RECENT DEVELOPMENTS IN PHARMACOGENETICS AND PHARMACOGENOMICS

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RECENT DEVELOPMENTS IN PHARMACOGENETICS AND PHARMACOGENOMICS

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REVIEW
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Germline and Somatic Pharmacogenomics to Refine Rectal Cancer Patients Selection for Neo-Adjuvant Chemoradiotherapy

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Neoadjuvant chemoradiotherapy (nCRT) followed by radical surgery is the standard of care for patients with Locally Advanced Rectal Cancer (LARC). Current selection for nCRT is based on clinical criteria regardless of any molecular marker. Pharmacogenomics may be a useful strategy to personalize and optimize nCRT in LARC. This review aims to summarize the most recent and relevant findings about the role of germline and somatic pharmacogenomics in the prediction of nCRT outcome in patients with LARC, discussing the state of the art of their application in the clinical practice. A systematic literature search of the PubMed database was completed to identify relevant English-language papers published up to January 2020. The chemotherapeutic backbone of nCRT is represented by fluoropyrimidines, mainly metabolized by DPD (Dihydro-Pyrimidine Dehydrogenase, *DPYD*). The clinical impact of testing *DPYD*2A, DPYD*13*, *c.2846A > T* and *c.1236G > A-HapB3* before a fluoropyrimidines administration to increase treatment safety is widely acknowledged. Other relevant target genes are *TYMS* (Thymidylate Synthase) and *MTHFR* (Methylene-Tetrahydro-Folate Reductase), whose polymorphisms were mainly studied as potential markers of treatment efficacy in LARC. A pivotal role of a *TYMS* polymorphism in the gene promoter region (*rs34743033*) was reported and was pioneeringly used to guide nCRT treatment in a phase II study. The pharmacogenomic analysis of other pathways mostly involved in the cellular response to radiation damage, as the DNA repair and the activation of the inflammatory cascade, provided less consistent results. A high rate of somatic mutation in genes belonging to PI3K (Phosphatidyl-Inositol 3-Kinase) and MAPK (Mitogen-Activated Protein Kinase) pathways, as *BRAF (*V-raf murine sarcoma viral oncogene homolog B1), *KRAS* (Kirsten Rat Sarcoma viral oncogene homolog), *NRAS* (Neuroblastoma RAS viral (v-ras) oncogene homolog), *PIK3CA* (Phosphatidyl-Inositol-4,5-bisphosphate 3-Kinase, Catalytic Subunit Alpha), as well as *TP53* (Tumor Protein 53) was reported in LARC. Their pharmacogenomic role, already defined in colorectal cancer, is under investigation in LARC with promising results concerning specific somatic mutations in *KRAS* and *TP53*, as predictors of tumor

response and prognosis. The availability of circulating tumor DNA in plasma may also represent an opportunity to monitor somatic mutations in course of therapy.

Keywords: pharmacogenomics, rectal cancer, neo-adjuvant chemoradiotherapy, germline, somatic, polymorphism, mutation

NEO-ADJUVANT THERAPEUTIC APPROACHES IN LOCALLY ADVANCED RECTAL CANCER

The standard of care for stage II/III Locally Advanced Rectal Cancer (LARC) is neo-adjuvant chemoradiotherapy (nCRT) followed by radical surgery including total mesorectal excision, with the possibility of an adjuvant chemotherapy. This program is based on a long-course radiotherapy (RT, 25-28 fractions in 5-6 weeks) and concomitant chemotherapy including fluoropyrimidines (FP), mainly capecitabine, administered daily for the whole course of treatment.

Despite the progress in the local disease control due to the introduction of nCRT in the treatment of patients with LARC, still there is a considerable proportion of patients (20%) that could be defined as non-responders, showing only minimal tumor response or poor prognosis mainly due to early progression. Another 40% of patients have a partial response and only a variable fraction of 8%–30% of patients achieve a pathological complete response at the time of surgery. The response to nCRT correlates with long-term patients' outcome considering disease-free survival (DFS) and overall survival (OS). Moreover patients achieving a major or complete clinical response after nCRT may be considered for an organ preservation strategy, (i.e., Local Excision, or Wait&Watch) and could avoid an adjuvant treatment (Van Der Valk et al., 2018). Furthermore, nCRT is not devoid of the risk of adverse drug reactions that could in some cases interfere with the treatment plans impacting not only patients' safety and quality of life, but also the success of the anti-tumor treatment.

In the clinical practice it is widely known the crucial importance of the risk stratification on patients. In order to reduce toxicity and improve the patients quality of life, a new generation of studies proposed risk adapted strategies including intensified programs in high risk patients and the possibility to avoid nCRT in low risk patients. Several trials were previously conducted in order to clarify the potential benefit of adopting a chemotherapy association regimen combining FP to other agents, as oxaliplatin or irinotecan (Valentini et al., 2019), or to anti-epidermal growth factor receptor (EGFR) monoclonal antibodies as cetuximab. On the other hand, nCRT intensification including a higher dose of RT appears as a promising approach (Burbach et al., 2014). More recently, Total Neoadjuvant Therapy strategies are hypothesised for high-risk sub-cohorts of patients. These programs are based on the integration of a neoadjuvant chemotherapy consisting in the administration of two or three different drugs, before or after the nCRT. The rationale for this strategy comes from the failure of adjuvant chemotherapy programs caused in most studies by the poor compliance of patients.

The selection of patients for these personalized treatment strategies is currently based essentially on clinical-pathological criteria, including tumor size, clinical T and N stages, distance of tumor from the anal verge, and interval from nCRT to surgery. In particular, some pathological features as circumferential tumor, tumor differentiation, mucinous histology and the presence of macroscopic ulceration, being associated with poor response to nCRT (Hur et al., 2011; Bitterman et al., 2015; Mccawley et al., 2016), demonstrated a detrimental effect in the neo-adjuvant treatment of LARC. Recently, the patients' stratification was shown to be improved using nomograms integrating clinical and radiomic features that may be also extracted from daily images acquired for image-guided RT (Dinapoli et al., 2018; Pirrone et al., 2019). However, despite the promising results, it would be necessary to deepen the knowledge on this topic, by conducting studies on larger

Abbreviations: 5-FU, 5-fluorouracil; AKT, v-akt murine thymoma viral oncogene homolog; AKT,v-akt murine thymoma viral oncogene homolog; APE1, apurinic/apyrimidinic endodeoxyribonuclease 1; AREG, amphiregulin; ATM, ATM serine/threonine kinases; ATR, ATR serine/threonine kinases; BCL2L10, Bcl-2-like protein 10; BER, base excision repair; BRAF, B-Raf proto-oncogene, serine/threonine kinase; CCDN1, cyclin D1; CORO2A, coronin 2A; COX-1, -2, cycloxygenase-1, -2; ctDNA, circulating tumor DNA; DFS, disease-free survival; DLC1, DLC1 Rho GTPase activating protein; DNAH14, dynein axonemal heavy chain 14; drosha, double-stranded RNA-specific endoribonuclease; DSB, double-strand breaks; DYPD (encoding DPD), dihydropyrimidine dehydrogenase; EGFR, epidermal growth factor receptor; ERCC1, -2, ERCC excision repair 1, -2; FAM101A, refilin A; FGFRs, fibroblast growth factor receptors; FP, fluoropyrimidine; GST, glutathione S-transferase; HIF1A (encoding HIF-1α), hypoxia inducible factor 1 subunit alpha; hOGG1, 8-oxoguanine DNA glycosylase; HR, homologous recombination; ICAM-1, Intercellular Adhesion Molecule 1; IL-X, interleukin-X; ITIH5, inter-alpha-trypsin inhibitor heavy chain 5; KRAS, KRAS proto-oncogene, GTPase; LARC, locally advanced rectal cancer; MAPK; mitogen activated protein kinase; MDM2, MDM2 proto-oncogene; MGMT, O6-methylguanine DNA methyltransferase; MLH1, mutL homolog 1; MPO, myeloperoxidase; MSH6, mutS homolog 6; MTHFR, methylenetetrahydrofolate reductase; mTOR, mammalian target of rapamycin; nCRT, neoadjuvant chemoradiation; NER, nucleotide excision repair; NFKB, nuclear factor κB; NHEJ, non-homologous end joining; NOS3, nitric oxide synthase 3; NRAS, NRAS proto-oncogene, GTPase; OS, overall survival; PAI-1, plasminogen activator inhibitor type 1; PAR-1, protease-activated receptor-1; PFS, progression-free survival; PI3K, phosphoinositide-3-kinase; PI3KCA, phosphoinositide-3-kinase catalytic subunit alpha; PTEN, phosphatase and tensin homolog; PTGS-1, -2, prostaglandin-endoperoxide synthase 1, - 2; RAD51, RAD51 recombinase; RAET1L, retinoic acid early transcript 1L; ROS, reactive oxygen species; RT, radiotherapy; SMAD-3, SMAD family member 3; SOD2, superoxide dismutase 2; TGF-β1, transforming growth factor beta 1; TNFA (encoding TNF-α), tumor necrosis factor-alpha; TP53 (encoding P53), tumor protein p53; TP73, (encoding P73) tumor protein p73; TRBP, trans-activation-responsive RNA-binding protein; TYMS (encoding TS), thymidylate synthase; UTR, untranslated; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; XPD, -A, xeroderma pigmentosum group D, -A; XRCC1, -3, x-ray repair cross-complementing 1, -3.

datasets, involving a wider range of MRI texture features to enhance the predictive value of these parameters, with further requirement of independent validation to improve the test sensitivity and specificity.

In this context, pharmacogenomics may be a useful strategy to personalize and optimize nCRT in patients with LARC (Agostini et al., 2014; De Mattia et al., 2015; Cecchin et al., 2018). The outcome of an anti-cancer treatment may depend on two genomes: the patient's germline and the tumor cell genomes. The genetic features of the cancer cells are generally related to disease aggressiveness and sensitivity to treatment, whereas the germline genetic variation can mostly impact drugs pharmacokinetics and pharmacodynamics. Consequently, this led to the hypothesis that some patients have germline polymorphisms or somatic mutations in genes encoding for protein with a major role in the nCRT LARC treatment as drug-target, drug-metabolizing enzymes, DNA-repair enzymes, and others that may affect response and safety profile of chemotherapy and radiotherapy in LARC. In this review, we will summarize recent advances in tissue and blood-based pharmacogenomic biomarkers for predicting nCRT response and toxicity in patients with LARC.

By a systematic literature search we reviewed the available pharmacogenomic studies that attempted to identify the role of germline and somatic pharmacogenomics in predicting chemotherapy and radiotherapy based nCRT outcome in patients with LARC.

MATERIALS AND METHODS

We performed a systematic literature search on January 2020 using the PubMed Medline Database and the combinations of the following terms: (chemotherapy OR radiotherapy OR chemoradiotherapy) AND "rectal cancer" AND (polymorphism* OR pharmacogenetic* OR pharmacogenomic* OR mutation*) AND (toxicity OR recurrence OR relapse OR survival OR progression). The literature search was completed by adding other articles that were identified with a hand search of the references of relevant works. As inclusion criteria the studies had to be published in English in a peer-reviewed journal. All the papers obtained by the PubMed search were reviewed for those specifically assessing the role on host and somatic genetic markers in predicting the clinical response in patients with LARC receiving pre-operative RT combined with FP-based chemotherapy, with or without other drugs (e.g., oxaliplatin, irinotecan, cetuximab). Eventually, nine and forty-two studies, addressing the host genetic profile's ability to predict the toxicity (**Supplementary Table 1**) and efficacy (**Supplementary Table 2**) of nCRT in patients with LARC, respectively, were selected. With regard to these specific papers, the attention was focused on details (**Supplementary Tables 1** and **2**) concerning the pharmacogenomic panel analyzed, the study population (e.g., sample size, ethnicity) and therapy (e.g., dose and schedule) characteristics, the clinical end-points evaluated and the adopted scoring system for toxicity or Tumor Regression Grade (TRG) classification, along with the main findings (e.g., statistical results). Additional fifteen works concerning the involvement of somatic mutations in determining the nCRT outcome in patients with LARC were selected and discussed in the manuscript. Seven out of these works, published between 2010 and 2012, are aggregated in a meta-analysis (Clancy et al., 2013) to which we referred in the text.

NEO-ADJUVANT RADIO-CHEMOTHERAPY COMBINATION TREATMENT IN LARC AND PHARMACOGENETIC MARKERS OF TOXICITY

FP-based nCRT is the standard backbone chemotherapy for pre-operative treatment of patients with LARC. FP exert their anti-tumor action as antimetabolite drugs through different mechanisms including impairment of thymidylate synthase (TS) activity, RNA synthesis and function leading to DNA strand breakage. FP are largely used in the treatment of several malignancies demonstrating a significant improvement in the patients' survival. However, these drugs present a narrow therapeutic index and around 15%–30% of patients suffer from severe toxicity such as diarrhea, nausea, mucositis, stomatitis, myelosuppression, neurotoxicity, and hand-foot syndrome, depending on the treatment regimen. These side effects lead to mortality in approximately 0.5%-1% of patients following 5-fluorouracil (5-FU) and capecitabine monotherapy, respectively (Lunenburg et al., 2016) (**Figure 1**).

FP are used in the neo-adjuvant treatment to increase the radiotherapy cytotoxic effect: this association can exacerbate the toxicity related to the chemotherapeutic treatment. The focused-damaging cytotoxic effect of radiotherapy is exerted on cell structures, proteins, and DNA of tumor cells. Particularly, the DNA is the main target of ionizing radiation. DNA structure is altered by the direct damage of radiation, that causes both single- and double-strand breaks in the DNA molecule, and by the indirect damage of generated free-radicals. The surrounding normal tissue is commonly also affected, resulting mainly in radiation-related intestinal injury, including acute inflammation and late fibrosis. Other radiation-induced early side effects include mucositis, vomiting, diarrhea, cystitis, perineal dermatitis, and hematological dysfunction. Bowel dysfunction, fecal incontinence, bleeding, perforation, genitourinary dysfunction, and pelvic fractures constitute most of the late toxicity (Joye and Haustermans, 2014). Severe adverse events related to the administration of nCRT require in many cases therapy delay, reduction or even termination; moreover, the patient's quality of life may be majorly affected, both acutely and chronically, by impaired organ functions. The literature data related to each gene or pathway investigated in the context of the neo-adjuvant treatment of LARC are reported in the following paragraphs (**Supplementary Table 1**, **Table 1**).

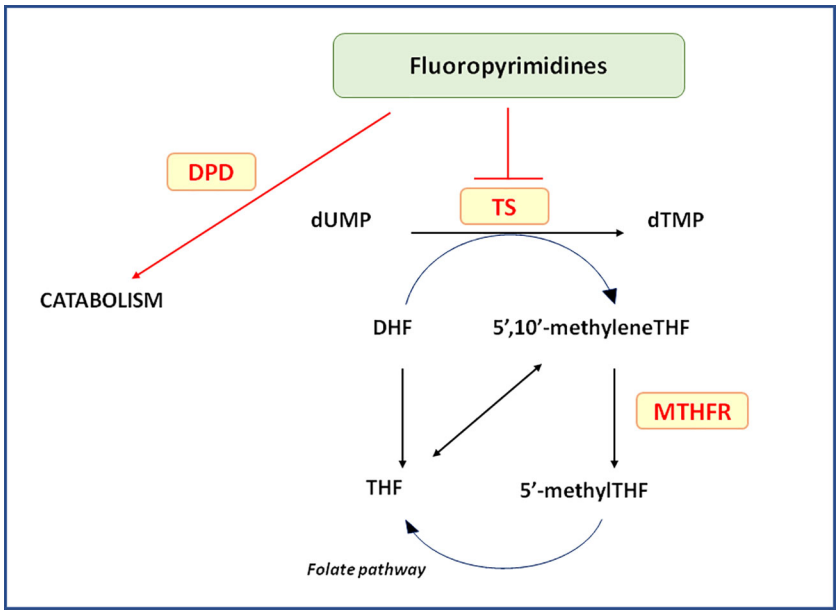

FIGURE 1 | Relevant pathways for pharmacogenetics of Fluoropyrimidines. In the Figure are pictured the most relevant proteins cited in the text and related to the clinical outcome of locally advanced rectal cancer patients receiving neo-adjuvant chemoradiotherapy. 5'-methylTHF, 5-methyltetrahydrofolate; 5',10'-methyleneTHF, 5,10-methylentetrahydrofolate; DHF, dihydrofolate; DPD, dihydropyrimidine dehydrogenase; dTMP, deoxythymidine 5'-monophosphate; dUMP, deoxyuridine 5'-monophosphate; MTHFR, 5,10-methylenetetrahydrofolate reductase; THF, tetrahydrofolate; TS, Thymidylate synthase.

Dihydropyrimidine Dehydrogenase

Dihydropyrimidine dehydrogenase (DPD, *DYPD*) is the enzyme responsible for the *in vivo* 80% detoxification of administered 5-FU, playing a major role as the rate-limiting step in FP metabolism. It has also been reported as the most important player in developing FP-related side effects in patients showing a deficient DPD activity and consequently more prone to the risk of toxicity. The prevalence of DPD partial deficiency in Caucasians is approximately 3%-5% (Etienne et al., 1994). A genetic background related to this inter-individual variability and its role in predicting toxicity occurrence have been described (Van Kuilenburg et al., 1999). Subjects who carry specific genetic variants related to a partial or complete DPD activity loss, may not be able to efficiently detoxify FP at normal rates, and are at greater risk of potentially life-threatening toxicity from standard doses.

The *DPYD* gene is highly polymorphic with 1,732 different genetic variants currently reported in GnomAD (https://gnomad.broadinstitute.org/), and 627 of those being missense variants impacting on the DPD protein sequence. Several studies investigating the role of specific *DPYD* polymorphisms on the risk of FP-related toxicity have been conducted in the past years. A number of them were conducted on large randomized clinical trials (Deenen et al., 2011; Lee et al., 2014; Boige et al., 2016) producing solid evidence on the predictive role of *DPYD**2A, *DPYD**13, *DPYD*-2846, and *DPYD*-HapB3 on treatment safety. The results were also confirmed in some large meta-analyses (Rosmarin et al., 2014; Meulendijks et al., 2015) and prospective pharmacogenetic guidelines were published providing guidance for dose adjustments based on *DPYD* genotype (Caudle et al., 2017). A first prospective study demonstrated in 2016 that a front-line FP dose adaptation based on the pre-emptive genotyping of *DPYD**2A, was effective in preventing toxicity (Deenen et al., 2016).

A gene activity score was proposed for translating *DPYD* genotype into a protein phenotype, considering the diplotype allelic combination of the *DPYD* variants according to a four polymorphisms panel (*DPYD* rs3918290, *DPYD* rs55886062, *DPYD* rs67376798, and *DPYD* rs56038477) (Henricks et al., 2015). A gene activity score was developed to provide a global DPD metabolizing status for each patient and to draw personalized dosing guidelines based on the patients' genotype for the four variants. Currently, the most recent version of shared pharmacogenetic guidelines is based on the definition of the patient gene activity score (Amstutz et al., 2018; Lunenburg et al., 2020). In late 2018 Henricks et al., published the results of a large prospective safety analysis providing strong evidences that the implementation of four *DPYD* polymorphisms genotyping is feasible in clinical practice and that genotype-guided individualized dosing improved patient safety of FP treatment (Henricks et al., 2018). Eventually, this study gave the final input for revising published pharmacogenetic guidelines (suggesting a 50% dose reduction for patients carrying any of the four *DPYD* variants at the heterozygous status) and for recommending *DPYD* genotyping among the strategies for a safer use of FP (https://cpicpgx.org/guidelines/guideline-for-fluoropyrimidines-and-dpyd/; November 2018 Update). Recently, testing of patients for DPD deficiency before starting treatment has been

TABLE 1 | Main findings from published works on germ-line variants and response to treatment (toxicity and efficacy) in locally advanced rectal cancer (LARC) patients receiving neoadjuvant chemoradiotherapy (nCRT).

Pathway/ *Gene*	Polymorphisms	Studies finding an association with the risk of toxicity	Studies that did not find any association with the risk of toxicity	Studies finding an association with the treatment efficacy	Studies that did not find any association with the treatment efficacy
Fluoropyrimidines Metabolism					
DPYD	DPYD*2A (rs3918290); DPYD*13 (rs55886062); c2846A > T (rs67376798); c.1236G > A/HapB3 rs56038477	Lunenburg et al., 2018			
DNA Repair					
ERCC1	rs11615			Paez et al., 2011	**Guo et al., 2015**;
	rs3212986			Sebio et al., 2015; Dreussi et al., 2016a; Sebio et al., 2015; Cecchin et al., 2011	**Salnikova and kolobkov, 2016**
ERCC2	rs13181	Duldulao et al., 2013a	Smith et al., 2017		**Guo et al., 2015**; **Salnikova and kolobkov, 2016**
XPA	rs3176683			Boige et al., 2019;	
XRCC1	rs25487	Duldulao et al., 2013a; Osti et al., 2017; Smith et al., 2017		Balboa et al., 2010; Grimminger et al., 2010; Paez et al., 2011; Cecchin et al., 2011; Lamas et al., 2012	Nicosia et al., 2018; **Guo et al., 2015**; **Salnikova and kolobkov, 2016**
XRCC3	rs1799794	Osti et al., 2017		Cecchin et al., 2011;	**Guo et al., 2015**; **Salnikova and kolobkov, 2016**
hOGG1	rs1052133			Cecchin et al., 2011	
RAD51	rs1801320	Osti et al., 2017		Gasinska et al., 2019	
Folate pathway/Fluoropyrimidines pharmacological target					
TYMS	rs34743033 and rs2853542 (TSER*2 and *3)			Villafranca et al., 2001; Spindler et al., 2007; Balboa et al., 2010; Paez et al., 2010; Hur et al., 2011; Tan et al., 2011; Paez et al., 2011; Lamas et al., 2012; **Salnikova and kolobkov, 2016** **Yang et al., 2017**	Stoehlmacher et al., 2008; Arrazubi et al., 2013; Ulrich et al., 2014;
	rs16430			Arrazubi et al., 2013; Stoehlmacher et al., 2008	Paez et al., 2010; **Yang et al., 2017**
MTHFR	rs1801131			Terrazzino et al., 2006;	
	rs1801133	Thomas et al., 2011 (in combination with rs1801131)		Terrazzino et al., 2006; Garcia-Aguilar et al., 2011; Cecchin et al., 2011; Nikas et al., 2015; Dreussi et al., 2016a; **Zhao et al., 2015**; **Salnikova and Kolobkov, 2016**	Ulrich et al., 2014
Oxidative Stress Detoxification					
GSTP1	rs1695		Osti et al., 2017	Nicosia et al., 2018; Gordon et al., 2006	
Cellular proliferation/Epidermal Growth Factor					
EGFR	rs712830			Spindler et al., 2006; Spindler et al., 2007 (in combination with rs4444903)	
	rs2227983			**Zhang et al., 2005**;	
	rs45608036			**Zhao et al., 2015**;	

(Continued)

TABLE 1 | Continued

Pathway/ *Gene*	Polymorphisms	Studies finding an association with the risk of toxicity	Studies that did not find any association with the risk of toxicity	Studies finding an association with the treatment efficacy	Studies that did not find any association with the treatment efficacy
				Salnikova and Kolobkov, 2016	
KRAS	rs61764370			Sclafani et al., 2015	
CCDN1	rs9344			Ho-Pun-Cheung et al., 2007	
AREG	rs11942466			Sebio et al., 2015	
Microenvironment-Related/Inflammation					
IL8	rs4073			Gordon et al., 2006	
IL13	rs1800925			Ho-Pun-Cheung et al., 2011;	Xiao et al., 2016
IL17F	rs641701			Cecchin et al., 2020	
NFKB1	rs28362491	Dzhugashvili et al., 2014		Dzhugashvili et al., 2014	
TGFB1	rs1800471	Smith et al., 2017; Schirmer et al., 2012			
	rs1800470			Dreussi et al., 2016a; Gordon et al., 2006	
PAI1	rs1050955	Zhang et al., 2015b			
	rs2227631	Zhang et al., 2015b			
PAR1	rs32934	Zhang et al., 2015b			
TNFA	rs1799964	Zhang et al., 2015a			

In bold are listed meta-analysis and in underlined contrasting results.

recommended by the European Medicines Agency's safety committee (Pharmacovigilance Risk Assessment Committee) either by measuring the level of endogenous uracil in the blood, or by checking for specific variations in *DPYD* associated with an increased risk of severe side effects (https://www.ema.europa.eu/en/documents/referral/fluorouracil-fluorouracil-related-substances-article-31-referral-ema-recommendations-dpd-testing_en.pdf).

