes REduction Assessment with ramipril and rosiglitazone Medication) trial that consist of 4,197 participants showed higher rate of roziglitazone-induced edema (OR = 1.89 [95% CI = 1.47–2.42], $P = 0.017$) in subjects homozygous for the C allele at rs6123045, a variant at the Nuclear Factor of Activated T-cells, Cytoplasmic, Calcineurin-Dependent 2 (*NFATC2*) locus (Bailey et al., 2010). We have previously showed association between the CYP2C8**3* variant with less weight gain compared to the wild

type ($P = 0.02$) in the GoDARTS (Dawed et al., 2016). Another study by Ruaño et al. investigated 384 SNPs in 87 subjects treated with TZDs and reported significant association between an intronic SNP, rs903361, in Adenosine A1 Receptor (*ADORA1*) and BMI after correcting for multiple testing (Ruaño et al., 2009). The A allele at Perilipin 1, PLIN 11482G>A (rs894160), was also associated with less weight gain in Korean subjects treated with rosiglitazone compared to the G allele (Kang et al., 2006). Given these findings replicate in well-powered independent studies, we could potentially identify individuals who can benefit from the considerable therapeutic advantages of TZDs and who are least at risk for the side effects.

DISCUSSION

This systematic review considers the adverse effects of glucose lowering drugs and their relationship to genetic variability. It presents the up-to-date knowledge of genetic variants that could influence ADRs related to drugs for the management of type 2 diabetes. Comprehensive understanding of genetic variants associated with ADRs have clinical utility in risk stratification of patients and precision therapeutics. Although numerous associations of genetic variants with ADR have been discovered, replication has proven difficult. This could mainly be due to a smaller sample which may not be sufficiently powered to detect the desired effect, the lack of consistent phenotypic definitions used, presence of possible drug-drug interactions, and related comorbidities.

This systematic review suggests that at present the clinical translation of genetic variants associated with ADR in diabetes therapy are limited.

Inter-ethnic differences in the susceptibility to ADRs and response to drugs are under-investigated. However, inter-ethnic differences have long been recognized as a crucial aspect of the genetics of variation in drug response. This could be due to differing background frequencies of risk alleles. For example, the frequency of 420del allele, that causes reduced OCT1 function and associated with higher odds of GI side effects of metformin, is much higher amongst European (∼17%) than East Asian (∼0.5%) or African (∼6%) populations (Karczewski et al., 2020). The same is true for the loss of function variant, CYP2C9*3 (European ∼6.7%, African ∼1.3%, South Asian 1.2%) (Karczewski et al., 2020).

It is also likely that adverse drug reactions are polygenic. In polygenic effects, risk is conferred by combinations between several variants each of which could have small individual effects that are summarized as polygenic risk scores. We have previously shown a better prediction of metformin-induced GI intolerance by combining SNPs in the OCT1 (*SLC22A1*) and PMAT (*SLC29A4*) (Dawed et al., 2019), pioglitazone-related weight gain and SNPs in the *SLCO1B1* and *CYP2C9* (Dawed et al., 2016). Comprehensive studies encompassing data from hypothesis free genome-wide associations are required to identify susceptibility loci. In addition, next generation sequencing that allows the analysis of rare variants that have been postulated to have larger effects are likely to reveal functionally relevant genomic variations for ADRs.

Clinical, anthropometric, and environmental factors such as age, sex, weight, concomitant use of other drugs was also shown to contribute to ADRs in diabetes. Older people, women, and concomitant use of gut metformin transporter inhibiting drugs were previously shown to increase the likelihood of GI side effects of metformin (Dujic et al., 2015; Dawed et al., 2019). In addition, longer diabetes duration, impaired renal function, lower body mass index, lower triglyceride levels and old age were identified as major risk factors for hypoglycemia in people with type 2 diabetes (Schloot et al., 2016).

Even though this review is comprehensive, it is subjected to limitations. First, the studies included were heterogeneous in design with regards to treatment, adverse effect outcomes definitions, and population (ethnicity). The timing to measure primary endpoint (adverse effects of oral glucose-lowering agents) is also not uniform.

Poor adherence to treatment is a well-known phenomenon in patients with diabetes and is associated with inadequate glycaemic control leading to rapid disease progression and complications (Polonsky and Henry, 2016). Moderate and severe ADRs such as hypoglycemia and GI intolerance are previously shown to be key contributors of poor adherence in diabetes.

In conclusion, there are few pharmacogenomic studies of ADRs in type 2 diabetes that have been undertaken. Most of the studies have not been externally replicated, except OCT1 and metformin induced GI intolerance, CYP2C9 and SU-induced hypoglycemia. In the future, well-powered pharmacogenomic studies in T2D should collect standardized ADR data in multi-ethnic populations.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

AMB and AYD conceived and designed this research, executed the analysis procedure, and analyzed the results. AMB, MKS, and AYD contributed to the writing of the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

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Further Evidence That OPG rs2073618 Is Associated With Increased Risk of Musculoskeletal Symptoms in Patients Receiving Aromatase Inhibitors for Early Breast Cancer

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Background: Aromatase inhibitors (AI) reduce recurrence and death in patients with early-stage hormone receptor-positive (HR +) breast cancer. Treatment-related toxicities, including AI-induced musculoskeletal symptoms (AIMSS), are common and may lead to early AI discontinuation. The objective of this study was to replicate previously reported associations for candidate germline genetic polymorphisms with AIMSS.

Methods: Women with stage 0-III HR + breast cancer initiating adjuvant AI were enrolled in a prospective clinic-based observational cohort. AIMSS were assessed by patient-reported outcomes (PRO) including the PROMIS pain interference and physical function measures plus the FACT-ES joint pain question at baseline and after 3 and 6 months. For the primary analysis, AIMSS were defined as $\geq$ 4-point increase in the pain interference T-score from baseline. Secondary AIMSS endpoints were defined as $\geq$ 4-point decrease in the physical function T-score from baseline and as $\geq$ 1-point increase on the FACT-ES joint pain question from baseline. The primary hypothesis was that *TCL1A* rs11849538 would be associated with AIMSS. Twelve other germline variants in *CYP19A1*, *VDR*, *PIRC66*, *OPG*, *ESR1*, *CYP27B1*, *CYP17A1*, and *RANKL* were also analyzed assuming a dominant genetic effect and prespecified direction of effect on AIMSS using univariate logistic regression with an unadjusted $\alpha = 0.05$. Significant univariate associations in the expected direction were adjusted for age, race, body mass index (BMI), prior taxane, and the type of AI using multivariable logistic regression.

Results: A total of 143 participants with PRO and genetic data were included in this analysis, most of whom were treated with anastrozole (78%) or letrozole (20%). On primary analysis, participants carrying *TCL1A* rs11849538 were not more likely to

develop AIMSS (odds ratio = 1.29, 95% confidence interval: 0.55–3.07, $p = 0.56$). In the statistically uncorrected secondary analysis, *OPG* rs2073618 was associated with AIMSS defined by worsening on the FACT-ES joint pain question (OR = 3.33, $p = 0.004$), and this association maintained significance after covariate adjustment (OR = 3.98, $p = 0.003$).

Conclusion: Carriers of *OPG* rs2073618 may be at increased risk of AIMSS. If confirmed in other cohorts, *OPG* genotyping can be used to identify individuals with HR + early breast cancer in whom alternate endocrine therapy or interventions to enhance symptom detection and implement strategies to reduce musculoskeletal symptoms may be needed.

Keywords: Pharmacogenetics, aromatase inhibitor, Musculoskeletal adverse events, *OPG*, *TCL1A*, breast cancer

INTRODUCTION

Adjuvant endocrine therapy for 5–10 years reduces recurrence and death after early-stage hormone receptor positive (HR +) breast cancer. Based on multiple trials demonstrating improved breast cancer outcomes when compared to treatment with adjuvant tamoxifen, a third-generation aromatase inhibitor (AI) (anastrozole, letrozole, or exemestane) is generally the preferred adjuvant endocrine therapy for postmenopausal women with early stage HR + breast cancer (Francis et al., 2018). Additionally, the use of an adjuvant AI in conjunction with ovarian suppression or ablation in high-risk premenopausal women with HR + breast cancer is associated with improved breast cancer outcomes (Hadji et al., 2013). The AI works by inhibiting the aromatase (CYP19A1) enzyme that is responsible for converting androgens to estrogens. This depletes the body of estrogens and starves the tumor of the estrogenic growth factor causing cellular replication.

Despite the established benefits of adjuvant endocrine therapy, up to 50% of patients are non-adherent or discontinue their treatment early, with some studies demonstrating higher rates of early discontinuation with AI therapy compared to tamoxifen (Partridge et al., 2003, 2008; Crew et al., 2007; Hershman et al., 2010, 2011, 2016, 2020; Henry et al., 2012, 2017; Murphy et al., 2012; Kemp et al., 2014; Kadakia et al., 2016). Risks of breast cancer recurrence and death are higher among individuals who are non-adherent to or who discontinue adjuvant endocrine therapy early (Kuba et al., 2016). Determinants of AI non-adherence and early discontinuation are multifactorial and include both baseline and treatment-emergent symptoms, especially AI-induced musculoskeletal symptoms (AIMSS) (Partridge et al., 2003, 2008; Felson and Cummings, 2005; Henry et al., 2012; Murphy et al., 2012; Chim et al., 2013; Bender et al., 2014; Kemp et al., 2014; Kidwell et al., 2014; Hershman et al., 2016; Neugut et al., 2016; Nabieva et al., 2018a,b; Wagner et al., 2018; Wulaningsih et al., 2018; Shinn et al., 2019; Wheeler et al., 2019).

Up to 50% of patients with breast cancer treated with adjuvant AI therapy experience AIMSS, a syndrome characterized by symptoms including joint pain and stiffness, myalgias, carpal tunnel syndrome, tenosynovitis, and/or reduced grip strength that is thought to be attributable to estrogen deprivation (Baum et al., 2003; Coates et al., 2007; Mao et al., 2009; Hershman et al., 2011; Hadji et al., 2014; Moscetti et al., 2015; Beckwée et al., 2017; Gupta et al., 2020). Among patients who discontinue adjuvant AI therapy due to symptoms or side effects, AIMSS are a leading reason for discontinuation (Felson and Cummings, 2005; Partridge et al., 2008; Murphy et al., 2012; Olufade et al., 2015). Musculoskeletal pain is associated with lower health-related quality of life in breast cancer patients receiving adjuvant AI therapy (Sitlinger et al., 2019), and endocrine therapy side effects, including pain, are associated with limitations of physical function (Sestak et al., 2008). Evidence-based interventions to manage AIMSS include strategies such as exercise, yoga, duloxetine, and acupuncture (Moscetti et al., 2015). Clinicians may also consider transitioning patients with AIMSS from one AI to another or switching to tamoxifen (Moscetti et al., 2015).

To date, accurate clinical predictors of AIMSS have not been identified. While some studies have demonstrated associations between breast cancer stage, body mass index (BMI), prior chemotherapy (especially taxanes), prior tamoxifen, and time since last menstrual period with AIMSS in postmenopausal women receiving AI therapy for early-stage HR + breast cancer, these associations have not been consistent across studies (Baum et al., 2003; Hershman et al., 2011; Hertz et al., 2017; Gupta et al., 2020). By identifying individuals at highest risk for AIMSS for enhanced symptom monitoring and management, an accurate pretreatment predictor of AIMSS has the potential not only to reduce pain and improve quality of life but also to improve endocrine therapy adherence and reduce early endocrine therapy discontinuation, thereby improving breast cancer outcomes.

Several studies have investigated potential physiological biomarkers for AIMSS including germline genetics [reviewed by Hertz et al. (2017)]. Many studies have used candidate-gene or candidate-single nucleotide polymorphism (SNP) approaches to investigate whether inherited germline variants affect AIMSS risk (Ingle et al., 2010; Park et al., 2011; Garcia-Giralt et al., 2013; Henry et al., 2013; Wang et al., 2013, 2015; Fontein et al., 2014; Leyland-Jones et al., 2015; Lintermans et al., 2016; Dempsey et al., 2018). These studies have reported several discovery-phase associations including inherited polymorphism in the genes that encode for the AI drug target CYP19A1

(Garcia-Giralt et al., 2013; Henry et al., 2013; Fontein et al., 2014; Leyland-Jones et al., 2015), proteins responsible for bone resorption *OPG*/*RANKL* (Wang et al., 2013; Lintermans et al., 2016; Dempsey et al., 2018), and the estrogen receptor *ESR1* (Ingle et al., 2010; Park et al., 2011). In addition, a hypothesis-agnostic genome-wide association study of the MA.27 clinical trial comparing anastrozole and exemestane reported that patients who carried rs11849538 near *TCL1A* had increased AIMSS risk (Liu et al., 2012). Although this association has not been successfully replicated, the well-done discovery and mechanistic support (Snyder et al., 2009) justifies further attempts to replicate the putative association in independent cohorts of AI-treated patients.

In order to pursue clinical translation of any of these potential genetic predictors of AIMSS, additional replication and retrospective validation of their association with AIMSS in independent patient cohorts is necessary. The primary objective of this study was to replicate the association of *TCL1A* rs11849538 with patient-reported AIMSS during the first 6 months of AI therapy in a prospectively accrued cohort of patients with early-stage HR + breast cancer initiating adjuvant AI therapy. The secondary objective was to utilize this cohort to attempt replication for other candidate SNPs that have been previously reported to be associated with AIMSS.

MATERIALS AND METHODS

Patients and AIMSS Data

This was a pharmacogenetic analysis of women with stage 0-III HR + breast cancer initiating adjuvant endocrine therapy who enrolled in a prospective observational clinic-based cohort at the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins from March 2012 through December 2016 (ClinicalTrials.gov Identifier: NCT01937052). Selection of the endocrine therapy regimen (tamoxifen versus AI with or without ovarian suppression) was determined by the treating clinician and recorded in the study database. Participants completed patient-reported outcome (PRO) measures assessing a range of symptom domains online using the PatientViewpoint interface at baseline and at 3, 6, 12, 24, 36, 48, and 60 months (Garcia et al., 2007; Snyder et al., 2013; Wu et al., 2016). This study was approved by the Johns Hopkins IRB, and all participants completed written informed consent prior to enrollment.

Participants included in this secondary analysis received a third-generation AI (anastrozole, letrozole, or exemestane) and completed the PRO measures at baseline and at 3 and/or 6 months. PRO measures related to AIMSS included the PROMIS pain interference and physical function questionnaires (Fallowfield et al., 1999; Jensen et al., 2017a) and a single item on the FACT-ES questionnaire addressing joint pain. The FACT-ES includes 19 items addressing a range of endocrine symptoms, including joint pain, to which respondents report symptom severity on a 5-point scale ranging from 0 ("not at all") to 4 ("very much") (Schalet et al., 2016). PROMIS measures are scored using a T-score metric with 50 representing the mean score in the United States population. Higher scores on PROMIS measures indicate more of the outcome measured (i.e., a higher pain interference T-score indicates more pain interference, and a lower physical function T-score indicates worse physical function). In patients with early-stage cancer, the minimal important difference (MID) on PROMIS measures, determined using a distribution-based method, is three to five points (Fallowfield et al., 1999; Rohlfs and Weir, 2008; Yost et al., 2011; Teresi et al., 2016; Jensen et al., 2017b). For this analysis, we selected the midpoint of the range reported for the MID for the PROMIS measures in patients with early-stage cancer, four points.

Our primary AIMSS endpoint was an increase of four or more points on the PROMIS pain interference T-score from baseline to 3 or 6 months. A secondary AIMSS endpoint was a decrease of four or more points on the PROMIS physical function T-score from baseline to 3 or 6 months. The other secondary AIMSS endpoint was an increase of one or more point on the FACT-ES question, "I have pain in my joints" from baseline to 3 or 6 months (Schalet et al., 2016). For patients who received multiple AIs due to switching treatment, only the data from their first AI treatment were included in the analysis.

Genotyping

A whole blood or a saliva sample was collected at baseline and stored at −80°C for germline DNA isolation. Germline DNA was isolated from whole blood using DNeasy Blood and Tissue Kits (Qiagen, Valencia, CA, United States) following the manufacturer's instructions. Germline DNA was isolated from saliva samples using prepIT-L2P (DNA Genotek, ON, Canada) following the manufacturer's instructions. Thirteen candidate gene variants of interest with a prespecified direction of effect on AIMSS were selected. The *a priori* determined primary analysis was an attempted replication of the increased AIMSS risk for carriers of *TCL1A* rs11849538. The other 12 SNPs and their predetermined direction of effect were tested in a secondary, statistically uncorrected analysis. Genotyping was performed using TaqMan™ Allelic Discrimination assays according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, United States). The SNP assay IDs used in this study were the following: rs10046 (C___8234731_30), rs11568820 (C___2880808_10), rs11849538 (C___1927667_30), rs16964189 (C__34453639_10), rs2073618 (C___1971047_40), rs2234693 (C___3163590_10), rs4646536 (C__25623453_10), rs6163 (C__12119916_1_), rs7176005 (C_189237142_10), rs7984870 (C__29811035_20), rs9322336 (C__29568677_10), rs9340799 (C___3163591_10), and rs934635 (C___8794643_10). PCRs were carried out using 10 ng of DNA with Genotyping Master Mix (Applied Biosystems) in a CFX96 real-time PCR detection system (Bio-Rad, Madison, WI, United States) for 35 cycles. Genotype quality assurance was assessed by random selection of 10% of DNA samples for re-genotyping, and the results were 100% concordant.

Statistical Analysis

The univariate association for each genetic predictor assuming a dominant genetic effect with AIMSS risk defined by the three previously described endpoints was tested using logistic

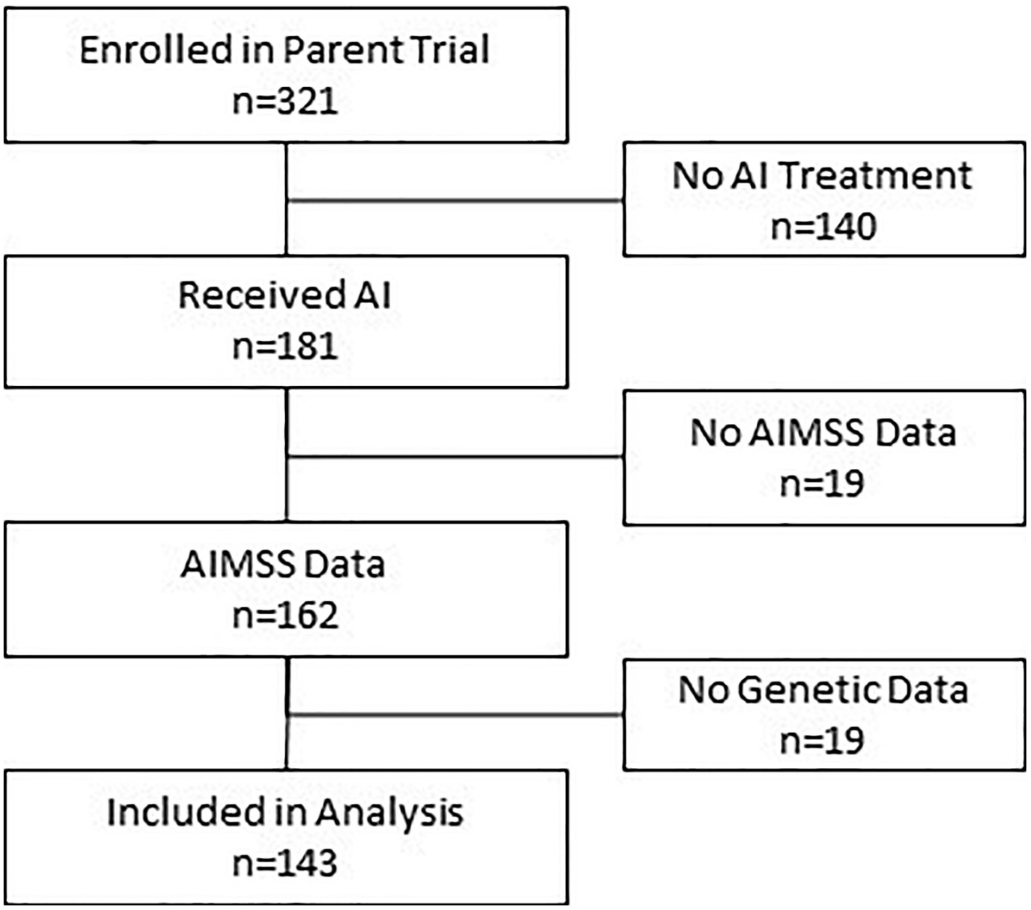

FIGURE 1 | Consort diagram describing patient flow into study and analysis. Of the 321 patients enrolled in the observational clinical study, 143 had all required data and were included in this analysis.

regression. Associations were only considered replicated if the direction of effect was consistent with our prespecified expected direction, based on previously reported associations. The primary hypothesis was that patients carrying *TCL1A* rs11849538 would have a higher risk of AIMSS, as defined by the primary endpoint of an increase of four or more points on the PROMIS pain interference measure from baseline to 3 or 6 months. The secondary analyses examined the association of the other 12 candidate variants with AIMSS defined by the pain interference measure and the association of all 13 candidate variants with AIMSS defined by either of the other two AIMSS definitions, for a total of 1 primary analysis and 38 statistically uncorrected secondary analyses. All associations were tested using an $\alpha = 0.05$, as appropriate for a single primary analysis and exploratory uncorrected secondary analyses. Any significant univariate associations were then corrected for relevant covariates: age in years (as a continuous variable), self-reported race (White versus other), baseline BMI (as a continuous variable), prior taxane chemotherapy (versus non-taxane chemotherapy or no chemotherapy), and aromatase inhibitor (anastrozole versus other due to low frequency of exemestane). In addition, any significant univariate associations assuming a dominant genetic model were then tested assuming recessive and additive genetic effects to identify the genetic model that best explained the association. Statistical analyses were conducted using R version 3.6.3.

RESULTS

Patients, AIMSS, and Genetics

The 143 AI-treated patients who completed the PRO measures assessing AIMSS at baseline and on-treatment at 3 and/or 6 months and with genetic information were included in this analysis (**Figure 1**). The patients were mostly white (85%) with a median age of 67 years (**Table 1**). Anastrozole (78%) and letrozole (20%) were used more frequently than exemestane (2%). AIMSS data from the PROMIS pain interference and physical function questionnaires and from the joint pain question on the FACT-ES were available for 99 and 90% of patients at 3 and 6 months, respectively. AIMSS were experienced by 31, 26, or 53% of patients when defined by the pain interference, physical function, or joint pain criteria, respectively. All genotypes were successfully determined in all patients, and all genetic data passed standard

quality control including assessment of call rate. All variant distributions were in Hardy–Weinberg equilibrium (Hardy, 1908; Deng et al., 2001) in the entire genotyped cohort ($p > 0.05$) except *VDR* rs11568820, which was within expected proportions in the self-reported White cohort ($p = 0.61$) indicating the presence of population admixture (Early Breast Cancer Trialists' Collaborative Group, 2015). The number of variant allele carriers is reported in **Table 2**.