Despite the large amount of published data on the effect of *DPYD* genetic variants on the risk of toxicity both in patients receiving FP monotherapy or different combination chemotherapies (Toffoli et al., 2015; Ruzzo et al., 2017; Dalle Fratte et al., 2018), no specific evaluation of the guidelines application was conducted in patients receiving a concomitant treatment with radiotherapy, as in patients with LARC receiving chemoradiation treatment. FP are commonly administered as short course treatment with lower dosage in the context of chemoradiation treatments, raising perplexities on the opportunity to apply the same genotype-related dose reductions that are prescribed by current guidelines. We demonstrated that the carriers of *DPYD* rs3918290, *DPYD* rs55886062, *DPYD* rs67376798, or *DPYD* rs56038477 variants are still at higher risk of toxicity when treated with chemoradiotherapy. The significant correlation between *DPYD* genotype and toxicity risk was investigated in 828 patients (including 93 patients with LARC) receiving a FP-based chemotherapy in combination with radiotherapy. This study suggests that FP dose reductions should also be applied in *DPYD* variant allele carriers who will start nCRT to prevent severe FP-induced toxicity (Lunenburg et al., 2020).

Among all the pharmacogenomic markers currently available for treatment personalization of patients with LARC, *DPYD* test for FP dosage adaptation is the only validated and recommended. However, clinical implementation of the *DPYD* test prior to FP treatment is not of common practice in most of the health care systems so far. One of the major concerns preventing the translation of *DPYD* pharmacogenetic guidelines in the clinical practice is the lack of formal health technology assessment studies, including cost-effectiveness and cost-consequences evaluation. Our group previously reported that the costs required to manage FP-related toxicity are associated to the patient's *DPYD* genotype. We demonstrated that the mean toxicity management cost per patient is related to the patients *DPYD* gene activity score (based on the four *DPYD* variants) (Fragoulakis et al., 2019; Toffoli et al., 2019). More recently a large prospective trial, provided evidence of the cost-effectiveness of an upfront *DPYD*-guided dose individualization (Henricks et al., 2019).

Probably the ultimate evidence needed to support the introduction of *DPYD* testing and, more generally, of pharmacogenomics in the clinical practice will derive from the ongoing implementation projects (Krebs and Milani, 2019), one of which in Europe (Ubiquitous Pharmacogenomics, U-PGx). U-PGx (www.upgx.eu) is led by a European Consortium of pharmacogenomics experts formed in 2016 with the aim of assessing the clinical utility of implementing a panel of pharmacogenomic markers into routine care. A prospective, block-randomized, controlled clinical study [PREemptive Pharmacogenomic testing for prevention of Adverse drug REactions (PREPARE)] was funded by the European Commission's Horizon-2020 program. In such study, a panel of clinically relevant pharmacogenomic markers will be preemptively genotyped and implemented in healthcare institutions across seven European countries and patients' outcome investigated (Cecchin et al., 2017; Van Der Wouden et al., 2017).

The study of rare genetic variants in drug-related "pharmacogenes" represents a promising innovative approach capable to explain and justify the observed variation in overall drug response, not currently explained by common genetic polymorphisms. Furthermore, exome sequencing can accelerate pharmacogenetic discovery by assessing both common (i.e. minor allele frequency, MAF >5%) and rare (MAF < 1%) mutations in virtually all genes in an individual at relatively low cost (Gordon et al., 2014; Ingelman-Sundberg et al., 2018). In the future, additional novel and rare variants significantly impacting the DPD activity could be integrated into available pharmacogenomic algorithms to further improve the safety of FP administration.

DNA Repair Pathway

RT exerts its cytotoxic action mainly by acting on DNA. If the DNA damage after irradiation is not completely repaired, cell death will occur either by apoptosis, mitotic catastrophe, or senescence (Maier et al., 2016) (**Figure 2**). At least five DNA repair molecular systems, each operating on a specific type of DNA damage, are involved in the repair of radiation-induced damage: direct reversal as the O6-methylguanine DNA methyltransferase–base repair; base excision repair (BER) including the repair of modifications generated by reactive oxygen as the 8-oxoguanine; nucleotide excision repair (NER); DNA mismatch repair; and double-strand breaks (DSB) repair that involved two main pathways, the non-homologous end joining (NHEJ) and homologous recombination (HR). A genetically determined defect in the capacity to repair RT induced damage has been reported (Lavin, 1998; Mckinnon and Caldecott, 2007; Mizutani and Takagi, 2013).

Three meta-analyses reported the role of DNA repair genetic variants as predictive markers of the risk to develop severe radiation-induced toxicity, although including patients withunselected solid cancers and not specifically LARC. including patients with unselected solid cancers and not specifically LARC. Particularly these meta-analyses demonstrated a significant involvement of polymorphisms in the ERCC excision repair 2 [*ERCC2,* also known as xeroderma pigmentosum group D (XPD), i.e., rs13181, Lys751Gln], x-ray repair cross-complementing 1 (*XRCC1,* i.e., rs25487, Arg399Gln), x-ray repair cross-complementing 3 (*XRCC3,* i.e., rs861539, Thr241Met; rs1799794, located in the 5'UTR) and ATM serine/threonine kinases (*ATM*, i.e., rs1801516,

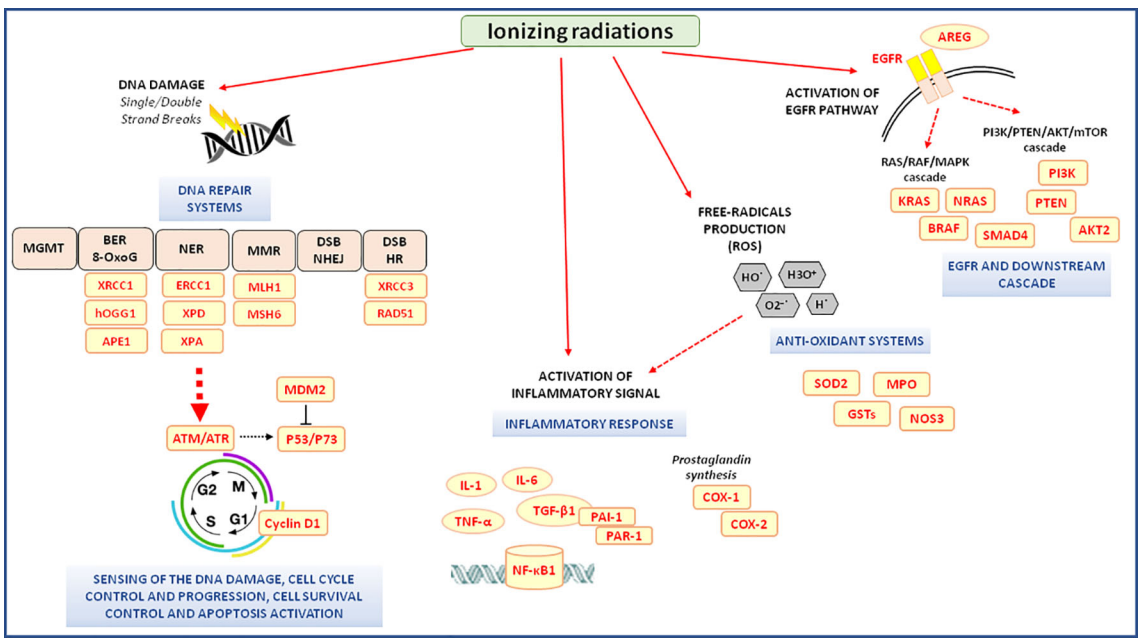

FIGURE 2 | Radiotherapy-related molecular pathways associated with the clinical outcome of neo-adjuvant chemoradiotherapy in locally advanced rectal cancer patients. 8-oxoG, 8-oxoguanine; AKT, v-akt murine thymoma viral oncogene homolog; APE1, apurinic/apyrimidinic endodeoxyribonuclease 1; AREG, amphiregulin; ATM, ATM serine/threonine kinases; ATR, ATR serine/threonine kinases; BER, base excision repair; COX-1, -2; cyclooxygenase 1, -2; DSB, double-strand breaks; EGFR, epidermal growth factor receptor; ERCC1, ERCC excision repair 1; GSTs, glutathione S-transferases; hOGG1, 8-oxoguanine DNA glycosylase; HR, homologous recombination; MDM2, MDM2 proto-oncogene; MGMT, O6-methylguanine DNA methyltransferase; MLH1, mutL homolog 1; MMR, DNA mismatch repair; MPO, myeloperoxidase; MSH6, mutS homolog 6; mTOR, mammalian target of rapamycin; NER, nucleotide excision repair; NF-κB1, nuclear Factor Kappa B Subunit 1; NHEJ, non-homologous end joining; NOS3, nitric oxide synthase 3; P53, tumor protein p53; P73, tumor protein p73; PAI-1, plasminogen activator inhibitor type 1; PAR-1, protease-activated receptor-1; PI3K, phosphoinositide-3-kinase; PTEN, phosphatase and tensin homolog; RAD51, RAD51 recombinase; ROS, reactive oxygen species; SMAD-4, SMAD family member 4; SOD2, superoxide dismutase 2; TGF-β1, transforming growth factor beta 1; TNF-α, tumor necrosis factor-alpha; TRBP, trans-activation-responsive RNA-binding protein; XPA, xeroderma pigmentoso complementation group A; XPD, xeroderma pigmentoso complementation group D; XRCC1, -3, x-ray repair cross-complementing 1, -3.

Asp1853Asn) genes in modulating the risk of radiotoxicity (Dong et al., 2015; Song et al., 2015a; Song et al., 2015b). However, the results of the meta-analyses are not fully transferable to the specific context of the radiation neo-adjuvant therapy of patients with LARC, where most promising data regard the *XRCC1* rs25487 variant. A concordant association between the presence of a polymorphic rs25487-A (Gln) allele and an increased risk to develop grade ≥3 toxicity, and skin toxicity of any grade was reported by three different research papers (Duldulao et al., 2013a; Osti et al., 2017; Smith et al., 2017). XRCC1 is one of the most relevant members of the BER pathway and its missense variation rs25487 was associated with a decreased DNA repair capacity, supporting an impact on the risk of toxicity (Duldulao et al., 2013a).

Genetic polymorphisms in *RAD51* recombinase (*RAD51*, i.e., rs1801320) and *XRCC3* (i.e. rs1799794), two members of the DSB-HR repair pathway, were also associated to toxicity risk. The polymorphic *RAD51* rs1801320-C allele resulted predictive of an increased risk of grade ≥3 abdominal/pelvis pain toxicity and acute skin toxicity of any grade, while the *XRCC3* rs1799794-G allele was associated with increased risk of grade ≥3 urinary frequency/urgency, acute skin toxicity of any grade and higher rates of fatigue (Osti et al., 2017). The *RAD51* rs1801320 G to C substitution in the untranslated (UTR) 5' region of the gene was shown to affect the mRNA stability leading to a lower protein expression and less effective DSBs repair (Gasinska et al., 2019). A genetically impaired *RAD51* expression has been reported to impact the individual radiosensitivity, and the rs1801320 variant was correlated with the risk of radiation-induced toxicity also in other tumor settings (Osti et al., 2017).

The missense variation rs13181 (Lys751Gln) in *ERCC2,* a member of NER system, was also investigated. The *ERCC2* rs13181-AA (Lys/Lys) genotype, resulted associated with an increased rate of grade ≥3 toxicity after a radiotherapy including treatment (Duldulao et al., 2013a). A following study aiming at validating the predictive role of *ERCC2*-rs13181 on radiotoxicity risk in patients with LARC (Smith et al., 2017) did not confirm those results.

Inflammation Pathway

In the context of chemoradiation treatment, inflammation-related genes have been considered as a target for the pharmacogenomic research, though the clinical setting of LARC has been poorly investigated up to date (**Figure 2**). The most promising results are related to the transforming growth factor beta 1 (TGF-β1), a cytokine that initiates and promotes acute and late radiation-induced side effects and is thus considered a major player in the inflammation process triggering. Exposure to radiation activates TGF-β1 *via* the generation of reactive oxygen species, and a sustained over-expression of TGF-β1 has been found in irradiated tissue. It was also investigated the role of rs1800471 in TGF-β1. The study of Schirmer et al., (2012), including two independent cohorts (discovery and replication) of Caucasian patients with LARC receiving neo-adjuvant radiation combined with 5-FU-based chemotherapy, evaluated the effect of *TGFB1* rs1800471 on the acute organ toxicity (quality of life-impairing acute organ toxicity, QAOT). In both cohorts, *TGFB1* rs1800471 resulted a significant predictor of QAOT, with all the patients carrying the polymorphic TGFB1 rs1800471-C (Pro25) allele experiencing QAOT. These data were confirmed by a subsequent study (Smith et al., 2017), performed in a similar cohort of Caucasian patients with LARC treated with FP-based CRT. The study reported that *TGFB1* rs1800471-C (Pro25) allele was associated with an increased risk of severe (grade ≥3) toxicity. The functional effect of the *TGFB1* rs1800471 polymorphism on the encoded protein has not been fully clarified yet. The polymorphism is in a genetic region encoding for the signaling portion of the peptide involved in the transport of the protein across the membrane of the endoplasmic reticulum. Preliminary *in silico* analysis has suggested that the presence of a proline at position 25, caused by rs1800471-C allele could affect the cleavage of the peptide, increasing TGFβ1 secretion and signaling (Schirmer et al., 2012). Another study including Chinese patients with LARC receiving nCRT demonstrated an effect on the toxicity risk of some polymorphisms in genes encoding for plasminogen activator (PA) inhibitor type 1 (PAI-1) and protease-activated receptor-1 (PAR-1). These two proteins, whose expression is activated by the TGFβ1-related pathway, contribute to acute and late radiation-induced injury (Zhang et al., 2015b). By multivariate analysis, *PAI-1* rs1050955-GG and *PAR-1* rs32934-CT genotypes were associated with lower risk of grade ≥2 diarrhea, fecal incontinence (i.e., anal toxicity) and other toxicities, while the *PAI-1* rs2227631-GG was associated with increased risk of grade ≥2 incontinence (Zhang et al., 2015b). Those polymorphisms are placed in the genes regulatory regions and may affect gene expression in different ways. The 3'UTR *PAI-1* rs1050955 variant is located at a microRNA binding site, whereas *PAI-1* rs2227631 and *PAR-1* rs32934 are located in the 5′-flanking region of the gene and may change the binding site of specific transcriptional factor (Zhang et al., 2015b). These data support a major causative role of TGFβ1 and its downstream mediators (i.e., PAI-1, PAR-1) on the risk of radiation–related toxicity, and a better elucidation of the predictive role of the related polymorphisms is warranted. However, the specific role of the genetic polymorphisms in the pathway mediators *PAI-1, PAR-1* is proved by a single study and the lack of independent validation results prevents to draw final conclusion.

A second cytokine of interest is the nuclear factor κB1 (NFKB1), a transcriptional factor that plays a key role in the activation of the inflammatory response and regulates various biological defense processes, including innate and adaptive immune responses, acute phase reaction and apoptosis (Karban et al., 2004). The *NFKB1* rs28362491 variant, consisting in an insertion/deletion of four bases in the promoter region of the gene, seems to decrease *NFKB1* transcription level by reducing the binding capacity of some transcription factors to the gene promoter (Karban et al., 2004; Riemann et al., 2007). Regarding the predictive role of *NFKB1* rs28362491 marker, literature provides only one study. The polymorphism was associated to the risk of radiation-induced toxicity. Particularly, the *NFKB1* rs28362491-DEL/DEL

genotype was associated with an increased risk of grade ≥2 dermatitis and proctitis. The rs28362491-DEL containing haplotype also showed a correlation with an increased risk to develop clinically significant grade≥2 acute organ toxicity (Dzhugashvili et al., 2014).

The pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α, encoded by *TNFA*) is another key molecule involved in the mediation of the inflammatory signal activated by ionizing radiation. As far as the predictive role of *TNFA* polymorphism in LARC is concerned, only one hypothesis generating study is currently available. The T allele of the *TNFA* rs1799964, located in the 5′-flanking region of the gene, was associated with increased TNF-α mRNA expression *in vitro*. It was reported to be associated with an increased risk of total grade ≥2 acute toxicity after chemoradiotherapy in Chinese patients with LARC (Zhang et al., 2015a).

Despite the promising aforementioned data about the potential involvement of some polymorphisms in genes implicated in the inflammation process in mediating the risk of toxicity in patients with LARC treated with nCRT, it must be underlined that most of the results are only reported in single exploratory studies, as for the case of polymorphisms in *PAI-1, PAR-1*, *NFKB1, TNFA*. Therefore, they should be further tested in independent validation studies in order to be then considered as effective biomarkers for a clinical translation.

NEO-ADJUVANT RADIO-CHEMOTHERAPY COMBINATION TREATMENT IN LARC AND PHARMACOGENOMIC MARKERS OF EFFICACY

The most compelling clinical need in patients with LARC is represented by a better understanding of the inter-individual unpredictable heterogeneity in the tumor response to nCRT. The prospective identification of patients who have a higher likelihood of responding to nCRT could be important for decreasing treatment morbidity and improving survival and local disease control. On the other hand, alternative intensified therapeutic approaches could be offered to patients who are unlikely to respond. Both germline and somatic genetic variants could play a role in defining the individual chance to get an effective response to treatment or the risk of disease recurrence (**Supplementary Table 2**, **Table 1**). A revision of the available literature evidence on genetic polymorphisms and somatic variants that could be considered to predict tumor response to treatment in LARC is reported below.

Thymidylate Synthase

Thymidylate synthase (TS, *TYMS*), an enzyme with a critical role in the DNA synthesis process, is the primary target of FP and its expression level was suggested to inversely correlate with the tumor sensitivity to FP (Johnston et al., 1995; Libra et al., 2004). Some common polymorphisms with an impact on the protein expression level and, in turn, with a hypothesized effect on an FP-based treatment outcome, were described in *TYMS*.

The most studied *TYMS* polymorphism consists of regulatory tandemly repeated sequences (VNTR) of 28–base pairs (rs34743033) in the 5' untranslated region of the gene and acts as an enhancer to the TYMS promoter (TYMS enhancer region [TSER]). TYMS expression is affected by the number of tandem repeats that are directly correlated with the enzyme expression level. Alleles with two or three repeats are the most common *(TSER*2* or *2R* and *TSER*3* or *3R)*. The second repeat of the 3R allele hosts a functional G > C single-nucleotide polymorphism (rs2853542). The 3R(G) allele has been correlated with a more efficient transcription activity while the transcription efficiency of the 3R(C) allele is the same as that of the 2R allele. TSER and rs2853542 are often combined to identify two groups of alleles: *TYMS* high-expression allele (2R/3RG, 3R/3RG and 3RG/3RG) and low-expression allele (2R/2R, 2R/3RC and 3RC/3RC) (Thomas et al., 2011). A third polymorphism that has been studied for its effect on the protein expression is a 6 bp insertion at the nucleotide 1494 in the 3′UTR of the gene. It was reported to be associated with an increased *TYMS* tumor expression that may decrease the chemosensitivity to FP (1494del6, rs16430, or rs34489327).

In the last 10 years, the association of *TYMS* polymorphisms with the tumor response to nCRT in LARC has been under investigation in literature. Although several studies showed an inconsistency of statistically significant results, in general *TYMS* high-expression alleles were associated with a worse response to nCRT and unfavorable prognosis. On the other side, *TYMS* low-expression alleles have been overall associated with better response and prognosis.

According to an abstract presented in 2001 from Villafranca et al., (2001) *TSER**3 homozygotes had a lower probability of downstaging compared with *TSER**2 homozygotes or *2/*3 heterozygotes (22% vs 60%) and a trend toward improved 3-year DFS was also detected in the *2/*2 and *2/*3 groups, compared with that in the *3/*3 group (81% vs 41%). The group of Spindler in 2007 demonstrated that 53% of *2/*2 patients experienced a 53% p-CR compared with 26% of *2/*3 and only 17% of patients with the *TSER**3/*3 variants (Spindler et al., 2007). The contribute of the 28-bp VNTR in the *TYMS* 5'UTR was considered along with the G > C SNP by Hur et al., reporting a significant difference in tumor response between low and high-expressing groups in 44 South Korean patients with LARC. Thirteen out of 14 patients in the low-expression genotype group exhibited a significantly greater tumor downstaging rate, as compared with 12 of 30 patients in the high-expression group (Hur et al., 2011). Based on such consistent results a prospective tandem phase II study was designed in 2011 by Tan and colleagues. *TYMS* genotyping was used to guide nCRT where carriers of three or more repeats in the *TYMS* 5'UTR VNTR were treated with a chemotherapy intensification program (Tan et al., 2011). *TSER**2/*2 or *TSER**2/*3, were treated with the standard of care hypothesizing a favorable response to 5-FU, while *TSER**3/*3 or *TSER**3/*4 genotypes who were unlikely to

derive significant benefit from a FP-based CT were treated with irinotecan in addition to standard 5-FU/CRT. This genotype-based strategy appeared to be successful, increasing treatment efficacy in poor-responder patients. Downstaging and complete tumor response rates reached 64.4% and 20% for standard of care and 64.5% and 42% for irinotecan-treated patients, respectively. However, this increase in efficacy came at the cost of higher rates of grade 3 to 4 toxicities, delay rates and hospitalization (Tan et al., 2011).

Only a few studies deriving from one research group reported contrasting results, highlighting an association between *3/*3 genotype and a higher response rate. The first study identified the *3/*3 genotype as an independent prognostic factor for improved survival in 2010 (Paez et al., 2010). A successive study conducted in 2011 on a larger group of patients reported as well a higher response rate (pathologic complete remission and microfoci residual tumor) for *TYMS* *3/*3 genotype compared to *2/*2 or *2/*3 in the amount of 59% vs 35%, and longer median progression-free survival (PFS) and OS (Paez et al., 2011). A significantly improved pathologic response among carriers of *TYMS* high-expressing alleles as compared with low-expressing alleles was also found by Lamas et al., (2012).

Regardless, two systematic reviews and metanalyses were published in 2016 (Salnikova and Kolobkov, 2016) and 2017 (Yang et al., 2017) assessing that *TYMS* *2/*3 genotype is associated with the response to nCRT in rectal cancer and that patients with a *2/*2 or *2/*3 genotype might benefit more from nCRT than others. The initial evidence of results heterogeneity for *TYMS* rs34743033 resolved after the exclusion of the data of Paez et al., (Paez et al., 2011; Salnikova and Kolobkov, 2016). However, the authors suggest that such results are not conclusive due to discrepancies among the studies analyzed in the nCRT administration and surgery strategies, sample size and ethnicities. A significant bias could also have derived from the source tissue (either tumor or normal) used for germinal DNA extraction.

Eventually, 3'-UTR 6-bp rs16430 was significantly associated with DFS by Arrazubi et al., (2013), while only a trend towards tumor response in patients receiving 5-FU-based nCRT, was described by Stoehlmacher et al., (2008). The metanalysis from Yang et al., (2017) did not found 1494del6 to be associated with the response to nCRT.

Methylenetetrahydrofolate Reductase

Methylenetetrahydrofolate reductase (encoded by *MTHFR* gene) is a fundamental enzyme in the folate cycle, important for DNA synthesis and repair (Toffoli et al., 2003) and hypothesized to be another important target of the FP pharmacological activity. MTHFR catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the methyl donor for methionine synthesis from homocysteine. Two common non-synonymous variants in *MTHFR*, rs1801133 (677C > T, Ala222Val) and rs1801131 (1298A > C, Glu429Ala), alone or in combination, were reported to affect the activity of the enzyme and hence the folate distribution (De Mattia and Toffoli, 2009; De Re et al., 2010). *MTHFR* rs1801133-CC wild genotype was associated with a major response to nCRT compared with *MTHFR* rs1801133-TT genotype (Salnikova and Kolobkov, 2016). The radiotherapy effect was also hypothesized to be affected by the action of MTHFR. Specifically, the reduced MTHFR activity, deriving from the aforementioned polymorphisms, could lead to an enhanced availability of non-methylated folate substrates for *de novo* synthesis of nucleotides, which could preserve DNA integrity from ionizing radiation, reducing genetic instability and thus preserving its efficacy. A number of studies were published with pretty consistent results about the detrimental role of *MTHFR* rs1801133 on the outcome of a CRT treatment. In particular, the *MTHFR* rs1801133-CC genotype was demonstrated to be more sensitive to the therapy, whereas the effect of *MTHFR* rs1801131 is still controversial.

In two studies on patients with LARC, *MTHFR* rs1801133-T allele was significantly associated with a lower chance to get a TRG ≤ 2 (Cecchin et al., 2011) and with non-pCR when in homozygosity (Garcia-Aguilar et al., 2011). In 2014 Urlich et al., documented a non-significant trend toward worse DFS and OS for *MTHFR* rs1801133-TT compared to patients with CC, although not supported by a significant association with OS or DFS. Such trend became significant for both endpoints in patients receiving 5-FU *via* protracted venous infusion 42 days before and 56 days after radiotherapy (Ulrich et al., 2014). Nikas et al., in 2015 reported that patients with *MTHFR* rs1801133-CC were 2.91 times more likely to benefit from nCRT, and 3.25 times more likely not to experience recurrence of the disease compared to heterozygous (CT) or homozygous (TT) genotypes (Nikas et al., 2015). The study from Terrazzino et al., identified the haplotype *MTHFR* rs1801133-T/rs1801131-A as an independent predictor of tumor regression at univariate and later confirmed at multivariate analysis. Patients not carrying the *MTHFR* rs1801133-T/rs1801131-A haplotype displayed a higher response rate (Terrazzino et al., 2006). Eventually the systematic review and metanalysis from Zhao et al., draw the line suggesting that *MTHFR* rs1801133 might be correlated with the tumor response under the recessive model (CC vs. CT/TT) in overall analysis, rectal cancer, and TRG1–2 vs. 3–5 group. *MTHFR* rs1801131 showed no significant association with the tumor response to nCRT (Zhao et al., 2015).

It has also been hypothesized that the effect of *MTHFR* SNPs could change according to specific combination drug therapies given with radiotherapy. A study by Dreussi et al., (2016a) compared the effect of a set of SNPs, including *MTHFR* rs1801133, in patients with LARC receiving an FP-based nCRT with or without oxaliplatin. They demonstrated a differential effect of the polymorphisms on the tumor response in the two subgroups, thus suggesting an effect of drug-drug interactions on the pharmacogenomic of LARC treatment.

MTHFR polymorphisms were also anecdotally associated with FP-related toxicity. A study published in 2011 by Thomas et al., investigated the effect of *MTHFR* rs1801133 and rs1801131 genetic markers with the outcome of a chemoradiation treatment. *MTHFR* haplotypes *(*rs1801133-*C/*rs1801131-C*)* and diplotypes (CA–TA and TA–TA) showed a protective or a detrimental effect on the incidence of severe diarrhea or mucositis, respectively (Thomas et al., 2011).

DNA Repair Pathway

The major role of germline variation in the DNA repair pathway on the response to radiotherapy has been already mentioned above for its association with a differential risk to develop adverse side effects after a chemoradiation treatment. The same genetic polymorphisms were investigated for their potential role in identifying good or poor responders to radiotherapy-based treatments. However, the results obtained up to date are far from their final conclusions. The missense *XRCC1*-rs25487 variant was investigated in a number of studies that generated heterogeneous results (Balboa et al., 2010; Grimminger et al., 2010; Cecchin et al., 2011; Paez et al., 2011; Lamas et al., 2012). The heterozygous rs25487-AG genotype was reported to be associated with both a higher (Grimminger et al., 2010) and a lower (Lamas et al., 2012) response rate. The *XRCC1* rs25487-AA genotype was associated either with an improved tumor response (Balboa et al., 2010) or with a decreased PFS by other authors (Paez et al., 2011). Interestingly, the work of Balboa et al., (2010) raised the problem of the source of genomic DNA used in the different studies for *XRCC1* genotyping (i.e. blood or tumor tissue). In their investigation, the authors demonstrated a discrepancy between the *XRCC1* rs25487-AA genotype as determined in healthy or tumor cells. The authors found an elevated rate of tumor specific genomic alterations (i.e., a loss of heterozygosity, gain of allele), in the chromosomal region containing DNA repair encoding genes (i.e., *XRCC1, ERCC1, ERCC2*), with the *XRCC1* locus presenting the highest percentage of aberrations. These findings highlighted a potential limitation in interpreting the results of the pharmacogenomic studies currently available.

The gene encoding polymorphisms for *ERCC1* (a NER member) were also analyzed by a number of studies, generating conflicting results. The TT genotype of the synonymous rs11615 (Asn118Asn) polymorphism, impacting the mRNA expression level of *ERCC1*, (Yu et al., 2000), resulted associated with a higher rate of R1-R2 circumferential rectal margin resection, and higher recurrence rate (Paez et al., 2011). Opposite results were produced by another study, where the same *ERCC1* rs11615-TT genotype resulted associated with higher rate of pathological complete response by multivariate analysis (Sebio et al., 2015). *ERCC1* polymorphism (i.e. rs3212986, located in the 3'UTR) was also highlighted for its association with complete tumor response to treatment (Cecchin et al., 2011; Dreussi et al., 2016a) but the results were not concordant with the findings of another published study (Sebio et al., 2015).

The impact of a set of DNA repair genetic markers (i.e., *XRCC1*-rs25487, *XRCC1*-rs179978, *XRCC3*-rs861539, *ERCC1*-rs11615, *ERCC2*-rs13181) on the tumor pathological response was analyzed by a meta-analysis (Guo et al., 2015), including five studies for a total of 265 Caucasian patients with LARC. The meta-analysis found no association between the polymorphisms and the response to radiotherapy in the context of LARC multimodality treatment. However, it should be considered that the small sample size of each single study, together with their significant methodological heterogeneity (genotyping strategy, study design, treatment schedule and dosage, combination with different chemotherapeutics drugs, clinical monitoring), could have affected the possibility to draw a common conclusion.

Additional studies were published exploring the predictive/prognostic effect of polymorphisms in other genes involved in the DNA repair pathways (i.e., *hOGG1*, *APE1*, *RAD51*, and *XPA*). Despite the encouraging results of the investigations on their potential impact on the tumor response phenotype, a final consensus was not achieved, and the clinical value of the markers should be independently validated in further studies.

The variant G allele of the missense rs1052133 (Ser326Cys) polymorphism, impairing the functionality of hOGG1 (Vodicka et al., 2007), was indicated to be a detrimental factor for the pathological tumor response to radiation therapy (Cecchin et al., 2011). The minor G allele of the missense rs1130409 (Asp148Glu) variation in *APE*-1, was instead associated with an increased response rate to radiotherapy at high dose (Dreussi et al., 2016a).

The functionally defective *RAD51* rs1801320-CC genotype was recently reported to be associated with a better outcome after short-course radiotherapy in term of both a longer OS and a lower risk to develop local recurrence and distant metastasis (Gasinska et al., 2019). This evidence supports the hypothesis that a defective repair of the radiation induced damage on DNA, determined by a genetic polymorphism, could increase the treatment efficacy with an improved patients' prognosis. Interestingly, patients with a *RAD51* rs1801320-CC genotype presented also a different tumor tissue phenotype with lower glucose transporter 1 expression, moderate differentiation, lower Ku70 expression, lower aneuploidy, and higher P53 protein expression. Moreover, patients' gender, in association with *RAD51* genotype and Ku70 expression, was shown to have an impact on OS. The findings of this study represent one of the first evidence of an association between a specific genetic background related to DNA repair (i.e., *RAD51* genotype) and a peculiar rectal cancer molecular phenotype and suggest a gender-related difference in the therapy outcome.

Another recent study by Boige et al., (2019) suggested that the *xeroderma pigmentosum group A (XPA)* rs3176683 polymorphism may help identifying patients who could benefit from adding oxaliplatin to a capecitabine-based nCRT due to its predictive effect for tumor response to oxaliplatin-based nCRT. The probability of tumor response was seven times higher in patient with *XPA* rs3176683-TT genotype treated with CAPOX (i.e., capecitabine and oxaliplatin). However, the polymorphism failed to be associated with the patients' prognosis.

Oxidative Stress/Detoxification Pathway

Since the radiotherapy exerts part of its cytotoxic effect by generating reactive oxygen species (ROS), genetic variations in oxidative stress-related enzymes have been also investigated as potential predictors of tumor response to treatment. However, the studies published up to date in LARC are sporadic and do not allow to draw final conclusion on the actual predictive or prognostic role of the markers investigated in *NOS3, MPO, SOD2*, and *GST*.

The missense rs1799983 (Glu298Asp) polymorphism in the gene encoding endothelial nitric oxide synthase (eNOS, *NOS3*), was related to ROS generation and patients OS. The same study also highlighted an association between the low ROS producing A-allele of the rs2333227 variant, located in the 5'flanking of the *myeloperoxidase (MPO)* gene, and a longer OS (Funke et al., 2009). Another potential predictive marker pointed out by literature is the missense rs4880 (Ala16Val) variant in SOD2 encoding gene. SOD2 plays a central role in the detoxification of ROS and its higher expression was reported to inhibit ROS-induced activation of NF-κB and to potentially increase the sensitivity to ionizing radiations (Ho-Pun-Cheung et al., 2011). In agreement with this hypothesis, the rs4880-T (Val), associated with lower SOD2 activity, was linked to a worse response to radiation therapy (Ho-Pun-Cheung et al., 2011).

Glutathione-S-transferases (GSTs) is a multigene family of phase II metabolic enzymes employed in the detoxification of reactive oxygen intermediates produced by a wide variety of potentially toxic and carcinogenic compounds, by conjugation with glutathione (Nicosia et al., 2018). The missense polymorphism 313A > G (Ile105Val, rs1695) in *GSTP1* was proposed, in a study from Nicosia et al., (2018) to be a predictive and prognostic marker. Among a population of 80 patients with LARC, those with a 313AA genotype for *GSTP1* rs1695presented a rate of 26.6% of pCR compared to 8.5% of the 313AG/GG population. Patients harboring at least a 313A allele presented a 5- and 8-year cancer-specific survival longer than those with 313GG genotype (87.7% and 83.3% vs. 44.4% and 44.4%, respectively). Only a trend for an improved OS in the rs1695-A allele carriers was reported.

Epidermal Growth Factor Receptor

EGFR was demonstrated to have a pivotal role in LARC as well as in the colorectal cancer carcinogenesis (Toffoli et al., 2007; Kim and Eng, 2012). Moreover, EGFR and related pathways, are activated by ionizing radiation, and have been suggested to be a druggable target for the neo-adjuvant treatment of LARC. However, the association of FP with cetuximab in the chemoradiation treatment was not successful. Nonetheless, germline polymorphisms in *EGFR* were investigated for their potential predictive or prognostic effect. The most studied functionally relevant *EGFR* polymorphisms in LARC are the following: 1. rs45608036, a (CA)n repeat in the intron 1 of the gene that alters EGFR expression *in vitro* and *in vivo*; 2. rs2227983, a missense substitution at codon 497 (Arg497Lys) that leads to attenuation in ligand binding, growth stimulation, tyrosine kinase activation, and induction of proto-oncogenes myc, fos, and jun; and 3. rs712830, a single nucleotide change located in the Sp1 binding site of the regulatory *EGFR* promoter region that impact *EGFR* transcription (Zhang et al., 2005; Spindler et al., 2006; De Mattia et al., 2015). The rs712830-GG genotype resulted associated with a poorer response to chemoradiotherapy (Spindler et al., 2006). In the same study, the rs712830-GG genotype was also associated with higher EGFR expression by immunohistochemistry.

In another exploratory study, both the *EGFR* rs2227983-GG (Arg/Arg) genotype and the rs45608036-CA repeats (genotype with both alleles <20 CA repeats) showed a tendency towards an increased risk of local recurrence after radiotherapy. The combination in the same patient of an rs2227983-G (Arg) and an rs45608036 < 20 CA repeats alleles was associated with the highest risk of local recurrence (Zhang et al., 2005). A subsequent meta-analysis, including about 350 Caucasian patients with LARC and evaluating the role of *EGFR* rs2227983 and rs45608036 on the response to chemoradiation therapy, showed a trend for a detrimental effect on the response to treatment of the *EGFR* shorter (S) alleles (Zhao et al., 2015). In another meta-analysis, including more than a thousand Caucasian patients with LARC, a different frequency of distribution of the *EGFR* rs2227983-A allele among responder and non-responder has been detected (Salnikova and Kolobkov, 2016). Further analyses are required to clarify whether specific *EGFR* polymorphisms can have a clinical utility in predicting the outcome of a chemoradiation therapy in LARC.

Within the EGFR-pathway, preliminary results have also been generated for some germline genetic markers in the downstream effector KRAS proto-oncogene (*KRAS*) and the EGFR-ligand amphiregulin (*AREG*). A 3'UTR *KRAS* variant, named LCS6 (rs61764370) was reported to alter the epigenetic transcriptional control of KRAS. The polymorphism was associated with an impaired capacity of the mature let-7 miRNA to bind the KRAS-encoding mRNA, potentially leading to KRAS up-regulation and downstream cellular pathway signaling interference (De Mattia et al., 2015). In patients with LARC, the results of the retrospective EXPERT-C trial evidenced that the minor *KRAS* rs61764370-G allele was associated with a higher rate of complete response after nCRT independently from cetuximab administration (Sclafani et al., 2015). The same polymorphic rs61764370-G-allele showed also an association trend for an improved 5-years PFS and OS. By a subgroup analysis, the favorable prognostic effect of the rs61764370 variant seemed to be limited only to patients with a *KRAS* mutated tumor, opening the possibility of identifying subgroups of good responder patients within that category of patients (*KRAS* mutated) presenting an overall unfavorable prognosis.

Some hypothesis-generating data were also published about the role of genetic polymorphisms in some downstream effectors and ligands cooperating in the EGFR pathway. These data are reported in single studies lacking independent validation and therefore requiring further investigation to assess their real clinical value.

Specifically, the intergenic rs11942466 polymorphisms located in the *AREG* gene region was associated with a higher rate of pCR. Through a classification and regression tree analysis, including some polymorphisms in the EGFR pathway and DNA repair genes, the *AREG* rs11942466 variant represented the most important marker for identifying the complete responder patients (Sebio et al., 2015). The phosphoinositide-3-kinase (PI3K)/phosphatase and tensin homolog (PTEN)/v-akt murine thymoma viral oncogene homolog (AKT)/mammalian target of rapamycin (mTOR) cascade is another downstream signaling pathway potentially activated by EGFR. The role of genetic polymorphisms in five genes belonging to the PI3K/PTEN/

AKT/mTOR pathway [i.e, *PI3K catalytic subunit alpha (PIK3CA), PTEN, AKT1, AKT2, FRAP1* encoding for mTOR] on the response to nCRT in LARC was recently investigated for the first time. The *PTEN* rs12569998-G allele was associated with an increased tumor response rate, while the *AKT2* rs8100018-C allele with a decreased recurrence risk and a higher 5-years DFS rate (Peng et al., 2018). *PTEN* plays a crucial role in the DNA damage repair and in the cellular response to DNA damage, whereas *AKT2* modulates the cell survival signals, important mechanisms for the response to nCRT in LARC.

Additional genetic markers affecting the cell cycle control, apoptosis and proliferation rate were evaluated in single exploratory studies. The rs9344 polymorphism in cyclin D1 (*CCND1)*, controlling the G_1/S checkpoint of the cell cycle, is located at codon 242 in the exon-4/intron boundary of *CCND1* gene and is responsible for alternate splicing of transcripts with different half-lives (Bitterman et al., 2015). The *CCDN1* rs9344 marker was found to be an independent predictor of response to neo-adjuvant radiotherapy, with the A-allele being associated with an increased response rate and a lower risk of local failure (Ho-Pun-Cheung et al., 2007). Moreover, the combination of *CCDN1* rs9344 with specific clinical-pathological parameters (i.e., post-therapeutic lymph node status) generated a prognostic index that accurately distinguished subgroups of patients with different recurrence-free survival and OS. Other data suggested that the p73 G4C14 → A4T14 marker could be an additional factor influencing the outcome of nCRT in LARC. G4C14 → A4T14 is a dinucleotide polymorphism consisting of two linked variants (rs2273953 and rs1801173, respectively) located at position 4 (G to A) and 14 (C to T) in the 5′-UTR of exon 2, just upstream the initial start codon of the *tumor protein p73* (*TP73*, encoding P73) gene. The G4C14 → A4T14 polymorphism was supposed to impact the gene expression of p73, implicated in the regulation of the balance between pro- and anti-apoptotic signals (Loof et al., 2009). The GC/GC genotype was showed to be associated with a lower expression of p53 and Survivin, another regulator of the apoptosis and cell-cycle, and to be tendentially related to a longer DFS. The combination of a GC/GC genotype with a negative p53 expression and a weak expression of Survivin, predicted a longer DFS as compared to other genotype/phenotype combinations (Loof et al., 2009). All of these markers could be considered of interest, but only future validation work will eventually assess their clinical value.

Microenvironment-Related Pathways

The role of the tumor microenvironment in the response to nCRT in LARC was largely explored (Perez-Ruiz and Berraondo, 2016; Zhang et al., 2019). In this context, germline

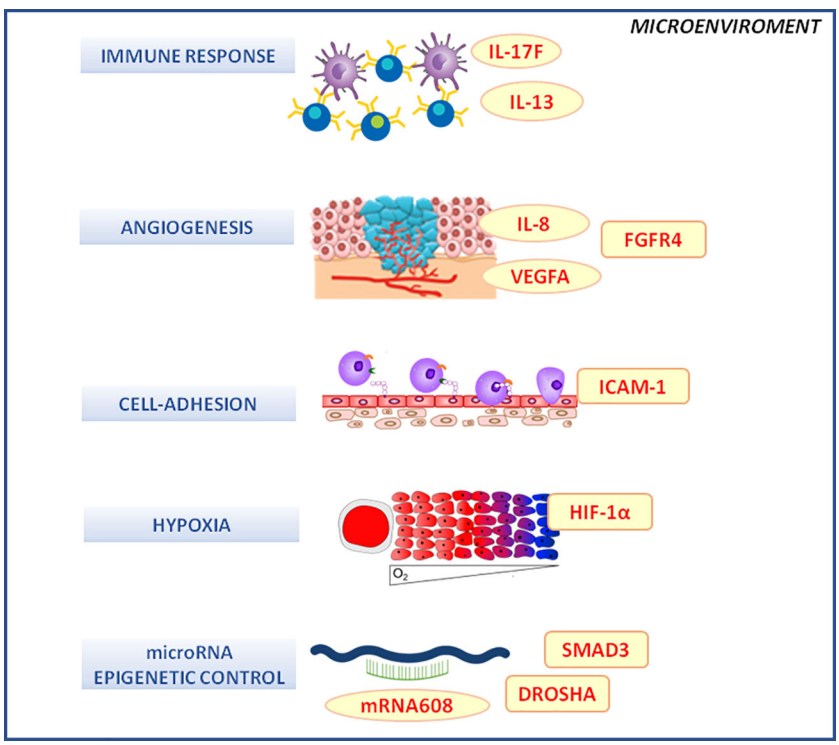

FIGURE 3 | Microenvironment molecular pathways associated with the clinical outcome of neo-adjuvant chemoradiotherapy in locally advanced rectal cancer patients. DROSHA, double-stranded RNA-specific endoribonuclease; FGFR4, fibroblast growth factor receptor 4; HIF-1α, hypoxia inducible factor 1 subunit alpha; ICAM-1, intercellular adhesion molecule 1; IL-1, -6, -13, -17F, interleukin -1, -6, -13, -17F; mRNA, microRNA; SMAD-3, SMAD family member 3; VEGF, vascular endothelial growth factor.

polymorphisms of proteins mediating inflammatory, angiogenic, hypoxia and cell adhesion phenomena were considered (Garziera et al., 2015; Garziera et al., 2017; Labriet et al., 2017; De Mattia et al., 2018a; De Mattia et al., 2018b; De Mattia et al., 2019; Labriet et al., 2019) (**Figure 3**). This is certainly a promising field of investigation in pharmacogenomics and this is proved by the number of studies published up to date analyzing different players and mediators of the complex inter-play between the microenvironment and the tumor tissue in determining the tumor response phenotype. However, despite many interesting results, no final consensus was reached up to date on the predictive role of each polymorphism. A lot of markers discussed below were investigated by single studies and lack of formal independent replication of the results prevents the possibility to draw final conclusion.

The promoter polymorphism rs4073 of interleukin 8 (*IL-8*), an interleukin with an important role in the angiogenic process, was linked to an increased IL-8 expression *in vitro* and was associated with a higher risk of LARC recurrence (Gordon et al., 2006). Another angiogenesis-related marker, the rs2010963 variant, located in the 5'UTR of *vascular endothelial growth factor (VEGFA)* gene and predictive of the protein serum level, was suggested to influence the probability to get a complete pathological response (Dreussi et al., 2016a). The missense variant rs351855 (Gly388Arg) in the gene encoding for fibroblast growth factor receptor 4 (FGFR4), a receptor tyrosine kinases involved in several cellular activities as angiogenesis, cell motility and inflammation, was also reported to contribute in determining the recurrence risk after radiotherapy by interacting with other genetic variants as *IL-8*-rs4073, *intercellular adhesion molecule 1 (ICAM-1)*-rs5498, and *TGFB1*-rs1800470 (Gordon et al., 2006). Among these markers, rs5498 is a missense variation (Glu469Lys) of *ICAM-1*, a cell adhesion molecule involved in the cell-to-cell interactions, another biological action under the control of the tumor microenvironment that deserves to be further investigated in the context of nCRT response in LARC.