Genetic Associations With AIMSS

In the primary analysis, patients carrying *TCL1A* rs11849538 did not have increased risk of AIMSS, as defined by the primary endpoint of the increased pain interference T-score from baseline to 3 or 6 months by at least four points (unadjusted odds ratio (OR) = 1.29, 95% confidence interval (95% CI): 0.55–3.07, $p = 0.56$, **Table 2** and **Figure 2**). None of the 12 secondary genetic predictors were associated with AIMSS as defined by the increase in the pain interference T-score (all $p > 0.05$). In the secondary analyses of the increase in joint pain as measured by the single question on the FACT-ES questionnaire, only *OPG* rs2073618 was associated with AIMSS in the expected direction of increasing risk (unadjusted OR = 3.33, 95% CI: 1.48–7.49, $p = 0.004$, **Figure 3**). This association persisted after adjustment for relevant covariates (adjusted OR = 3.98, 95% CI: 1.61–9.84, $p = 0.003$, **Table 3**), none of which had significant univariate associations with AIMSS in this relatively small cohort (all $p > 0.05$). This association would also be significant if a Bonferroni correction were applied to the secondary analyses (0.05/12 = 0.0042). In *post hoc* analyses, the effect of *OPG* rs2073618 could also be explained by an additive genetic model [OR = 2.06 (1.22–3.50), $p = 0.007$]. None of the other secondary analyses had significant univariate associations in the expected direction (**Table 2**).

TABLE 1 | Clinical information of subjects included in the analysis.

Characteristic		*n* = 143
Race	White	122 (85%)
	Black	15 (11%)
	Other/Unknown	6 (4%)
Age	Years	67.0 (47.0–86.0)
Body mass index	kg/m^2	27.8 (19.1–45.3)
Aromatase inhibitor	Anastrozole	112 (78%)
	Letrozole	28 (20%)
	Exemestane	3 (2%)
Prior chemotherapy	Taxane chemotherapy	38 (27%)
	Non-taxane or no chemotherapy	105 (73%)
On-treatment PRO questionnaires Completed	3 and 6 months	127 (89%)
	Only 3 months	14 (10%)
	Only 6 months	2 (1%)
Pain Interference	≥4 point increase*	45 (31%)
Physical Function	≥4 point decrease*	37 (26%)
Joint pain	≥1 point increase**	74 (52%)

**PROMIS T-score change from baseline to 3 and/or 6 months.*
***FACT-ES score change from baseline to 3 and/or 6 months for item, "I have pain in my joints."*
Numbers presented as n (%) or median (range).

DISCUSSION

AI-induced musculoskeletal symptoms are one of the most common adverse effects of adjuvant AI treatment and are a primary reason for early discontinuation of adjuvant endocrine therapy, a treatment that reduces risks of recurrence and improves survival (Baum et al., 2003; Felson and Cummings, 2005; Coates et al., 2007; Partridge et al., 2008; Mao et al., 2009; Hershman et al., 2011; Murphy et al., 2012; Hadji et al., 2014; Moscetti et al., 2015; Olufade et al., 2015; Basch et al., 2017; Beckwée et al., 2017; Gupta et al., 2020). The objective of this study was to replicate associations with AIMSS risk in an independent patient cohort for SNPs that have been previously associated with AIMSS. Although we could not successfully replicate an increased AIMSS risk for carriers of *TCL1A* rs11849538, we demonstrated that patients who carried *OPG* rs2073618 had increased AIMSS risk.

Our finding that patients who carried the *OPG* rs2073618 variant G allele have increased AIMSS risk is consistent with several prior retrospective pharmacogenetic analyses. The rs2073618 variant is a C > G substitution with a minor allele frequency close to 0.5 in Caucasians, making it possible that researchers could inadvertently swap the wild-type and variant alleles depending on which strand was genotyped (the allele frequencies for this variant and all other variants in this analysis can be viewed in dbSNP[1]). Therefore, the resulting amino acid change for this missense variant (Asp3Lysine or N3K) can be used to definitively refer to the patient's phenotype. In our study, we genotyped the *OPG* forward strand where a wild-type C allele results in AAC (asparagine) and a variant G allele results in AAG (lysine). Wang et al. initially hypothesized that SNPs in OPG, which encodes osteoprotegerin, could be associated with risk of AIMSS. Using a large cohort ($n = 420$) of AI-treated patients, these investigators found that patients who carried the allele encoding lysine had lower OPG expression and higher AIMSS risk (Dempsey et al., 2018). They also found that carriers of this variant had greater levels of the bone turnover markers carboxy terminal telopeptide and procollagen type I N-terminal propeptide and greater reduction in bone mineral density at the lumbar spine. Wang et al. also summarized the prior evidence that this SNP is associated with many bone-related conditions, likely due to enhanced bone resorption. This association was then partially replicated in a smaller ($n = 154$) cohort of AI-treated patients, in which rs2073618 variant (lysine) carriers reported greater musculoskeletal symptoms and pain severity (dominant $p = 0.046$). Interestingly, in this previous study, the increased AIMSS risk was restricted to the heterozygous carriers (Mao et al., 2011; Wang et al., 2013). In our cohort, the risk of AIMSS was greater in patients who were heterozygous (40/62 = 65%) or homozygous (21/30 = 70%) lysine carriers compared with homozygous asparagine patients (13/35 = 37%),

[1]https://www.ncbi.nlm.nih.gov/snp/rs2073618

TABLE 2 | Unadjusted genetic associations for each variant with AIMSS endpoints.

				Pain interference[a]			Physical function[b]			Joint pain[c]		
SNP	**Gene**	**Risk effect[c]**	**Carriers[d]**	**OR**	**95% CI**	***p*-value[e]**	**OR**	**95% CI**	***p*-value[e]**	**OR**	**95% CI**	***p*-value[e]**
rs11849538[f]	*TCL1A*	Higher	30	1.29	0.55, 3.07	0.56	1.29	0.52, 3.17	0.59	0.73	0.30, 1.75	0.48
rs2073618	*OPG*	Higher	102	1.36	0.60, 3.10	0.46	0.94	0.41, 2.16	0.88	**3.33**	**1.48, 7.49**	**0.0036**
rs7984870	*RANKL*	Higher	91	0.99	0.46, 2.10	0.98	0.98	0.44, 2.18	0.97	0.67	0.32, 1.42	0.30
rs11568820	*VDR*	Higher	64	0.94	0.46, 1.95	0.88	0.85	0.39, 1.82	0.67	0.51	0.25, 1.05	0.068
rs2234693	*ESR1*	Higher	106	1.46	0.63, 3.39	0.38	1.07	0.46, 2.51	0.87	0.97	0.44, 2.15	0.94
rs9322336	*ESR1*	Higher	37	1.04	0.46, 2.36	0.92	1.04	0.44, 2.44	0.93	1.45	0.64, 3.26	0.38
rs9340799	*ESR1*	Lower	89	1.10	0.52, 2.31	0.81	1.14	0.52, 2.50	0.74	1.08	0.52, 2.22	0.84
rs10046	*CYP19A1*	Lower	99	0.67	0.31, 1.46	0.32	0.92	0.41, 2.07	0.83	1.19	0.55, 2.57	0.65
rs934635	*CYP19A1*	Higher	38	1.32	0.58, 2.99	0.50	1.59	0.69, 3.68	0.28	0.96	0.43, 2.15	0.93
rs16964189	*CYP19A1*	Lower	61	1.67	0.80, 3.47	0.17	1.09	0.51, 2.36	0.82	0.96	0.47, 1.97	0.91
rs4646536	*CYP27B1*	Higher	75	0.89	0.43, 1.83	0.74	0.91	0.43, 1.95	0.81	1.44	0.71, 2.93	0.31
rs6163	*CYP17A1*	Lower	94	1.49	0.68, 3.24	0.32	0.74	0.34, 1.63	0.46	1.05	0.51, 2.19	0.89
rs7176005	NA/Chr15	Lower	40	1.03	0.46, 2.30	0.94	1.34	0.59, 3.07	0.48	0.74	0.34, 1.64	0.46

OR: Odds ratio, 95% CI: 95% Confidence interval.
Bold denotes p < 0.05 in unadjusted analysis.
[a] *≥4-point increase PROMIS T-score from baseline to 3 and/or 6 months.*
[b] *≥4-point decrease in PROMIS T-score from baseline to 3 and/or 6 months.*
[c] *≥1-point increase on FACT-ES item, "I have pain in my joints" from baseline to 3 and/or 6 months.*
[c] *Prespecified expected direction of effect on AIMSS risk in patients carrying the variant allele compared to the homozygous wild-type.*
[d] *Number of individuals who were heterozygous or homozygous carriers of the variant allele.*
[e] *p-values are unadjusted for multiple comparisons.*
[f] *Prespecified primary hypothesis was that carriers of TCL1A rs11849538 had greater risk of AIMSS, as defined by the pain interference endpoint.*

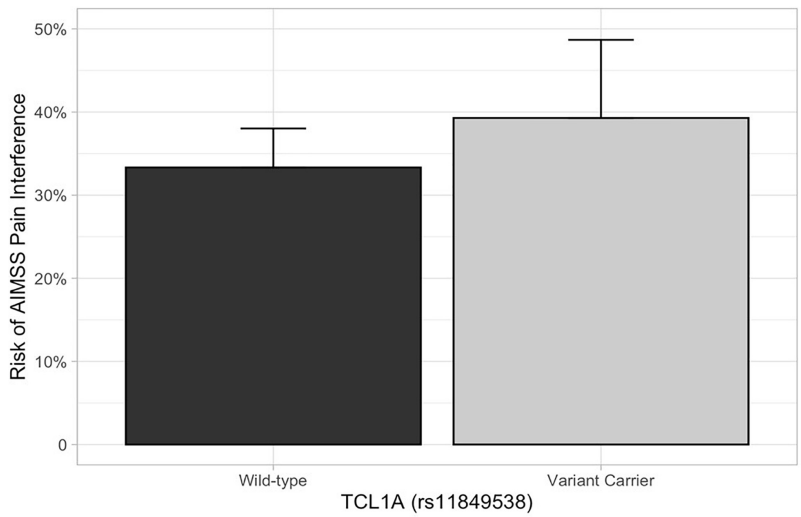

FIGURE 2 | AIMSS risk stratified by *TCL1A* rs11849538. The proportion of patients who experienced AIMSS (*Y*-axis), as defined by a ≥ 4-point increase in PROMIS T-score from baseline to 3 or 6 months, is indicated by the box (standard error indicated by the vertical line). In the primary analysis, there was no increased AIMSS risk, as defined by an increase in the pain interference score from baseline to 3 or 6 months by at least four points, in patients carrying *TCL1A* rs11849538 (n = 30, unadjusted odds ratio = 1.29, 95% confidence interval: 0.55–3.07, p = 0.56).

consistent with an additive or dominant genetic effect. However, not all studies have successfully replicated this association, including our prior replication attempt in the ELPh cohort where we were unable to detect an association of rs2073618, perhaps due to the use of a different AIMSS endpoint defined by a musculoskeletal symptom cluster (Lintermans et al., 2016). Our current analysis supports the initial discovery and replication attempts and further suggests that carriers of lysine at rs2073618 have increased AIMSS risk.

None of the other candidate SNPs that we selected for this replication study were associated with our AIMSS phenotypes. This includes *TCL1A* rs11849538, which was originally discovered in a genome-wide association study conducted in a nested case–control study of patients

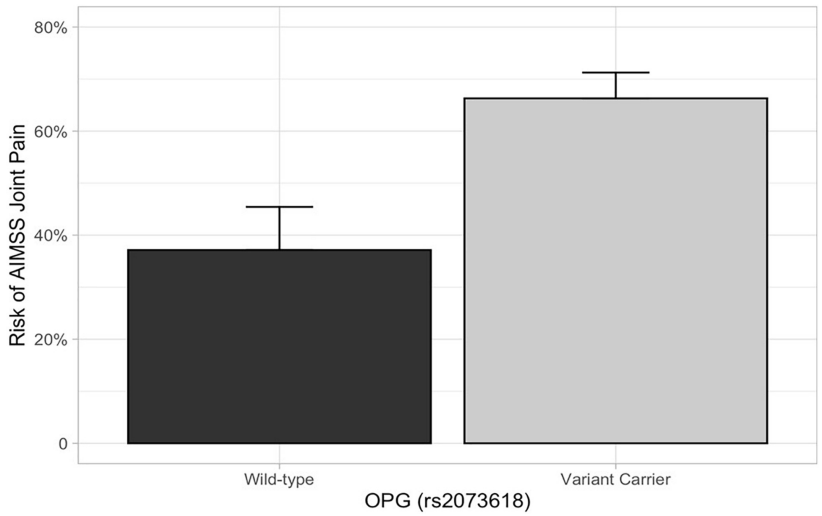

FIGURE 3 | AIMSS risk stratified by *OPG* rs2073618. The proportion of patients who experienced AIMSS (*Y*-axis), as defined by a ≥ 1 point increase in FACT-ES from baseline to 3 or 6 months, is indicated by the box (standard error indicated by the vertical line). In the secondary analysis, patients carrying *OPG* rs2073618 (n = 102) had a greater risk of AIMSS, defined by the increase in joint pain measured by the FACT-ES (unadjusted OR = 3.33, 95% CI: 1.48–7.49, p = 0.004).

who experienced AIMSS while receiving anastrozole or letrozole on the MA.27 clinical trial (Liu et al., 2012). Previous attempts and this current study have not replicated this association in a cohort of patients treated with AIs (Park et al., 2011).

Our results indicate that *OPG* rs2073618 may be a risk factor for AIMSS. If successfully validated in additional breast cancer cohorts, this genetic variant could potentially be used to identify patients at risk for musculoskeletal toxicity during AI therapy for personalized treatment, symptom monitoring, and symptom management that could lead to improve breast cancer outcomes. The approach to personalizing treatment based on the presence of this variant will require further understanding of the risk status in variant carriers. If this variant only increases AIMSS risk from a specific AI, it could provide the rationale for choosing that AI over the others. Alternatively, if carriers of this variant have higher AIMSS risk regardless of which AI they receive, the presence of this variant may justify enhanced side-effect monitoring (Early Breast Cancer Trialists' Collaborative Group Davies et al., 2011) and symptom management or consideration of alternative treatment options such as tamoxifen (Sheppard et al., 2020). Finally, rs2073618 is very common in Caucasians and Asians, with minor allele frequencies of ∼45 and ∼25% in these groups, respectively, which will be much more efficient for preemptive genetic testing compared to other low-frequency variants that require screening many patients to identify the small number of patients at risk. The potential clinical benefit of preemptive testing to guide personalized treatment selection would likely need to be demonstrated in prospective clinical trials prior to clinical translation.

TABLE 3 | Multivariable association of *OPG* rs2073618 with worsening of joint pain measured on FACT-ES.

Variable	OR	95% CI	*p*-value
OPG rs2073618 (dominant model)	3.98	1.61, 9.84	0.003
Age	0.97	0.91, 1.02	0.21
White Race (vs. other)	0.70	0.21, 2.27	0.55
BMI	1.04	0.97, 1.13	0.25
Taxane Chemotherapy (vs. non-taxane or no chemotherapy)	0.94	0.39, 2.30	0.90
Anastrozole (vs. other AI)	0.54	0.22, 1.36	0.19

BMI, Body mass index; AI, aromatase inhibitor; OR, odds ratio; 95% CI, 95% confidence interval.

This pharmacogenetic study was conducted in a prospectively accrued real-world clinic-based cohort of patients from whom treatment-related AIMSS data were collected via validated PRO outcome measures prior to and at predefined time points during treatment. This analysis used clinically relevant definitions of AIMSS, and all analyses were conducted with a predefined expected direction of effect. While these analytical decisions improved the likelihood that our finding is valid, the lack of statistical correction for the 38 unique secondary analyses conducted increases the chances that this was a false-positive replication and therefore additional validation studies are needed. Our study had several additional limitations that need to be considered. This cohort was relatively small for a retrospective pharmacogenetic analysis, which precludes further analyses to determine whether the effect is exclusive to a single AI or shared among two or all three of the agents. Although there is evidence that AIMSS is more common in African Americans (67) our cohort was insufficiently large or diverse (85% Caucasian) to conduct analyses within individual racial subcohorts. Additionally, this study was likely underpowered to detect associations for less common variants or those with smaller effect sizes, and we were not able to explore haplotypes for genes

with multiple variants including *CYP19A1* and *ESR1*. Moreover, the PRO outcome measures used in this study were not specific to AIMSS. The PROMIS pain interference measure addresses pain of any type, and multiple factors, such as other medications or physical injuries, could contribute to pain interference or physical function limitations. Although we did not exclude patients taking other medications with musculoskeletal side effects or patients with preexisting musculoskeletal conditions from participation, the fact that we compared PRO data from 3 and/or 6 months to baseline makes it likely that that AIMSS we detected using the PRO measures are attributable to AI treatment.

In conclusion, in our secondary analyses of a prospectively accrued cohort of HR + patients with early-stage breast cancer treated with an adjuvant AI, patients carrying lysine at *OPG* rs2073618 had greater risk of treatment-related AIMSS. Further retrospective pharmacogenetic analyses are needed to validate this clinical association, and preclinical functional studies are needed to further validate the mechanisms underlying this association. Upon validation, prospective genotyped-guided treatment trials are necessary to demonstrate that preemptive genotyping of rs2073618 can improve clinical outcomes in patients with HR + early-stage breast cancer receiving adjuvant endocrine treatment by guiding enhanced symptom monitoring and symptom management interventions with the goal of improving treatment adherence and persistence.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Johns Hopkins IRB. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DH, KS, JR, and VS contributed to conception and design of the study. YZ and KK conducted the data analysis. CG and AP conducted the genetic analysis. JL, AB, and VS enrolled participants. DH wrote the original draft of the manuscript, which was subsequently reviewed and edited by all co-authors. All authors contributed to the article and approved the submitted version.

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Polymorphism in *INSR* Locus Modifies Risk of Atrial Fibrillation in Patients on Thyroid Hormone Replacement Therapy

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Aims: Atrial fibrillation (AF) is a risk for patients receiving thyroid hormone replacement therapy. No published work has focused on pharmacogenetics relevant to thyroid dysfunction and AF risk. We aimed to assess the effect of L-thyroxine on AF risk stratified by a variation in a candidate gene.

Methods and Results: A retrospective follow-up study was done among European Caucasian patients from the Genetics of Diabetes Audit and Research in Tayside Scotland cohort (Scotland, United Kingdom). Linked data on biochemistry, prescribing, hospital admissions, demographics, and genetic biobank were used to ascertain patients on L-thyroxine and diagnosis of AF. A GWAS-identified insulin receptor-*INSR* locus (rs4804416) was the candidate gene. Cox survival models and sensitivity analyses by taking competing risk of death into account were used. Replication was performed in additional sample (The Genetics of Scottish Health Research register, GoSHARE), and meta-analyses across the results of the study and replication cohorts were done. We analyzed 962 exposed to L-thyroxine and 5,840 unexposed patients who were rs4804416 genotyped. The rarer G/G genotype was present in 18% of the study population. The total follow-up was up to 20 years, and there was a significant increased AF risk for patients homozygous carriers of the G allele exposed to L-thyroxine (RHR = 2.35, P = 1.6e–02). The adjusted increased risk was highest within the first 3 years of exposure (RHR = 9.10, P = 8.5e–04). Sensitivity analysis yielded similar results. Effects were replicated in GoSHARE (n = 3,190).

Conclusion: Homozygous G/G genotype at the *INSR* locus (rs4804416) is associated with an increased risk of AF in patients on L-thyroxine, independent of serum of free thyroxine and thyroid-stimulating hormone serum concentrations.

Keywords: atrial fibrillation, insulin receptor, thyroid hormone replacement therapy, hypothyroidism, genetics

INTRODUCTION

Hypothyroidism is the most common thyroid disorder affecting about 3–5% of the general population, and patients are nearly always treated with L-thyroxine (Flynn et al., 2004; Wiersinga et al., 2012). Patients usually respond well to treatment, and dosage is monitored in response to a combination of serum thyroid stimulating hormone (TSH) concentration and patients' symptoms (Wiersinga et al., 2012). However, increased risk of cardiovascular disease, atrial fibrillation (AF) and bone fractures have been described in patients receiving long-term replacement thyroxine therapy (Flynn et al., 2010; Chaker et al., 2015; Floriani et al., 2017).

Atrial fibrillation is the most common cardiac dysrhythmia and a leading cause of cardiovascular and cerebrovascular morbidity and mortality (Chugh et al., 2014). Risk of AF for patients taking L-thyroxine is partly related to their dose and serum TSH concentration (Flynn et al., 2010). Over the last decade, progress has been made in defining the genetic basis of AF, and there is now evidence that genetic factors may play a role in its pathogenesis. Most published work has focused on identifying genetic variants (common and rare) associated with AF (Fatkin et al., 2017), less on pharmacogenetics relevant to AF management (Huang and Darbar, 2016), few on genetic determinants of thyroid function/dysfunction associated with AF (Ellervik et al., 2019; Larsson et al., 2019; Salem et al., 2019), but none on pharmacogenetics relevant to thyroid dysfunction on AF risk.

We have previously replicated in a Scottish population a number of GWAS-identified loci associated with serum TSH concentrations including the insulin receptor-*INSR* locus (Soto-Pedre et al., 2017). Insulin resistance and serum TSH concentration have both been highlighted as being possible underlying mechanisms for AF (Flynn et al., 2010; Bell and Goncalves, 2019; Bohne et al., 2019; Ellervik et al., 2019; Salem et al., 2019). We aimed to assess the effect of L-thyroxine replacement therapy on AF stratified by a variation at an *INSR* locus.

MATERIALS AND METHODS

Discovery Cohort

A retrospective follow-up study was performed among patients from the Genetics of Diabetes Audit and Research Tayside Scotland (GoDARTS) study. All subjects in this population are of white European ethnicity, the period of follow-up was defined from 1994 to March 2014 and all patients with at least one serum TSH recording were considered for inclusion. For each individual the date of entry into the study was the first date of thyroid replacement therapy prescription (exposed cohort) or the date at first serum TSH recording (unexposed cohort). Each eligible patient was followed from the date of entry until either occurrence of AF or withdrawal from observation (i.e., the earliest of three dates: date of death, last date under observation, or 1 April 2014).