Genetic polymorphisms in NFKB1, a key transcriptional factor for the activation of the inflammatory signaling cascades, has been also investigated. The *NFKB1* rs28362491-DEL allele, alone or in haplotype combination, was reported to be predictive of an increased rate of pathological complete response. An association trend was also observed between the rs28362491-DEL allele and a longer DFS and OS (Dzhugashvili et al., 2014). This preliminary finding suggests how an altered triggering of the inflammatory response could be related to the resistance to treatment. *TGFB1*-rs1800470 and *prostaglandin-endoperoxide synthase 2* (*PTGS2)*-rs20417 are other inflammatory-related genetic markers that have been suggested to contribute in determining the response to chemoradiation treatment in term of recurrence rate (Dreussi et al., 2016a). IL-13 is involved in the modulation of the immune system and the tumor immunosurveillance, which polymorphisms were related to the outcome of LARC patients treated with nCRT. The T-allele of the rs1800925 variant, located in the promoter region of *IL13*, was shown to be associated with a poorer response to nCRT in a cohort of Caucasian LARC patients (Ho-Pun-Cheung et al., 2011). The polymorphic T-allele was described to increase the transcription of *IL-13* that is involved in a down-regulation of tumor immunosurveillance. This is in line with the hypothesis that an impaired chemoradiation-induced tumor immunosurveillance could decrease the efficacy of the chemoradiotherapy. However, the impact of rs1800925 variant on response to radiation therapy was not confirmed in another investigation performed in a cohort of patients with LARC of Chinese ethnicity (Xiao et al., 2016). Since the genotype frequency of rs1800925 varies significantly by ethnicity, further studies are required to better clarify the predictive value of this marker in the different ethnic groups.

Our group recently produced promising data for some polymorphisms (i.e., 3'UTR rs641701, 5' UTR rs9463772) in the IL-17F encoding gene. IL-17 is an effector of the immune system that displays anti-tumor proprieties by acting on tumor angiogenesis and by improving the host inflammatory response against neoplastic cells. IL-17F rs641701-C and rs9463772-A alleles were associated with a poor prognosis in term of higher risk of disease recurrence after surgery, of distant failure of the treatment, and of death (Cecchin et al., 2020). Within a subgroup analysis, the two polymorphisms seemed to identify subcohorts of patients with a poorer long-term prognosis within homogeneous TRG strata, making them suitable to be integrated with already available prognostic clinical parameters. A hypoxic microenvironment represents another factor that was suggested to cause both a deficiency in DNA repair and a genetic instability.

The hypoxia inducible factor 1 subunit alpha (HIF-1α, encoded by HIF1A) is an important mediator of hypoxia-induced radio-resistance. The association between hypoxia-related markers (i.e., HIF1A rs11549465, rs11549467, and rs2057482 polymorphism) and response to nCRT was analyzed but no significant association were highlighted (Havelund et al., 2012).

Germline variants impacting the activity of microRNAs, with a potential epigenetic control on the overall cellular gene expression pattern were also investigated. A single variation affecting the microRNAs activity could have a downstream down-regulation effect on a large number of genes, including those involved in crucial pathways for the response to radiotherapy as DNA repair, angiogenesis, and inflammation (Dreussi et al., 2012). The work of Dreussi et al., (2016b) evaluated a set of miRNA-related TagSNPs, potentially affecting miRNA maturation and activity, in a cohort of 270 Caucasian patients with LARC stratified in two subgroup according to the radiation dose (50.4Gy or 55.0Gy). *SMAD family member 3 (SMAD3)*-rs744910, *SMAD3*-rs745103, and *trans-activation-responsive RNA-binding protein (TRBP)*-rs6088619 were associated to an increased chance of pCR, while *double-stranded RNA-specific endoribonuclease (DROSHA)*-rs10719 and *SMAD3*-rs17228212 had an opposite detrimental effect on pathological tumour response. A classification and regression tree analysis highlighted that specific combination of *SMAD3*-rs744910 and *TRBP*-rs6088619

genotypes together with clinical features (i.e., longer interval time between the end of radiotherapy and surgery) increases the chance of pCR. The finding of three independent variants (i.e., located in different haploblocks) in *SMAD3* provides a strong support for the involvement of this protein in the response to chemoradiotherapy. Another study (Sclafani et al., 2016) focused instead on a specific polymorphism, the rs4919510 C to G substitution that affects the mature microRNA 608 previously associated with response to treatment in patients with CRC. Within a retrospective analysis of the EXPERT-C phase II trial, the rs4919510-CC genotype was associated with worse 5 years PFS and OS after neo-adjuvant CAPOX followed by capecitabine-based chemoradiotherapy. The polymorphism probably impacts the interaction between miR-608 and its target mRNAs in a tissue-specific manner.

Most of the pharmacogenomic studies that investigated the response to nCRT in LARC have adopted a candidate gene or a pathway-based approach. Alternatively, two works opted for an unbiased strategy, performing a genome-wide analysis including thousands of polymorphisms. The study of Kim and colleagues (Kim et al., 2013), including 113 Korean patients with LARC receiving FP-based CRT, implemented a 3-step genome-wide strategy, based on genome-wide screening, clinical association, and biological validation of predictive polymorphisms. At the end, two novel markers were identified as potential predictors of response to treatment, *coronin 2A (CORO2A)* rs1985859 and *refilin A (FAM101A*) rs7955740. The reference *CORO2A* rs1985859-C allele was associated with higher rate of positive response. Moreover, an *in vitro* assay highlighted that the downregulation of CORO2A, linked to the variant rs1985859-T allele, was associated with reduced early apoptosis, increased cell survival or viability, and lower radiosensitivity. Even if *FAM101A* rs7955740 was not related with therapy outcome in the clinical association study, its downregulation, linked to the minor G-allele, was associated with a reduced early apoptosis and lower radiosensitivity similarly to *CORO2A* rs1985859. Another study (Lee et al., 2018), including a similar cohort of Korean patients with LARC and adopting a discovery/validation design, performed a whole-exome sequencing analysis identifying some further potential novel markers. Overall, five candidate variants emerged, *Bcl-2-like protein 10 (BCL2L10)* rs2231292, *DLC1 Rho GTPase activating protein (DLC1)* rs3816748, *dynein axonemal heavy chain 14 (DNAH14)* rs3105571, *inter-alpha-trypsin inhibitor heavy chain 5 (ITIH5)* rs3824658, and *retinoic acid early transcript 1L (RAET1L)* rs912565. Particularly, *DLC1*-rs3816748-C allele, *DNAH14* rs3105571-C allele, and *RAET1* rs912565-TT genotypes were associated with a higher rate of pCR according to a dominant model, while *BCL2L10* rs2231292-CC genotype and *ITIH5* rs3824658-T allele according to recessive model. In the co-dominant model, four candidate variants (all except *BCL2L10* rs2231292) were significantly correlated with pCR. The identified polymorphisms potentially impact the functionality of the encoded proteins that are involved in crucial biological pathway as tumor suppression, transport along microtubules, and regulation of cell apoptosis, extracellular matrix stability, tumor invasion and metastasis. The markers of radiosensitivity emerged from genome-wide analyses, could represents an interesting candidate that require to be validated by larger independent studies.

SOMATIC PHARMACOGENOMIC PROFILE

Several studies investigated the potential predictive role of some somatic mutations in patients with LARC treated with a nCRT but their predictive/prognostic value in this setting remains uncertain. The most studied candidate genes are those belonging to PI3K cancer-related pathway, and *KRAS* in particular, due to its primary role in colorectal cancer. KRAS plays a major role in two tumor-related cellular pathways, mitogen activated protein kinase (MAPK) and PI3K/AKT, by regulating their activation in response to cellular stimuli. Mutant forms of KRAS can cause a stable activation of those cellular pathways conferring a more aggressive tumor phenotype and resistance to anti-EGFR agents as cetuximab or panitumumab.

To this regard a meta-analysis published in 2013, analyzing the results of seven studies published between 2010 and 2012, concluded that somatic mutations in *KRAS* were neither predictive nor prognostic pharmacogenomic markers in LARC patients treated with nCRT (Clancy et al., 2013). Going deeply in the aforementioned studies, we could notice that just two of them included more than 100 patients, that the pharmacological treatments received were quite heterogeneous with three out of seven studies including cetuximab as a co-treatment, and that the frequency of *KRAS* mutation was lower than expected, probably due to the use of low sensitivity genotyping techniques. The last observation could be related to the sequencing method used (Sanger direct sequencing approach), sometimes focused only on hot-spot regions.

A number of studies have been conducted and published afterwards providing some more convincing evidence of an actual role of somatic mutations in *KRAS* and other related genes [*NRAS proto-oncogene, GTPase (NRAS), B-Raf proto-oncogene, serine/threonine kinase (BRAF)*, and *PIK3CA*] in the identification of low responder/bad prognosis patients. Most recent studies are characterized by the use of more sensitive DNA sequencing/genotyping approaches that led to the identification of a higher rate of tumors with mutation in *KRAS*, with an average frequency of about 40% of patients carrying at least one somatic mutation. Moreover, larger studies including a higher number of patients and combining the *KRAS* genetic information with other relevant genetic alterations in the same pathway or in *tumor protein p53 (TP53)* gene, allowed to provide a better idea of the actual predictive/prognostic role of somatic pharmacogenomics in LARC.

In 2013 a study by Duldulao and colleagues (Duldulao et al., 2013b) reported the results of a prospective multicenter clinical trial investigating the effect of increasing the nCRT-to-surgery time interval and adding chemotherapy during the waiting period (ClinicalTrials.org Identifier: NCT00335816). In a group

of 148 stage II-III rectal cancer patients undergoing *KRAS* and *TP53* genotyping on pretreatment tumor biopsies, they demonstrated that patients with *KRAS* mutated tumors had a decreased chance to get a pathological complete response compared to wildtype *KRAS* tumors, and specifically, no tumor with a KRAS mutation in codon 13 had a complete response.

The following year, another group (Abdul-Jalil et al., 2014) performed the genotyping of 234 potentially clinically relevant nonsynonymous mutations in 33 PI3K and MAPK pathway-related genes, including *PIK3CA, PIK3R1* (encoding phosphoinositide-3-kinase regulatory subunit 1)*, AKT, STK11* (encoding serine/threonine kinase 11)*, KRAS, BRAF, MEK* (encoding mitogen-activated protein kinase kinase)*, CTNNB1* (encoding catenin beta 1), EGFR, MET (encoding MET proto-oncogene, receptor tyrosine kinase), and NRAS, using the Sequenom platform on pretreatment LARC biopsy samples from 201 patients with LARC treated with nCRT. Patients without mutations in PI3K pathway-related genes, thus including RAS/RAF, were more likely to have pCR after nCRT. The same association was not observed for mutations in MAPK pathway-related genes.

In 2016, Chow and colleagues (Chow et al., 2016) published the results of a retrospective analysis performed on 229 pretreatment biopsies from patients with stage II/III rectal cancer receiving FOLFOX (i.e., folinic acid, fluorouracil, and oxaliplatin) treatment either before or after nCRT, but prior to surgical excision. Tumor DNA samples were sequenced to highlight the presence of either *TP53* or *KRAS* mutations. It was demonstrated that 34% of patients with a *KRAS* wild-type tumors had a pCR in comparison to 15% of *KRAS* mutated tumors (p=0.001). When specifically focusing on *KRAS* Gly12Val or Gly13Asp mutations, the percentage of complete responders lowered to 7%. When considering the combination of mutations in both *KRAS* and *TP53* in the same tumor, the risk of lymph node metastasis was also increased.

More recently a comprehensive and well conducted study was published by Sclafani et al. (2020) reporting on 210 patients with LARC treated with neoadjuvant CAPOX (i.e., capecitabine and oxaliplatin) followed by capecitabine-based chemoradiotherapy with or without cetuximab based on a prospective clinical trial (PAN-EX study). The mutational status of *KRAS, NRAS, BRAF, PIK3CA,* and *TP53* was assessed on the pre-treatment biopsy. The presence of *TP53* mutations was a risk factor for extramural venous invasion, poor pathological response and 5-year PFS. Even if similar validated data are not up to date available for *LARC*, very promising results have been published about the role of the same molecular markers, and specifically of somatic mutation in *KRAS* and *TP53*, as predictors of tumor response and prognosis. Tumors simultaneously carrying a mutation in *TP53* and either *KRAS* or *NRAS*, had a worse prognosis than those with a *TP53/KRAS/NRAS* wild genotype. This study suggests how integrating different molecular markers could better refine the predictive/prognostic value of specific pharmacogenomic features of patients with LARC. Other examples of such an approach can be found in the study by Kamran et al., (2019) that in a small set of well-studied LARC, treated with nCRT demonstrated that concurrent *KRAS/TP53* mutations were associated with a non-responder tumor phenotype and were enriched for an epithelial–mesenchymal transition transcriptional profile.

Another small study by Krajnovic and colleagues (Krajnovic et al., 2016) investigated the mutational status of *KRAS* gene in the pre-treatment biopsies of 63 patients with LARC. The genetic information was integrated with the immunohistochemical analysis of VEGF and Ki67. Even if *KRAS* mutation status by itself was not predictive of any clinical outcome evaluated, including pathological response, a simultaneous high VEGF expression was related to worse response to nCRT, higher risk of local recurrences and distant metastasis, and shorter OS. The predictive effect of the combination of somatic mutations in *BRAF* and *SMAD family member (SMAD4)* was investigated by Jiang and colleagues in 2019 (Jiang et al., 2019) in 74 patients with LARC treated with a FP-based induction chemotherapy followed by nCRT. It was demonstrated that *BRAF* and *SMAD4* were more frequently mutated among non-responder patients, and that the mutations were negative prognostic factors.

Interestingly some studies reported, as previously observed for colorectal cancer, a specific role of different *KRAS* somatic mutations, suggesting that a different contribute to tumor aggressiveness and treatment sensitivity could derive from different alterations in the *KRAS* protein structure. The previously mentioned study by Abdul-Jalil et al. (2014) reported that in patients not achieving a pCR, only mutations in codon 12 (Gly12Asp/Gly12 Val/Gly12Ser) and codon 13 mutations in *KRAS* were associated with poor recurrence-free survival. Gaedke and colleagues (Gaedcke et al., 2010) demonstrated that in 94 patients with LARC, tumors bearing a Gly12Val mutation were related to significantly higher rates of tumor regression than those with a Gly13Asp mutation, despite the overall presence of *KRAS* mutations did not correlate with tumor response and patient's outcome after preoperative chemoradiotherapy. In the previously mentioned study by Krajnovic et al. (2016) patients with a Gly12Ala mutation in *KRAS* had a significantly improved tumor response to CRT than those with any other type of *KRAS* mutation. Despite the heterogeneity of these results, probably driven also by the low numerosity of patients in each subgroup, it appears evident that not all mutations have the same value when considering the impact on the tumor phenotype and specific studies should be performed in order to assess the predictive/prognostic value of each mutation.

Most recent studies highlighted that in the oncological setting the immune system could play a pivotal role also in the tumor response to nCRT in patients with LARC. In one of the above mentioned study, published in 2019 by Kamran and colleagues (Kamran et al., 2019), 34 pre- and post-nCRT–matched tumor samples from 17 patients with LARC who received FP-based nCRT, followed by surgical resection, were analyzed. The authors demonstrated that the non-responder phenotype was associated with reduced CD4/CD8 T-cell tumor infiltrates and with a post-CRT M2 macrophage phenotype. These results highlighted how local tumor immune escape together with specific genomic features can contribute to the efficacy of chemoradiotherapy in the control of distant disease progression, paving the way to new

therapeutic approaches and a new generation of predictive/prognostic markers in the neo-adjuvant treatment of LARC.

CONCLUSION AND FUTURE PERSPECTIVES

Important refinements to the patients' stratification based on clinical, pathological and radiological parameters have been reached. Recently, the advanced analysis of medical images and their correlation with patient's outcome, using machine learning techniques (radiomics) were demonstrated to be helpful in predicting tumor response to pCRT in LARC (Dinapoli et al., 2018; Pirrone et al., 2019). Still significant inter-individual differences in patient's outcome, not captured by currently employed risk algorithms solely based on patients clinicopathological risk features are observed. Despite extensive research programs developed with the aim of identifying new criteria to adapt neo-adjuvant treatment programs based on the patient's molecular profiles, still too little is known about the predictive/prognostic effect of germline and somatic pharmacogenomic variants.

Considering that FP represent the backbone of each nCRT regimen in LARC, the most relevant drug-gene interaction that should be considered when planning a treatment is related to the study of *DPYD* polymorphisms in the context of increasing treatment safety. The clinical impact of testing the four polymorphisms panel (*DPYD*2A, DPYD*13*, *c.2846A > T,* and *c.1236G > A-HapB3)* on reducing the risk to develop severe toxicity and the costs associated to the treatment of the adverse events has been fully elucidated. The test still struggles to be integrated in the routine clinical practice of oncological treatments. However, the available pharmacogenetic guidelines on *DPYD* testing have been recently endorsed by some international regulatory agencies, as European Medicines Agency, that recently recommended to perform the test prior a FP administration. This will probably represent the ultimate step towards the clinical implementation of this life-saving analysis in the clinical management of LARC and of many other human malignancies.

In the context of FP treatment, other gene-drug interactions have been studied with the aim to improve treatment efficacy, by the analysis of polymorphisms in *TYMS* and *MTHFR*. The identification of germline genetic markers predictive of tumor response or prognosis is in general more difficult to be accomplished due to the complexity of the tumor response phenotype and to the contribution of the tumor genome that could significantly differ from the germline. A pivotal role of the *TYMS* polymorphism in the gene promoter region (*TSER*, rs34743033) has been reported by several studies, demonstrating that alleles associated with an increased target protein expression seems to lower the efficacy of a FP based treatment. The interventional phase II study based on those evidences demonstrated that the *TSER* polymorphism may be used as a baseline selection criterion in LARC patients to personalize nCRT by chemotherapy intensification in patients with a high TYMS expressing tumors. The study published in 2011 was not followed by further similar experiences at our knowledge. The validation of the prospective use of TSER, possibly in combination with other more recently developed predictive markers could represent a further step towards a personalization of LARC multimodality treatment. As far as *MTHFR* is concerned, several studies were concordant in assessing a detrimental role of the rs1801133 polymorphism on patients with LARC tumor response and survival and further effort should be made to test its effect in prospective interventional studies in order to eventually define its clinical applicability.

More exploratory data are available on other cellular pathways that demonstrated a primary role in the clinical outcome of patients undergoing chemoradio combination treatments as DNA repair and inflammation. Those pathways, that control the repair of radiation generated damage and the consequent activation of the inflammatory cascade, represent an important source of inter-individual variability in the outcome of nCRT. Most consistent data are related to the role of polymorphisms in *TGF-β* (particularly rs1800471) on the risk of developing treatment-related toxicity and a differential chance of tumor response. The anti-tumor effect of ionizing radiation is known to be linked not only to a direct damaging effect on tumor cells DNA and to the generation of free oxygen radicals, but also to the priming effect of radiotherapy on the immune system. Administration of nCRT treatment in patients with LARC can significantly modify the tumor tissue immune-profile and stimulate the release of cancer derived neo-antigens, resulting in the so-called "immunogenic cell death". The effect of radiotherapy on the activation of an immunogenic cytotoxic effect is mediated by a complex network of cytokines and chemokines that are released in the tumor microenvironment attracting dendritic cells. By reviewing literature, it appeared that genetic polymorphisms in those immune mediators can affect the risk of toxicity, tumor response and patients prognosis. Genetic polymorphisms in DNA repair genes as *XRCC1, ERCC1*, and *RAD51* have been studied for a long time as potential germline markers of radio sensitivity. Nonetheless, even if an overall effect of these genes is likely, no candidate markers with a promising application in the patients with LARC treatment scheduling is currently identifiable.

Globally, the results of available pharmacogenetic studies on germ-line predictive markers in patients with LARC published over the past 10-years have produced findings that in the majority of the cases did not allow a translation in the clinical practice, suggesting that a critical revision of research strategies is required. The impossibility to demonstrate a strong clinical validity of the investigated markers was in most cases the primary reason that preventing them to be transferred to the clinical practice. The high heterogeneity among published studies and the low number of patients included surely account for part of the difficulty to compare data and find reliable markers of clinical utility. Future investigations aimed at discover solid predictors of radio sensitivity should be performed on larger cohorts of patients homogeneous for

ethnicity and treatment modalities, including dosages, schedules and formulations used for radiotherapy and drugs administration (e.g., total radiation and FP dose; administration of 5-FU or capecitabine that presents different radio-sensitizing activity; other co-administered chemotherapeutics, as platinum derivates, irinotecan, cetuximab; time frames between preoperative therapy end and surgery). Standardization of the clinical monitoring strategies, clinical end-point assessment (i.e., clinical endpoints and methods to evaluate and score tumor response), as well as genotyping methods are additional aspects to be standardized (Di Francia et al., 2010).

The effect of the somatic pharmacogenomic profile was extensively investigated in advanced colorectal cancer and in stage II-III colon cancer, and the clinical significance of mutations in cancer-related genes such as *BRAF, KRAS, NRAS,* and *TP53* is widely acknowledged. The analysis of the mutational status of *RAS/RAF* genes represents a mandatory test in the metastatic colorectal cancer to assess tumor sensitivity to anti-EGFR monoclonal antibodies. Even if similar validated data are not currently available for LARC, very promising results were published about the role of the same molecular markers, and specifically of somatic mutation in *KRAS* and *TP53*, as predictors of tumor response and prognosis. The somatic pharmacogenomic profile of the tumor represents a useful piece of information in the oncologist toolbox that can be helpful in the outline of the most appropriate therapeutic approach also in this clinical context.

Indeed, the tumor genomic landscape is not stable and the disease evolution, as well as the selective pressure of the radio and chemotherapy can modify its characteristics. In this context a dynamic monitoring of the somatic genomic profile could anticipate the occurrence of tumor pharmaco-resistant or more aggressive clones overcoming. The discovery of the presence of circulating tumor DNA (ctDNA) in patients' blood and the possibility to analyze it through digital next-generation sequencing technologies led to important advances in the field. Several studies currently demonstrated a good correlation between the information derived from tumor tissue DNA sequencing and circulating tumor DNA (Coombes et al., 2019; Mezzalira et al., 2019; Siravegna et al., 2019; Dalle Fratte et al., 2020) paving the way to the use of ctDNA as a new candidate biomarker to be used either as a diagnostic or as a predictive/prognostic tool in cancer.

A number of researchers investigated the role of ctDNA as a biomarker of the therapeutic outcome in LARC and the results were recently reviewed by Massinhia and colleagues (Massihnia et al., 2019). Particularly a study by Tie et al. (2019) analyzed in a large group of 159 patients with LARC by sequencing cell free DNA in serial plasma samples at the time of diagnosis, after nCRT and after surgery demonstrating that the tumor DNA fraction level in plasma was significantly related to the risk of treatment failure. Most recently Khakoo et al., (2020) demonstrated how an integrated approach integrating standard clinical monitoring of tumor response with information deriving from the study of ctDNA in plasma can better identify patients at risk of developing metastases after surgery. This tool could represent an easily translatable test in the clinical practice helping the selection of patients for more conservative surgical approaches or less invasive therapeutic options after surgery.

In conclusion, despite the advancements in the field of personalized medicine and availability of treatments based on specific molecular targets, still the great molecular heterogeneity among patients and tumors represents a barrier to reach the final goal of a precision medicine in oncology. Different predictive methods have been explored, but none of these has showed enough accuracy to be used in the clinical setting. This is likely due to the high level of heterogeneity of the disease that considers complex interaction of genetic, molecular, and physiological features. The application of germline and somatic pharmacogenomics in the context of nCRT in LARC has provided up to date few validated markers as *DPYD* polymorphisms for preventing toxicity and *TYMS-TSER* or *KRAS* somatic variants to identify poor responders to treatment. A number of additional germline and somatic markers are under investigation and have already demonstrated encouraging clinical evidence of predictive and prognostic value and are likely to get in the future a clinical application. To identify in advance the toxicity and efficacy outcome to nCRT is an "unmet clinical need" in LARC to avoid under- or over-treatment. This would result into an optimal treatment approach, increasing treatment efficacy, minimizing surgery-related morbidity, avoiding unnecessary side-effects, improving quality of life, and reducing healthcare costs.

AUTHOR CONTRIBUTIONS

EM performed the literature review and analysis and contributed to writing the manuscript. RR contributed to writing the manuscript and elaborated the tables. EP contributed to writing the manuscript. GT edited the manuscript. EC conceptualized and contributed to writing the manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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The Clinical Impact of the C_0/D Ratio and the CYP3A5 Genotype on Outcome in Tacrolimus Treated Kidney Transplant Recipients

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Tacrolimus is metabolized by CYP3A4 and CYP3A5 enzymes. Patients expressing CYP3A5 (in Caucasian patients about 15% of the population but more frequent in African Americans and Asians) have a dose requirement that is around 50% higher than non-expressers to reach the target concentration. CYP3A5 expressers can be considered fast metabolizers. The trough concentration/dose (C_0/D) ratio of tacrolimus has recently been proposed as a prognostic marker for poor outcome after kidney transplantation. Patients with a low C_0/D ratio (also referred to as fast metabolizers) seem to have more tacrolimus-related nephrotoxicity, more BK-viremia, and a lower graft survival. At first sight, the expression of CYP3A5 and a low C_0/D ratio seem to be overlapping factors, both pointing towards patients in whom a higher tacrolimus dose is needed to reach the tacrolimus target concentration. However, there are important differences, and these differences may explain why the impact of the C_0/D ratio on long term outcome is stronger than for CYP3A5 genotype status. Patients with a low C_0/D ratio require a high tacrolimus dose and are exposed to high tacrolimus peak concentrations. The higher peak exposure to tacrolimus (and/or its metabolites) may explain the higher incidence of nephrotoxicity, BK-viremia and graft loss. A potential confounder is the concurrent maintenance treatment of corticosteroids, as steroids are sometimes continued in patients at high immunological risk. Steroids induce the metabolism of tacrolimus *via* pregnane X receptor mediated increased CYP3A4 expression, resulting in lower tacrolimus C_0/D ratio in high risk patients. Also non-adherence may result in lower C_0/D ratio which is also associated with poor outcome. The C_0/D ratio of tacrolimus does seem to identify a group of patients with increased risk of poor outcome after kidney transplantation. Our recommendation is to monitor tacrolimus peak concentrations in these patients, and if these are high then target slightly lower pre-dose concentrations. Another possibility would be to switch to a prolonged release formulation or to dose the drug more frequently, in smaller doses, to avoid high peak concentrations.

Keywords: tacrolimus, transplantation, kidney, CYP3A5, pharmacogenetics

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INTRODUCTION

Tacrolimus is the first choice calcineurin inhibitor (CNI) in kidney transplant patients. Maintaining the tacrolimus exposure within the therapeutic window is considered to be essential to prevent the development of cellular and antibody-mediated rejections and to minimize drug-related toxicity.

The pharmacokinetics (PK) of tacrolimus is characterized by poor and highly variable oral bioavailability (mean 25%, range 5–90%) (Shuker et al., 2015). Also drug–drug interactions, epigenetic changes in the expression of metabolizing enzymes and patient adherence contribute to a large inter-patient and intra-patient variability in tacrolimus exposure (Vanhove et al., 2016). An important part of the inter-patient variability is explained by the presence of a single nucleotide polymorphism (SNP) in the gene encoding for the cytochrome P450 (CYP) 3A5 enzyme (6986A>G). Patients expressing CYP3A5 (those carrying the A nucleotide, defined as the *1 allele) have a dose requirement that is around 50% higher than non-expressers (those homozygous for the G nucleotide, defined as the *3 allele) (Hesselink et al., 2014). Also the CYP3A4 gene carries a SNP (SNP in intron 6 (rs35599367C>T)) that is significantly associated with the tacrolimus dose requirement, but to a lesser degree than the CYP3A5 gene polymorphism (Elens et al., 2011). Genome wide association studies have shown that there are no other common single genetic variants aside from the CYP3A gene that significantly influences the tacrolimus PK (Oetting et al., 2018; Oetting et al., 2019).

Intra-patient variability in tacrolimus exposure is easily identified by repetitive measurement of drug concentrations in patients on maintenance treatment. Ten years ago Borra et al. demonstrated that patients with high intra-patient variability more often reached a composite endpoint consisting of graft loss, biopsy-proven chronic allograft nephropathy, and 'doubling in plasma creatinine concentration in the period between t = 12 months post-transplantation and last follow-up' (Borra et al., 2010). A high intra-patient variability in tacrolimus exposure is now recognized as a predictor of poor clinical outcome (van Gelder, 2014; Mendoza Rojas et al., 2019). Rodrigo et al. found that a higher intra-patient variability was independently related to development of donor-specific antibodies and graft-loss (Rodrigo et al., 2016). Identification of patients with a high intra-patient variability is therefore important as it is a modifiable risk factor, and interventions may improve long-term outcomes (Neuberger et al., 2017).

CONCENTRATION/DOSE (C_0/D) RATIO AND OUTCOME

More recently the concentration/dose (C_0/D) ratio of tacrolimus has also been proposed as a prognostic marker for poor outcome. The C_0/D ratio can be calculated by dividing the tacrolimus pre-dose concentration (C_0) by the corresponding daily tacrolimus dose (D).

Formula: $C_0/D\ ratio = tacrolimus\ trough\ concentration\ (ng/mL)/daily\ dose\ (mg)$

Thölking et al. were the first to hypothesize that the metabolization rate of tacrolimus, expressed as the C_0/D ratio, would be a prognostic factor of clinical outcome (Thölking et al., 2014). The mean of the C_0/D ratios calculated at months 1, 3, and 6 after renal transplantation was used to categorize patients as fast, intermediate, and slow metabolizers. The incidence of T cell-mediated rejection or antibody-mediated rejection was not related to the tacrolimus metabolizer status, but in the group of fast metabolizers more often CNI nephrotoxicity ($p = 0.015$) and BK-virus associated nephropathy ($p = 0.024$) were observed. They concluded that the tacrolimus C_0/D ratio is a simple and inexpensive tool to identify patients at risk for the development of CNI nephrotoxicity or BK nephropathy. Moreover, a C_0/D ratio < 1.05 was also associated with a higher mortality in a 24 months follow-up. In a five-year follow-up study from the same group the patient survival was noticeably reduced in fast metabolizers as compared to intermediate/slow metabolizers (89.9 *vs.* 95.3%, log-rank $p = 0.036$), and in a Cox regression analysis fast metabolizer status was an independent predictor of both graft and patient survival (Schütte-Nütgen et al., 2019). The suggested intervention could be to switch fast Tac metabolizers from tacrolimus based therapy to treatment with a mammalian target of rapamycin inhibitor (mTORi) or cyclosporine, but there is no evidence that this intervention does improve long term outcome. Although the authors do acknowledge that tacrolimus metabolism is also related to the CYP3A5 genotype, they claim that the prognostic value of the C_0/D ratio is stronger than that of the genotype.

In a subsequent study, patients were categorized into three metabolizer groups based on the same cut-off values. Patients with a tacrolimus C_0/D ratio < 1.05 ng/ml/mg were characterized as fast metabolizers, patients with a C_0/D ratio of 1.05–1.54 ng/ml/mg as intermediate metabolizers, and those with a C_0/D ratio ≥ 1.55 ng/ml/mg were defined as slow metabolizers (Thölking et al., 2016). Also in this study a fast tacrolimus metabolism was associated with increased risk of BK viremia. The potential explanation for the effect of the metabolizer status on outcome is that in patients with a faster metabolism the drug dose required to reach the target tacrolimus trough concentration is higher. As a result, the tacrolimus peak levels in the first hours after oral administration are higher. Evidence for this hypothesis was obtained in an additional study in 56 renal transplant recipients, in whom the tacrolimus concentrations 2 h after drug intake (C_2) in patients with a low C_0/D ratio (high metabolizers) were increased compared to the other patients (20.2 ± 10.3 ng/ml *vs.* 9.8 ± 4.2 ng/ml, respectively; $p = 0.004$) (Thölking et al., 2019). In daily practice most centers only monitor pre-dose tacrolimus concentrations, and the higher peak levels often go unnoticed.

A Polish group recently also reported impaired outcome data in patients with a low C_0/D ratio from a large group of 571 renal transplant patients (Kwiatkowska et al., 2019). In these patients the C_0/D ratio was calculated at their most recent out-patient appointment (mean time after transplantation = 84 months), and this ratio was then correlated with the change in renal function from transplantation to last follow-up. Also in this study a higher

metabolization status was associated with a significantly greater drop in the eGFR. In a smaller study, also from Poland, the highly significant relationship between C_0/D ratio at 6 months and kidney function 2 years after transplantation was confirmed in a linear regression model ($p = 0.007$) (Nowicka et al., 2019).

In 2020 Jouve et al. published a retrospective study on more than 1,000 kidney transplant patients treated with tacrolimus and with more than 1 year follow-up (Jouve et al., 2020). This study was called the TOMATO study, which stands for TacrOlimus MetAbolization in kidney TransplantatiOn. In a multivariate analysis the C_0/D ratio at month 3 and month 6 proved to be independent of early predictors of death-censored kidney graft survival. The authors stressed the importance of mechanistic studies to understand how the C_0/D ratio causes its effect.

Taber et al. studied the impact of tacrolimus pharmacokinetics in African Americans (AAs) (Taber et al., 2015). In AAs with sub-therapeutic tacrolimus concentrations, the incidence of acute cellular rejection and of antibody-mediated rejection was increased. But also patients who did achieve therapeutic tacrolimus concentrations were at an increased risk of developing interstitial fibrosis and tubular atrophy (IF/TA), reflecting nephrotoxicity. Most likely, in these AA patients therapeutic tacrolimus concentrations are reached at the cost of high drug doses, and the high peak concentrations inherent to these dosages result in more nephrotoxicity.

A faster metabolization of tacrolimus will result in higher concentrations of tacrolimus metabolites. These metabolites may accumulate in the blood and or renal tubular cells and cause nephrotoxicity. Compared to nonexpressers, the CYP3A5 expressers have a 2.0- to 2.7-fold higher metabolite/parent AUC ratio for these metabolites (Zheng et al., 2012). As several immunoassays suffer from cross-reactivity of these metabolites (without reporting a concentration of the individual metabolites), the mean of the tacrolimus concentrations measured with these immunoassays is higher than if measured with mass spectrometry based assays (Akamine et al., 2018). A survey in 2015 showed that for TDM of tacrolimus 53% of the laboratories used LC–MS/MS and 47% immunoassays ($n = 72$) (Christians et al., 2015). Accumulation of tacrolimus metabolites in the blood may go unnoticed if TDM is done exclusively on the basis of LC–MS/MS.

CYP3A5 GENOTYPE AND C_0/D RATIO

As already mentioned, there are multiple studies that show that renal transplant patients who express the CYP3A5 enzyme need a higher dose to reach the target tacrolimus concentration compared to non-expressers (Hesselink et al., 2003; Thervet et al., 2003; Haufroid et al., 2004). Thus, CYP3A5 expressers in general have a low C_0/D ratio. In a Caucasian population the prevalence of patients expressing CYP3A5 is low (10%) (van Schaik et al., 2002). In contrast, in patients from African descent about 40–50% express CYP3A5, and in Asian patients the prevalence of CYP3A5 expressers is even higher (50–70%) (Andrews et al., 2016).

In most centers the starting dose of tacrolimus is based on body weight. Standard tacrolimus dosing is 0.2 mg/kg bodyweight, divided in two doses. The guideline of the Clinical Pharmacogenetics Implementation Consortium (CPIC) recommends to increase the starting dose in CYP3A5 expressers 1.5–2 fold, but not to exceed 0.3 mg/kg/day (Birdwell et al., 2015). The higher starting dose is meant to avoid early underexposure to tacrolimus in the first days after transplantation.

CYP3A5 AND OUTCOME

In 1994 an analysis was published on differences in outcome of black and white patients after kidney transplantation based on a large dataset from the United Network for Organ Sharing (UNOS). An impaired graft survival in black patients was found. The difference in outcome was attributed to poor socioeconomic factors and reduced access to health care in African Americans and to poor HLA-matching (Koyama et al., 1994). In this study CYP3A5 genotype data were not available. A French study demonstrated that ethnic origin did not affect outcome after renal transplantation in France, and it was suggested that the poor results of renal transplantation in patients of African origin in the US could be improved with universal immunosuppressive drug coverage (Pallet et al., 2005). Also in this French study CYP3A5 genotype data were not available.

In a meta-analysis Rojas found that the CYP3A5 expresser genotype might be associated with a higher risk of acute rejection (OR 1.32, 95% CI 1.02–1.71) and a trend towards more chronic nephrotoxicity (OR 1.81, 95% CI 0.89–3.68) (Rojas et al., 2015). However, a large-scale genome-wide association study did not identify strong donor or recipient genetic predictors of allograft survival or renal function outside the HLA region (Hernandez-Fuentes et al., 2018; Stapleton et al., 2019). Also a candidate gene association study of allograft loss in renal transplant recipients CYP3A5 was not related to outcome (Woillard et al., 2018).

In a recently published meta-analysis, the effect-size of CYP3A5 polymorphism on the risk of rejection was estimated in Asian and European kidney transplant populations (Khan et al., 2020). In European populations no significant association was found with rejection episodes between expressers and nonexpressers (OR: 1.12; $p = 0.47$). However, in Asian patients a higher risk of rejection was found after follow-up of 3 years post-transplantation (OR: 1.68; $p < 0.05$), respectively. An explanation for this difference, other than a higher prevalence of CYP3A5 expressers in Asian populations could not be given. Data on longer term follow-up are scarce, and this higher risk estimate would need confirmation in larger datasets.

C_0/D RATIO AS PROGNOSTIC FACTOR FOR OUTCOME

In daily practice tacrolimus pre-dose concentrations are being monitored, and routinely monitoring tacrolimus peak concentrations or AUC is unusual since it is more cumbersome both for patients and clinicians. Whether or not the patient needs a high tacrolimus dose to reach the target concentration is not taken into account. However, patients who need a high tacrolimus dose

may be exposed to substantially higher tacrolimus peak concentrations and tacrolimus-AUC compared to patients who reach the same pre-dose concentrations with lower dosages. A study of 46 African Americans, of whom 35 were CYP3A5-expresser, showed that the tacrolimus pre-dose concentrations were similar in CYP3A5-expressers and non-expressers (6.26 and 6.24 ng/ml respectively) (Trofe-Clark et al., 2018). In the expressers group a much higher tacrolimus dose was required to reach these pre-dose concentrations (10.1 *vs* 6.3 mg/day), and as a result the C_{max} was also higher in the expressers (25.5 ng/ml and 19.5 ng/ml respectively; p = 0.04). The higher peaks and higher AUC may lead to chronic nephrotoxicity and ultimately contribute to graft loss. In a study on chronic irreversible drug-induced nephrotoxicity Kuypers et al. also found that especially patients with a high early tacrolimus dose requirement developed this form of toxicity (Kuypers et al., 2010). In their study this was predominantly but not exclusively encountered in CYP3A5 expressers.

At present tacrolimus is being dosed based on trough concentrations in the vast majority of centers. There is limited experience with dosing tacrolimus based on AUC. In Leiden the tacrolimus target AUC_{0-12h} for maintenance treatment (>6 months post-transplantation) is 80 ug*h/L. Based on a pharmacokinetic model the upper threshold of this target relates to a peak concentration of 22 ng/ml (Scholten et al., 2005). In case of high peak concentrations and high AUC the tacrolimus dose can be reduced. If peaks are high but AUC is within the target, then dose could be divided into smaller portions (from twice daily to three times daily), but this will negatively affect adherence. An alternative option would be to change from a twice daily immediate release formulation to a once daily prolonged release formulation. Besides avoiding high peaks this will also improve adherence. Another option would be to switch to mTOR-inhibitors as suggested by Thölking et al. (Schütte-Nütgen et al., 2019). There are no studies available that show that either of these options is beneficial.

At first sight the expression of CYP3A5 and a low C_0/D ratio seem to be overlapping factors, both pointing towards patients in whom a higher tacrolimus dose is needed to reach the tacrolimus target concentration. However, there are important differences, and these differences may explain why the impact of the C_0/D ration on long term outcome is stronger than for CYP3A5 genotype status.

First of all, on average the CYP3A5 genotype does lead to a higher dose requirement, but there is considerable overlap between expressers and non-expressers (Thervet et al., 2003). Some of the CYP3A5 expressers do not have a fast metabolizer phenotype, and they reach target concentrations with conventional tacrolimus doses. In these patients there is not a high peak concentration after drug intake. In contrast, patients with a low C_0/D ratio all have a fast metabolizer phenotype and invariably need high doses of tacrolimus. As a result, the impact of the C_0/D ratio may be stronger than the CYP3A5 genotype.

Another factor that may impact on the prognostic significance of the C_0/D ratio is the use of corticosteroids in patients at increased risk of rejection. For patients at low-immunological risk the avoidance, or early withdrawal of steroids, is well tolerated, but in patients with higher risk this may lead to an increased incidence of acute cellular rejections or late antibody medicated rejections (Pascual, 2011). Therefore, continued exposure to steroids may be linked to a higher immunological risk. As steroids are known to induce CYP3A enzymes the tacrolimus metabolism is faster in patients treated with steroids, and C_0/D ratio is lower (Hesselink et al., 2003; van Duijnhoven et al., 2003). Steroid use may thus be a confounder by indication, affecting the C_0/D ratio. However, in the recently published TOMATO-study the C_0/D ratio was independently associated with death-censored kidney-graft survival, even when corrected for continued corticosteroid use (Jouve et al., 2020).

An important limitation is that the number of studies on this topic is still limited and that several of these studies have been published by one center (Thölking et al.). In order to be more certain on the clinical relevance of the C_0/D ratio it would be good if more well documented analyses in larger data sets, including correction for potential confounding factors such as ethnicity, steroid use, and intra-patient variability, would be performed. Furthermore, interventions such as dose reductions and switching to prolonged release formulations should ideally be tested in prospective controlled studies. Novel developments in therapeutic drug monitoring, including home-based dried blood spot (DBS) sampling, offer the potential to facilitate repetitive large scale AUC assessment as an alternative to the conventional TDM sampling at the (out-patient) clinic. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays have been developed to quantify tacrolimus in DBS samples (Veenhof et al., 2017; Zwart et al., 2018), and at home sampling has been shown to be feasible in the kidney transplant population. Adherence to immunosuppressive therapy has been shown to decrease over time (Massey et al., 2015). Furthermore, non-adherence is a strong predictor for poor outcome and is related to development of donor specific antibodies and a higher risk of rejection (Takemoto et al., 2007). Patients who do not take (part of) their daily drug dose seemingly have a fast metabolism and a low C_0/D ratio. In these patients the poor outcome is not due to the faster metabolism, or due to high peak concentrations but to non-adherence.

CONCLUSION

The C_0/D ratio of tacrolimus does seem to identify a group of patients with increased risk of poor outcome after kidney transplantation. Our recommendation is to check tacrolimus peak concentrations in these patients, and if these are high then target for slightly lower pre-dose concentrations. Assessment of limited sampling AUC can assist in finetuning the tacrolimus dose.

AUTHOR CONTRIBUTIONS

TG and DM developed the idea for this manuscript. The first draft version was written by TG. All authors contributed to the article and approved the submitted version.

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REVIEW
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Pharmacogenomics as a Tool to Limit Acute and Long-Term Adverse Effects of Chemotherapeutics: An Update in Pediatric Oncology

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In the past decades, new cancer treatments have been introduced in pediatric oncology leading to improvement in clinical outcomes and survival rates. However, due to inter-individual differences, some children experience severe chemotherapy-induced toxicities or a poor clinical outcome. An explanation for the diversity in response to chemotherapy is genetic variation, leading to differences in expression and activity of metabolizing and transport enzymes as well as drug targets. Pharmacogenetic testing has emerged as a promising tool to predict and limit acute and long-term adverse effects in patients. However, in pediatric oncology, limited number of patients and a considerable diversity in study results complicate the interpretation of test results and its clinical relevance. With this review, we provide an overview of new developments over the past four years regarding relevant polymorphisms related to toxicity in pediatric oncology. The following chemotherapeutics and associated toxicities are discussed: alkylating agents, anthracyclines, asparaginase, methotrexate, platinum compounds, steroids, thiopurines, topoisomerase inhibitors, and vinca alkaloids. Our review identifies several questions regarding the role of genetic variants in chemotherapy-induced toxicities. Ambiguities in the literature stem from small population sizes, differences in (statistical) interpretation and variations in sequencing technologies as well as different clinical outcome definitions. Standardization of clinical outcome data and toxicity definitions within electronic health records combined with the increased availability of genomic sequence techniques in clinical practice will help to validate these models in upcoming years.

Keywords: pediatric oncology, chemotherapeutic agents, drug toxicity, adverse effects, pharmacogenomics

INTRODUCTION

Over the past decades, the 5-year survival rate for childhood cancer improved from 58% for children diagnosed during 1975 to 1977 to 83% for those diagnosed during 2005 to 2015 (O'Leary et al., 2008; Pui et al., 2011; Siegel et al., 2019). This improvement is mainly driven by risk stratification and intensification of cytotoxic chemotherapy. As survival is increasing, the focus has shifted to decreasing serious toxicities of chemotherapy without losing anti-tumor effectiveness of

multimodality treatment. Inter-individual differences in drug response have been implicated as an important consequence of chemotherapy-induced toxicities (Roden et al., 2019). As a result, genetic predisposition has been proposed as an explanation for individual variation in chemotherapeutic response and toxicity.