Each patient has a unique identification number (Community Health Index) which facilitates data linkage across all available electronic medical records (EMRs) by the Health Informatics Centre of the University of Dundee[1]. Linked data on biochemistry, prescribing, hospital admissions, demographics, and genetic biobank were used to ascertain patients on L-thyroxine and diagnosis of AF (see **Supplementary Material** for a detailed description of linked datasets).

Replication Cohort

The Genetics of Scottish Health Research register (GoSHARE) was used to perform the replication analyses (McKinstry et al., 2017). In brief, participants anywhere in Scotland are asked to allow their information held within NHS Scotland EMRs to be used for research, and to give consent for blood remaining from diagnostic tests to be used. Any participant included also in the discovery cohort was removed from GoSHARE. The same data linkage procedure and phenotype definition criteria for the discovery cohort were applied to this dataset.

Phenotype Definition Criteria

A phenotype of thyroid replacement therapy was defined as having been issued at least two prescriptions of L-thyroxine (British National Formulary codes-BNF 6.2.1) during the study period. Patients with any prescription of liothyronine and/or with history of thyroid cancer or probable hyperthyroidism were excluded. Unexposed patients never received a prescription for L-thyroxine, and had a serum TSH within the reference range. There was no distinction between AF and atrial flutter when identifying AF phenotypes because the conditions are similar with respect to risk factors and possible complications (Chaker et al., 2015). See **Supplementary Material** for more detailed information on phenotype definition.

Serum TSH and free thyroxine (FT4) were taken as the median of these measures recorded throughout the study period for each patient.

Genetic Data

A GWAS-identified *INSR* locus (rs4804416) associated with average serum TSH concentrations and replicated in the GoDARTS cohort (Soto-Pedre et al., 2017) was the candidate gene. To strengthen the choice of this candidate, additional single-nucleotide polymorphisms (SNPs) associated with TSH were also considered regarding AF in patients on L-thyroxine (see **Supplementary Table 1**). Genotype data was available from several platforms as previously described (Soto-Pedre et al., 2017; see **Supplementary Material**).

As serum TSH has been identified as a possible underlying mechanism for AF (Ellervik et al., 2019; Salem et al., 2019), a genetic risk score was developed using a weighted sum of TSH increasing alleles (wGRS) reported by Teumer et al. (2018). The

[1]http://www.dundee.ac.uk/hic

purpose of this was to explore whether any possible association with the insulin receptor was driven by serum TSH.

Statistical Analysis

ANOVA and chi-square tests were used to compare means and frequencies among subgroups of patients, respectively, and non-parametric tests were used where appropriate. Single-locus tests of association with rs4804416 (*INSR*) were performed under the assumption of an additive model in the discovery cohort, with the T/T genotype (ancestral allele) as the reference. Cross-sectional analysis was done by logistic regression models to strengthen the choice of the candidate gene.

Longitudinal analyses to explore the relationship between exposure to L-thyroxine and AF by genotype over time were performed by Cox proportional hazards regression models. The effects of the exposure were estimated by including in models gender, age, and body mass index (BMI) at baseline, and terms for average serum TSH (or FT4) and a diagnosis of diabetes mellitus at any time over the study period were considered as strata. A two-way interaction term between L-thyroxine and genotype was included in all models. To further explore the hypothesis that serum TSH might be related with the development of AF in the context of L-thyroxine therapy, Cox survival models were fitted instead with a two-way interaction term between L-thyroxine and the generated TSH-wGRS (Teumer et al., 2018). The estimates of interaction effects are based on a ratio of hazard ratios (RHR); for each Cox model, ratios between treated (i.e., L-thyroxine) and untreated hazard ratios (HR) were computed. Sensitivity analyses to assess the robustness of the findings were conducted by competing risk regression models where death was the competing event. Model specification was evaluated using goodness of fit diagnostics by computing Harrell's *C* coefficient, and a test of the proportional hazards assumption was performed for each covariate and globally using a formal significance test based on Schoenfeld residuals. Replication analyses in GoSHARE consisted of repeating same statistical models that were fitted in GoDARTS. Data were entered into a STATA/SE® version 13.1 package (StataCorp, TX, United States) for statistical analysis.

The results from the discovery and replication cohorts were combined in a fixed-effect meta-analysis by using R-package "metaphor"[2], and heterogeneity was quantified using the I-squared measure. Meta-analyses were based on the assumption of an underlying recessive genetic model. Evidence for this effect came from our primary analyses (see **Supplementary Tables 2–4**), where the effect of the genotype on AF was visibly similar in participants homozygous for the ancestral allele (T/T) and heterozygous carriers (T/G), while the only clear effect was observed in homozygous carriers of the rare allele (G/G). This was supplemented by mRNA expression data available from the Genotype-Tissue Expression (GTEx) portal, where the association of the genotype with *INSR* expression in the thyroid tissue showed a decreased expression only for those with the G/G genotype, while the other two groups showed very marginal increase (see **Figure 1**).

[2]https://www.r-project.org

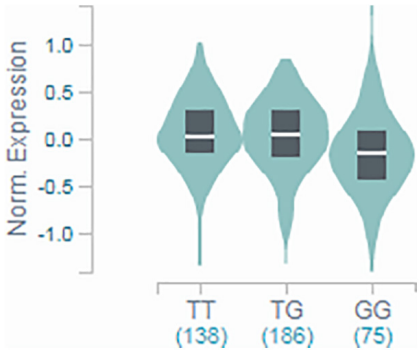

FIGURE 1 | GTEx portal data showing the association of rs4804416 on *INSR* expression in thyroid tissue. Normalized expression in tissue with T/T genotype was 0.017, in T/G was 0.036, and G/G was –0.146 (*T*-test statistic = –3.0, $P = 0.0034$).

RESULTS

We identified 6,802 patients eligible for the study cohort who were *INSR*-rs4804416 genotyped. During a median follow-up of 12.1 years (interquartile range 7.9–15.8 years) a total of 535 AF events occurred. A comparison of the baseline characteristics of the exposed and unexposed cohorts is shown in **Table 1**. Although those exposed to L-thyroxine had a higher average serum TSH (2.7 vs 1.8 mIU/L, $P < 1e\text{-}03$), average TSH (and FT4) levels were within the biochemical reference range. Preliminary cross-sectional analyses strengthen the choice of the candidate gene by showing association signal only for rs4804416 among other SNPs related also to average serum TSH concentration (see **Supplementary Table 1**).

Survival analyses showed that for patients taking L-thyroxine there was a significant increased risk of AF for homozygous carriers of the G allele at any time during the follow-up compared to the other genotypes. **Table 2** shows the unadjusted and adjusted RHR for the interaction effects between L-thyroxine treatment and genotype on AF risk by time to follow-up. The increased risk was highest within the first 3 years after starting on treatment when it was over nine times higher than in non-carriers (RHR = 9.10, $P = 8.5e\text{–}04$). However, heterozygous carriers did not show a significant increased risk compared to non-carriers. Similar results were obtained after adjusting for height instead of BMI or stratifying for serum FT4 instead of TSH (see **Supplementary Tables 2, 3**, respectively). **Figure 2** graphically shows the difference in AF-free survival by genotype within 3 years of treatment. Survival models fitted instead with a two-way interaction term between L-thyroxine and the increasing TSH-wGRS (i.e., weighted TSH-based genetic risk score) showed no associated risk of AF per genetically predicted increase of TSH levels for those treated (see **Supplementary Table 5**).

Sensitivity analyses using competing risk regression models with death as a competing event are shown in **Supplementary Table 4** and yielded similar results to the survival models showed in **Table 2**. A competing risk is an event that either hinders the observation of the outcome of interest or

TABLE 1 | Description of patients on thyroid replacement therapy (L-thyroxine) and their comparison cohort at study entry (*n* = 6,802).

Characteristic	L-thyroxine (*n* = 962)	Comparison cohort (*n* = 5,840)	*P*
n (%)			
Gender-female	644 (66.9)	2,205 (37.7)	<0.001
SIMD quintile:			
1 Most deprived	180 (18.9)	1,048 (18.2)	=0.968
2	157 (16.5)	958 (16.7)	
3	155 (16.3)	958 (16.7)	
4	294 (30.9)	1,755 (30.5)	
5 Most affluent	164 (17.2)	1,031 (17.9)	
Diabetes mellitus	236 (24.5)	584 (10.0)	<0.001
Genotype rs4804416:			
TT	323 (33.6)	1,955 (33.5)	=0.962
TG	466 (48.4)	2,813 (48.1)	
GG	173 (18.0)	1,072 (18.4)	
Mean (SD)			
Age-years	58.1 (12.5)	59.6 (12.4)	<0.001
BMI (Kg/m^2)	31.1 (6.6)	30.6 (5.6)	<0.001
Height (cm)	163.9 (9.3)	168.3 (9.6)	<0.001
Serum TSH (mIU/L)*	2.7 (1.7–3.6)	1.8 (1.3–2.4)	<0.001
Serum FT4 (pmol/L)*	14.6 (13.2–16.3)	14.7 (13.3–16.5)	=0.415

BMI, Body mass index; FT4, free thyroxine; SIMD, Scottish Index of Multiple Deprivation; and TSH, Thyroid-stimulating hormone.
** median (interquartile range) of measures recorded throughout the study period.*

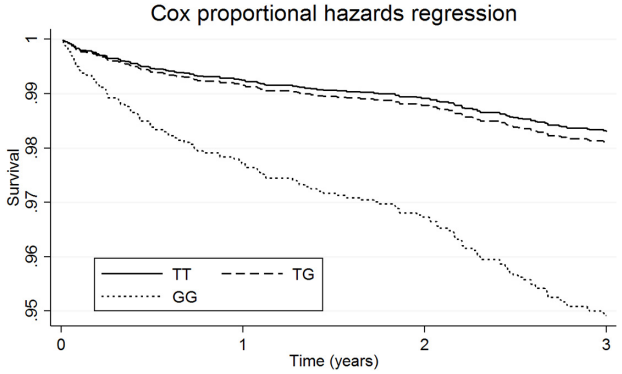

FIGURE 2 | Survival functions of atrial fibrillation in patients on L-thyroxine by genetic variation at *INSR*- rs4804416 within 3 years of follow-up.

modifies the chance that this outcome occurs. Thus, our results were not confounded by death.

The replication study consisted of additional 3,190 eligible individuals of white ethnicity recruited from GoSHARE from 1995 to 2018. During a median follow-up of 10.2 years (interquartile range 6.1–14.5 years) a total of 220 AF events occurred. A comparison of the baseline characteristics of the exposed and unexposed groups to L-thyroxine is shown in **Supplementary Table 6**. Survival analyses showed that for patients taking L-thyroxine there was a significant increased risk of AF for homozygous carriers of the G allele at any time during the follow-up compared to the other genotypes (see **Supplementary Figure 1**) similar to the discovery cohort. Sensitivity analyses using competing risk regression models with death as a competing event yielded similar results. **Supplementary Table 7** shows the increased AF risk in unadjusted and adjusted genetic recessive models, although it was not significant due to less number of AF events in this smaller dataset.

The results of the adjusted genetic recessive models from the study (i.e., GoDARTS) and replication (i.e., GoSHARE) were further combined in fixed-effect meta-analyses. We reported the *p*-values for the two-tailed test on the combined effect. **Figure 3** shows a decreased AF risk for homozygous carriers of rs4804416 G allele in both unexposed cohorts with a significant summary estimate (HR = 0.66, *P* = 1.1e–02), and an increased risk for the exposed cohorts that was not significant due to the sample size (HR = 2.34, *P* = 2.5e–01). To overcome the sample size issue in the exposed groups, a two-way interaction term between L-thyroxine

TABLE 2 | Pharmacogenetics interaction between exposure to L-thyroxine and INSR-rs4804416 on developing atrial fibrillation by follow-up time (*n* = 6,802).

Follow-up	At risk (p-y)	Events (*n*)	Genotype	RHR (95% CI)[a]	*P*[†]	RHR (95% CI)[b]	*P*[†]	RHR (95% CI)[c]	*P*[†]
3 years	19,652	128	TG	1.13 (0.38–3.38)	8.2e-01	1.17 (0.39–3.51)	7.7e-01	1.19 (0.39–3.58)	7.5e-01
			GG	7.34 (2.11–25.53)	1.7e-03*	7.48 (2.15–26.06)	1.6e-03*	9.10 (2.48–33.33)	8.5e-04*
5 years	31,837	184	TG	1.04 (0.41–2.60)	9.4e-01	1.06 (0.42–2.68)	8.9e-01	1.10 (0.43–2.78)	8.3e-01
			GG	4.40 (1.62–11.94)	3.6e-03*	4.48 (1.65–12.17)	3.2e-03*	4.70 (1.70–12.95)	2.7e-03*
10 years	57,984	347	TG	1.45 (0.74–2.82)	2.7e-01	1.43 (0.73–2.80)	2.9e-01	1.40 (0.71–2.74)	3.2e-01
			GG	2.77 (1.24–6.19)	1.3e-02*	2.83 (1.26–6.32)	1.1e-02*	2.93 (1.30–6.58)	9.1e-03*
15 years	74,153	470	TG	1.37 (0.75–2.48)	3.0e-01	1.29 (0.71–2.35)	4.0e-01	1.30 (0.71–2.37)	3.9e-01
			GG	2.39 (1.17–4.86)	1.6e-02*	2.36 (1.16–4.81)	1.8e-02*	2.45 (1.20–5.01)	1.4e-02*
20 years	79,301	535	TG	1.49 (0.84–2.65)	1.7e-01	1.45 (0.81–2.57)	2.0e-01	1.45 (0.81–2.58)	2.1e-01
			GG	2.25 (1.12–4.50)	2.2e-02*	2.27 (1.13–4.55)	2.0e-02*	2.35 (1.17–4.72)	1.6e-02*

INSR = insulin receptor-polymorphism. INSR effect allele = G; coding TT = 0, TG = 1, GG = 2. RHR = Ratio of hazard ratios. TSH = thyroid-stimulating hormone.
**P < 0.05, †P value for the interaction term (L-thyroxine*INSR).*
[a]Unadjusted Cox survival models.
[b]Adjusted Cox models for age and gender, and stratified by average serum TSH during follow-up and history of diabetes mellitus.
[c]Adjusted Cox models for age, gender and BMI, and stratified by average serum TSH during follow-up and history of diabetes mellitus.

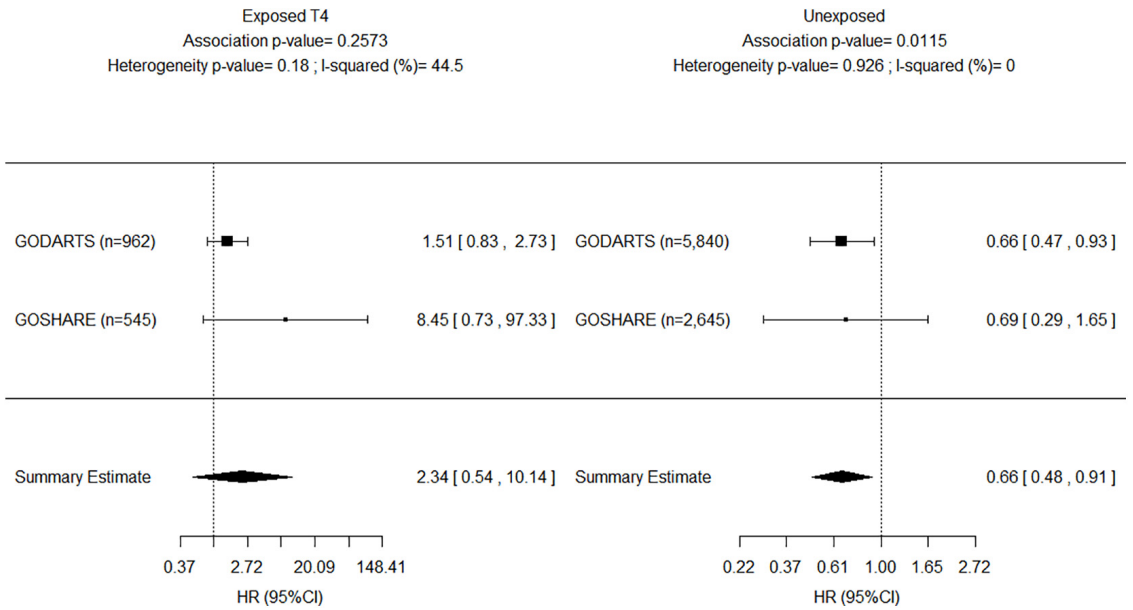

FIGURE 3 | Forest plot for meta-analyses of *INSR*-rs4804416 on developing atrial fibrillation by exposure to L-thyroxine within 10 years of follow-up. HR, Hazard ratio.

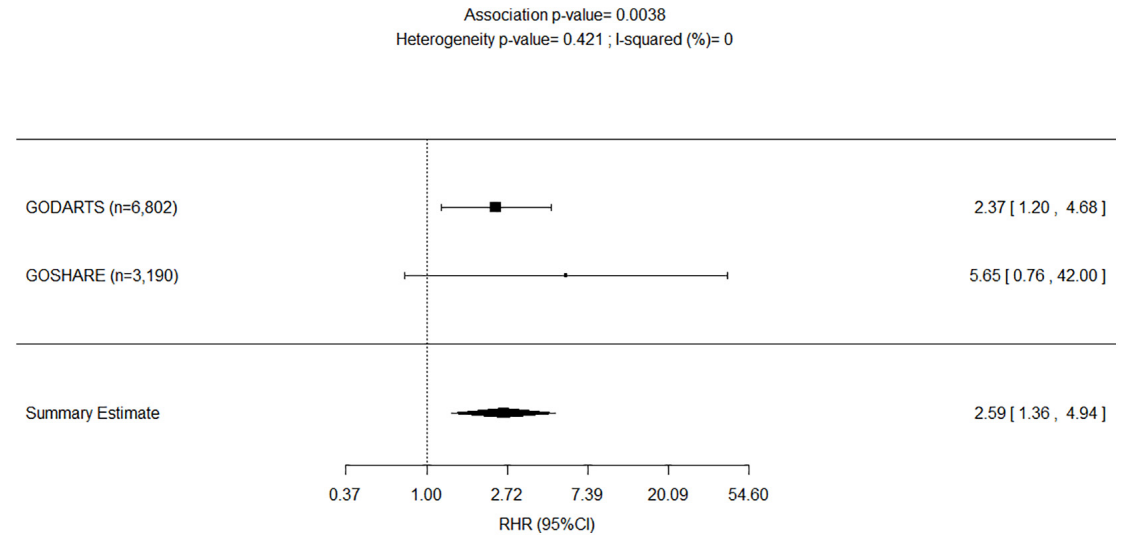

FIGURE 4 | Forest plot for meta-analyses of pharmacogenetics interaction between exposure to L-thyroxine and *INSR*-rs4804416 on developing atrial fibrillation within 10 years of follow-up. RHR, Ratio of hazard ratios.

and genotype was included in the model as mentioned earlier. **Figure 4** shows a significant summary estimate of interaction effects across the studies (P = 3e–03), meaning that homozygous carriers of G allele exposed to L-thyroxine have an increased AF risk 2.59 (95% CI 1.36–4.94) times higher than unexposed individuals. The consistency of the SNP effects direction across the studies (i.e., study and replication) was also graphically shown in **Figures 3**, **4**. Although no significant heterogeneity was detected, a value close to 44% was found in the meta-analysis across the exposed cohorts (P = 1.8e–01).

DISCUSSION

We have undertaken a large follow-up study to assess the association between thyroid hormone replacement therapy, the SNP rs4804416 and risk of developing AF over a 20 year period. We have shown that AF risk is up to nine times higher in patients taking L-thyroxine that are also homozygous carriers of the minor G allele of rs4804416. The impact of L-thyroxine upon AF was evident independent of average serum TSH (or FT4) concentration, BMI (or height) and diabetes status at any point

during the follow-up period, which have been associated with AF (Flynn et al., 2010; Rosenberg et al., 2012; Chaker et al., 2015; Baumgartner et al., 2017; Bell and Goncalves, 2019).

Replication was performed in an additional comparable sample (i.e., GoSHARE) where identical phenotype definition criteria were applied. The replication sample was smaller and younger, and thus had fewer AF events during similar average follow-up. Nonetheless, replication results showed same direction of effects, similar effect size when there was no exposure to L-thyroxine, but larger effect size when there was exposure to L-thyroxine. Survival analyses of the discovery and replication data showed similar functions for non-carriers and heterozygous carriers compared to homozygous carriers. Meta-analyses of the association across the discovery and replication were based on underlying recessive genetic models, which did not assume that the underlying genetic model was known in advance but made use of the information available on all genotypes. The association test results observed indicate that variability between estimated effects from the study and the replication can be explained by chance only.

Recently, Salem et al. (2019) and Ellervik et al. (2019) researched the association between thyroid function and AF using genetic data, and provided support for the observational association between thyroid function and AF. They showed that the risk of AF seems to vary throughout the spectrum of thyroid function as measured by TSH, including the reference range. Thus, serum TSH concentration may explain only some of the risk of AF. Salem et al. reported a negative association with AF for carriers of the increasing TSH rs4804416 G allele in a phenome-wide association study of individuals unexposed to L-thyroxine ($\beta = -0.0185$, $P = 0.15$; Salem et al., 2019). Ellervik et al. (2019) conducted a Mendelian randomization analysis and reported a protective association of their genetic increasing TSH predictor on AF (OR = 0.88; 95% CI 0.84–0.92). These studies support our finding of decreased AF risk among homozygous carriers of rs4804416 G allele unexposed to L-thyroxine (HR = 0.66; 95% CI 0.48–0.91). Our data provides evidence for the first time that a genetic variation impacts on AF risk in patients treated with L-thyroxine for hypothyroidism. Genetic polymorphisms may increase susceptibility to environmental changes in the pathogenesis of AF (Fatkin et al., 2017). Exposure to L-thyroxine could reverse the protective association of rs4804416 G allele observed in individuals with normal thyroid function.

Candidate genes from GWAS are considered an unbiased approach to identifying or validating genetic influences on drug response (Roden et al., 2011). This approach focuses on associations between genetic variation within pre-specified genes of interest and phenotypes. In this study, a GWAS-identified *INSR* locus (rs4804416) replicated in a Scottish population was the candidate gene (Soto-Pedre et al., 2017). This gene encodes a preproprotein that is processed to generate two alpha and two beta subunits that work together as a functioning insulin receptor. *INSR* gene mutations also underlie the type A insulin resistance syndrome and leads to diabetes mellitus (Semple et al., 2011). Although this SNP has been associated with average serum TSH concentrations, our results are suggestive that the observed increased AF risk for homozygous carriers of the increasing TSH allele (i.e., G/G) in the context of L-thyroxine therapy might not be dependent on serum TSH.

The GTEx Project has created a reference resource of gene expression levels from non-diseased tissues (Carithers and Moore, 2015). Studies of tissue-specific gene expression across human tissues provide useful insights into how genes can affect disease. Data from the GTEx shows that the SNP rs4804416 is a strong expression quantitative trait locus for *INSR* mRNA expression, and it is associated with a statistically significant expression at several tissues (see **Supplementary Figure 2**). In thyroid and aortic artery tissue, carriers of the rare allele (G) have lower expression of *INSR* mRNA. It is possible that exposure to L-thyroxine associated with impaired expression of *INSR* in patients with the G/G genotype in some of these tissues may explain the mechanism of increased AF risk. This indirectly supports the hypothesis that altered insulin resistance due to reduced mRNA product of *INSR* might be associated with the development of AF in the context of L-thyroxine therapy.

Our results were adjusted for known potential confounders and the impact of missing values is considered low. The data on morbidity and AF mainly related to hospital admission data and would have missed out-patient activity, and thus missed some events of AF underestimating overall AF risk. An apparent limitation of the study is that criteria for individual date of entry into the study differed between exposed and unexposed cohorts; the first date of prescription for exposed to L-thyroxine and the date at first serum TSH recording for those unexposed. However, these criteria do not differ much in clinical practice because serum TSH is nearly always measured before starting on L-thyroxine. A major strength of this study is that it is a longitudinal study and that sensitivity analysis using competing risk regression models with death as a competing event for AF confirmed the results across the discovery and replication datasets.

In summary, genetic polymorphisms in the *INSR* gene may affect disease outcomes in patients on L-thyroxine replacement therapy and support to predict those who will have higher risk of AF. The results of the present study may help to customize L-thyroxine prescribing for patients to improve safety. Further studies are needed to reassure our findings.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: This is consented data and due to sensitive nature is store in secure computing environments. Data can be shared based on specific requests but as such is not publicly available. Requests to access these datasets should be directed to Christopher Hall, C.Hall@dundee.ac.uk.

ETHICS STATEMENT

All analyses were performed on anonymized datasets. The studies involving human participants were reviewed and approved by Tayside Medical Ethics Committee (Scotland, United Kingdom). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ES-P researched/analyzed data and wrote the manuscript. MS researched data and wrote the manuscript. CM and AYD researched data and reviewed the manuscript. ASD, CP, and EP contributed to the discussion and reviewed/edited the manuscript. GL planned the study, researched the data, contributed to the discussion, and reviewed/edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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CYP2B6 Functional Variability in Drug Metabolism and Exposure Across Populations—Implication for Drug Safety, Dosing, and Individualized Therapy

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Adverse drug reactions (ADRs) are one of the major causes of morbidity and mortality worldwide. It is well-known that individual genetic make-up is one of the causative factors of ADRs. Approximately 14 million single nucleotide polymorphisms (SNPs) are distributed throughout the entire human genome and every patient has a distinct genetic make-up which influences their response to drug therapy. Cytochrome P450 2B6 (CYP2B6) is involved in the metabolism of antiretroviral, antimalarial, anticancer, and antidepressant drugs. These drug classes are commonly in use worldwide and face specific population variability in side effects and dosing. Parts of this variability may be caused by single nucleotide polymorphisms (SNPs) in the *CYP2B6* gene that are associated with altered protein expression and catalytic function. Population variability in the *CYP2B6* gene leads to changes in drug metabolism which may result in adverse drug reactions or therapeutic failure. So far more than 30 non-synonymous variants in *CYP2B6* gene have been reported. The occurrence of these variants show intra and interpopulation variability, thus affecting drug efficacy at individual and population level. Differences in disease conditions and affordability of drug therapy further explain why some individuals or populations are more exposed to CYP2B6 pharmacogenomics associated ADRs than others. Variabilities in drug efficacy associated with the pharmacogenomics of *CYP2B6* have been reported in various populations. The aim of this review is to highlight reports from various ethnicities that emphasize on the relationship between CYP2B6 pharmacogenomics variability and the occurrence of adverse drug reactions. *In vitro* and *in vivo* studies evaluating the catalytic activity of CYP2B6 variants using various substrates will also be discussed. While implementation of pharmacogenomic testing for personalized drug therapy has made big progress, less data on pharmacogenetics of drug safety has been gained in

terms of CYP2B6 substrates. Therefore, reviewing the existing evidence on population variability in CYP2B6 and ADR risk profiles suggests that, in addition to other factors, the knowledge on pharmacogenomics of CYP2B6 in patient treatment may be useful for the development of personalized medicine with regards to genotype-based prescription.

Keywords: drug metabolism, adverse drug reaction, cytochrome P450 2B6, genetic polymorphism, drug safety, pharmacogenetics, pharmacogenomics, personalized medicine

INTRODUCTION

Adverse drug reactions (ADRs) are globally one of the major causes of morbidity and mortality (Giardina et al., 2018; Patel and Patel, 2018). According to the World Health Organization (WHO), ADR is a noxious and unintended response to a medication (Agency, 2017). ADRs can range from mild to severe causing ∼6.5% of all visits to the emergency department and longer duration of hospitalization (Giardina et al., 2018; Patel and Patel, 2018; Schurig et al., 2018). Drug clearance may vary up to 10-fold between two individuals of the same weight taking the same drug dosage (Stingl et al., 2013). This variation can be influenced by pathophysiological, physiological, or environmental factors. However, genetic polymorphisms in drug transporters, drug targets and most importantly drug metabolizing enzymes have been emphasized over the years as one of the major factors causing variability in drug response (Weinshilboum, 2003; Evans and Relling, 2004).

The cytochrome P450 (CYPs) enzymes are involved in phase I drug metabolism. Variability in patient exposure and response to various medication have been associated to genetic variants in genes that code for CYP enzymes (Lynch and Price, 2007; Zanger and Schwab, 2013). The human genome harbors 18 CYP families divided into 41 subfamilies encoding 57 genes. Specifically, CYP1, CYP2, and CYP3 families catalyze the biotransformation of many xenobiotic agents. *CYP2B6* is the only gene in the human CYP2B subfamily encoding a functional enzyme (Nebert et al., 2013).

The *CYP2B6* gene which consists of nine exons is located on chromosome 19 at position 19q13.2. It is highly expressed in the liver, and to a certain extent in the extrahepatic tissues such as brain, kidney, digestive tract and the lungs (Lonsdale et al., 2013). *CYP2B6* is a polymorphic cytochrome P450 enzyme with many single nucleotide polymorphisms (SNPs) encoding thirty-eight variants. These variants are referred as star alleles on the Pharmacogene Variation website with designated clinical function as normal, decrease, increase, no or uncertain function (Thorn et al., 2010). Compared to other well-studied phase I enzymes such as CYP2D6, CYP2C19 and CYP2C9, CYP2B6 at first had been thought to play a minor role in human drug metabolism (Desta et al., 2021). However, with the increase in techniques to evaluate its regulation, relative hepatic expression and function, it became evident that CYP2B6 constitutes up to 10% of the functional CYP enzymes in the liver. It is involved in the metabolism of 10–12% of all drugs commercially available in the market (Hanna et al., 2000; Rendic, 2002) and accounts for the metabolism of 4% of top 200 drugs in the market (Zanger et al., 2008). Specifically, it is fully or partially involved in the catalytic biotransformation of at least 90 drugs. **Table 1** shows selected drug substrates which are metabolism by CYP2B6.

Interestingly, variability in the expression and function of the CYP2B6 enzyme alters the metabolism of these substrates leading to altered pharmacokinetics and therapeutic efficacy. Abnormal drug efficacy associated with patient CYP2B6 genotype has been reported in various populations (Sarfo et al., 2014; Kharasch and Greenblatt, 2019; Chaivichacharn et al., 2020). The scope of this review is to report the evidence on ADRs of CYP2B6 substrates and elucidate possible functional mechanisms of the influence of CYP2B6 polymorphisms on enzyme function and ADRs. Population disparity in the use of CYP2B6 substrates and consequent exposure to substrate-related ADRs are discussed. Serious ADRs due to high-risk pharmacogenetic variants might be avoided by the use of preemptive genotyping (Dolgin, 2011; Bielinski et al., 2014; Kim et al., 2017; Bank et al., 2019) and the use of pharmacogenetic testing has greatly improved the lives of many patients (Lonsdale et al., 2013; Drozda et al., 2018). The knowledge on pharmacogenomics of CYP2B6 in patient treatment may be useful for the development of personalized medicine with regards to genotype-based prescriptions.

FACTORS THAT INFLUENCE CYP2B6 EXPRESSION AND FUNCTION

A significant interindividual variability in the mRNA expression, protein levels and activity of CYP2B6 has been reported in human liver microsomes (Ekins et al., 1998; Lang et al., 2001; Hesse et al., 2004). This variability is caused by the following factors; transcriptional regulation involving inhibition or induction of *CYP2B6* expression via the constitutive androstane receptor (CAR) and/or pregnane X receptor (PXR) (Wang et al., 2003a), inductive expression via glucocorticoid receptor (GR) (Lee et al., 2003; Wang et al., 2003b), inhibition of *CYP2B6* by cytokines through CAR and PXR (Aitken and Morgan, 2007; Liptrott et al., 2009), induction of *CYP2B6* by estrogen via the estrogen responsive element (ERE) (Faucette et al., 2004; Lo et al., 2010) and most importantly genetic polymorphism in the *CYP2B6* gene itself (Lang et al., 2001). Developmental regulation (age), gender and disease condition are other confounders of *CYP2B6* differential expression and function (Pearce et al., 2016). It is estimated that genetic polymorphisms and/or gene regulation are the major factors that impact variability in *CYP2B6* expression and function.

TABLE 1 | Drug substrates known for metabolism by the CYP2B6 enzyme.

Therapeutic class	Substrate[a]	Reaction (type)[b]	Other CYPs[c]	References
Antiretroviral	Efavirenz	8-hydroxylation (major reaction)	CYP3A4, 3A5, 1A2, 2A6	Patel and Patel, 2018
	Nevirapine	Hydroxylation (major reaction)	CYP3A4 3A5, 2C9, 2D6	Giardina et al., 2018
Antimalarial	Artemether	Demethylation (minor reaction)	CYP3A4, 3A5	Agency, 2017
	Artemisinin	Unknown (major reaction)	CYP3A4	Agency, 2017
Anticancer	Cyclophosphamide	4-hydroxylation (major reaction) N-dechloroethylation (minor reaction)	CY2C19, 3A4, 2E1	Stingl et al., 2013; Schurig et al., 2018
	Ifosfamide	4-hydroxylation N-dechloroethylation (major reaction)	CYP3A4, 2C9, 2C19, 2C8	Stingl et al., 2013
	Tamoxifen	4-hydroxylated (minor reaction)	CYP2D6, 3A4, 2C9, 2C19	Weinshilboum, 2003; Evans and Relling, 2004
Antidepressants	Bupropion	Hydroxylation (major reaction)	CYP3A4, CYP2D6,	Lynch and Price, 2007; Zanger and Schwab, 2013
	Esketamine	N-demethylation (major reaction)	CYP3A4, 2C9, 2C19	Lonsdale et al., 2013; Nebert et al., 2013
	Vortioxetine	Hydroxylation (minor reaction)	CYP2D6, 3A4/5, 2C19, 2C9, 2A6, 2C8	Thorn et al., 2010
	Amitriptyline	N-demethylation (minor reaction)	CYP2C9, 2C19, 1A2, 3A4, 2D6, 2B6, 2C8	Desta et al., 2021
	Fluoxetine	N-demethylation (minor reaction)	CYP2D6, 2C9, 3A4	Rendic, 2002
	Mianserin	N-demethylation (minor reaction)	CYP3A4, 2C19, 1A2	Hanna et al., 2000
	Sertraline	N-demethylation (minor reaction)	CYP3A4, 2C19, 2D6, 2C9	Zanger et al., 2008; Sarfo et al., 2014
Anticonvulsants	Phenytoin	Oxidation (minor reaction)	CYP1A2, 2A6, 2C19, 2C8, 2C9, 2D6, 2E1, CYP3A4	Kharasch and Greenblatt, 2019
	S-mephenytoin	N-demethylation (minor reaction)	CYP2C9, 2C19	Chaivichacharn et al., 2020
	Carbamazepine	3-hydroxylation (minor reaction)	CYP3A4, CYP2C8, CYP3A5	Bank et al., 2019
	Valproic acid	4, 5-hydroxylation (minor reaction)	CYP2C9, 2A6, 3A5	Kim et al., 2017
Anxiolytics, anticonvulsants	Diazepam	N-demethylation (minor reaction)	CYP2C19, 3A4, 3A5, 2C9	Bielinski et al., 2014
	Clotiazepam	N-demethylation (minor) reaction	CYP3A4, 2C18, 2C19,	Dolgin, 2011
	Clobazam	N-demethylation (minor reaction)	CYP3A4, 2C19	Drozda et al., 2018
	Temazepam	N-demethylation (minor reaction)	CYP2C19, 3A4	Bielinski et al., 2014
Anesthetics	Kitamine	N-demethylation (major reaction)	CYP3A4, 2C9	Lonsdale et al., 2013
	Propofol	4-hydroxylation (major reaction)	CYP2C9	Hesse et al., 2004; Zanger and Schwab, 2013
Opioid	Methadone	N-demethylation (major reaction)	CYP3A4, 2C19, 2C9, 2C8, 2D6,	Ekins et al., 1998; Lang et al., 2001
MAOI	Selegiline	N-demethylation (major reaction)	CYP2C19, 3A4, 1A2, 2D6, 2C9	Wang et al., 2003a,b
Antiplatelet	Clopidogrel	Oxidation (minor reaction)	CYP1A2, 2B6, 2C19, 2C9, 3A4	Lee et al., 2003
Smoking cessation agent	Nicotine	N-demethylation (minor reaction)	CYP2A6, 3A4	Aitken and Morgan, 2007; Liptrott et al., 2009
Analgesics	Tramadol	N-demethylation (minor reaction)	CYP2D6, 3A4,	Faucette et al., 2004; Lo et al., 2010
	Diclofenac	5-hydroxylation (minor reaction)	CYP2C8, 2C19, 2C9	Pearce et al., 2016
Gastro-intestinals	Loperamide	N-demethylation (minor reaction)	CYP219, 3A4, 2D6, 2C8,	Lewis and Lake, 1997
Steriod	Testosterone	Hydroxylation (minor reaction)	CYP3A4, 2C9, 2C19	Ekins et al., 2008
Anticoagulant	Coumarin	Aromatic hydroxylation (minor reaction)	CYP2A6, 1A1, 1A2, 3A4	Xie et al., 2003
SERM	Ospemifene	Hydroxylation (minor reaction)	CYP3A4, 2C9, 2C19,	Hidestrand et al., 2001

[a] *According to the drug Bank (https://go.drugbank.com/categories/DBCAT002619) and online literature.*
[b] *CYP2B6 is either the major enzyme or the minor enzyme in the biotransformation of the drug. The type of metabolic reaction according to the information from Pharmacogenomics Knowledge base (PharmGKB) website, drug bank and online literature.*
[c] *Other cytochromes that are involve in the metabolism of the drug, taken from PharmGKB website, drug bank and online literature.*
SERM, Selective estrogen receptor modulators.
MAOI, Monoamine oxidase inhibitors.

SUBSTRATES OF CYP2B6

Previous investigations revealed diversity in the structure among CYP2B6 substrates (Lewis and Lake, 1997). They also confer differences in the site of metabolism (Lewis and Lake, 1997). Typically substrates of CYP2B6 are hydrophobic small molecules, neutral or weak bases, very lipophilic with one or two hydrogen-bond acceptors (Ekins et al., 2008). **Table 1** indicates that CYP2B6 catalyzes demethylation, hydroxylation and oxidation reactions to form active or inactive metabolites (Hidestrand et al., 2001; Xie et al., 2003; Ekins et al., 2008; Zhang et al., 2017). Substrates of CYP2B6 are found in ∼23 different therapeutic classes (**Table 1**). It is predicted that CYP2B6 is the major catalytic enzyme in the biotransformation of important drugs commonly used worldwide. In combination with other cytochromes, CYP2B6 also plays a minor role in the metabolism of other xenobiotics. Notably, common metabolism of drugs is mediated by CYP2B6, CYP2C19, and CYP3A4 in terms of metabolism of clinically relevant therapeutics (**Table 1**). The CYP2B6 enzyme confers stereoselectivity, showing higher K_M for certain enantiomers such as S-efavirenz, S-mephenytoin, S-fluoxetine, S-ifosfamide, S-methadone, S-ketamine, S-bupropion (Coles and Kharasch, 2008; Ekins et al., 2008; Rakhmanina and van den Anker, 2010). CYP2B6 variants are substrate specific in their metabolic function. Thus, evaluating the impact of each enzyme variant on the metabolism of a specific substrate is of clinical importance (Ariyoshi et al., 2011). For example, recombinant CYP2B6*6 showed a decreased metabolism for both efavirenz and cyclophosphamide, while CYP2B6*4 showed increased metabolic activity toward efavirenz but less efficient metabolic activity toward cyclophosphamide (Ariyoshi et al., 2011).

CYP2B6 ALLELE VARIABILITY ACROSS POPULATIONS

Wide variability in CYP2B6 allelic frequencies is reported across populations. There exists intra and interethnic variability in the frequency of CYP2B6*6 and CYP2B6*18 variants. For example, at global level, the minor allele frequency of the CYP2B6*6 ranged from 0.33 to 0.5 in African Americans and Africans, 0.10–0.21 in Asians, 0.14–0.27 in Caucasians and 0.62 in Papua New Guineans (Zanger and Schwab, 2013; Rajman et al., 2017). Furthermore, allele frequency at the global level may not represent intraethnic differences within a specific population (**Table 2**). Though the CYP2B6*6 allele is more frequent in Africans and people of African descent, large intraethnic variability within these populations is observed (Rajman et al., 2017). For instance, the frequency of CYP2B6*6 in the Nigerian Yoruba population was reported to be 42%, Kenya Kikuyu 34% and Tswana of Bostwana 22% (**Table 2**). Also, the frequency of CYP2B6*9 (G516T) which forms part of the CYP2B6*6 variants was reported to be 55% in the Congolese, 20% in South African Xhosa and 37% in the Cameroonian population (**Table 2**). CYP2B6*4 is more frequent in the African, American, and Asian compared to the European population (**Table 2**). Meanwhile CYP2B6*2 is more frequent in the European and African compare to the Asian population (**Table 2**). This differences are associated with CYP2B6 functional variability and the occurrence of substrate specific ADR.

POPULATION DISPARITY IN THE USE OF CYP2B6 SUBSTRATES AND CONSEQUENT EXPOSURE TO SUBSTRATE-SPECIFIC ADVERSE DRUG REACTION (ADR)

Efavirenz and Nevirapine

Efavirenz (EFV) is classified as an effective non-nucleoside reverse transcriptase inhibitor used in the treatment of HIV infection[1,2]. As part of the highly active antiretroviral therapy, EFV is used in combination with other nucleoside reverse transcriptase inhibitors (NRTIs)[1,2]. EFV, which is presented in the form of 600 mg once daily or in a reduced dose of 400 mg oral tablets, is metabolized mainly by hepatic CYP2B6. EFV therapy is limited due to its narrow therapeutic window, thus, there exist a small difference between therapeutic and toxic doses. ADRs which are linked to the use of EFV includes increased risk of neurotoxicity, neuropsychiatric disorders, sleep disorders, high cholesterol level and drug induced liver disease (Cohen et al., 2009; Yimer et al., 2011; Aminkeng et al., 2014; Sarfo et al., 2014; Dhoro et al., 2015).

CYP2B6 genotype is a strong predictor of high systemic exposure to EFV in HIV infected patients. **Table 3** presents studies on associations between CYP2B6 genotype and the occurrence of CYP2B6 substrate related ADRs in various ethnicities. According to reports across ethnicities, patients harboring the CYP2B6*6 (516G>T, 785A>G*)* and the CYP2B6*18 (983T>C) variants experience reduced metabolism of EFV and increased exposure to the drug (**Table 3**). Patients with the homozygote 516TT and/or 785GG genotype as well as those with the 983CC genotype (poor metabolizers) experience significant increase in EFV plasma concentration and reduced clearance (**Table 3**). High exposure to EFV increases the risk of ADRs in these patients (Gounden et al., 2010; Mukonzo et al., 2013; Sarfo et al., 2014). Amongst all CYP2B6 substrates, EFV is the most studied drug with diverse forms of ADRs reported from various ethnicities. EFV is among top drugs causing ADRs in HIV patients in Africa, some part of Eastern Europe, and Asia (Manosuthi et al., 2013; Birbal et al., 2016; Rajman et al., 2017).

Nevirapine (NVP) based antiretroviral therapy is also used as a first line regimen for HIV infection. Its usage is limited due to side effects including hepatotoxicity, fever, Steven-Johnson syndrome and toxic epidermal necrolysis (Ciccacci et al., 2013; Aminkeng et al., 2014). In addition to other factors such as weight and gender, *CYP2B6* genotype influences patient response to NVP (Srivastava et al., 2010; Rajman et al., 2017; Yoon et al., 2020). These variants, CYP2B6 516G>T and 983T>C (CYP2B6*18) also impact patient exposure to NVP as reported

[1]Drugbank. Available online at: https://go.drugbank.com/drugs/DB00625.
[2]FDA. Available online at: https://www.accessdata.fda.gov/drugsatfda_docs/nda/2019/210649Orig1s000TOC.cfm.

TABLE 2 | Selected studies revealing variability in allele frequency of CYP2B6 alleles in different ethnicities.