Pharmacogenomics includes all studies assessing genetic variations in patients influencing pharmacokinetics (drug absorption, metabolism, excretion, cellular transport) and pharmacodynamics. Pharmacogenetic testing has emerged as a promising tool to predict and limit acute and long term adverse effects in individual patients, and is widely investigated in pharmacogenomics (PGx) and genome-wide association studies (GWAS) (Relling et al., 2020). At present, the Clinical Pharmacogenetics Implementation Consortium (CPIC) has provided guidelines for the implementation of pharmacogenetics in practice, which led to 23 clinical guidelines, comprising 19 genes and 46 drugs (Haidar et al., 2019; Relling et al., 2020). In the Netherlands, The Dutch Pharmacogenetics Working Group (DPWG) has reviewed 97 gene-drug interactions, leading to multiple recommendations for clinical practice (KNMP apothekersorganisatie; van der Wouden et al., 2019). However, these guidelines are predominantly based on research in adults and exclude some pediatric cancer treatment drugs (e.g. asparaginase).

The rarity of childhood cancer and inherently small patient populations combined with diversity in outcome measurements led to uncertainties regarding the clinical relevance of genetic testing in pediatric oncology as well as difficulties with the interpretation of test results (Relling and Klein, 2011). Given that high survival rate in pediatric cancer is dependent on intensive chemotherapy, clinicians are hesitant to preemptively reduce dosages due to genetic variants, as retaining the beneficial outcome is of utmost importance in pediatric cancer.

The availability and affordability of genomic technologies have improved greatly in the past years, resulting in many studies, albeit of varying quality. In this literature review we provide an overview of developments and new insights over the last years regarding relevant genes and polymorphisms as well as their role in acute and long-term adverse effects of drugs in pediatric oncology. The review is limited to pediatric age group (0–18 years). To address the recent developments, we reviewed recent publications (from 2016 onward) using terms related to pediatric oncology, pharmacogenomics and pharmacogenetics, and toxicities of (pediatric) cancer drugs. While a wide variety of supportive care drugs (e.g. anti-infective drugs, analgesics drugs, and antiemetic drugs) and immunotherapy are also used in pediatric oncology, we focus exclusively on the association between genetic variants and chemotherapy-induced toxicities.

ROLE OF PHARMACOGENETIC VARIATIONS IN CHEMOTHERAPEUTIC RELATED TOXICITIES

The following chemotherapeutics and their toxicities have been selected based upon extensive use in pediatric oncology: alkylating agents, anthracyclines, asparaginase, methotrexate, platinum compounds, steroids, thiopurines, topo-isomerase inhibitors, and vinca alkaloids. For each chemotherapeutic agent, the relationship between various genetic variations and chemotherapy-induced toxicity are discussed. An overview of recent studies included in this review can be found in **Supplementary Table 1**.

Alkylating Agents

Alkylating agents are widely used antitumor prodrugs deriving their cytotoxic effect from adding an alkyl group to the guanine base of the DNA molecule. This alkylation results in abnormal nucleotide sequences, miscoding of messenger RNA, blockade of DNA replication and breakage of DNA strands and eventually tumor cell death. Most common alkylating agents in pediatric oncology are nitrogen mustards like cyclophosphamide and ifosfamide, alkyl sulfonates like busulfan and triazenes such as dacarbazine and temozolomide. Alkylating agents show a wide range of toxicity including myelosuppression, kidney and gastrointestinal toxicities. Due to limited data, only cyclophosphamide, ifosfamide, and busulfan are updated in this review. In addition to toxicity studies, we also included studies analyzing the association between pharmacokinetics of alkylating agents and genetic variants, while limited studies were available which directly investigated the association between genetic variants and alkylating-induced toxicities.

Cyclophosphamide

Metabolism and Transport

Activation of cyclophosphamide is catalysed by the hepatic cytochrome P450 (CYP) isozymes *CYP2B6, CYP2C19*, and *CYP3A4*. The overall metabolism of cyclophosphamide is complex, with numerous enzymes involved which vary in expression and activity.

Genetic Variances and Toxicity

In the past, *CYP2B6* and *CYP2C19* have shown to influence cyclophosphamide pharmacokinetics in adult patients (Helsby et al., 2010). Recently, the influence of *CYP2B6* on cyclophosphamide clearance was confirmed in the pediatric population of 49 B-cell Non Hodgkin Lymphoma (NHL) patients. Patients carrying *CYP2B6*6* had significant lower cyclophosphamide clearance (Veal et al., 2016). This is in line with previous research showing a decreased function of *CYP2B6*6* (Lang et al., 2001; Hesse et al., 2004; Zukunft et al., 2005; CYP2B6 P, 2020).

Ifosfamide

Metabolism and Transport

Ifosfamide requires activation by *CYP3A4* and *CYP2B6* to active metabolites. Variation in the renal expression of *CYP2B6* leads to higher rates of ifosfamide metabolite chloroacetaldehyde (CAA), which is nephrotoxic. Increasing evidence suggests that CAA is also involved in ifosfamide-induced encephalopathy.

Genetic Variances and Toxicity

Very limited data is available regarding the influence of genetic variants on toxicity of ifosfamide. *CYP2B6*6* carriers have been

linked with ifosfamide-induced encephalopathy in a report of three pediatric cases (Duflot et al., 2018). Earlier, this genotype has been linked with lower catalytic activity and protein expression in the liver, higher concentrations of ifosfamide and higher rates of CAA associated toxicity (Wang and Tompkins, 2008). This could be a mechanism for ifosfamide-induced encephalopathy, though more extensive studies are needed to confirm this assumption.

In conclusion, prospective studies are needed to further elucidate the role of CYP2B6 polymorphism in the metabolism and toxicity of cyclophosphamide and ifosfamide.

Busulfan

Metabolism and Transport

Busulfan, widely used in conditioning regimens before hematopoietic stem cell transplantation, has a narrow therapeutic window and demonstrates wide interpatient variability in pharmacokinetics. High drug exposure is associated with increased risk of toxicities, such as veno-occlusive disease, while low drug exposure is associated with treatment failure. Busulfan is metabolized in the liver by glutathione S-transferase isoenzymes (*GSTs*). *GSTA1* is the predominant GST isoenzyme in the metabolism of busulfan. *GSTM1* and *GSTT1* are involved to a lesser extent.

Genetic Variances and Toxicity

In the past, several studies in adult and pediatric patients showed a higher busulfan clearance in patients with *GSTA1*A/*A* genotype (with consequent lower AUC), while patients with *GST*B/*B* genotype had lower clearance (with consequent higher AUC) (Myers et al., 2017). While this association has been found, it is noteworthy that not all studies found clinical correlations. Recently, one study has successfully incorporated *GSTA1* genotype into a pharmacokinetic model for busulfan in a group of 112 pediatric patients. In this study, *GSTA1*A2* or **A3* homozygote or heterozygote carriers showed a 7% higher clearance. Also, clearance of patients carrying *GSTA1*B1b*B1b* was 12% lower. Based doses in this study resulted in a better achievement of AUC targets (see **Supplemental Material** of Nava et al. for gene expression information) (Nava et al., 2018). However, another recent study showed no significant association with *GST* polymorphisms and busulfan pharmacokinetics (Nishikawa et al., 2019). These contradictory data may be attributed due to small study cohorts and variation in study design. Further basic research and clinical investigative efforts are required to fully understand the key factors determining busulfan PGx characteristics (Myers et al., 2017).

Anthracyclines

Anthracyclines are widely used in many pediatric cancers, including leukemia, lymphomas, and solid tumors. Anthracyclines agents are doxorubicin, daunorubicin, idarubicin, epirubicin, and mitoxantrone. While their mechanism of action is not fully known, it is believed anthracyclines interfere with DNA metabolism (including inhibition of topoisomerase II) and damage DNA through reactive oxygen species (ROS) (McGowan et al., 2017; Anthracyclines and related substances, 2020). Notorious for their severe cardiotoxicity, anthracycline cumulative doses are monitored closely during treatment.

Anthracycline-Induced Cardiotoxicity

Anthracycline-induced cardiotoxicity can be acute and reversible (within the first weeks of treatment) or develops one or more year(s) after treatment discontinuation and causes chronic cardiotoxicity. There are various theories with regard to the development of anthracycline-induced cardiotoxicity. One theory discusses the formation of ROS and topoisomerase II alterations which causes damage to cardiomyocytes and mitochondria in cells. ROS is mainly formed during anthracyclines metabolism. Also, risk factors such as sex, age, comorbidities, and cumulative dose of anthracyclines (>350 mg/m^2) play a relevant role in anthracycline-induced cardiotoxicity (see for more in-depth information on cardiotoxicity Bansal et al.'s review) (Bansal et al., 2017). However, ROS formation, topoisomerase II alterations and the mentioned risk factors do not fully explain the inter-individuals differences in the severity of cardiotoxicity among children (Huang et al., 2017). This led to the assumption that genes involved in metabolism and transport of anthracyclines as well as genes associated with the prevention of ROS and genes involved in iron homeostasis could also influence anthracycline-induced cardiotoxicity (see **Figures 1** and **2**) (Thorn et al., 2011; Aminkeng et al., 2016; Doxorubicin Pathway, 2020; Doxorubicin Pathway (Cardiomyocyte Cell) P, 2020).

Metabolism and Transport

Anthracyclines are metabolized through three pathways: hydroxylation, semiquinone formation, and/or deoxyaglycone formation. Hydroxylation is mediated by NADPH-dependent carbonyl (*CBR*) and aldo-keto (*AKR*) reductases. Enzymes known for catalyzing semiquinone formation are CYP reductase (*CYPR*), NADH dehydrogenase (*NDUFS*), nitric oxide synthase (*NOS*), and xanthine oxidase. These conversions also increase the formation of reactive oxygen species (ROS). The final metabolizing step is deoxyaglycone formation. This is catalyzed by NADPH quinone oxidoreductases (*NQO1*), *CPR*, and xanthine dehydrogenase (*XDH*) (see **Figures 1** and **2**) (Thorn et al., 2011; Edwardson et al., 2015; Doxorubicin Pathway, 2020; Doxorubicin Pathway (Cardiomyocyte Cell) P, 2020).

While the transport of anthracyclines is not fully known, acknowledged genes involved in transport of anthracyclines are ATP-binding cassette (*ABC*) *ABCB1, ABCC1, ABCC2, ABCG2*, and solute carrier (SLC) *SLC22A16* (see **Figures 1** and **2**).

Genetic Variants in Metabolizing and Transport Enzymes

Past studies showed a strong association between genetic variations *SLC28A3* (rs7853758 and rs885004) and detoxifying uridine diphosphate glucuronosyl transferase *(UGT) UGT1A6*4* (rs17863783) and anthracycline-induced cardiotoxicity (Visscher et al., 2013; Aminkeng et al., 2016). Also genetic variations in *CBR3* (Blanco et al., 2012; Visscher et al., 2012),

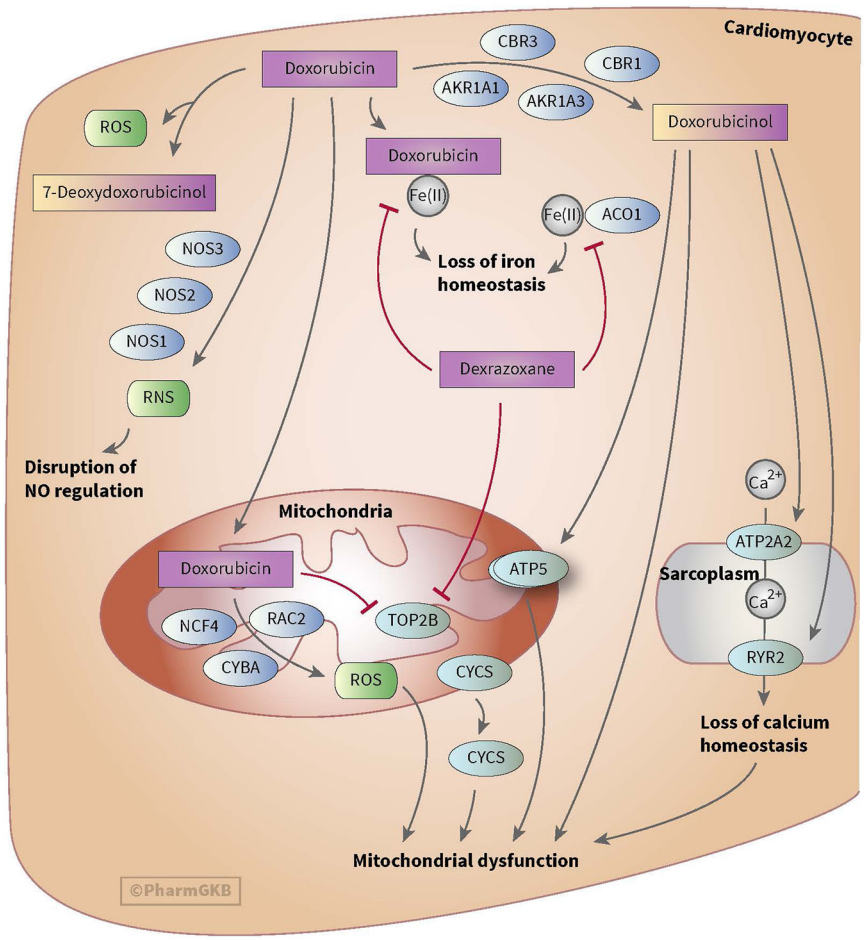

FIGURE 1 | Figure used with permission of PharmGKB (Thorn et al., 2011; Doxorubicin Pathway (Cardiomyocyte Cell) P, 2020). This is an example of genes involved in doxorubicin-induced cardiotoxicity. The presented genes (e.g. CBR, NOS, and AKR) are involved in the metabolizing and transport pathways of anthracyclines. The formation of ROS during the metabolism of anthracyclines is thought to play an important role in anthracycline-induced cardiotoxicity.

ABCC1 (rs3743527, rs246221) (Blanco et al., 2008), *SLC28A3* (rs885004 and rs4877847), *SLC22A17* (rs4982753, rs4149178) (Visscher et al., 2015), and sulfotransferase (*SULT) SULT2B1* (rs12882406 and rs12896494) (Visscher et al., 2015) have been associated with cardiotoxicity during anthracycline treatment (Conyers et al., 2018). In recent years, three studies have focused on *ABCB1, ABCC1, ABCC2, ABCC5, ABCG2, SLC28A3*, and *CYP3A5* (Krajinovic et al., 2016; Huang et al., 2017; Ruiz-Pinto et al., 2017).

A small Chinese study including 36 ALL patients showed no association between potential metabolizing *CYP3A* polymorphisms (*CYP3A5*1-*1, CYP3A5*1-*3, CYP3A5*3-*3*) and daunorubicin-induced cardiotoxicity (Huang et al., 2017). A study by Ruiz-Pinto et al. (2017) performed a GWAS with 93 pediatric cancer patients who had used doxorubicin, daunorubicin or epirubicin in the past. In this study, no association was found with transporter *ABCB1* and *SLC28A3* polymorphisms and chronic anthracycline-induced cardiotoxicity. A retrospective cohort study by Krajinovic et al. (Krajinovic et al., 2016) included 251 cALL patients and 44 cALL patients (validity set). Multiple polymorphisms in transporter genes *ABCC1, ABCC2, ABCC5, ABCB1*, and *ABCG2* were investigated for associations with doxorubicin-induced cardiotoxicity. The *ABCC5* (rs7627754) was found to be significant associated with a lower ejection fraction (EF) and shortening fraction (SF) 3 years after diagnosis, suggesting a possible higher risk for cardiotoxicity with patients carrying this polymorphism.

Genetic Variants Involved in ROS Prevention

Genetic variants involved in ROS prevention have received much attention in past studies (Aminkeng et al., 2016; Bansal et al., 2017; Chang and Wang, 2018). These include genetic variations in NADPH oxidase, Ras-related C3 botulinum toxin substrate 2 (*RAC2)* (Aminkeng et al., 2015), neutrophil cytosolic factor 4 (*NCF4)* (Visscher et al., 2012), Cytochrome B-245 Alpha Chain (*CYBA)* (Visscher et al., 2012; Windsor et al., 2012; Armenian et al., 2013) and catalase *(CAT)* (Rajić et al., 2009; Aminkeng

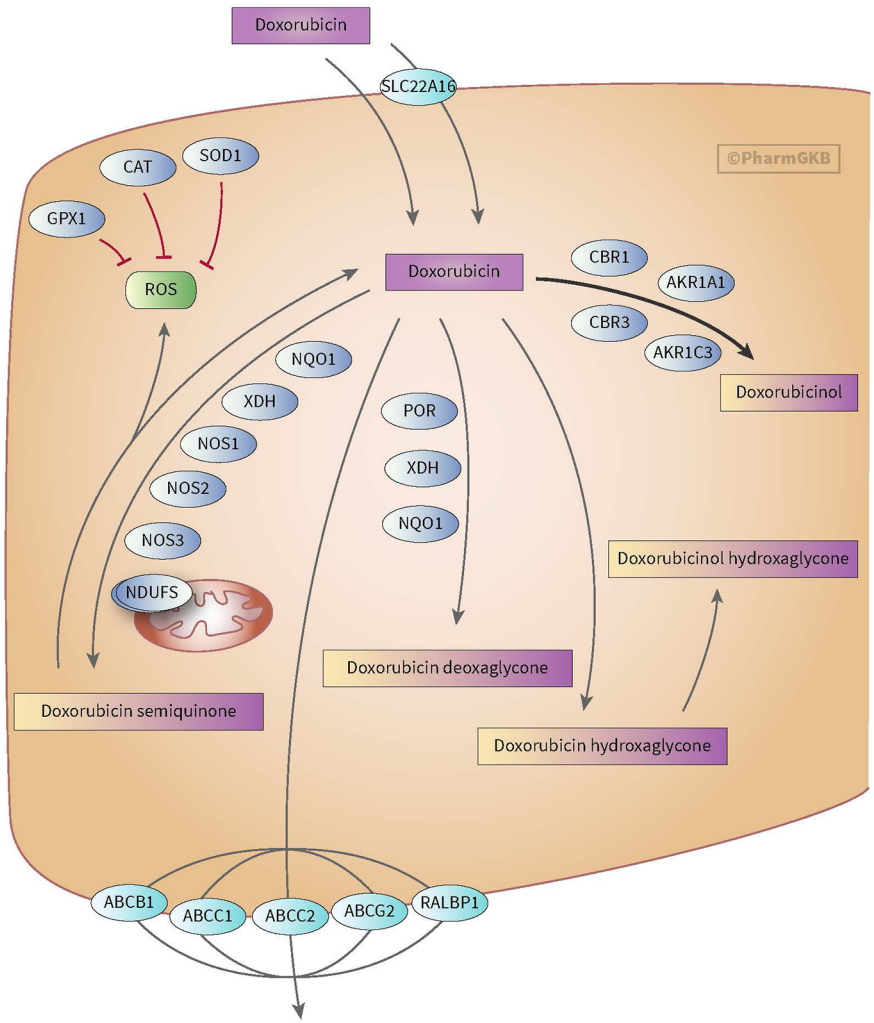

FIGURE 2 | Figure used with permission of PharmGKB (Thorn et al., 2011; Doxorubicin Pathway, 2020). This is an example of genes involved in doxorubicin pharmacokinetic pathway. These include metabolizing genes such as CBR, AKR, and NOS as well as SLC and ABC transporters.

et al., 2015; Aminkeng et al., 2016; Conyers et al., 2018). However, results in relation to these gene variants and anthracycline-induced cardiotoxicity remain inconsistent. One recent study investigated the role of polymorphisms involved in ROS prevention. A study by Krajinovic et al. (2016) found a possible protective effect of *NOS3* (rs1799983) leading to lower risk of developing chronic doxorubicin-induced cardiotoxicity.

Other Polymorphisms

Four recent studies discussed polymorphisms in genes not clearly relatable to the known mechanisms of action of anthracyclines.

Wang et al. (2016) found an association between CUGBP Elav-Like Family Member 4 (*CELF4*) (rs1786814) and anthracycline-induced cardiotoxicity. *CELF4* is involved in cardiac dysfunction and fibrosis. In this study, patients carrying *CELF4* (rs1786814) had a higher risk of developing anthracycline-induced cardiotoxicity. This risk increased significantly when patients received an anthracycline dose above 300 mg/m^2. Hildebrandt et al. (2017) showed a significant protective effect of 1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase epsilon-1 (*PLCE1*) (rs932764) and ATPase Plasma Membrane Ca2+ Transporting 1 (*ATP2B1*) (rs17249754) in chronic anthracycline-induced cardiotoxicity. The anthracyclines used were not specified in this study. *PLCE1* and *ATP2B1* are involved in calcium signaling in cells. Krajinovic et al. (2016) showed no significant associations between polymorphisms in MutL homolog 1 (*MLH1*), *MLH2* and *GSTs* and chronic doxorubicin-induced cardiotoxicity. *MLH1* and *MLH2* are genes playing a role in DNA repair and *GSTs* are detoxifying enzymes. Another recent study by Singh et al. (2020) investigated the role of *GSTM1* in chronic anthracycline-induced cardiotoxicity. They found that patients carrying *GSTM1* null genotype (i.e. enzyme activity is absent) had an increased risk of cardiomyopathie. This risk did not increase with patients who received anthracycline-doses of ≥250 mg/m^2. Ruiz-Pinto et al.

(2017) showed a significant association between chronic anthracycline (i.e. doxorubicin, daunorubicin or epirubicin)-induced cardiotoxicity and G protein-coupled receptor family 35 (*GPR35*) (rs12468485) gene. Patients carrying *GRP35* (rs12468485) developed cardiotoxicity more frequently.

Other Anthracycline-Induced Toxicities

One recent study investigated the role of Glucose-6-fosfaat-dehydrogenase (*G6PD*) (gene involved in the pathway of detoxifying ROS) normal or deficient enzyme function with daunorubicin-induced hematotoxicity. A retrospective study of Robinson et al. (2019) showed no association between *G6PD* normal of deficient function and daunorubicin-induced hematotoxicity.

Most recent studies focused on polymorphisms in metabolism and transport genes. More recently, other genetic variants are discovered through GWAS which play a possible role in toxicities of anthracyclines. However, there is no consensus on the role of metabolism, transport and other gene variants in anthracycline-induced toxicities, and more research is needed to confirm associated findings and propose dose adjustments to minimalize anthracycline-induced (cardio)toxicity.

Asparaginase

Asparaginase is a chemotherapeutic agent derived from bacteria *E. Coli* (Oncaspar®) and *Erwinia chrysanthemi* (Erwinase®) and is used in the treatment for acute lymphatic leukemia (Verma et al., 2007). Asparaginase catalyzes the deamination of asparagine to aspartic acid and ammonia, leading to a reduced serum asparagine concentration and leukemic cell death (Hijiya and van der Sluis, 2016). Unfortunately, asparaginase causes severe toxicities such as hypersensitivity, hepatotoxicity, pancreatitis, and thrombosis. These toxicities lead to therapy resistance, treatment discontinuation and eventually poor clinical outcomes (Hijiya and van der Sluis, 2016; Rank et al., 2019). A study by Rank et al. (2019) showed that pancreatitis occurred in up to 11% of children treated with asparaginase and 44% of patients re-exposed to asparaginase experienced a second episode of pancreatitis.

Genetic Variances and Toxicity

Over the last years, an increasing number of studies have reported associations between genetic variants and asparaginase toxicities (Abaji and Krajinovic, 2016; Lee and Yang, 2017; Lopez-Santillan et al., 2017; Rank et al., 2019). Genetic variants in asparagine synthase (*ASNS*), human leukocyte antigens (*HLA*) (Abaji and Krajinovic, 2016) and the glutamate Ionotropic Receptor AMPA Type Subunit 1 (*GRIA1*) have been found to influence asparaginase toxicity (Lee and Yang, 2017; Lopez-Santillan et al., 2017). Recently, eight studies investigated four asparaginase-induced toxicities (hypersensitivity, pancreatitis, thrombosis, and hepatotoxicity).