Country/region/population	*N*	CYP2B6*2	CYP2B6*4	CYP2B6*6	CYP2B6*9	References
Europe						
German	430	5.3	4	32.1	28.6	Giardina et al., 2018; Patel and Patel, 2018
Spaniard	360	-	6.2	21.5	1.4	Agency, 2017
Swiss	226		3.9	24.8	26	Giardina et al., 2018; Schurig et al., 2018
British	270	3.7	2.2	28.15	28.6	Stingl et al., 2013
Ukraine	102	-	-	_	25	Weinshilboum, 2003
Turkish	344	-	6.4	25.3	2	Giardina et al., 2018
Africa		-		-	-	
West Africa	153	4	42	-	50	Evans and Relling, 2004
Congo	418	-	-	-	55¥	Lynch and Price, 2007
Ghana	800	-	-	-	48	Zanger and Schwab, 2013
Botswana	570	-	-	22		Nebert et al., 2013
Mozambique	360	5.7	41	-	42.6¥	Lonsdale et al., 2013
Nigeria (Hausa)	100	-	-	42	-	Thorn et al., 2010
Nigeria (Igbo)	100	-	-	38	-	Thorn et al., 2010
Nigeria (Yoruba)	100	-	-	42	-	Thorn et al., 2010
Tanzania	256	-	-	-	36¥	Desta et al., 2021
Ethiopia	285	-	-	-	31.4¥	Rendic, 2002
Kenya (Kikuyu)	102	-	-	34	-	Thorn et al., 2010
Kenya (Luo)	100	-	-	37	-	Thorn et al., 2010
Kenya (Maasai)	152	-	-	35	-	Thorn et al., 2010
Zimbabwe (San)	64	-	-	40	-	Thorn et al., 2010
Zimbabwe (Shona)	100	-	-	38	-	Thorn et al., 2010
Cameroon	75	-	-	-	37¥	Hanna et al., 2000
South Africa	163	-	-	-	36¥	Hanna et al., 2000
South Africa (Venda)	81	-	-	36		Thorn et al., 2010
South Africa (Xhosa)	109	-	-	-	20¥	Zanger et al., 2008
South Africa (MA)	67	-	-	-	23¥	Zanger et al., 2008
Uganda Bantus	58	-	-	25.9	-	Sarfo et al., 2014
Asia		-	-	-		
Thai	100	6	3.6	-	32¥	Kharasch and Greenblatt, 2019
Chinese	567	2.8	3.2	-	25.9¥	Chaivichacharn et al., 2020
Japanese	530	4.7	9.3	16.4	-	Bank et al., 2019
Han Chinese	386		9.1	18.4	1.8	Giardina et al., 2018
Malaysian Malay	196	0.8	7.6	25.4	4.6	Kim et al., 2017
Malaysian Chinese	165	1.3	6.4	13.9	10.2	Kim et al., 2017
Malaysian Indian	63	4.1	9.9	18.5	5.9	Kim et al., 2017
South Indians	135	-	-	-	44	Bielinski et al., 2014
Koreans	374	-	-	16.4	-	Dolgin, 2011
Indonesia (Timorian)	109	-	56.8	41.7	46.9	Drozda et al., 2018
Taiwanese	68	-	11.8	16.2	0	Hesse et al., 2004
Mongolian	200	-	9	21	-	Ekins et al., 1998; Giardina et al., 2018
Papa New Guinea	172	0	0	65	0	Lang et al., 2001
America		-	-	-	-	
Columbian Mestizo	250	4.4	15.2	18	14.4	Wang et al., 2003a
Central American Mestizo	362	-	7.3	23.1	2.3	Wang et al., 2003a
Chilean Mestizo	438	-	-	35	-	Wang et al., 2003a
Hispanic–American	77	3	35	-	37	Evans and Relling, 2004
African American	85	2	37	-	36	Evans and Relling, 2004

¥ *In the manuscript, it is referred to as CYP2B6*6 but according to the Pharmacogene Variation Consortium (Pharmvar) website it should be CYP2B6*9, CYP2B6*6 is a combination of CYP2B6*4 & CYP2B6*9. (-) Allele not verified.*

TABLE 3 | CYP2B6 polymorphisms and adverse drug reactions reported amongst patients in various ethnicities.

Substrate	Subjects	*N*	CYP2B6 genotype	Predicted functional effect on CYP2B6 enzyme activity	Patient exposure to the drug	Frequency of allelic variants (%)	Population	Adverse drug reaction	References
Efavirenz	HIV/TB	185	CYP2B6*6/*6	↓ Activity	Higher exposure	45	Zimbabwe	Central nervous system adverse events(CNS) including insomnia, severe headaches, vivid nightmares, drowsiness, ataxia, dystonia and dizziness.	Patel and Patel, 2018
	HIV, TB-HIV co-infected patient	353	CYP2B6 516TT	↓ Activity	Higher exposure	31.6	Ethiopians	Anti-retroviral and anti-tuberculosis drug induced liver injury in TB-HIV co-infected patients.	Agency, 2017; Giardina et al., 2018
	HIV	285	CYP2B6 516TT	↓ Activity	Higher exposure	31	Ethiopians	Higher risk of drug induced liver injury (DILI)	Agency, 2017; Schurig et al., 2018
	HIV	800	CYP2B6 516TT	↓ Activity	Higher exposure	48	Ghanaian	Neuropsychiatric toxicity	Stingl et al., 2013
	HIV	134	CYP2B6*6/*6	↓ Activity	Higher exposure	8.2	Thai	Increase risk of hepatotoxicity	Weinshilboum, 2003; Evans and Relling, 2004; Lynch and Price, 2007
	HIV/AIDS	1,147	CYP2B6 G516TT	↓ Activity	Higher exposure	38, 21.9	Mixed population European American, African American, Hispanics	Central nervous system toxicity	Zanger and Schwab, 2013
	HIV/AIDS	373	CYP2B6 516TT	↓ Activity	Higher exposure	30, 37	Mixed population (Black & White)	Central nervous system related effects and 131 patients withdrew from therapy within the first 3 months	Nebert et al., 2013
	HIV	197	CYP2B6 516TT	↓ Activity	Higher exposure	30	Ugandans	Neuropsychiatric symptoms. High incidence of vivid dream, sleepwalking, insomnia and tactile hallucination	Thorn et al., 2010; Lonsdale et al., 2013
	HIV/TB patients	473	CYP2B6 516GT CYP2B6 516TT	↓ Activity	Higher exposure	35.5	Tanzanians	Development of efavirenz based HAART liver injury	Agency, 2017; Desta et al., 2021
	HIV adults	142	CYP2B6 516GT CYP2B6 516TT	↓ Activity	Higher exposure	32	South Africans	High efavirenz level associated with severed sleep disturbance	Rendic, 2002

(Continued)

TABLE 3 | Continued

Substrate	Subjects	*N*	CYP2B6 genotype	Predicted functional effect on CYP2B6 enzyme activity	Patient exposure to the drug	Frequency of allelic variants (%)	Population	Adverse drug reaction	References
	HIV	80	CYP2B6 516GT CYP2B 516TT	↓ Activity	Higher exposure	43	South Africans	Higher EFV concentration and early neuropsychiatric side effects (presence of hallucinations or psychotic episodes)	Hanna et al., 2000
	HIV	191	CYP2B6 516GT, CYP2B6 516TT	↓ Activity	Higher exposure	49	Mixed population (Caucasian 162, African 23, Asiantic four, others two)	CNS related symptoms such as disturbances in consciousness, mood disorders, headaches, sleep disturbances, cognitive and attention disturbances, eating disturbances and dizziness	Zanger et al., 2008
	HIV/AIDS	1330	CYP2B 516GT/TT	↓ Activity	Higher exposure	77, 25	Mixed population African ancestry (372), European ancestry (958)	Increase in cholesterol levels, Increased risk of neurotoxicity, CNS depression and neuropsychiatric disorders, Increased risk of fatigue and sleep disorder and Increased risk of hepatotoxicity and drug-induced liver injury	Sarfo et al., 2014
	HIV	32/90	CYP2B6 516TT	↓ Activity	Higher exposure	32	Mixed population	Increased likelihood of central nervous system disease	Kharasch and Greenblatt, 2019
	HIV	235	CYP2B6 516TT	↓ Activity	Higher exposure	26	Swiss	Neuropsychological toxicity	Chaivichacharn et al., 2020
	HIV patients	105	CYP2B6*1/*18, CYP2B6*18/*18	↓ Activity	Higher exposure	5.1	Mozambicans	Associated with severe cutaneous adverse event such as Steven-Johnson syndrome and toxic epidermal necrolysis	Bank et al., 2019
Nevirapine	HIV	672	CYP2B6*18	↓ Activity	Higher exposure	18	Malawians Ugandans	Hypersensitivity such as nevirapine induced-Stevens–Johnson syndrome (SJS)	Kim et al., 2017
	HIV	105	CYP2B6 516TT CYP2B6 983CC	↓ Activity	Higher exposure	55.6 18.5	Mozambicans	Patients with Nevirapine-induced SJS/toxic epidermal necrolysis (TEN)	Bank et al., 2019
Cyclophosphamide	non-Hodgkin's lymphoma	567	CYP2B6 516TT CYP2B6 785AG (*4)	↓ Activity	Reduce exposure to 4-hydroxycy-cyclophosphamide	25.9 32	Chinese	Lower risk of grade 2–4 toxicities and poor treatment outcome	Bielinski et al., 2014
	Breast Cancer	230	CYP2B6*2 CYP2B6*4 CYP2B6*5 CYP2B6*9	↑ Activity	High exposure	227 15 30	Mixed population (European 97, South Asian 2, East Asian < 1)	Leucopenia and neutropenia associated with dose delay	Hesse et al., 2004; Dolgin, 2011; Drozda et al., 2018
	Breast cancer	Case report	CYP2B6*7	↑ Activity	Higher exposure to 4-hydroxycy-cyclophosphamide	18.4	Han Chinese	Severe and prolonged hepatotoxicity	Ekins et al., 1998; Dolgin, 2011; Drozda et al., 2018

(Continued)

TABLE 3 | Continued

Substrate	Subjects	*N*	CYP2B6 genotype	Predicted functional effect on CYP2B6 enzyme activity	Patient exposure to the drug	Frequency of allelic variants (%)	Population	Adverse drug reaction	References
	Chronic lymphocytic leukemia (CLL)	428	CYP2B6*1/*6 CYP2B6*6/*6	↓ Activity	Reduced exposure	22	UK clinical trial	Decrease risk of drug toxicity. Toxicity included neutropenia, thrombocytopenia, anemia, mucositis, and alopecia	Lang et al., 2001
	Leukemia patients on stem cell transplant	107	CYP2B6*4 CYP2B6*2 CYP2B6*6 (donor GG genotype)	↑ activity	Higher exposure	53 16, 50	Mixed population	Oral mucositis, (*4), hemorrhagic cystitis (*2),Veno-occlusive disease of the liver (*6),	Wang et al., 2003a
Methadone	Breast cancer	166	CYP2B6 516GT, CYP2B6516TT	↓ Activity	High exposure	27	Brazilian	High risk of severe levels of asthenia and arthralgia	Ekins et al., 1998; Wang et al., 2003b
	Fatalities cases due to methadone	380	CYP2B6*9 CYP2B6*5	↓ Activity	High exposure	27	Caucasians	Methadone fatalities	Lee et al., 2003; Wang et al., 2003b; Liptrott et al., 2009
	Opioid dependent males	148	CYP2B6*6	↓ Activity	High exposure	25.4	Malays	Lower pain threshold, increase severity of pain	Lee et al., 2003; Liptrott et al., 2009

↓, *Decrease;* ↑, *Increase.*

by few studies from different ethnicities (Uttayamakul et al., 2010; Calcagno et al., 2012; Vardhanabhuti et al., 2013; Mhandire et al., 2015; Giacomelli et al., 2018). A reduced metabolic activity of the CYP2B6 enzyme leading to increased exposure of the patient and subsequent ADRs are observed (Yoon et al., 2020) (**Table 3**). NVP induced hypersensitivity reactions in HIV patients (Ciccacci et al., 2010; Carr et al., 2014). The variant allele of CYP2B6*18 and CYP2B6*4 were associated with the occurrence of Stevens-Johnson syndrome (SJS) among HIV patients (Ciccacci et al., 2013; Carr et al., 2014). Studies suggest dose reduction in patients harboring CYP2B6 poor metabolizer variants (Schipani et al., 2011; Morales-Pérez, 2021).

Interestingly, the use of EFV and NVP for HIV treatment is variable among populations. Highly active antiretroviral therapy (HAART) that includes EFV or NVP is mostly used in low and middle income countries (LMICs) including countries in Africa, Asia and some part of Eastern Europe (Gokengin et al., 2018; Ndashimye and Arts, 2019). Some studies have examined the reasons for such disparity in the use of EFV. It was observed that countries with higher income had greater access to novel drugs and new drug classes as first-line treatment than countries with low income (Gokengin et al., 2018; Ndashimye and Arts, 2019). Although most HAART regimens are given free to patients in all countries, the high level of donor dependency in LMICs might be a major obstacle to accessibility to new, expensive, more quality and less toxic drugs (Gokengin et al., 2018). The high cost of new first-line drugs with better tolerability and lower toxicity influences the choice of treatment regimen, with high income countries (HICs) choosing regimens that include new drugs compared to LMICs who depend on cheaper regimens (Gokengin et al., 2018). We observe an association between countries depending on cheaper drugs (EFV) and a high frequency of CYP2B6*6 which enhances the risk of EFV related ADRs in these populations (Africans, Papua New Guineans and African Americans with low income/incomplete insurance coverage). Intraethnic variability of CYP2B6 alleles within the African population, further indicates differences in the risk profiles of occurrence of CYP2B6*6 and CYP2B6*9-EFV-related ADRs between these African countries. This genomic diversity of African populations has been observed for many genes (Rajman et al., 2017) and highlights the potential error in generalizing the likely response to medicines of populations on the African continent (Ampadu et al., 2016). HIV infected individuals in LMICs are more likely to suffer from EFV-related ADRs than those in HICs.

Even though many LMICs have transited from the use of EFV and NVP to the use of the HIV-1 integrase inhibitor dolutegravir (DTG) which is assumed to be less expensive and less toxic (Vitoria et al., 2018), DTG has its own limitations as confirmed by the U.S. Food and Drug Administration (FDA). DGT may be unsuitable for patients with hepatitis B and C infection due to elevated levels of hepatic enzyme (Vitoria et al., 2018). Risk of hypersensitivity reactions and development of renal failure may be observed (Vitoria et al., 2018). New-onset or worsening hepatic or renal toxicity with longer cumulative exposure is a potential risk (Vitoria et al., 2018). In pregnancy, there are concerns that the baby may be harmed, thus women are recommended to take effective birth control while on the drug (Vitoria et al., 2018; Zash et al., 2019; Alhassan et al., 2020). Weight gain was observed in patients treated with DTG compared to EFV in clinical trials performed in sub-Saharan Africa and studies in Europe and North America. Therefore, long term use of DTG is still under concern (Kouanfack et al., 2019; Bourgi et al., 2020; Sax et al., 2020). A recent communication in December 2020 by Siedner et al. found that patients with drug resistant mutation in the reverse transcriptase are unlikely to benefit from the HIV integrase inhibitor DTG (Siedner et al., 2020). That implies EFV and NVP might still be in use for specific group of patients (Vitoria et al., 2018).

The occurrence of other diseases, including hepatitis and liver disease, are more common among HIV patients in LMICs (Labarga et al., 2007; Su et al., 2018). Unfortunately, much care is not given to HIV patients who have comorbid conditions, thus increasing the risk of ADRs amongst these patients (Labarga et al., 2007; Wu et al., 2017; Su et al., 2018). For example, patients with liver disease and hepatitis may experience enhanced hepatotoxicity due to EFV (Agbaji et al., 2013; Wu et al., 2017; Nampala et al., 2018; Su et al., 2018). Certain preexisting conditions, such as mild psychiatric disorder, may be exacerbated by EFV. Patients in LMICs may be vulnerable to ADRs not just because of the choice of regimen or their CYP2B6 genotype, but also because of comorbidity conditions. As such individualized prescriptions taking into account the pharmacogenetic profile, patient vulnerability caused by comorbid disease, and the toxicity profile of the drug will help to reduce ADR amongst these group of individuals in any part of the world.

Cyclophosphamide

Cyclophosphamide (CP) is an anticancer agent that is widely used in the treatment of pediatric and adult malignancies. CP is also known as an antirheumatic and anti-inflammatory drug (Brummaier et al., 2013; Sevko et al., 2013). CP is a prodrug that requires enzymatic bioactivation to produce its therapeutic function (Raccor et al., 2012). The formation of 4-hydroxycyclophosphamide (4-OHCP) is catalyzed by CYP2B6, CYP2C9, CYP2C19, CYP3A4 and CYP3A5. CYP2B6 and CYP2C19 confer the highest metabolic activity for the bioactivation of CP (Roy et al., 1999; Griskevicius et al., 2003; Raccor et al., 2012).

There is a significant variation in the plasma levels of CP ranging from 1.0 to 12.6 L/h (Batey et al., 2002; de Jonge et al., 2005). According to *in vitro* studies, it is clear that genetic polymorphisms in *CYP2B6* gene and interindividual differences in the CYP2B6 enzymatic activity are associated with variability in CP efficacy (Roy et al., 1999; Xie et al., 2003; Hofmann et al., 2008). Very limited clinical studies have evaluated the impact of CYP2B6 variants on patient exposure to CP, instead, many studies have investigated the influence of patient genotype on therapeutic outcome or treatment efficacy of CP. However, Shu et al., showed a significant association between CYP2B6 genotype and exposure to 4-OHCP amongst 567 patients with non-Hodgkin lymphoma (**Table 3**). Reduced expression and catalytic activity of CYP2B6 protein was found among homozygous and heterozygous carriers of CYP2B6*6,

*29, and *30 (**Table 3**). Patients with the 785G allele (*6) showed the worst treatment response (Shu et al., 2017). In breast cancer patients, a higher incidence of dose delay due to toxicity was observed among carriers of the CPY2B6 *2 and *5 (Bray et al., 2010). Furthermore, CYP2B6*2, *4, *8, and *9 alleles where associated with worse treatment outcome (Bray et al., 2010). In another study, an association was observed between CYP2B6*6 allele and inferior treatment response in patients with chronic lymphocytic leukemia. Patients harboring at least one CYP2B6*6 allele were less likely to achieve complete response to CP and fludarabine combination therapy due to impaired cytoreduction (Johnson et al., 2013).

Decreased risk of drug toxicity (neutropenia, thrombocytopenia, anemia, mucositis and alopecia) was observed in carriers of CYP2B6 *1/*6 and CYP2B6*6/*6 genotypes (**Table 3**). Considering the fact that *CYP2B6*6* is a decreased function allele, these patients seem to experience less toxicity and impaired cytoreduction due to the inability of the variant enzyme with reduced activity to convert CP to the active anticancer metabolite which is also responsible for toxicity. A significant association was observed between CYP2B6 785A>G (*4) and shorter progression free survival (Falk et al., 2012) in patients with multiple myeloma. Patients with the 785GG genotype had the worse outcome compared to patients with the wild type allele. In another study involving lymphoma patients who were placed on high dose CP prior to hematopoietic stem cell transplant, patients with the CYP2B6*1/*5 genotype had a higher 2 years relapse and decrease overall survival than patients with the wild type genotype (Bachanova et al., 2015). Though the CYP2B6*5 variant is designated as normal function, *in vitro* studies of CP revealed reduced conversion of CP in human liver microsomes and recombinant expressed CYP2B6*5 variants confer 50% decrease in the formation of 4-OHCP compared to the wild type enzyme (Helsby et al., 2010; Raccor et al., 2012). Most recently, CP related toxicity was associated with *CYP2B6* variants in patients with leukemia after HLA-identical hematopoietic stem cell transplantation (Rocha et al., 2009). Donor CYP2B6*6 was associated with Veno-occlusive disease of the liver meanwhile recipient CYP2B6*2 and *4 were associated with hemorrhagic cystitis and oral mucositis correspondingly (Rocha et al., 2009). High risk of severe levels of asthenia and arthralgia was observed amongst breast cancer women with CYP2B6 516GT and 516TT genotype placed on FAC-D combination therapy (Paula et al., 2020). Some studies also evaluated the role of CYP2C19 variants alongside with CYP2B6 in the efficacy and toxicity of cyclophosphamide. Even though few studies could not find any association between CYP2B6 or CYP2C19 and treatment outcome and/or toxicity of CP, differences in the study design, ethnicity, physiological or disease condition as well as power of the studies might have a role in the experimental outcome. However, it appears that these two pharmacogenes (*CYP2B6* & *CYP2C19*) play a significant role in the bioactivation and efficacy of CP.

Variability in *CYP2B6* polymorphisms between populations indicates that cancer patients in various ethnicities will respond variably to CP therapy. For example, CYP2B6*9 and CYP2B6*2 variants which are associated with toxicity and worse treatment outcome were not detected among 172 individuals from Papua New Guinea (Mehlotra et al., 2006; Bray et al., 2010), thus, cancer patients in this region are less likely to experience CYP2B6 pharmacogenetic associated-CP toxicity. Meanwhile, in Mozambique and Germany, patients are more likely to experience toxicity and worse treatment outcome due to the frequency of CYP2B6*9 (42.6 and 28.6%) in these populations (**Tables 2**, **3**). Furthermore, reduced CP toxicity and impaired cytoreduction are likely to occur in populations with high frequency of CYP2B6*6 (**Tables 2**, **3**).

According to research, the number of cancer survivals vary between and within countries. These differences are partly due to limited access to high quality and effective treatment in some part of the world (Newton et al., 2010; Eniu et al., 2019). CP is included in the WHO list of essential medicine (Bazargani et al., 2015). However, the majority of cancer patients living in LMICs have limited access to essential medicine and sometimes imported medication are either counterfeits or of poor quality (Fernandez et al., 2011; Ruff et al., 2016). Cancer care is not included in most resource limited settings due to high cost, as such, patients have to pay for cancer treatment out of their pocket (Eniu et al., 2019). In Asia, the number of death in a year ranged from 12% in Malaysia to 45% in Myanmar amongst cancer patients and those with low income were at risk of adverse outcomes even at early disease stages (ACTION Study Group, 2017). Important barriers to availability and affordability of anticancer drugs include lack of governmental reimbursement, allocation of healthcare budgets, generic and biosimilar products as well as the right to patent (Renner et al., 2013; Eniu et al., 2019). Therefore, patients in these regions are likely to experience drug resistance, treatment failure and more ADRs than those in HICs (Newton et al., 2010).

Additionally, increase number of adverse outcome is expected to occur in Africa, Asia and some parts of Europe, where other diseases such as HIV, tuberculosis and hepatitis are found amongst cancer patients who are self-sponsored without any health care coverage and reduced compliance to medication due to lack of funds and timely treatment (ACTION Study Group, 2017; Eniu et al., 2019). Therefore, in addition to other confounders, increased accessibility to essential medicine, availability of health care coverage, special care for patients with comorbidity and genotyping of patients may improve therapy.

Bupropion

Bupropion, generally used as an antidepressant, was later recommended as a non-nicotine treatment for smoking cessation. It is approved for treatment of nicotine dependence in patients with tobacco use disorder (Jorenby et al., 1999; Schnoll and Lerman, 2006). It acts by increasing dopaminergic and noradrenergic transmission via the blockage of neurotransmitter reuptake at the synapse thereby antagonizing the effects of nicotine acetylcholine receptor leading to nicotine withdrawal (Paterson et al., 2007). Also, bupropion is prescribed alone or in combination with other antidepressants for treatment of major depressive disorder and seasonal affective disorder. It is used as an adjunctive medication to reverse antidepressant-associated sexual dysfunction and to improve the efficacy of other antidepressants in partial or non-responders (Fava et al., 2005).

Both bupropion and the active metabolite S-hydroxybupropion are responsible for the antidepressant and smoking cessation effect of bupropion (Zhu et al., 2012; Laib et al., 2014).