One GWAS by Abaji et al. (2017) assessed the association between asparaginase-induced hypersensitivity, pancreatitis, and thrombosis and polymorphisms. Three genetic variants, transporter *SLC7A13* (rs9656982), Myb-binding protein 1A *(MYBBP1A)* (rs3809849) (involved in embryonic and cellular development such as mitosis) and YTH Domain Containing 2 (*YTHDC2)* (rs75714066) (involved in regulate mRNA translation and stability), were associated with a higher risk of developing hypersensitivity. Three polymorphisms, ADAM Metallopeptidase With Thrombospondin Type 1 Motif 17 (*ADAMTS17)* (rs72755233) (function unknown), *MYBBP1A* (rs3809849) (involved in many cellular processes such as syntheses of ribosomal DNA) and Sperm Antigen With Calponin Homology And Coiled-Coil Domains 1 (*SPECC1*) (rs9908032) (function unknown), were associated with a higher risk of pancreatitis. Six polymorphisms were associated with a higher risk of thrombosis. These were Polycystin 2 Like 1, Transient Receptor Potential Cation Channel (*PKD2L1*) (rs6584356) (involved in cell-cell/matrix interactions), Ras And Rab Interactor 3 (*RIN3)* (rs3742717) (functions as a guanine nucleotide exchange for genes *RAB5B* and *RAB31*), Sperm Flagellar 2 (*SPEF2)* (rs34708521) (involved in axoneme development), Macrophage Expressed 1 (*MPEG1)* (rs7926933) (involved in cell cycle), interleukin-16 (*IL16)* (rs11556218) (involved in immune system), and *SLC39A12* (rs62619938).

A GWAS by Højfeldt et al. (2019) found a significant higher risk of hypersensitivity with CCR4-NOT Transcription Complex Subunit 3 (*CNOT3)* (rs73062673). Among other functions, this gene is involved in the regulation of human leukocyte antigen (HLA) gene transcription. While no significance was reached on a genome-wide significance level, Højfeldt et al. also discovered two gene risk variants (i.e. *HLA-DQA1* (rs9272131) and the antigen peptide transporter 2 (*TAP*2) which showed a higher frequency of asparaginase hypersensitivity with patients carrying these variants. These results show that variants in the HLA regions as well as genes regulating expression of these variants are involved in asparaginase hypersensitivity. To further investigate the role of HLA gene variants and asparaginase hypersensitivity, a study by Kutszegi et al. (2017) investigated *HLADRB1, HLADQB1*, and *HLADQA1* alleles (both HLA class II alleles). They showed a significant higher risk of developing hypersensitivity for *HLA-DRB1*07:01*, *HLA-DBQ1*02:02*, and *HLA-DQA1*02:01* carriers. Also, 27 amino acid positions in HLA class II alleles were found to be significant association with a higher risk for hypersensitivity as well as two haplotypes. These findings are replicated and also confirmed by Gagné et al. (2020).

Three GWAS studies analyzing multiple polymorphisms showed significant associations with asparaginase-induced pancreatitis (Liu et al., 2016; Liu et al., 2017; Wolthers et al., 2017). A GWAS by Liu et al. (2016) generated sixteen carboxypeptidase A2 (*CPA2)* single-nucleotide polymorphisms (SNP) that were associated with pancreatitis (highest association with rs199695765). However, this could not be reproduced by Wolthers et al. (2017). Wolthers et al. (2017) found associations with fourteen SNPs in the theunc-51-like kinase 2 (*ULK2*) gene (highest association with rs281366) and one SNP in G-protein signaling 6 (*RGS6*) gene (rs17179470), nuclear factor of activated T cells 2 (*NFATC2*, rs62228256), pancreatic secretory trypsin

inhibitor (*SPINK1*, rs17107315), chymotrypsin C (*CTRC*, rs10436957), and Claudin-2 (*CLDN2*, rs4409525) (Wolthers et al., 2019). The proteases cationic and anionic (*PRSS1-PRSS2*, rs13228878, and rs10273639) reduced the risk of pancreatitis (Wolthers et al., 2019).

One GWAS by Liu et al. (2017) investigated the role of polymorphisms in hepatotoxicity during asparaginase treatment. They found that higher alanine aminotransferase (ALT) levels were associated with patatin-like phospholipase domain-containing protein 3 *(PNPLA3)* (rs738409) (involved in the balance of energy usage and storage in adipocytes).

Studies included in this review added new knowledge to genes and polymorphisms that could play a role in asparaginase toxicity. Previous studies found associations between polymorphisms in asparagine synthase (*ASNS* gene), human leukocyte antigens (*HLA* gene) (Abaji and Krajinovic, 2016) and the glutamate receptor (*GRIA1* gene) and asparaginase toxicity (Lee and Yang, 2017; Lopez-Santillan et al., 2017; Hojfeldt et al., 2019).

Methotrexate

Methotrexate (MTX) is widely used in pediatric oncology treatment protocols of both hematological malignancies (including ALL) as well as solid tumors. MTX is administrated through various ways of administrations and dosages. These include high intravenous (IV) dosage (>500 mg/m^2, up to 12 g/m^2), low oral dosages during the maintenance phase as well as intrathecal administration. The drug has shown great benefit in many cancer treatments, but is also associated with various toxicities, ranging from gastro-intestinal toxicity (including severe mucositis), hepatic toxicity, neurotoxicity, nephrotoxicity, and hematological toxicity. These toxicities show large inter-individual differences in pediatric patients. Not only toxicity has been found to be unpredictable, also variations in MTX activity and resistance or reduced sensitivity have been seen in clinical settings.

Metabolism and Transport

MTX acts by inhibiting the folate acid cycle, resulting in impairing nucleic acid syntheses. Its pharmacological action follows a complex pattern, with many metabolic enzymes, transporters, and targets. Genetic variants may influence the pharmacological action of MTX in several ways, and many candidate polymorphisms have been studied in relation to folate pathways or MTX metabolism, in search for correlation with response or toxicity of MTX.

Genetic Variances and Toxicity

MTX enters the cell through active transport through reduced folate carrier (*SLCO1B1/RFC1*). Efflux transporter gene belongs to the ABC superfamily, including ABC transporters such as *ABCB1*. The OATP transporter family is expressed in a variety of tissues and organs important for, among others, MTX elimination. Across the blood-brain barrier, MTX undergoes saturable efflux, presumably through *ABCG2* and organic anion transporter OAT3 (see **Figure 3**) (Mikkelsen et al., 2011; Franca et al., 2017; Methotrexate Pathway, 2020). MTX is intracellularly metabolized to its active polyglutamate form (MTX-PGs) by folylpolyglutamate-synthetase (*FPGS*) and gamma-glutamyl hydrolase (*GGH*) enzymes. GGH enzymes catalyzes the removal of polyglutamates

Polymorphism in genes, coding for these transporters, have been studied for associations with an altered clearance and sensitivity of MTX (e.g. *ABCB1* and *ABCC4* genes), but results have been inconsistent (Ramírez-Pacheco et al., 2016; Hegyi et al., 2017). Genetic polymorphism in the ABC-transporter genes are believed to result in failure of the excretory system, prolonged MTX exposure and have been associated with higher incidence of myelosuppression during MTX treatment (Mlakar et al., 2016).

Recently, *SLCO1B1* gene (encoding for OATP1B1 transporter) has gained interest. The OATP1B1 transporter, located on the membrane of human hepatocytes, mediates disposition of many medications. *SLCO1B1* polymorphisms have been associated with lower MTX clearance, nephrotoxicity, and GI toxicity (Liu et al., 2017). While the association for *SLCO1B1* could not be replicated by Razali et al. (2020), they did show a possible increased risk of leukopenia with *ABCC2* (rs717620) or the transcriptional factor of B lymphocyte progenitors (*ARID5B*, rs4948496) carriers. Patients with genetic variants in *SLCO1B1* may benefit from increased precautionary measures like more aggressive hydration and alkalization (Mlakar et al., 2016).

SLCO1A2 (encoding for OATP1A2 transporter) plays a role in MTX elimination. A microRNA (miR) binding site polymorphism in *SLCO1A2* (rs4149009) showed delayed MTX excretion in Chinese population (Wang et al., 2018).

Preliminary evidence showed that *SLC19A1* polymorphism (*SLC19A1* 80G>A), an influx transporter involved in MTX uptake in gut and liver cells, may have a protective effect on the occurrence of mucositis (Park and Shin, 2016; Kotnik et al., 2017). *GGH* polymorphism (*GGH_16T/C*) has been described in relation to MTX associated hepatotoxicity in osteosarcoma patients (Hattinger et al., 2016).

Based on recent studies, genetic variance in transporter or metabolizing enzymes is expected to have influence on the exposure of MTX and its toxicity. However, given the complex route of metabolism and transport of MTX, a simple PGx model will probably not suffice.

Genetic Variances and Target

Both MTX and MTX-PGs inhibit dihydrofolate reductase (*DHFR*), an enzyme that catalyzes the conversion of dihydrofolate to its active form tetrahydrofolate. Tetrahydrofolate deficiency leads to the depletion of intracellular folates, resulting in decreased synthesis of nucleic acids and cell death. MTX-PGs also interfere with methylenetetrahydrofolate reductase (*MTHFR*), an enzyme which plays a major part in intracellular folate metabolism. Another enzyme target of MTX is thymidylate synthetase (*TYMS*), responsible for synthesis of a precursor of DNA synthesis.

Variants in *MTHFR* activity have been described and the role of *MTHFR* polymorphism (mainly C677T and A1298C genotypes) in relation to toxicity has been studied by several groups (Campbell et al., 2016; Ramírez-Pacheco et al., 2016;

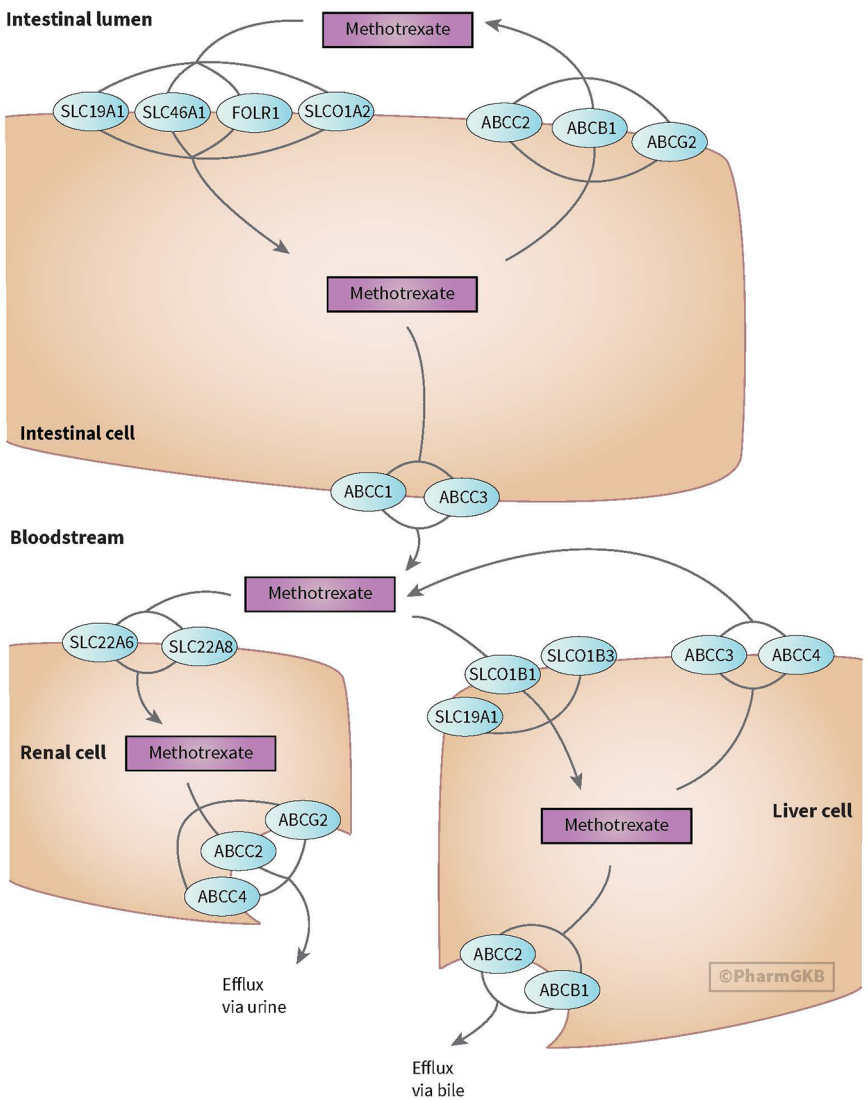

FIGURE 3 | Figure used with permission of PharmGKB (Mikkelsen et al., 2011; Methotrexate Pathway, 2020) Genes involved in transport of methotrexate (Mikkelsen et al., 2011; Methotrexate Pathway, 2020).

Mahmoud et al., 2018; Zhu et al., 2018; Yousef et al., 2019). However, recent reviews summarized the available data and showed ambiguous results (Umerez et al., 2017; Yao et al., 2019), concluding no clear correlation could be established between *MTHFR* polymorphism and MTX toxicity or relapse data.

DHFR and *TYMS* genes have also been studied in smaller cohorts in relation to hematological toxicity of MTX and to intrinsic resistance to MTX (Yousef et al., 2019). However, these results have also been inconclusive.

Neurotoxicity, Hepatotoxicity, and Mucositis

MTX and MTX-polyglutamates (MTX-PGs) interfere with the adenosine pathway by inhibiting 5-aminoimidazole-4-carboxamide ribonucleotide formiltransferase (*ATIC*) and promoting adenosine release. This pathway has been implicated in MTX-associated neurotoxicity. Adenosine receptor *ADORA2A* polymorphisms have been associated with MTX related leukoencephalopathy in a small cohort study (Tsujimoto et al., 2016) and with an increased risk on hepatotoxicity (Franca et al., 2017). A study performed by Gutierrez-Camino et al. (2017b) replicated results showing an association between MTX-induced mucositis and miR-1206 variant (rs2114358) in ALL patients.

In summary, many efforts have been undertaken to associate polymorphism in enzymes involved in metabolic routes or targets of MTX with toxicity or activity. These were mainly based upon pediatric cohorts of patients with ALL or osteosarcoma. Despite several positive associations, replication in other cohorts has been

difficult and evidence for the association between polymorphism and MTX toxicity is still inconsistent. Currently, pharmacokinetic (PK)/pharmacodynamic (PD) monitoring of MTX treatment is still mandatory as the genomic complexity associated with MTX treatment hampers the preemptive use of PGx to predict variability in toxicity or response in individual patients.

Platinum Compounds

In pediatric oncology, platinum compounds such as cisplatin and carboplatin are widely used in solid malignancy and neuro oncology treatments. Similar to alkylating agents, they cause DNA damage by establishing crosslinks within and between DNA strands. Characteristic toxicities of platinum compounds include nephrotoxicity, neurotoxicity, and ototoxicity.

Metabolism and Transport

Platinum compound transport is handled by several enzymes, including copper uptake protein 1 (*CTR1*), *ABCC2*, copper-transporting P-type ATPase (*ATP7A*), and *ATP7B*. Inside the nucleus, platinum-DNA adducts are formed. Several mechanisms influence the impact of DNA damage caused by platinum compounds, including recognition of platinum-DNA adducts (*HMGB1*), DNA mismatch repair (*MSH2, MSH6, MLH1*, and PMS1 Homolog 2, Mismatch Repair System Component (*PMS2*), nucleotide excision repair (X-Ray Repair Cross Complementing 1 (*XRCC1*), ERCC excision repair 1, endonuclease non-catalytic subunit (*ERCC1*), *ERCC2, ERCC3, ERCC4, ERCC6*, DNA Damage Recognition and Repair Factor *(XPA)* and SWItch/Sucrose Non-Fermentable (*SWI/SNF*) and translesion synthesis (DNA Polymerase Eta *(POLH)* and DNA polymerase Beta (*POBL*). Furthermore, several genes (Myeloperoxidase (*MPO*), superoxide dismutase (*SOD1), GSTM1, NQO1, GSTP1, GSTT1, MT1A*, and *MT2A*) may lower the intracellular concentration of platinum compounds (Platinum Pathway Pharmacokinetics/Pharmacodynamics, 2020).

Genetic Variances and Toxicity

Nephrotoxicity

Platinum compounds, mainly cisplatin, cause damage to the proximal tubules in the kidney, leading to acute kidney injury and electrolyte disturbances. Variants in *ERCC1* (rs3212986) (Khrunin et al., 2010; Tzvetkov et al., 2011), *EPHX1* (rs1051740) (Khrunin et al., 2014), organic cation transporter-2 *(OCT2)* (rs596881) and *CTR1* (rs12686377 and rs7851395) (Chang et al., 2017) have been associated with a reduced risk of renal toxicity in adult patients. All these genes play a role in platinum compound uptake and handling. No recent PGx studies were found regarding platinum compound nephrotoxicity in pediatric oncology populations.

Neurotoxicity

Platinum-induced peripheral neuropathy may lead to (irreversible) sensory and motor dysfunction. It is a dose-dependent phenomenon, which could be progressive months after treatment, leading to significant long-term disability. In a cohort of adult testicular cancer survivors, a GWAS revealed a correlation between reduced expression of Regulation Of Nuclear Pre-MRNA Domain Containing 1B *(RPRD1B)* and cisplatin-induced peripheral neuropathy (Dolan et al., 2017). *RPRD1B* is thought to play an important role in several DNA repair mechanisms. There are no data available on PGx of platinum-induced neuropathy in children.

Ototoxicity

Among platinum compounds, cisplatin imposes the highest risk of irreversible sensorineural hearing loss. Known concomitant risk factors include higher cumulative dose, younger age, treatment with additional ototoxic drugs and cranial irradiation. Several genetic variants related to cisplatin ototoxicity, includes rs12201199 in *TPMT*, rs9332377 in catechol-O-methyltransferase (*COMT*) (Ross et al., 2009), and rs62283056 in Wolframin ER Transmembrane Glycoprotein (*WFS1*) (Wheeler et al., 2017). However, results concerning these genes are conflicting (Thiesen et al., 2017).

A genetic variant (rs1872328) in Acylphosphatase 2 (*ACYP2*), coding for a protein thought to be responsible for hair cell development, was found to be significantly associated with hearing loss in pediatric patients treated with cisplatin for embryonal tumors (Xu et al., 2015) and osteosarcomas (Vos et al., 2016). Recently, a variant in *GSTP1* (rs1695), coding for a detoxification enzyme, was found to be associated with an elevated risk of hearing loss in pediatric patients treated with cisplatin or carboplatin (Liberman et al., 2018). Another recent study investigated the role of genes responsible for nucleotide excision repair in DNA (Turan et al., 2019). However, no significant association between ototoxicity and *ERCC1* (rs25487), *ERCC2* (rs13181), and *XRCC1* (rs11615) was found.

Apart from genetic factors, epigenetic factors influencing cisplatin ototoxicity have recently come to attention. In a pediatric medulloblastoma and primitive neuroectodermal tumor patient cohort, Brown et al. (2017) found an association between increased cisplatin ototoxicity susceptibility and a methylation locus (cg14010619) in the P21 (RAC1) Activated Kinase 4 (*PAK4* gene). This gene is responsible for stereociliary bundle migration in inner and outer cochlear hair cells.

In conclusion, PGx studies concerning platinum compound toxicity in pediatric populations have so far focused on ototoxicity. The results of these studies, however, have not yet resulted in PGx based dosing recommendations.

Glucocorticosteroids

Glucocorticosteroids (GCs) play a major role in the treatment of pediatric cancer. Despite significant benefits of high-dose GCs, treatment is associated with toxicities like hepatotoxicity, hypertension, muscle wasting, metabolic effects, neuro-psychiatric effects, osteonecrosis, and osteoporosis.

Metabolism and Transport

Glucocorticoids (GC) exert their activity by reducing cell proliferation and promoting apoptosis or cell arrest by binding to intracytoplasmic glucocorticoid receptors (*GR/NR3C1*). Three polymorphisms in the *NR3C1* gene are known to be associated

with reduced sensitivity of GCs: *TthIII* (rs10052957), *ER22/23K* (rs6189/rs6190), and *GR-9β* (rs6198). In contrast, *N363S* (rs6195) and *BC1I* (rs41423247) are associated with an increased sensitivity to GC (van Rossum and Lamberts, 2004).

GCs are metabolized in the liver primarily *via CYP3A4*. Expression of *CYP3A4* varies between individuals and has been associated with outcome in adult cancers (Miyoshi et al., 2002). Differences in expression of *CYP3A4* may be explained by polymorphisms in nuclear receptors (*NR112*) that are involved in the transcriptional regulation of *CYP3A4* (Lamba et al., 2010).

GCs are mainly transported by a multidrug resistance protein encoded by *ABCB1*. Inter-individual variability in the expression of multidrug resistant gene (*MDR1*) is observed and may influence the efficacy of GCs. Furthermore, *GSTs* are involved in several cellular processes and genes of the BCL2 family are involved in the apoptotic response of GCs.

Genetic Variances and Toxicity

Numerous studies have investigated the potential link between polymorphisms in the *NR3C1, CYP3A4, ABCB1, GST*, and *BCL2* genes and GC response and toxicity, but have yielded conflicting results and so far, none of the studied genetic variants has been implemented in the treatment of cancer. In the past four years only a few new studies have been published.

A recent study including 346 pediatric ALL patients showed that patients with N363S genotype in the *NR3C1* gene were more prone to steroid-induced toxicities during ALL treatment. Hepatotoxicity was significantly more frequent among patients with N363S genotype than non-carriers (Eipel et al., 2016). This underlines the hypothesis that increased GC sensitivity due to a polymorphism might lead to increased susceptibility to steroid-induced toxicity. A study by ElFayoumi et al. (2018) associated a polymorphism in the *ABCB1* (rs1045642 C3435T) gene with life-threatening infections due to GC treatment.

Bone fractures and osteonecrosis have most frequently been attributed to exposure to GCs. Two polymorphisms in the *BCL2L11* gene (891T>G rs2241843 and 29201C>T rs724710), encoding Bim protein, were significantly associated with steroid-induced osteonecrosis in children with ALL. The 891T>G was also confirmed in a replication cohort and influenced *in vitro* Bim gamma isoform levels. Bim proteins are believed to be involved in the sensitivity of ALL cells to corticosteroid-induced apoptosis (Plesa et al., 2019). A GWAS in children with ALL and osteonecrosis showed that the SNP rs10989692 near the glutamate receptor GRIN3A locus, was associated with osteonecrosis (Karol et al., 2015). Glutamate receptor variants were previously associated with arterial embolism and thrombosis (Lin et al., 2013). GCs have been shown to induce the expression of glutamine synthetase in osteoblasts (Olkku et al., 2004). Hence, variations in the glutamate receptors may contribute to vascular events leading to osteonecrosis in patients exposed to GC therapy (Karol et al., 2015). Meanwhile bone toxicity has mainly been considered a consequence of exposure to corticosteroids during ALL therapy (Mattano et al., 2000; Girard et al., 2013). Recently, the TS variant 2R/2R was associated with increased rise of osteonecrosis among children younger than 10 years at diagnosis suggesting that MTX may play a pathophysiologic role in the development of osteonecrosis (Finkelstein et al., 2017). Although evidence is limited, published data describe a positive association between polymorphisms in GC pathways and the efficacy and toxicity of GCs. Its impact on outcome is debatable since resistance to GC might be overcome by the effect of combination drug therapy. Concerning GC-induced toxicity larger studies are needed to investigate the role of genetic polymorphisms in the development of GC-induced toxicity to avoid severe complications.