In vitro studies revealed variability in the rate of metabolism of S-bupropion by various CYP2B6 variants (Wang et al., 2020). Studies from different ethnicities show that the CYP2B6 allelic variants *4, *6, and *18 are associated with altered bupropion metabolism (Kirchheiner et al., 2003; Zhu et al., 2012; Benowitz et al., 2013). Depending on the patient's genotype, they can experience either increased or decreased exposure to the active metabolite hydroyxbupropion compared to wild type (Kirchheiner et al., 2003; Benowitz et al., 2013). Reportedly, patients harboring at least one reduced function allele had reduced exposure to the active metabolite and a lower clearance of the parent drug (Kirchheiner et al., 2003). Meanwhile, those with at least one increased function allele have increased exposure to hydroxyl active metabolites and a higher clearance of the parent drug (Kirchheiner et al., 2003).

Some studies have linked patient genotype to the efficacy of bupropion as a smoking cessation agent. In a smoking cessation trial involving 326 Europeans, a significant rate of abstinence was observed among patients with the CYP2B6*1/*6 and CYP2B6*6/*6 genotype (Lee et al., 2007). The CYP2B6*4 variant allele was associated with lower success rate in bupropion therapy. Specifically, patients with the AA genotype (wild type) succeeded in ceasing smoking compared to those with the variant genotype 785AG and 785GG (Tomaz et al., 2015). The increase function alleles might have led to higher clearance of the drug as well as reduced efficacy. In another clinical trial, bupropion drug gene interaction indicated that individuals with at least one T allele of CYP2B6*5 (1459CT, TT) and DRD2-Taq1 A2/A2 genotype had higher odds of abstinence (David et al., 2007). There are some differences in experimental outcome, for example, in a clinical trial involving 540 African American light smokers, a direct association between CYP2B6 genotype and smoking cessation was not observed, according to the authors, poor adherence amongst the participants might have led to the observed results. Most studies did not assess the impact of CYP2B6 polymorphisms on the antidepressant effect of bupropion. Instead, many focused on the CYP2B6 pharmacogenetics of bupropion in smoking cessation (Pharmgkb, 2000; Clark et al., 2012).

Compared to HICs, mental health in LMICs is still limited with low number of clinical trials and extremely low number of patients receiving treatment (Sankoh et al., 2018; Chibanda et al., 2020). Individuals with serious mental health problems in Nigeria cannot have access to health care due to the limited number of resources and mental health staff (Chibanda et al., 2020). Approximately, 300 psychiatric doctors serve a population of about 200 million people. Currently, these issues are being addressed by the Nigerian government via the forthcoming Mental Health and substance abuse bill (Brathwaite et al., 2020; Ugochukwu et al., 2020). Another study involving 683 prescriptions and case records of patients who were placed on antidepressants showed that tricyclic antidepressants were the most prescribed drugs (61.3%), followed by selective serotonin re-uptake inhibitors (38.7%) in Nigeria (Oyinlade, 2017). Amitriptyline hydrochloride, fluoxetine, clomipramine, escitalopram, and imipramine hydrochloride are the antidepressants found on the WHO list of essential medicine for Nigeria (WHO, 2017)[3]. In Zimbabwe, a research made in 2017 indicated that psychiatrists sometimes experience shortage of drugs which results in relapse among patients (Kidia et al., 2017). The antidepressants available were old generation drugs with many side effects (Kidia et al., 2017). According to one of the psychiatrists, more attention is being given to people with HIV than those with mental health, furthermore mental health policies were never implemented because of inadequate funding (Kidia et al., 2017). The African Mental Health Research Initiative group aims to create awareness and strengthen the mental health sector via research and capacity building in Sub-Saharan Africa (Chibanda et al., 2020; Langhaug et al., 2020).

The Global Health Observatory (GHO) data indicates the availability of bupropion in various countries (GHO/WHO, 2007). In some countries bupropion is available in the pharmacy with prescription. In others, it is found in general stores without prescription (Langhaug et al., 2020). Meanwhile, for some countries, data for the use of bupropion is not available. For example, GHO data shows that in Germany, France, Australia, Canada, United States of America, Congo, and India bupropion is available in pharmacies with prescription, whereas in Nigeria, it is found in general stores without prescription, in Malaysia, Ukraine, Zimbabwe, and Cameroon, GHO 2012 data shows that bupropion was present in pharmacy with prescription. In Hungary, Algeria, Tanzania, Mali, no data was available from 2007 to 2018 (GHO/WHO, 2007). Compared to HICs, the shortage of medication faced in LMIC is likely to increase relapse and the use of old drugs with more side effects can aggravate ADRs among patients.

Though bupropion is cost effective and widely used in most countries, its application as a smoking cessation agent is still very limited in some LMICs. The MPOWER tobacco control measures put in place by the World Health Organization (WHO) in 2005 have greatly helped many countries to discourage the use of tobacco and to help users quit tobacco addiction[4]. MPOWER measure is a tobacco free initiative established by WHO for defeating the global tobacco epidemic via **M**onitoring tobacco use, **P**rotecting people from tobacco use, **O**ffering help to quit, **W**arning about dangers of tobacco, **E**nforcing bans on advertising and sponsorship and **R**aising taxes on tobacco in all parts of the world (Batini et al., 2019). According to a recent report by Batini et al., the application of the MPOWER measures vary across continents and countries with a marked discrepancy observed between LMICs, and HICs, where cost, governmental buy in and resources influence implementation of the various measures (Batini et al., 2019). Using the African continent as an example, authors report that pharmacotherapy for smoking cessation is scarce in Nigeria with few pharmacies

[3]WHO. Available online at: https://digicollections.net/medicinedocs/#/d/s19018en.2010.

[4]WHO. *Tobacco Free Initiative*. Available online at: https://www.who.int/tobacco/mpower/en/.

offering nicotine replacement therapy. In Zimbabwe, there are currently no smoking cessation services in the public sector except for private sectors, which is offered at very high cost (NRT 130USD/week, varenicline 250USD/month). In Tanzania, nicotine replacement therapy is available and smoking cessation therapy has been introduced into the methadone clinic in Dar es Salaam (Batini et al., 2019). However, the use of non-nicotine therapy such as bupropion is still lacking in these countries.

Depression seems to coexist with many diseases that are common in both high and LMICs. In Sub Saharan Africa, depression is common among HIV patients and people with disability (Mayston et al., 2020). In India, high prevalence of depressive episodes were found among diabetic patients (Kanwar et al., 2019). In a comparison between Ethiopian and German cancer patients, depression and anxiety were found in both groups, but a little higher among uneducated Ethiopian patients (Wondie et al., 2020). Considering the fact that some HIV and cancer patients in LMICs are not able to afford treatment, depression might be high among these group of patients. It is well-established that CYP2B6 genotype is a significant contributor to variability in hydroxybupropion and bupropion levels, influencing the efficacy of bupropion therapy in smoking cessation patients. Knowledge on CYP2B6 genotype in consideration with other factors might give a clue on patients who are likely to benefit from therapy. Constant supply of bupropion could help to reduce relapse among patients in most LMICs. Treatments of other comorbidities that are risk factors of depression have the potential to improve therapy and reduce the number of depressive patients worldwide. There is need to create awareness in the area of mental health via research and innovation, including capacity building, training more mental health staff and psychiatric doctors in LMICs.

Ketamine

Ketamine is a World Health Organization Essential Medicine widely used for perioperative, acute and chronic pain, and sedation. It is used either solely or in combination with opioids for the management of acute post-operative and chronic refractory pain (Laskowski et al., 2011). Its use in the management of status epilepticus, bipolar disorder, suicidal behavior, major depressive disorder and treatment resistant depression has been demonstrated (Yeh et al., 2011; Synowiec et al., 2013; Pizzi et al., 2017; Borsato et al., 2020). Ketamine acts as a non-competitive antagonist of the N-Methyl-D-aspartic acid receptor blocking its action, thereby preventing the development and chronification of pain (Noppers et al., 2010). CYP2B6 is among other enzymes (CYP2C9, CYP3A4) involved in the hepatic N-demethylation of ketamine to norketamine (Yanagihara et al., 2001; Hijazi and Boulieu, 2002; Portmann et al., 2010; Desta et al., 2012; Palacharla et al., 2018). Although ketamine has a large therapeutic window, its use is limited due to low efficacy and huge interindividaul variability in treatment response including ADRs that require cessation of therapy (Kvarnström et al., 2004; Noppers et al., 2010; Laskowski et al., 2011; Hardy et al., 2012; Perez-Ruixo et al., 2020). Ketamine has been associated with increased blood pressure, alteration of speech, muscular discoordination, euphoria, hallucination, loss of consciousness, seizure, nausea, out of body experience, hypothermia, traffic accident or drowning and irrational behavior (Iyalomhe and Iyalomhe, 2014; Lonnée et al., 2018; Gajewski et al., 2020).

Variability in the expression and catalytic activity of CYP2B6 variant enzymes results in differences in the hepatic clearance of ketamine (Wang et al., 2018). Individual differences in hepatic blood flow leading to differences in patient clearance of the drug has also been implicated (Yanagihara et al., 2001). The presence of the CYP2B6*6 allele led to a decrease in the intrinsic clearance of ketamine of up to 89% in human liver microsomes and 55% in cDNA-expressed CYP2B6 protein *in vitro* (Yanagihara et al., 2001). The CYP2B6*1/*1 (wildtype) genotype confers a 6-fold higher clearance of both enantiomers of ketamine compared to the CYP2B6*6/*6 genotype (Li et al., 2013). In a study involving patients with chronic opioid-refractory pain, the plasma clearance of ketamine was lower in patients with CYP2B6*6/*6 and CYP2B6*1/*6 genotype compared to patients with CYP2B6*1/*1 genotype (Li et al., 2015). Although there were no direct associations between the genotype of the patients and the occurrence of ADRs, drowsiness and hallucination were more often observed in patients with lower clearance than those with higher clearance. The authors hypostatized that higher plasma concentrations due to reduced clearance may have predisposed patients to ketamine ADRs. *In vitro* studies further demonstrate that CYP2B6 variants confer variability in the metabolism of ketamine. S-ketamine metabolism ranged from CYP2B6*1 (wildtype) > CYP2B6*4 > CYP2B6*26, CYP2B6*19, CYP2B6*17, CYP2B6*6 > CYP2B6*5, CYP2B6*7 > CYP2B6*9, respectively (Wang et al., 2018). This indicates that patients harboring different CYP2B6 variants will respond differently to the drug (Borsato et al., 2020). Genetic variations in *CYP2B6* and other CYPs including *CYP3A4* might result in different plasma concentration of ketamine and its metabolites. Therefore, knowing the genotype of the patient prior to prescription could help to address individual needs and reduce ADRs.

There is a huge difference in availability and application of anesthesia between high and LMICs as reviewed by Dolman et al. Factors influencing these differences include limited physician anesthesiologists and nurse anesthetists, insufficient anesthesia equipment and infrastructure, patient comorbidities and late presentations (LeBrun et al., 2014; Dohlman, 2017). In LMICs, ketamine is the only anesthetic drug in many hospitals as compared to HICs, where it is used as an adjunct in combination with other anesthetics (Dohlman, 2017). LMICs are lacking in the provision and training for safe anesthesia practice. In a cross-sectional survey made in Zimbabwe involving 42 hospitals, the number of specialist physician anesthetics were limited (Lonnée et al., 2018). Further, 19% of the nurse anesthetists have had no formal training (Lonnée et al., 2018). In Nigeria, intravenous ketamine is used as general anesthesia in parts of the country where anesthesiologist's services are scarce. However, few patients experience adverse effects including high blood pressure, priapism, emergent delirium, tachycardia, disorientation and confusion (Iyalomhe and Iyalomhe, 2014). In a recent survey conducted in Tanzania, Malawi and Zambia, ketamine was widely used in many of the hospitals to compensate for shortages of

other forms of anesthesia. Anesthesia care in these countries were performed by non-physician anesthetists, some of whom had no formal training. Shortage of staff, interrupted access to electricity and water for some facilities and lack of functional anesthesia machines were reported (Gajewski et al., 2020). Though ketamine is considered safe, its application by untrained personal might lead to abnormal doses and contribute to adverse effects in these populations.

Methadone

Methadone is a synthetic opioid utilized for the treatment of chronic, acute and neuropathic pain. It is also used as an analgesic to treat pain in cancer patients and as a maintenance therapy for opiate addiction (Chou et al., 2009; Parsons et al., 2010; Kharasch, 2011). In opioid use disorder, methadone reduces the painful symptoms of opiate withdrawal and relieves drug craving by acting on the opioid receptor in the brain. Methadone is administered as a racemate consisting of the *S*-and *R*-enantiomers (Crettol et al., 2005; Ansermot et al., 2010). Methadone is metabolized in the liver via N-demethylation performed by various cytochromes including CYP3A4 and CYP2B6. CYP2B6 confers enantioselectivity for the S-enantiomer (Yang et al., 2016), further, the influence of CYP2B6 variants on the S-enantiomer has been reported (Kharasch and Crafford, 2019).

In vitro studies demonstrate differential metabolism and clearance of methadone by CYP2B6 variants ranging from CYP2B6*4 ≥ CYP2B6*1 > CYP2B6*5 > CYP2B6*9 ≥ CYP2B6*6 (Gadel et al., 2015). CYP2B6 genotype influences the plasma levels of both enantiomers (Victorri-Vigneau et al., 2019). Also, decreased clearance and high plasma concentration of methadone enantiomers was observed in patients with CYP2B6*1/*6 and CYP2B6*6/*6 compared to controls (Eap et al., 2007; Kharasch et al., 2015; Kringen et al., 2017; Talal et al., 2020). In a study involving 125 methadone fatality cases, the frequency of CYP2B6*9 was high in the methadone group compared to the control groups. High plasma levels of methadone were observed in individuals with the CYP2B6*5 homozygous genotype compared to the wild type and heterozygous genotype (Ahmad et al., 2017). Studies also show that CYP2B6 genotype influences the severity of neonatal abstinence syndrome (Mactier et al., 2017) in infants of methadone-maintained opioid-dependent mothers. Infants who needed treatment were more likely to carry the wild type genotype for CYP2B6*6 allele (516GG, 785AA) (Mactier et al., 2017). Studies involving Malay opioid dependent males revealed association between CYP2B6*6 and increased severity of pain (Zahari et al., 2016). The presence of concomitant diseases such as HCV infection influence methadone therapy. Reportedly, HCV patients often require higher doses of methadone. Most studies have reported the inability of patients, harboring the CYP2B6*6 allele to metabolize and clear the drug (Crettol et al., 2005; Hung et al., 2011; Bart et al., 2014; Csajka et al., 2016). Thus, patients are likely to experience unwanted effects of the drug. Therefore, dose reduction in this group of patients may yield methadone safety. Meanwhile, others have shown that the CYP2B6*4 allele results in increased metabolism of the drug, thus patients might require increased dose. Patient genotyping for CYP2B6 variants may be of importance when considering dose requirement in methadone maintenance treatment most especially among HCV and HIV patients.

Even though methadone maintenance therapy is effective and affordable, it is still unavailable in many LMICs where it is highly needed. High prevalence of HIV, hepatitis C virus (HCV) and tuberculosis is reported among people with drug use disorder (Wu and Clark, 2013; Larney et al., 2017). As reported in 2018, out of 179 countries with evidence of drug use disorder, opioid substitution therapy (OST) was available in only 86 countries (Avert, 2016; International HR, 2019). OST is a replacement therapy whereby prescribed medications such as methadone and buprenorphine are given to opioid dependent patients, which enables them to reduce or cease from injecting drugs. OST is not found in Nigeria and Zimbabwe despite the presence of drug addicts and HIV patients. However, the Nigerian government has initiated guidelines on the use of methadone for treatment of drug rehabilitation (International HR, 2018). OST is available in South Africa and on a smaller scale in Tanzania, Uganda, Senegal, and is highly expanding in Zanzibar and Kenya (International HR, 2019). In Asia, OST is present in most countries with the highest number in China and the least in Cambodia (International HR, 2019). In Eastern Europe and Central Asia, OST is applied in many countries but coverage is limited. In Western Europe and North American countries, OST is vastly available. However, in Germany opiate substitution treatment is variable between people in prison and those living outside of prison. According to a report by Stöver et al., the application of OST in German prisons depends on the federal state, the prison and prison doctors (Stöver et al., 2019). Existing barriers to accessing OST in both high and LMICs include criminalization and financial barrier, for example, OST is forbidden in Russia and Uzbekistan. Though OST is free in Australia, most people still buy it at a minimum cost of AU35 per week. If people with opiate addiction are not treated, the prevalence of HIV will continue to rise in LMICs. There is a need for more countries to provide OST with good coverage so as to reduce HIV, hepatitis C and mortality among drug users. There is need for the government to provide funds for the health care in some LMICs.

Artemisinin

Artemisinin-based combination therapy (ACT) is the basis of treatment and considered first-line for the majority of malaria infection cases (WHO, 2015). ACT, presently approved for treatment of uncomplicated *Plasmodium falciparum* malaria in many malaria-endemic countries, are substrates of CYP enzymes (Svensson and Ashton, 1999). Combining artemisinin agents, which are fast acting with a short half-life with partner drugs that have long half-life enables optimization of parasite killing and greatly protects against reinfection (White et al., 2014). Patient's response to antimalarial treatment can be influenced by factors such as quality of the antimalarial agent, the natural immune system of the host, parasite resistance, concomitant diseases and the pharmacokinetics of the malaria drug (Travassos and Laufer, 2009). Most studies on the efficacy of antimalarial agents

have focused more on understanding the resistant mechanism of the parasite by investigating the parasites multi drug resistant genes (Travassos and Laufer, 2009). However, studies have shown interindividual variations in the concentration of artimisinin, dihydroartemisinin, artesunate, and their anti-malarial effect among malaria infected individuals. Underdosing seems to enhance parasite resistance to the therapeutic agents. Due to the lack of efficacy, optimization of antimalarial treatment is the main factor considered toward global eradication of the diseases (WHO, 2015).

Among many other factors, host genetics, especially polymorphisms in CYP enzymes involved in the metabolism of the drugs, may be one of the confounders, causing variability in ACT drugs levels and treatment failure. *In vitro* studies show that artemisinin metabolism in human liver microsomes is mediated primarily by CYP2B6 with a contribution of CYP3A4 in individuals with low expression of CYP2B6 (Svensson and Ashton, 1999). So far, a report from Tanzania indicated their concern about metabolism of ACT and high prevalence of CYP2B6 G516T and other CYP polymorphisms in the population. The prevalence of CYP2B6 G516T in the Tanzanian population was 36% (Marwa et al., 2014). Authors did not evaluate the direct impact of these polymorphisms on treatment outcome or safety of the drugs (Marwa et al., 2014). Another study in the malaria-endemic population of Timor Leste indicated that the prevalence of CYP2B6*4, *9, and *6 might impact the metabolism and efficacy of artemisinin and its derivatives among the Timorians (Hananta et al., 2018). Another study amongst Nigerian HIV-malaria infected subjects explored the impact of CYP2B6 516GT polymorphism on NVR and artemether-lumefantrine drug-drug interaction. The authors showed that decreased exposure to artemether and desbutyl-lumefantrine caused by NVR was further enhanced by patients with CYP2B6 516GG genotype (ultrarapid metabolizers) (Abdullahi et al., 2020). Again, the CYP2B6 516TT genotype (poor metabolizers) also influenced increased exposure to dihydroartemisinin and lumefantrine caused by NVR (Abdullahi et al., 2020). According to the authors, the inductive effect of NRV on CYP2B6 and CYP3A4 enzymes, both of which are involved in the metabolism of these antimalarial drugs, might have caused this variability (Abdullahi et al., 2020). An Iranian study also indicated high prevalence of CYP2B6*2, *4, *5, *6, and *7 alleles among the Iranian Baluchi, which may affect patient response to artemisinin and derivatives (Zakeri et al., 2014).

Limited effort has been employed to determine genetic polymorphisms in CYP enzymes, which may lead to therapeutic failure in patients who are extensive metabolizers or cause toxicity and resistance in patients who are slow metabolizers (Gil, 2013). ACT is an effective treatment for resistant malaria, knowledge on polymorphisms influencing their efficacy may help to improve malaria therapy. The fight against over the counter drugs will help to reduce relapse among patients and improve the lives of people in the rural population.

The quality of malaria care offered in many malaria pandemic LMICs is still very poor, presumption diagnosis is common whereby treatment is provided to patients without any malaria test inspite of the WHO recommendation of "test and treat" (Macarayan et al., 2020). Over the counter poor quality malaria drugs, some of which have no active ingredients, are offered especially by smaller vendors (Bassat et al., 2016; Walker et al., 2018). In Uganda, compared to the urban population, the rural populations spent more money and experienced 97.9% of deaths due to poor quality antimalarial drugs.

DISCUSSION AND FUTURE PERSPECTIVE

CYP2B6 Pharmacogenetics and Drug Response

This article provides evidence on CYP2B6 functional variability in drug metabolism and exposure across populations. The impact of CPY2B6 variant on patient response to various substrates is evident in most ethnicities involved in this study. Depending on CYP2B6 genotype, patients may be vulnerable to ADRs ranging from mild to severe due to increased exposure to active oral drugs, or otherwise experience therapeutic failure due to reduced exposure to active metabolite in the case of prodrugs. Poor metabolizers are likely to experience more ADRs (active compounds) or treatment failure (prodrugs) than intermediate or normal metabolizers. According to literature, CYP2B6*6, which is a haplotype of two variants, CYP2B6*4 and CYP2B6*9, is the most studied allele (Li et al., 2012; Desta et al., 2021). Due to the increasing knowledge on the role of CYP2B6 enzyme in drug metabolism, there is the need to evaluate other CYP2B6 variants on substrate metabolism in various populations or ethnicity. According to the information gathered, some studies considered the G516T (*4) variant as CYP2B6*6 allele, to maintain a common style of variant nomenclature in articles. Researchers can make use of the publicly available pharmacogene variation (PharmVar) website, which clearly defines each haplotype or alleles (Desta et al., 2021). CYP2B6 loss of function alleles (poor metabolizer genotypes), which lead to an increase in individual active drug exposure and toxicity are frequent in some populations leading to high risk of ADRs in these populations.

In order to achieve the Joint United Nations Programme on HIV/AIDS (UNAIDS) 90-90-90 target and complete eradication of AIDS by 2030, pharmacogenetics testing for CYP2B6 to assist EFV therapy in patients who are unlikely to benefit from dolutegravir is of urgent need (Masimirembwa et al., 2016; Mukonzo et al., 2016). The Clinical Pharmacogenetics Implementation Consortium guideline for CYP2B6 and EFV-containing antiretroviral therapy might serve as a basis for implementation of CYP2B6 pharmacogenetic testing for EVF therapy (Desta et al., 2019). This may help to reduce ADRs and increase patient compliance with subsequent reduction in drug resistance due to lack of patient compliance. At the moment, many pharmacogenomics related ADRs are noticed only after the drugs have been administered to the patients. Population specific pharmacogenomics approaches at the level of drug development can be used to address differences in susceptibility to ADRs between populations. The inclusion of pharmacogenomics in clinical trials could give a clue on populations that are likely to benefit or suffer from adverse effects. This could guide dose adjustments for some populations.

Previous clinical trials included individuals from African descent to represent the African population, while it is clear that the African continent demonstrates high genetic diversity. Likewise, Asian Americans or Asian Europeans cannot represent the Asian population. Therefore, the inclusion of a diverse population in clinical trials is inevitable. Interestingly, intrapopulation as well as inter-individual variability in CYP2B6 alleles further complicates the efficacy of many of its substrates. This could potentially be improved by the application of patient genotyping prior to prescription in clinical practice. Thus, a robust and more personalized therapy could be provided to the patients.

Availability and Accessibility of Medication

According to our findings, HAART combination therapy that includes EFV or NVP is mostly used in LMICs despite the high frequency of CYP2B6 loss of function alleles in these populations. Thus, pharmacovigilance is urgently needed in these populations for the detection and subsequent prevention of ADRs. Contrary to LMICs, in HICs more potent, less toxic and novel antiretroviral drugs are used quite often. Higher donor dependency and cost of medication has been highlighted as barriers to the accessibility of quality and less toxic drugs in LMICs. Donor funding has saved many lives in LMICs for the past decades. The help from richer countries is provided in the form of finance or medication via Government officials or private agencies in health sectors. With the growing population in LMICs, it is evident that donor funding can no longer benefit every individual. Therefore, in this era, donors should focus more on human capacity building and establishment of infrastructures that will help LMICs become independent or self-sponsored (Pillai et al., 2018). For example, antiretroviral drugs are manufactured and sold at a cheaper rate in India compared to African countries, where drugs are mostly imported at a very high cost (Dickson, 2001). The African pharmaceutical industry, for example, could be expanded and strengthened. This could help to remove financial barriers to medicines as well as to improve access to more potent, expensive, less toxic medication. The public health sector needs to fully support organizations such as the African Pharmacogenomics Consortium (APC), which seeks to address the issue of drug safety, financial problems in the health care sector, disease burden, research training and implementation of pharmacogenomics in Africa (Dandara et al., 2019). There is a need for creating awareness, funding and provision of medication in countries where mental health has been neglected. For example, a significant reduction of heroin use and improvement of mental health was observed among participants who were retained for methadone therapy for 6 months in South Africa (Scheibe et al., 2020).

Health Polices and Patent

There is a need for amendments of foreign and government policies, that limit the growth of health care. According to Tomlinson et al., amending the patent law could improve affordability and accessibility of medicines in South Africa (Tomlinson et al., 2019). The government in LMICs needs to develop strategies to raise internal funds to support health care rather than solely depending on foreign aid. There is a need for allocation of healthcare budgets in both public and private sectors. Additionally, accountability and better amendment of funds in healthcare can help to improve health care services.

Comorbidities

People with two or more diseases need special attention and health care coverage, for example, people with HCV and HIV coinfection. The treatment of HIV might worsen HCV infection in such individuals. Thus, increasing health coverage and accessibility to diagnosis and counseling by trained medical staffs can help patients to receive the right therapy and avoid drug-drug interactions due to concomitant use of HIV and HCV drugs.

CONCLUSION

In conclusion, there is a high level of CYP2B6 genetic variability between and within ethnicities. In addition to other confounders that can affect the pharmacokinetics and pharmacodynamics properties of a drug, CYP2B6 genotyping could be considered in regards to all CYP2B6 substrates prescriptions in populations with expected high variability and drugs with narrow therapeutic window.

AUTHOR CONTRIBUTIONS

JS developed the presented idea, supervised the manuscript progress and data analysis, and published own data on CYP2B6 pharmacogenetics. SY, KJ, JB, and CM contributed to the manuscript content, data analysis, and literature search. IL did the systematic review of the literature, evaluated, analyzed the impact of the CYP2B6 alleles on drug safety in a global view, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Common Statin Intolerance Variants in *ABCB1* and *LILRB5* Show Synergistic Effects on Statin Response: An Observational Study Using Electronic Health Records

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Background: Statin intolerance impacts approximately 10% of statin users, with side effects ranging from mild myalgia to extreme intolerance resulting in myopathy and rhabdomyolysis. Statin intolerance results in poor adherence to therapy and can impact statin efficacy. Many genetic variants are associated with statin intolerance. The effect of these variants on statin efficacy has not been systematically explored.

Methods: Using longitudinal electronic health records and genetic biobank data from Tayside, Scotland, we examined the effect of seven genetic variants with previously reported associations with simvastatin or atorvastatin intolerance on the outcome of statin response. Statin response was measured by the reduction achieved when comparing pre- and post-statin non-high-density lipoprotein-cholesterol (non-HDL-C). Post-treatment statin response was limited to non-HDL-C measured within 6 months of therapy initiation. Univariate and multivariable linear regression models were used to assess the main and adjusted effect of the variants on statin efficacy.

Results: Around 9,401 statin users met study inclusion criteria, of whom 8,843 were first prescribed simvastatin or atorvastatin. The average difference in post-treatment compared to pre-treatment non-HDL-cholesterol was 1.45 (±1.04) mmol/L. In adjusted analyses, only two variants, one in the gene ATP-binding cassette transporter B1 (*ABCB1*; rs1045642), and one in leukocyte immunoglobulin like receptor B5 (*LILRB5*; rs12975366), were associated with statin efficacy. In *ABCB1*, homozygous carriers of the C allele at rs1045642 had 0.06 mmol/L better absolute reduction in non-HDL-cholesterol than carriers of the T allele

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(95% CI: 0.01, 0.1). In *LILRB5* (rs12975366), carriers of the C allele had 0.04 mmol/L better absolute reduction compared to those homozygous for the T allele (95% CI: 0.004, 0.08). When combined into a two-variant risk score, individuals with both the rs1045642-CC genotype and the rs12975366-TC or CC genotype had a 0.11 mmol/L greater absolute reduction in non-HDL-cholesterol compared to those with rs1045642-TC or TT genotype and the rs12975366-TT genotype (95% CI: 0.05, 0.16; $p < 0.001$).

Conclusion: We report two genetic variants for statin adverse drug reactions (ADRs) that are associated with statin efficacy. While the *ABCB1* variant has been shown to have an association with statin pharmacokinetics, no similar evidence for *LILRB5* has been reported. These findings highlight the value of genetic testing to deliver precision therapeutics to statin users.

Keywords: pharmacogenomics, non-HDL-cholesterol, *ABCB1*, *LILRB5*, statins, hyperlipidaemia

INTRODUCTION

Statins, or 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, are the most commonly prescribed cholesterol-lowering therapy (Ward et al., 2019). Statin at maximum doses can reduce low density lipoprotein-cholesterol (LDL-C) levels by 50% (Schachter, 2005; Mangravite et al., 2006; Taylor and Thompson, 2018; Newman et al., 2019). Large randomized clinical trials have reported a 20–30% reduction of cardiovascular diseases (CVD) among statin users (Baigent et al., 2005; Mangravite et al., 2006; Mihaylova et al., 2012).

However, there are interindividual differences in response to statin treatment: both in the ability of statins to reduce the LDL-C levels and in observed statin-related adverse drug reactions (ADRs) (Turner and Pirmohamed, 2019). It is estimated that 30% of statin users cease therapy by the end of the first year of treatment. Approximately 50% of patients at high risk of developing CVD discontinue taking their statins (Pedro-Botet et al., 2019; Bair et al., 2020). Among those who withdraw from treatment, about 1–7% discontinue taking statins due to ADRs (Vermes and Vermes, 2004; Oh et al., 2007; Donnelly et al., 2011).

Stain-induced ADRs can range from complaints of muscle pain referred to as myalgia to the more severe cases of myopathy, and finally, in extreme cases, can result in rhabdomyolysis (Alfirevic et al., 2014; Selva-O'Callaghan et al., 2018; Newman et al., 2019). Almost 60% of adults who discontinue statin therapy report muscle pain as the major cause of non-adherence (Pedro-Botet et al., 2019). It is understood that myalgia, whether or not associated with an elevation in the creatine kinase (CK) level, is the most common statin-induced ADR and is included in definitions of statin intolerance (Bair et al., 2020). The risk of ADR is greater during the first year of therapy (Armitage et al., 2010) and can be exacerbated by increases in statin dose, interacting concomitant medications, advanced age, or comorbidities (Newman et al., 2019). The exact prevalence of statin intolerance is difficult to estimate. It has recently been reported that myopathy is increased by <0.1% in individuals on statins than those on placebo (Amarenco et al., 2006; Ridker et al., 2008; Armitage et al., 2009; Newman et al., 2019). Randomized controlled trials using strict criteria to define myopathy suggested that prevalence is 1–3%. In studies with a more inclusive definition of statin intolerance, prevalence could be as high as 10–25% of cases (Oh et al., 2007).

Several genetic variants have been identified to be potentially associated with statin ADRs through genome-wide, exome-wide, and candidate gene studies. However, the impact of these variants on cholesterol reduction on a population level has not been understood (Canestaro et al., 2014; Brunham et al., 2018; Turner and Pirmohamed, 2019; Ward et al., 2019). In the present retrospective observational study, single nucleotide polymorphisms (SNPs) in the genes of ATP-binding cassette transporter B1 (*ABCB1*), Solute Carrier Organic Anion Transporter Family Member 1B1 (*SLCO1B1*), Leukocyte Immunoglobulin Like Receptor B5 (*LILRB5*), and Cytochromes P450 (*CYP*) family having known associations with statin ADRs were selected to assess their statin efficacy using electronic health records.

ABCB1

Polymorphisms in *ABCB1* play a vital role in the lipid-lowering response of statins. Variants such as rs1128503 (Gly412Gly, 1236C>T), rs2032582 (Ser893Ala, 2677G>A/T), and rs1045642 (Ile1145Ile, 3435C>T) have been linked to statins pharmacokinetics and statin tolerability (Fiegenbaum et al., 2005; Becker et al., 2009; Hoenig et al., 2011). In a study by Fiegenbaum et al. (2005), the 3435T variants at rs1045642 were associated with decreased risk of myalgia for people treated with simvastatin compared to allele C.

In another study, the T allele variants in rs1045642 were more frequently present in patients on atorvastatin who experienced muscle symptoms compared to those without the variant allele (Hoenig et al., 2011).

LILRB5

Leukocyte immunoglobulin like receptor B5 is highly expressed in skeletal muscle, liver, and gallbladder. *LILRB5* rs12975366 (Asp247Gly, T>C) was associated with important indicators of muscle damage such as serum CK (creatinine phosphokinase) and lactate dehydrogenase (LDH) levels as well as with statin

intolerance and statin-induced myalgia. Individuals homozygous for the wild Asp247 (TT) genotype were more likely to experience elevated CK and LDH levels as well as statin intolerance (Dubé et al., 2014; Kristjansson et al., 2016; Siddiqui et al., 2017).

SLCO1B1

Many studies extensively focused on *SLCO1B1* polymorphisms and their association with statin-induced myopathy (Puccetti et al., 2010; Donnelly et al., 2011; Brunham et al., 2012).

The *SLCO1B1* rs4149056 (Val174Ala, 521T>C) reduces hepatic uptake of statins. Recessive carriers of the variant experience a higher rate of ADRs (Link et al., 2008; Donnelly et al., 2011). *SLCO1B1* rs2306283 (Asp130Asn, 388 A>G) is a gain of function variant and is associated with statin tolerance (Donnelly et al., 2011).

Cytochrome P450 enzyme: *CYP3A4* and *CYP3A5*

Cytochromes P450 is a superfamily of enzymes involved in the metabolism of several drugs including statins. Variants in *CYP3A4* (rs2740574), *CYP3A5* (rs776746) have been shown to affect statin intolerance (Wilke et al., 2005; Becker et al., 2010).

Statin Response

Statin response is measured by reduction of cholesterol, typically LDL cholesterol. Recently, research has determined that non-high-density lipoprotein (non-HDL) cholesterol rather than LDL cholesterol is a better predictor of long-term residual cardiovascular risk (CV) risk in statin-treated individuals (Johannesen et al., 2021). Calculating non-HDL concentration provides a simple way to assess the total amount of pro-atherogenic lipoproteins (apolipoprotein B, i.e., *apoB*). Guidelines from the American Heart Association (AHA), European Society of Cardiology (ESC), and European Atherosclerosis Society (EAS) indicate using non-HDL cholesterol (non-HDL-C) calculated as total cholesterol minus HDL cholesterol to estimate the CV risk (Grundy et al., 2019; Mach et al., 2020; Johannesen et al., 2021).

There remains scepticism around ADRs to statin therapy. A recently concluded cross-over trial has found non-specific complaints of intolerance, i.e., equivalent rates of adverse effects reported, while on statins or placebo (Herrett et al., 2021). However, if ADR and indeed the associated genetic variants result in poor compliance or adherence to statin therapy, a knock-on effect would be observed on cholesterol reduction. Here, we examine variants associated with statin ADRs to determine if they impact non-HDL cholesterol response in the 6 months following commencement of statin therapy. We hypothesize that these variants would impact statin efficacy by lowering compliance with statin use.

MATERIALS AND METHODS

Study Design

This study utilizes data from two cohorts that are part of the Tayside Bioresource, University of Dundee: The Genetics of Diabetes Audit and Research in Tayside Scotland (GoDARTS) and Scottish Health Research Register and Biobank (SHARE). Both cohorts are based in the Tayside Region of Scotland, United Kingdom. Both cohorts have genetic biobanks alongside linked electronic health records and community prescribing records. All participants in GoDARTS and SHARE provide informed consent for their medical records to be anonymized and linked to biobanks for clinical and epidemiological research. The cohorts have been used extensively for pharmacogenetic research: to establish associations between statin intolerance and genetic variants, such as *SLCO1B1* and *LILRB5* genotypes (Donnelly et al., 2011; Siddiqui et al., 2017). These cohorts were also used in the discovery of the association between variants of the *F5* gene and an increased risk of ADRs to ACE-I therapy (angiotensin-converting enzyme inhibitors; Maroteau et al., 2020).

These cohorts comprise a consented bioresource with longitudinal follow-up containing complete electronic health records from the same local population. Details of the individual cohorts have been described elsewhere (McKinstry et al., 2017; Hébert et al., 2018). For the purposes of the current study, these cohorts were analyzed collectively as they are from the same base population, data are sourced identically and held in the same International Organization for Standardization 27,001 – and Scottish Government accredited secure safe haven. This approach substantially improves the statistical power of this analysis and overcomes the obstacle faced by most pharmacogenetic studies of insufficient power to detect effects.

Study Population

The study period was from 1st January 1990 to 31st January 2018. Prescribing and clinical data of cohorts were available from 78,534 individuals. The data linkage includes basic demographics, community prescribing records, biochemistry data from the region-wide clinical laboratory, Scottish Morbidity Records (SMR), detailing International Statistical Classification of Diseases and Related Health Problems (ICD) 9 and 10 codes for hospital admissions. The use of electronic linkage allows access to automatically updated NHS data, which includes hospital admissions, laboratory results, and the provision and fulfilment of prescriptions. Together these were used to characterize statin usage patterns, non-HDL cholesterol response, comorbidities such as CV disease, type 2 diabetes.

Study Definitions

Data for non-HDL-C was sourced from biochemistry files. Sex and age were determined from demographic data. Type 2 diabetes status from the Scottish Care Information – Diabetes Collaboration (Scottish Diabetes Survey Monitoring Group, 2011). Major adverse cardiovascular events (MACE) were determined using hospital admissions data. All prescribing features such as statin type, dose, statin switching, duration of therapy, and adherence were determined using community prescribing data.

Statin Efficacy Using Non-HDL-C Response to Therapy

Baseline non-HDL-C (pre-treatment value) was calculated as the nearest value available before statin initiation. The first

available non-HDL-C measurement available between 28 and 180 days after statin initiation was used. The non-HDL-C reduction was calculated as the difference between post-statin and pre-statin non-HDL-C (mmol/L). Absolute reductions are quoted in the text and tables throughout.

Statins

Individuals who changed statin type before the non-HDL-C measurement were defined as switchers. Duration of statin therapy was defined as the period between the first statin prescription and the follow-up non-HDL-C measure. The duration of therapy was calculated in days and then divided into 28 days to reflect the standard pack size of dispensed statin. To account for differences in potency among statin types, we used a simvastatin equivalent daily dose (Maron et al., 2000), and the mean of all doses during the follow-up was used as a covariate in the analysis. Any reduction or increase of the dose was also identified. Dose reduction before the first non-HDL-C reading was used as one of the predictors of statin intolerance. The percentage of daily coverage (PDC) was used as an indicator of adherence to medication, which can also indicate tolerability of statins. To do this, the quantity of dispensed pills (using pack size information) was calculated. Then the number of days of coverage was calculated based on dates of the first and last prescribed statins. Finally, using prescribing directions (e.g., 1/day or 2/day), we determined if the number of pills dispensed was sufficient for coverage over the period of study. The formula used has been described and used previously (Siddiqui et al., 2017).

Selection of Statin ADR Variants

Seven SNPs from five different genes were identified through recent systematic reviews (Canestaro et al., 2014; Turner and Pirmohamed, 2019; Ward et al., 2019; Kee et al., 2020) and were selected based on their association with simvastatin and atorvastatin ADRs.

In order to detect genotyping errors, all SNPs were tested for the Hardy-Weinberg equilibrium. We considered the following variants: *ABCB1* rs1128503, *ABCB1* rs1045642, *SLCO1B1* rs4149056 and rs2306283, *LILRB5* rs12975366, *CYP3A4* rs2740574, and *CYP3A5* rs776746.

Post hoc, on the basis of the variant effects (dominant, recessive, etc.) and their association with non-HDL-C response to statins, we developed a two-SNP unweighted risk score by considering risk alleles from both *ABCB1* rs1045642 and *LILRB5* rs12975366. There are two levels of this risk score; the protective genotypes were grouped into level 0 (individuals with *LILRB5* rs12975366 genotypes CC or TC and *ABCB1* rs1045642 genotype CC were classified as protected), while individuals with risky genotypes were grouped into level 1 (*LILRB5* rs12975366 genotype TT+ABCB1 rs1045642 genotypes CT or TT) and were classified as at risk of poor response to statins.

Statistical Methods

Continuous data were presented as a mean and SD; categorical data were expressed as counts and proportions. Analyses were carried out in the entire study population and then was restricted to simvastatin and atorvastatin users only. The association of non-genetic covariates with the outcome of non-HDL-C response was examined using univariate linear regression. Next, the univariate effect of the genetic variants with non-HDL-C response was examined in additive, recessive, and dominant models to determine the genetic effect model based on value of p and in concordance with literature. Subsequently, the appropriate genetic effect was examined in models adjusted for features of statin intolerance and in a model adjusted for all measured potential confounders. In the first adjusted model, features of statin intolerance were adherence to therapy (PDC was used as surrogate), switching to another type of statins, and dose reduction. In the second multivariable model, covariates added were the average dose of statin, duration of therapy, the diabetic status of the participant, a history of MACE, and finally, the model was adjusted for baseline level non-HDL-C. Analyses were conducted for each variant, with the hypothesis that they would be associated with statin response. However, these associations are likely to be confounded by statin intolerance and other measured confounders. Therefore, we selected variants that were significant after adjustment for all measured confounders. This included testing for epistasis and non-additive effects. Given the *a priori* hypothesis, results for SNP-wise association testing were considered statistically significant at a 5% level of significance. However, a correction for multiple testing (seven SNPs, three genetic models resulting in 21 independent test) was applied for the two-SNP risk score and results in a threshold of value of $p < 0.002$ for significance.

Guidelines and Guidance STrengthening the REporting of Genetic Association Studies (STREGA) were used to report this study (Little et al., 2009). All Statistical analysis was performed with SAS statistical software version 9.4 (SAS Institutes, Cary, NC, United States).

RESULTS

A total of 9,401 statin users with genotypic information met study inclusion criteria. A population flow chart details the definition of the study population and reasons for exclusion (**Supplementary Figure 1**). Briefly, of a total of 37,990 statin users, only 19,280 had the necessary baseline and follow-up non-HDL-C measured, of which 9,401 had genotype data available.

Demographics and Clinical Characteristics

At the time of commencement of statin therapy, the mean age of the participants was 63 years (SD ± 10.97). Females in the cohort constituted 45.3% of the total population (**Table 1**). About 71.4% of participants had type 2 diabetics and 18.6% had a history of prevalent CV disease before starting statin therapy. The majority of participants were

initiated on simvastatin (74.7%) or atorvastatin (19.4%) therapy, of which 3.1% switched therapy to another type of statin. About 38.6% of cases were prescribed a starting dose of 20 mg of simvastatin or an equivalent dose of other statins.

Statin Mediated Non-HDL-C Response

Pre-treatment non-HDL-C levels were measured at a median of 12 days (IQR: 4–35 days) before statin initiation. Post-treatment non-HDL-C measures were taken at a median of 75 days (IQR: 49–112 days) after commencing therapy. The mean baseline non-HDL-C level was 4.43 (±1.19) mmol/L, and the mean on-treatment change of non-HDL-C levels was calculated as an absolute reduction of 1.45 (±1.0) mmol/L. The difference in non-HDL-C levels was also calculated as percentage change from pre-treatment, where the median percentage reduction was 35.7% (IQR = 21.1–45.5%; **Table 1**).

Non-genetic Predictors of Non-HDL-C Response to Statins

Multiple covariates were significantly associated with non-HDL-C response to statin therapy; baseline non-HDL-C level was the major predictor of non-HDL-C reduction within 6 months of commencing statin therapy (beta 0.53 CI: 0.51, 0.54; $p < 0.001$). PDC, a surrogate for adherence to therapy, was also a significant predictor of non-HDL-C reduction (beta 0.26 CI: 0.23, 0.28; $p < 0.001$). The significant results of univariate regression of non-genetic variables and non-HDL cholesterol response are presented in **Supplementary Table 1**.

Association of Statin ADR Variants With Non-HDL-C Cholesterol Response to Statins

Minor allele frequencies of the variants were found to be similar to a reference white European population (Karczewski et al., 2020; **Supplementary Table 2**). The allele frequencies were in Hardy-Weinberg equilibrium for all seven SNPs.

We analyzed the effect of the variants on non-HDL-C in recessive, dominant, and additive genetic models, and the appropriate model was selected for further analyses (**Supplementary Table 3**). We examined the association of all the ADR variants with statin response in models adjusted for all confounders (**Table 2**). The only variants associated with statin response were in *ABCB1* rs1045642 (Ile1145Ile, 3435C>T; **Table 3**) and *LILRB5* rs12975366 (Asp247Gly, T>C; **Table 4**). Other selected variants did not show any significant association with change in non-HDL-C response in main effects or adjusted models (**Supplementary Tables 4–9**).

ABCB1 and *LILRB5* Effects

We found that the *ABCB1* rs1045642 (Ile1145Ile, 3435C>T) genotype as a recessive trait was associated with a significant reduction in non-HDL-cholesterol levels (beta 0.09 CI: 0.04, 0.14; $p = 0.001$). In models adjusted for features of statin usage, baseline non-HDL-C, type 2 diabetes, CVD, the outcome estimates were still significant. Individuals homozygous for the minor (C) allele had 0.08 mmol/L greater reduction of non-HDL-C (CI: 0.03, 0.13; $p = 0.003$) compared to carriers of the (T) allele (**Table 3**). The effect of the *LILRB5* rs12975366 variant was found to be dominant. In an adjusted model, carriers of (C) allele

TABLE 1 | Demographic and clinical descriptions of the study population.

Variables	First measurement within 6 months post-statin therapy (*n* = 9,401)	Simvastatin and atorvastatin users (*n* = 8,843)
Age at starting therapy, mean (SD)	63.06 (10.97)	63.03 (11.01)
Sex		
Female, *n* (%)	4,262 (45.3)	4,023 (45.5)
BMI kg/m^2, (*n* = 8,107), mean (SD)	30.49 (6.06)	30.49 (6.08)
Pre-statin non-HDL-C, mmol/L mean (SD)	4.43 (1.19)	4.42 (1.19)
Post-statin non-HDL-C, mmol/L mean (SD)	2.98 (1.03)	2.94 (1.01)
Mean absolute reduction in non-HDL-C, mmol/L (SD)	1.45 (1.04)	1.47 (1.04)
Median percentage reduction of non-HDL-C, % (IQR)	35.7 (21.1–45.5)	36.5 (22.4–45.9)
The first statin prescribed		
Simvastatin, *n* (%)	7,020 (74.7)	7,020 (79.4)
Atorvastatin, *n* (%)	1,823 (19.4)	1,823 (20.6)
Starting Simvastatin equivalent dose, mg (%)	20 (38.6)	20 (39.8)
Statin switchers before the measurement, *n* (%)	294 (3.1)	268 (3)
Mean duration of statin therapy/28 days, periods (SD)	2.96 (1.43)	2.96 (1.43)
Records of dose change before measurement		
Dose reduction	4,589	4,224
Dose increase	5,868	5,393
Mean adherence (SD)	1.54 (0.79)	1.54 (0.78)
Type 2 diabetes mellitus		
Yes, *n* (%)	6,715 (71.4)	6,285 (71.1)
History of MACE		
Prior to statin therapy, *n* (%)	1,749 (18.6)	1,603 (18.1)

TABLE 2 | Univariate effects of statin ADR variants on non-HDL-C reduction.

Gene/SNP	Genetic effect	Statin specificity	*p* (adjusted model)*
ABCB1/rs1128503	Dominant	Simvastatin/Atorvastatin	0.278
ABCB1/rs1045642	Recessive	Simvastatin/Atorvastatin	0.017
SLCO1B1/rs4149056	Recessive	Simvastatin/Atorvastatin	0.56
SLCO1B1/rs2306283	Dominant	Simvastatin/Atorvastatin	0.380
LILRB5/rs12975366	Dominant	Not specific	0.03
CYP3A4/rs2740574	Recessive	Simvastatin/Atorvastatin	0.140
CYP3A5/rs776746	Dominant	Simvastatin/Atorvastatin	0.534

**Model adjusted for all measured confounders.*

TABLE 3 | Effect of *ABCB1* (rs1045642686 3435C>T) on the absolute reduction in non-HDL-cholesterol in simvastatin and atorvastatin users.

Variables	Effect estimate (95% CI)		
	Univariate analysis (Model 1)	Model 2	Model 3
ABCB1 rs1045642	0.09 (0.04, 0.14)**	0.08 (0.03, 0.13)**	0.05 (0.01, 0.1)*
Percentage of daily coverage	-	0.27 (0.25, 0.30) **	0.22 (0.19, 0.24)**
Switching	-	−0.21 (−0.35, −0.08)**	−0.21 (−0.33, −0.09)**
Dose reduction	-	−0.25 (−0.36, −0.13)**	−0.18 (−0.27, −0.08)**
Mean dose	-	-	0.006 (0.005, 0.007)**
Duration of statin therapy	-	-	−0.04 (−0.06, −0.03)**
Type 2 diabetes	-	-	−0.13 (−0.17, −0.09)**
History of mace	-	-	−0.04 (−0.09, 0.01)
Non-HDL-C (baseline)	-	-	0.46 (0.45, 0.48)**

*Model 1: univariate effect, Model 2: features of statin intolerance, and Model 3: features of statin intolerance and important comorbidities. *p < 0.05; **p < 0.005.*

TABLE 4 | Effect of *LILRB5* (rs12975366) with the absolute reduction in non-HDL cholesterol to all statin treatment.

Variables	Effect estimate (95% CI)		
	Univariate analysis (Model 1)	Model 2	Model 3
LILRB5 rs12975366	0.04 (−0.01,0.08)	0.04 (−0.01,0.09)	0.05 (0.01,0.08)*
Percentage of daily coverage		0.27 (0.24,0.30)**	0.21 (0.19,0.24)**
Switching		−0.31 (−0.43,−0.19)**	−0.25 (−0.36,−0.14)**
Dose reduction		−0.08 (−0.12,−0.04)**	−0.18 (−0.22,−0.14)**
Mean dose		-	0.006 (0.005,0.007)**
Duration of statin therapy		-	−0.04 (−0.06,−0.03)**
Type 2 diabetes		-	−0.12 (−0.16,−0.08)**
History of mace		-	−0.04 (−0.09,0.01)
Non-HDL-C (baseline)		-	0.47 (0.45,0.49)**

*Model 1: univariate effect, Model 2: features of statin intolerance, and Model 3: features of statin intolerance and important comorbidities. *p < 0.05; **p < 0.005.*

TABLE 5 | Two-variant risk score for percentage reduction in non-HDL cholesterol.

	Statin type	Effect estimate (95% CI)		
		***LILRB5* rs12975366 *n* = 8569**	***ABCB1* rs1045642 *n* = 9256**	**Two-SNP risk score *n* = 8569**
Percentage reduction of non-HDL-C in adjusted models	All statins	0.45 (−0.45,1.35)	0.5 (−0.5,1.5)	1.61 (0.35,2.87)**
	Simvastatin + atorvastatin	0.44 (−0.48,1.35)	0.79 (−0.25,1.8)	1.82 (0.54,3.11)**
		n = 8070	*n* = 8709	

*Models shown were adjusted for all features of statin intolerance, sex, age, BMI, daily dose, duration of therapy, switching therapy, prevalent type 2 diabetes, history of MACE, and baseline non-HDL cholesterol.**p < 0.005.*

at rs12975366 had a significantly greater reduction of non-HDL-C (beta 0.04 CI: 0.004, 0.08; $p = 0.03$) compared to non-carriers (**Table 4**).

We tested the interaction between variants in *ABCB1* and *LILRB5* in a model also adjusted for the main effect of these variants. The interaction term was found to be significant ($p = 0.001$). The most significant effect was observed in carriers of both variants (beta 0.14, CI: 0.08, 0.21; $p < 0.001$) compared to non-carriers. Based on the significant interaction, we developed a two-variant risk score by combining the recessive *ABCB1* and dominant *LILRB5* variants. Carriers of both *ABCB1* (CC) variant and the protective variants for *LILRB5* (C allele) carriers had 0.1 mmol/L (CI: 0.05, 0.16; $p < 0.001$) reduction in non-HDL-C compared to non-carriers of the *ABCB1* and *LILRB5* variants (**Supplementary Table 10**). The combined effect of the *ABCB1* rs1045642 and the *LILRB5* rs12975366 variants was 1.61% of non-HDL-C reduction. In comparison, the expected additive effect would be 0.95% (**Table 5** and **Figure 1**), suggesting that the genetic effects are synergistic. Since *ABCB1* is involved in the pharmacokinetics of simvastatin and atorvastatin only, we restricted our analyses to individuals prescribed those two statins. We found that the main effect of the two-SNP risk score was strongest in subjects prescribed simvastatin (beta 0.16, $p < 0.001$, $n = 6{,}411$; **Supplementary Table 11**) and slightly weaker in those prescribed either simvastatin or atorvastatin (beta 0.14, $p < 0.001$, $n = 8{,}070$; **Table 6**). In this sub-group, the two-SNP risk score in an adjusted model improved non-HDL-C response by 1.82%, whereas the expected additive effect would be 1.23% (**Table 5**), confirming the

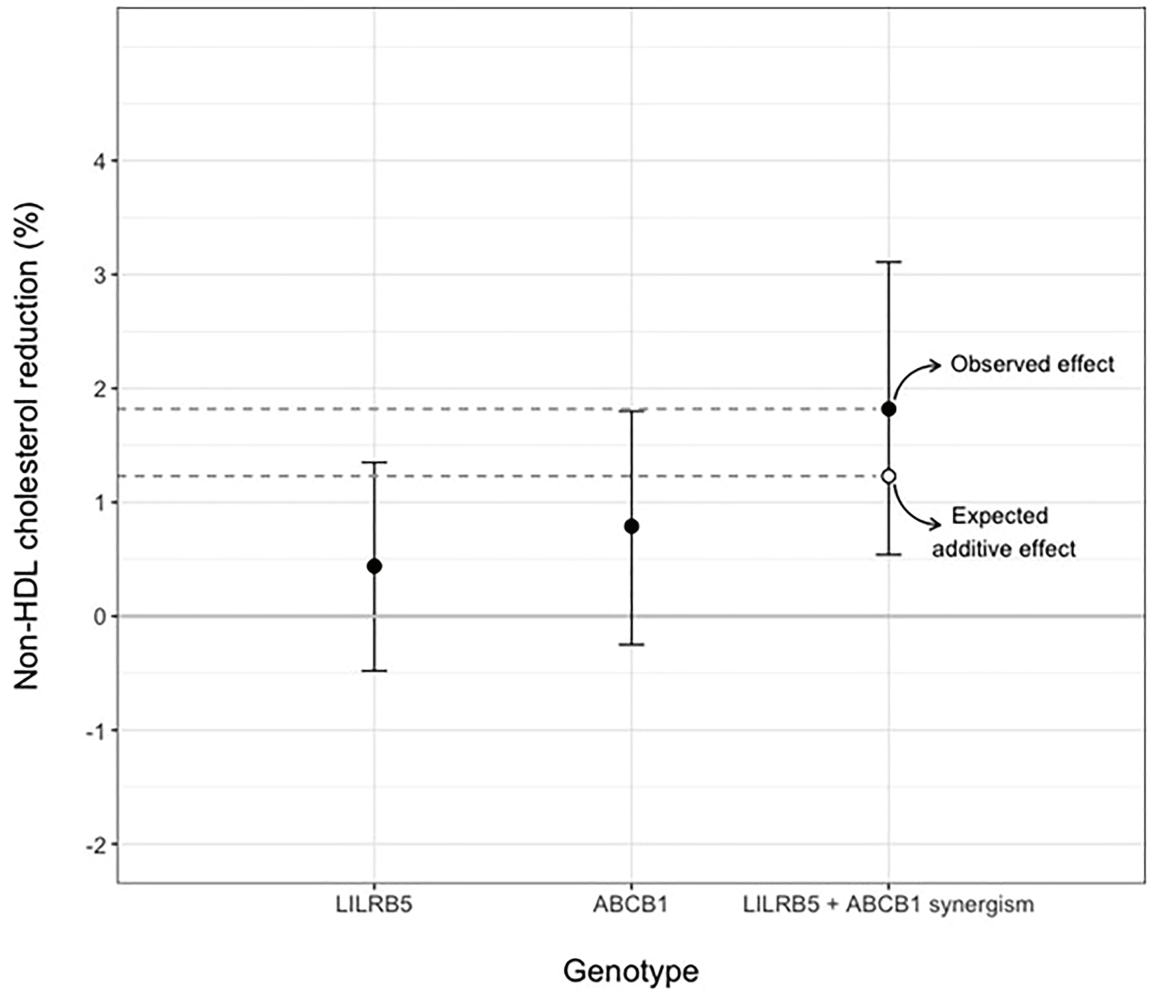

FIGURE 1 | Synergistic effect of *LILRB5* and *ABCB1* two-variant risk score on percent reduction of non-HDL cholesterol in simvastatin and atorvastatin users. The observed effect was a reduction of 1.82% whereas the expected effect was 1.23%.

synergistic nature of the interaction in adjusted and statin-specific models.

DISCUSSION

This study, leveraging detailed genetic, clinical, and drug dispensing data from nearly 9,000 statin users, finds that two statin ADR variants in *ABCB1* and *LILRB5* are associated synergistically with non-HDL-cholesterol response to statin therapy. Together, individuals homozygous for the C allele in rs1045642 *ABCB1* and carriers of the C allele in rs12975366 *LILRB5* were associated with 0.14 mmol/L greater reduction of non-HDL-C in response to simvastatin or atorvastatin therapy compared to those carriers of both the T allele in rs1045642 and those homozygous for the T allele in rs12975366. In main effects analyses, the actual observed effect was greater than the expected additive effect of these two variants. This effect was more pronounced when considering the percentage reduction of non-HDL-C as opposed to the absolute difference. The expected additive effect would be 1.23%, whereas the observed effect was a 1.82% better reduction in variant carriers. Crucially, there was no significant association between these variants and baseline non-HDL-cholesterol or the duration of statin therapy.

Although, some previous studies have found a higher post-treatment reduction of LDL-C in individuals carriers of the T variant genotype at rs1045642 (Kajinami et al., 2004; Kadam et al., 2016), results were inconclusive and a metanalysis indicated that CC variant was associated with decreases in LDL-C levels upon statin treatment when compared to the TT variation (Su et al., 2015). We report that individuals with the homozygous CC variant had 0.09 mmol/L higher reduction of non-HDL-C in comparison to those carriers of the T allele.

LILRB5 rs12975366 did not significantly predict the absolute non-HDL-C reduction univariately, but controlling for

TABLE 6 | Effect of *LILRB5* and *ABCB1* two-variant risk score on the absolute reduction of non-HDL cholesterol in simvastatin and atorvastatin users (n =8,070).

Variable	Effect estimate (95% CI)		
	Univariate analysis (Model 1)	Model 2	Model 3
LILRB5 rs12975366 (CC or TC) + *ABCB1* rs1045642 (CC) vs. *LILRB5* rs12975366 (TT) + *ABCB1* rs1045642 (CT or TT)	0.14(0.08,0.21)**	0.13(0.07,0.19)**	0.10(0.04,0.15)**
Percentage daily coverage	-	0.27(0.24,0.30)**	0.22(0.19,0.24)**
Switching	-	−0.31(−0.44,−0.18)**	−0.24(−0.35,−0.13)**
Dose reduction	-	−0.06(−0.11,−0.02)*	−0.15(−0.19,−0.12)**
Mean dose	-	-	0.006(0.005,0.007)**
Duration of statin therapy	-	-	−0.04(−0.06,−0.03)**
Type 2 Diabetes	-	-	−0.12(−0.17,−0.08)**
History of MACE	-	-	−0.04(−0.09,0.01)
Non-HDL-Cat baseline	-	-	0.48 (0.46,0.49)**

*Model 1: univariate effect, Model 2: features of statin intolerance, and Model 3: features of statin intolerance and important comorbidities. *p < 0.05; **p < 0.005.*

confounders and crucial covariates including baseline non-HDL-C in multiple regression models allowed us to estimate a less biased association between the Asp247Gly variant and the absolute reduction of non-HDL-C level. The genotype significantly predicted the percentage reduction of non-HDL-C in both univariate and adjusted models. We hypothesize that together carriers of the C allele of rs12975366 in *LILRB5*, which has been shown to increase statin tolerance, and the CC genotype of rs1045642 in *ABCB1*, which impairs statin excretion from the liver leading to a higher hepatic concentration, result in an enhanced response to the drug.

A limitation of the study is that over 94% of the population were simvastatin or atorvastatin users. Therefore, the results can only be generalizable to populations prescribed either of these drugs. Since these two statins share pharmacokinetic pathways, particularly since they are both substrates for the hepatic efflux transporter *ABCB1*, the results are likely to apply to users of either statin. However, the effects observed for the *LILRB5* variant are not specific to the type of statin as the original effects of the variant were observed in users of simvastatin, atorvastatin, and rosuvastatin and since this is not a pharmacokinetic variant. Further analyses in large observational cohorts are necessary to understand the relationship of statin ADR variants with other statins such as rosuvastatin and pravastatin.

Furthermore, these results would need to be replicated in *post hoc* analyses of randomized clinical trials and in pharmacokinetic studies in order to assess the value of clinical implementation.

Additionally, due to insufficient high-quality genetic data, a polymorphic variant in *ABCB1* (rs2032582) was not examined in this study. This variant forms a haplotype along with the two other *ABCB1* variants examined in this study. However, as documented, the haplotype effect is largely driven by the variant, we have examined, rs1045642.

The lack of association with *SLCO1B1* is surprising as it is the best-documented statin ADR variant. A *SLCO1B1* risk score was also created based on the described haplotype effect by Donnelly et al. (2011), who also did not find the genetic risk score to be associated with LDL-c response in adjusted models. This gene risk score was also not associated with differential response to statins. Similar to our findings, no significant differences in lipid-lowering effect between different *SLCO1B1* genotypes were reported in different studies including genome-wide association studies conducted in white Europeans (Turner and Pirmohamed, 2019; Chen et al., 2020). In a meta-analysis of 13 studies of the association between *SLCO1B1* polymorphisms and the effectiveness of statin in lipid reduction, it was concluded that both 521C and 388G do not affect the lipid-lowering effects of statins. However, in two different sub-analysis one for subjects on a long term treatment of statins (>6 months), and another for individuals of non-Asian ethnicities, results showed that those with the wild variant TT had a significant more LDL reduction compared with CC and TC variants (Dai et al., 2015). Similarly, no significant association between haplotype and mean percentage reduction in lipid and lipoprotein levels after simvastatin treatment for 6 months was reported in a study by Sortica et al. (2012).

A potential explanation for this lack of association is that the total hepatic exposure to a statin may not be significantly decreased by the change of hepatic uptake in the carriers of the alternative allele and that the effect is more significant on plasma exposure. Therefore, carriers of the minor allele have an increased risk of ADR without a remarkable change in efficacy. Hence, the association between the *SLCO1B1* genotype and ADR is more consistent than its association with the cholesterol-lowering effect of statins. It is also possible that hepatic concentration of statin and statin metabolites for *SLCO1B1* variant carriers is enough to show a lipid-lowering effect at higher daily doses and that the effect of the genetic variant may only appear at lower daily doses. Donnelly et al. (2011) reported a significant association of rs4149056 (Val174Ala) with a higher incidence of statin intolerance and lower LDL-C response. However, when adjusted for features of statin intolerance, the effect was non-significant. Further, once statin-intolerant individuals were removed from

the analysis, the association between *SLCO1B1* genotypes and LDL-C response remained non-significant. This result highlights the possibility that variants in this gene have a non-pleiotropic effect on statin ADRs (Donnelly et al., 2011).

A *post hoc* power analysis shows that the study is sufficiently powered to detect non-HDL-C changes as small as 0.07 mmol/L for genetic variants with MAF greater than 0.42. Whereas, for variants such as rs4149056 (Val174Ala; MAF = 0.16) the minimum detectable difference would be 0.2 mmol/L. Therefore, it is possible that this study is insufficiently powered to detect effects for rs4149056 (Val174Ala) variant in *SLCO1B1* or for rs2740574 in *CYP3A4*.

It is also likely that individuals who were prescribed low doses of statins do not have a high non-HDL-cholesterol lowering requirement. While, we have adjusted for dose, history of MACEs, and baseline non-HDL-C, there may still be residual confounding diluting the genetic effects we report. In our data, the median simvastatin equivalent daily dose was 20 mg, and only 5% of patients started on a therapeutic dose less than 10 mg daily, which implies that our analysis lacks the statistical power to detect differences in these groups.

The study demonstrates real-world prescribing, behaviors, and effects. The duration of follow-up allows us to avoid heterogeneous effects associated with differential lengths of statin use. With longer follow-up, other confounding factors arise – changes to, e.g., diet, exercise, changes to statin type, and dosing regimens. Some of these are hard to measure. It also reflects the first clinical interaction after the commencement of statin use, where a medical professional assesses the observed efficacy of the statin. This time point is crucial as 66% of the population in our cohort is assessed by the end of these 6 months.

CONCLUSION

These results highlight the value in genotyping statin ADR variants, as they affect tolerance to statins and statin efficacy. Even though, some of these variants have proven evidence of association with statin ADRs (e.g., variants in SLCO1B1), genetic testing is still limited. Li et al. (2014) compared a group of genotyped patients to a non-genotyped group. They found a significantly greater reduction in LDL-C within the genotyped group compared to non-genotyped. The same group also had more new statin prescriptions as well as better adherence. Interestingly in this study both carriers and non-carriers of the risk alleles benefited from genetic testing, which may suggest that genotyping may even provide benefits to the patient regardless of the test result.

Our two-SNP risk score was associated with a 1.82% change in statin treated individuals. Oni-Orisan et al. (2018) recently demonstrated that doubling of statin dose was associated with an approximately 5–10% reduction in non-HDL cholesterol. Thus, our observed reduction due to the two-SNP risk score is equivalent to a 36–73% increase in statin dose. With the polemics around the nocebo effect in statin-treated individuals (Herrett et al., 2021), such findings carry weight as they demonstrate an effect on statin efficacy independent of poor adherence.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: Restrictions applied to datasets. The datasets presented in this article are not readily available as they contain individual-level identifiable information. All analyses of anonymized data are performed in an International Organization for Standardization 27,001– and Scottish Government–accredited secure safe haven. Data requests can be initiated by contacting the corresponding author. Requests to access these datasets should be directed to MS (m.k.siddiqui@dundee.ac.uk).

ETHICS STATEMENT

The GoDARTS studies involving human participants were reviewed and approved by Tayside Medical Ethics Committee 053/04 and East of Scotland Ethics committee NHS REC 13/ES/0020. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AM, MC, MB, CP, and MS contributed to the conception and design of the study. AM and MC performed the data cleaning and statistical analysis. MB assisted with statistical analyses and interpretation. CM, AT, AD, RP, AT, and CP assisted with data curation, interpretation, and critical revision of the manuscript. AM and MS wrote the first draft of the manuscript and critically revised the manuscript. All authors contributed to the article 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/fgene.2021.713181/full#supplementary-material

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