Thiopurines

6-Mercaptopurine (6MP) is the cornerstone of the maintenance phase of ALL treatment in children and is used more often than its analogue tioguanine (TG). 6MP is required continuously for 2 to 3 years in leukemia treatment and used in oral dosages varying between 25 and 75 mg/m^2. Known for its narrow therapeutic window, 6MP is able to cause severe toxicities including myelosuppression, hepatotoxicity, and GI toxicity. 6MP treatment interruption is known to increase the risk of relapse. It is therefore of great importance to find and maintain the optimal 6MP dosage in ALL patients.

Metabolism and Transport

6MP is converted intracellularly by hypoxanthine guanine phosphoribosyl transferase (HPRT) into active 6-thioguanine nucleotides (6TG) which are incorporated into DNA, causing cell death. 6MP is methylated by the enzyme thiopurine S-methyltransferase (TPMT) into 6-methylmercapturine (6-MMP). Methylated 6MP metabolites also contribute to the cytotoxic effects of 6MP by inhibiting *de novo* synthesis of purines. Increased levels of 6-TGN and 6-MMP have been associated with an increased risk of toxicity (Koutsilieri et al., 2019).

TPMP activity is well studied and shown to be highly variable among individuals, although the incidence of genetic variants differs between ethnic populations (Jimenez-Morales et al., 2016). In Caucasians, 90% to 95% of subjects have a normal/high TPMT activity, 5% to 10% reduced and around 0.5% an absent enzymatic activity (Franca et al., 2019). In Asian and Hispanic population, the incidence of variant TPMT genes is much lower (Koutsilieri et al., 2019).

Three variant alleles *TPMT*2* (G238C), *TPMT*3A* (G460A and A719G) and *TPMT*3C* (A719G), account for more than 95% of the inherited variability in TPMT enzyme activity (Conyers et al., 2018), although more TPMT deficient variants have recently been identified, with a less frequent occurrence (Koutsilieri et al., 2019).

Genetic Variances and Toxicity

Polymorphisms in TPMT are well studied in relation to the risk of toxicity (mostly severe myelosuppression) and corresponding dosage adjustments (Jimenez-Morales et al., 2016). This has lead to implementation of Federal Drugs Authority (FDA) and European Medical Agency (EMA) supporting clinical

guidelines for preemptive testing of TPMT, corresponding with lower starting dosages of 6MP for poor and intermediate metabolizers (Relling et al., 2020). In this review, one study examining the tolerable dose and treatment outcome within patients carrying *TPMT* and nudix hydrolase 15 (*NUDT15*) genetic variants is included, while dose adjustments for *TPMT* and *NUDT15* intermediate and/or poor metabolizers are already used in practice (Liang et al., 2016).

The discovery of frequent thiopurine-induced myelosuppression in Asian populations, while *TPMT* variants rarely occur in these ethnic populations, has led to several studies on the role of *NUDT15* and *ITPA* gene variants (Liang et al., 2016; Milosevic et al., 2018). *NUDT15* dephosphorylates thiopurine active metabolites and reduces the formation of active 6TG nucleotides, whereas *ITPA* is assumed to stimulate the formation of 6TG nucleotides. Results with respect to the influence of *ITPA* polymorphism in relation to myelosuppression, hepatotoxicity or TGN formation have not been consistent and its clinical relevance is still controversial (Gerbek et al., 2018; Milosevic et al., 2018; Soler et al., 2018; Zhou et al., 2018; Khera et al., 2019; Wahlund et al., 2020). In contrast, in many recent studies, *NUDT15* gene defects (rs116855232, rs186364861, rs869320766, rs201094029) have shown to result in increased TG availability for incorporation into DNA and have consistently been associated with TG toxicity including early myelosuppression (Chiengthong et al., 2016; Moriyama et al., 2016; Lee and Yang, 2017; Moriyama et al., 2017; Moriyama et al., 2017; Soler et al., 2018; Choi et al., 2019; Khera et al., 2019; Koutsilieri et al., 2019). The incidence of *NUDT15* gene defects is higher in Asian and Hispanic populations, but *NUDT15* genetic polymorphism has also been described in European patients (Schaeffeler et al., 2019). Therefore, the most recent CPIC guideline on thiopurines recommends both TPMT and NUDT genotyping (Koutsilieri et al., 2019; Relling et al., 2019).

Other SNPs that have been studied in relation to thiopurine sensitivity are variants within the phosphoribosylglycinamide formyltransferase *(GART)* gene (involved in folate cycle), Molybdenum Cofactor Sulfurase *(MOCOS)* gene (involved in thiopurine metabolism) and Protein Kinase C And Casein Kinase Substrate In Neurons 2 *(PACSIN2)* gene (involved in thiopurine metabolism (Smid et al., 2016; Franca et al., 2019). Polymorphisms in *PACSIN2* have been associated with an increased risk of GI and hematologic toxicity during 6MP treatment. However, results have not been as convincing as *TPMT* and *NUDT15* polymorphism (Choi et al., 2019). Genetic variants in Apurinic/Apyrimidinic Endodeoxyribonuclease 1 *(APEX1)* have been studied in relation to a more general sensitivity to antimetabolite active drugs, as human *APEX1* is involved in DNA base excision repair pathway. In Asian populations *APEX1* (rs2307486) variant resulted in an increased risk of 6MP-related early onset neutropenia (Kim et al., 2018).

Transporter genes have also been studied and variants in the *ABCB1* gene (C1236T) have found to be associated with increased sensitivity to 6MP (Gervasini et al., 2017), as well as variant genes in efflux transporter *ABCC4* (Kim et al., 2018; Tanaka et al., 2018). These findings have been reported in small studies and need confirmation before being introduced in clinical practice.

In conclusion, in recent years evidence for preemptive TPMT and NUDT15 genotyping in facilitating tailored thiopurine treatment has been firmly established.

Topoisomerase Inhibitors

Topoisomerase I Inhibitors

Topoisomerase I (Top 1) inhibitors act by preventing ligation of single strand breaks in DNA. In pediatric oncology, topoisomerase I inhibitors (such as irinotecan and topotecan) are used in the treatment of multiple solid malignancies. Topoisomerase I inhibitors are known to cause serious toxicities, such as myelosuppression and severe diarrhea. As not much is known on pharmacogenetic mechanisms involved in topotecan metabolism, this section will focus on irinotecan.

Metabolism and Transport of Top 1 Inhibitors

Irinotecan is a prodrug that is transported over hepatocyte cell membranes by several transporters (*SLCO1B1, ABCB1, ABCC1, ABCC2*, and *ABCG2*). In the cytosol, it is converted to active compound SN-38 *via* carboxylesterase (*CES1*) and *CES2*. SN-38 actively inhibits topoisomerase I, forcing cells to arrest in S phase and leading to cell death. UGT1A1 detoxifies SN-38 through glucuronidation. UGT1A1 converts SN-38 into soluble and non-toxic SN-38 glucuronic acid, which is released into the intestines. As diarrhea is caused by accumulation of SN-38 in intestinal mucosa, detoxification to SN-38 glucuronic acid is important for limiting this toxicity. Furthermore, irinotecan itself can be converted to inactive metabolites APC and NPC by *CYP3A4* and *CYP3A5*. NPC can be converted to SN-38 by *CES1* and *CES2* (Mlakar et al., 2016).

Genetic Variances and Toxicity in Top 1 Inhibitors

Two genetic variants in *UGT1A1* are known to significantly influence irinotecan metabolism. *UGT1A1*28* allele (rs8175347) is more prevalent in Caucasian and African American populations compared to East Asian populations (Zhang et al., 2007). It is a thymidine-adenosine (TA) repeat in the *UGT1A1* promoter region, impairing transcription and therefore UGT1A1 enzyme function and SN-38 detoxification. This variant is associated with severe neutropenia and late diarrhea. According to the FDA drug label for irinotecan, dose reduction is recommended in adult *UGT1A1*28* poor metabolizers. However, evidence in a pediatric population showed no severe toxicity when a low dose of irinotecan was used (Stewart et al., 2007). *UGT1A1*6* allele (rs4148323) mainly occurs in East Asian populations. It is characterized by G71 to R substitution and causes decreased UGT1A1 enzyme function similar to the *UGT1A1*28* allele (Etienne-Grimaldi et al., 2015).

Efforts to identify genetic variants that influence irinotecan toxicity are ongoing. Trubicka et al. (2017) found that pediatric medulloblastoma patients with variants in DNA repair genes (such as *MSH2*, *RAD50*, nibrin (*NBN*), Fanconi anemia complementation group (*FANCM*), and exonuclease 1 (*EXO1*)) experienced significantly more adverse effects from treatment containing irinotecan. However, as these patients were treated with multiple drugs concomitantly, functional studies will be needed to elucidate the underlying mechanisms.

Topoisomerase II Inhibitors

Topoisomerase II (Top 2) inhibitors, such as etoposide, prevent ligation of double strand breaks in DNA. Etoposide is widely used in the treatment of both solid and hematological malignancies in pediatric oncology. Toxicities include myelotoxicity and increased long-term risk of secondary malignancies (such as myeloid leukemia).

Although many enzymes are known to play a role in metabolism and transport of Top 2 inhibitors (among which are CYP3A4, CYP 3A5, and UGT1A), no variants of significant clinical impact have been identified to date (Khera et al., 2019).

Vinca Alkaloids

Vinca alkaloids are included in chemotherapy regimens of hematologic malignancies, solid tumors, and neuro-oncology. Their main mechanism lies in the disruption of microtubule function during cell division, leading to a metaphase arrest in the cell cycle and apoptosis (Moudi et al., 2013; van de Velde et al., 2017; Vinka Alkaloid Pathway, 2020). Peripheral neurotoxicity is a well-known side effect of vinca alkaloids, while primarily of vincristine (VCR). VCR-induced peripheral neuropathy has an incidence rate between 78% and 100% (Kandula et al., 2016) leading to muscle weakness and pain in hand and feet (van de Velde et al., 2017). It is dose-dependent and develops most severe at doses above 2 mg/m^2 (Park et al., 2013). Therefore, in pediatric oncology a dose maximum of vincristine is fixed at 2 mg to prevent severe neurotoxicity (Kandula et al., 2016).

Genetic polymorphisms are believed to play a role in a patients' sensitivity for VCR-induced neurotoxicity. These include genes involved in metabolism and transport out of (hepatic) cells of vinca alkaloids as well as genes involved in pharmacodynamics, stabilization, and formation of microtubules and nerves and inherited neuropathy genes (van de Velde et al., 2017). In this review we were only able to update vincristine (VCR) PGx in ALL patients as our literature search revealed no recent studies regarding vinblastine or vinorelbine or other pediatric cancer populations.

Metabolism

CYP3A4 and *CYP3A5* enzymes are involved in the metabolism of vinca alkaloids in the liver and particularly known for VCR metabolism (Vinka Alkaloid Pathway, 2020). Four recent studies showed no association between vincristine (neuro)toxicity and polymorphisms in metabolizing enzymes.

In a retrospective study by Franca et al. (2017) 28 single nucleotide polymorphisms (SNPs) and two deletions genes involved in efficacy and adverse effects were collected to find an association between the polymorphisms and grade III/IV gastrointestinal, hepatic, and neural toxicity. Before adjusting for multiple variables, four genes were found to be associated with vincristine related toxicities. *ITPA* (rs1127354) increased the risk of neurotoxicity and gastrointestinal toxicity, while *ADORA2A* (rs2236624) was found the associated with hepatic toxicity. After adjustment for multiple variables, none of the associations remained significant (Franca et al., 2017). McClain et al. (2016) performed a retrospective study with 239 Hispanic ALL patients, analyzing *CYP3A5* extensive (*1/*1), *CYP3A5* intermediate (*1/*3, *1/*6, *1/*7) and *CYP3A5* poor metabolizers (*3/*3, *3/*6, *3/*7). No significant association was found between *CYP3A5* polymorphisms and vincristine-induced peripheral neuropathy (VIPN). Skiles et al. (2018), included a cohort of 78 Kenyan children to find an association between *CYP3A5* and VIPN. Ninety one percent of the children were *CYP3A5* high-expresser genotypes and none developed neuropathy, leading to no conclusive results.

Results in the past are conflicting, showing associations as well as no associations between *CYP3A4, CYP3A4*1B, CYP3A5, CYP3A5*3*, and VIPN (Kandula et al., 2016; Mora et al., 2016; van de Velde et al., 2017; Conyers et al., 2018).

Transport

Vinca alkaloids are transported out of cells through *ABCB1, ABCC1, ABCC2, ABCC3, ABCC10*, and RalA-binding protein 1 (*RALBP1*). Seven recent studies investigated polymorphisms in transport genes and the risk on developing vincristine neurotoxicity. One study showed a significant association between *ABCC1* (rs3784867) and VIPN.

As stated above, Franca et al. (2017) investigated 28 SNPs and two deletions, including *ABCC1* (rs35592, rs246240, rs3784864, rs11075291) and *ABCC2* (rs17222723). Before adjustment for multiple variables, *ABCC1* (rs246240) was associated with increased the risk of neurotoxicity. After adjustment, this result did not reach significance. Lopez-Lopez et al. (2016) performed a retrospective study including 152 B-cell ALL Spanish patients. In this study 150 genetic variants involved in VCR pharmacokinetics and 13 microRNAs were analyzed. A significant higher risk of developing neurotoxicity grades 1 to 4 during vincristine treatment was found for rs3740066 and rs12826 in the *ABCC2* gene. Zgheib et al. (2018) assessed the association between grade II or higher VIPN and *ABCB1* (rs1045642), *ABCB1* (rs1128503) and *ABCC2* (rs717620). The study included 133 Arab ALL patients, where 19.5% developed VIPN. None of the polymorphisms showed a significant association with VIPN. In a retrospective study by Wright et al. (2019), a higher risk of VIPN was associated with *ABCC1* (rs3784867).

Gutierrez-Camino et al. (2017a) performed a retrospective study including 179 Spanish children with B-cell ALL. In this study, the authors analyzed 154 microRNAs (miRNAs) to find an association between the miRNAs and vincristine neurotoxicity. Three miRNAs were found most significant before corrections. These were miR-3117 (rs12402181) involved in *ABCC1* and RalA-binding protein 1 (*RALPBP1*) expression, miR-4481 (rs7896283), and miR-6067 (rs35650931). miR-3317 and miR-6067 were associated with a decrease risk of neurotoxicity during vincristine treatment, while miR-4481 was associated with a higher risk of neurotoxicity. After multivariable correction, none of the miRNAs produced a significant result.

Pharmacodynamics, Stability of Microtubules and Neurotoxicity Sensitivity

Polymorphisms in pharmacodynamics (i.e. actin gamma 1 (*ACTG1*) (Ceppi et al., 2014), formation and stabilization of microtubules (i.e. Centrosomal Protein 72) (*CEP72*) (Diouf et al.,

2015), Microtubule Associated Protein 4 (*MAP4*) (Ceppi et al., 2014), Capping Actin Protein, Gelsolin Like (*CAPG*) (Ceppi et al., 2014) Tubulin Beta 1 Class VI (*TUBB1*), *TUBB2A, TUBB2B, TUBB3, TUBB4*) (Ceppi et al., 2014) and genes known to influence neurotoxicity sensitivity (i.e. Charcot-Marie-Tooth disease confirmed in adults) have in the past been associated with vincristine toxicology (Kandula et al., 2016; Lee and Yang, 2017). A special attention is drawn to the *CEP72* gene. In a past preliminary study with ALL children, genetic variants in the promotor region of *CEP72* were associated with a higher risk of developing VIPN (Diouf et al., 2015). This investigation has recently been replicated in more studies. Seven recent studies investigated genetic variations in pharmacodynamics, microtubules stability, and neurotoxicity sensitivity. Two studies found an association with VIPN.

Gutierrez-Camino (2016) showed no significant association between VIPN and *CEP72* (rs924607) in a retrospective Spanish cohort with 142 B-cell ALL patients. McClain et al. (2016) also found no significance with polymorphisms in the *CEP72* gene, while Wright et al. (2019) showed that *CEP72* (rs924607), *SLC547* (rs1013940) (choline transporter), and Alpha Tocopherol Transfer Protein (*TTPA*, binding to vitamin E) (rs1050436) were significant associated with a higher risk of developing neuropathy. No association was found by Zgheib et al. (2018) between VIPN and *CEP72* (rs924607), Ewing's tumor-associated antigen 1 *(ETAA1)* (rs17032980) and Melatonin Receptor 1B *(MTNR1B)* (rs12786200).

Li et al. (2019) investigated two independent cohorts and used a meta-analysis to assess the association between multiple SNPs and VIPN. One SNP, rs1045644 (encoding for protein cochlin, which is associated with progressive hearing loss and vestibular imbalance), showed a protective effect against neuropathy, while rs7963521 (gene involved in angiogenesis) was associated with a higher risk of VIPN. Martin-Guerrero et al. (2019) used a retrospective cohort with 152 B-cell ALL patients to analyze 24 polymorphisms (in *TUBB1, TUBB2A, TUBB2B, TUBB3, TUBB4, MAPT*, *MIR146a*, *MIR202*, and *MIR411*). Before adjusting for false discovery rate, several gene variants have been associated with vincristine-induced neurotoxicity. Patients carrying *MAPT* (rs11867549), Mir-202 (rs12355840), and *TUBB3* (rs4395073) had a higher risk of developing neurotoxicity. Also several haplotypes were associated with neurotoxicity.

Abaji et al. (2018) screened retrospectively WES data of ALL patients to find possible new gene variants which could be associated with VIPN. In this study, three new gene variants are association with VIPN: Spectrin Repeat Containing Nuclear Envelope Protein 2 (*SYNE2*) (rs2781377), Mitochondrial Ribosomal Protein L47 (*MRPL47*) (rs10513762) and Bromo Adjacent Homology Domain Containing 1 (*BAHD1*) (rs3803357). While *SYNE2* (rs2781377) and *MRPL47* (rs10513762) were associated with a higher risk of developing VIPN, *BAHD1* (rs3803357) showed a protective effect. *SYNE2* is a protein involved in cellular cytoskeletons, DNA damage repair and other cellular processes. *MRPL47* plays a role in the mitochondrial protein synthesis. *BAHD1* is a protein involved in gene silencing

Studies investigated polymorphisms in metabolism, transport, pharmacodynamics, and sensitivity genes of patients to VIPN shows conflicting results. While CEP72 has drawn more attention, its role has yet to be confirmed. The new gene variants associated with VIPN showed by Abaji et al. needs to be replicated by other studies to include these gene variants as risk factors for developing VIPN.

DISCUSSION

Chemotherapeutics are known for their narrow therapeutic window. While it is of utmost importance to use the right dose to sustain a favorable outcome in pediatric oncology, toxic doses cause poor outcomes, and a worse quality of life for pediatric cancer survivors. Based upon inter-individual differences in occurrence and severity of toxicity between pediatric cancer patients, the need for personalized treatment is apparent. PGx has been introduced to understand and facilitate individual treatments in pediatric oncology.

With this review, we present new developments over the past years concerning PGx within pediatric oncology. We included relevant literature from 2016 onward, revealing new genetic variations as well as new evidence for already known associated genetic variations with chemotherapeutics' toxicity.

Most studies have focused on genetic variations within metabolism or transport genes. Surprisingly, while CYP enzymes influence drug exposure significantly within other drug categories [e.g. selective serotonin reuptake inhibitors (Relling et al., 2020)], associations with toxicities of alkylating agents, anthracyclines, topoisomerase inhibitors and vinca alkaloids were not consistently found in studies included in this review. ABC transporters play a role in the transport of anthracyclines, methotrexate, platinum compounds, glucocorticosteroids, thiopurines, topoisomerase inhibitors, and vinca alkaloids. SLC transporters are thought to play a role in the transport of anthracyclines, asparaginase, methotrexate, topoisomerase inhibitors, and vinca alkaloids. However, there is slight evidence for robust and reproducible correlations between transporter genes and efficacy or toxicity of chemotherapeutics.

The role of ontogeny in the activity of metabolic enzymes or transporters may play a part when studying patient populations of different age groups, especially in neonates and infants (Mlakar et al., 2016). In our review, we focused on pediatric populations but found no indication that (young) age was considered an independent factor that influences the role of PGx in toxicity of chemotherapeutic drugs. However, neonates and infants are underrepresented in pediatric oncology studies, because of limited occurrence of malignancies in these age groups. Strong evidence for associations between genetic variations in *NUDT15* and *TPMT* and 6MP toxicity have been confirmed in our review and preemptive genetic testing for NUDT15 and *TPMT* variants should be implemented in standard clinical care. Future attention should be focused on standardizing corresponding dosing guidelines and exploring the relevance of less frequent occurring variants of *TPMT* and

NUDT15. Evidence has been established for the role of *UGT1A1* polymorphisms in irinotecan toxicity in adults, but this still need confirmation in pediatric populations.

Due to improvements in genomic sequencing technologies, research has shifted to genetic variations in pharmacodynamics and cytostatic targets as well as less apparent gene polymorphisms as an explanation for efficacy or toxicity differences of chemotherapeutics in pediatric cancer patients. Variations in genes coding for cytostatic targets (e.g. microtubule stabilization by VCR, glucocorticoid sensitivity, folate activity by MTX) have been included in this review and showed probable contribution to individual differences in response and toxicity. However, there were no decisive conclusions that could be drawn from these studies and recommendations on dose adjustments are not yet established.

We did not include studies on immunotherapy or supportive care drugs. The use of immunotherapy in pediatric oncology has increased in the past years, showing promising results. However, as with chemotherapeutics, individual differences in response and toxicity of immunotherapy within children are reported. In future years, the role of genetics will need to be further elucidated. Supportive care drugs are also of great importance in limiting chemotherapeutics' toxicities (e.g. neuropathy, diarrhea, infections due to neutropenia). Therefore, optimalization of supportive care dosages is needed and PGx may be beneficial in assessing the correct dosages. Currently, adult guidelines are used to assess the impact of PGx in dosing supportive care drugs in pediatric oncology.

Our review shows that pharmacogenomics in pediatric oncology is still facing challenges. The main problem is inconsistency in results, caused by small population sizes, differences in (statistical) interpretation, variations in sequencing technologies as well as in differences in definition of clinical outcomes. Lack of information on how to use genetic test results to adjust the use or dose of chemotherapeutics hinders broad introduction in clinical practice (Relling et al., 2020). Also, pediatric cancer patients receive combinations of chemotherapeutics, making it difficult to discern the impact of individual genetic variances. Finally, most studies are performed in pediatric ALL patients, leading to limited data on other pediatric cancer types.

In conclusion, pharmacogenomics of chemotherapeutics is complex with multiple genes involved in the process of metabolism, transport and its target mechanisms. Future studies should focus on establishing comprehensive models, integrating pharmacogenomics with pharmacokinetics and pharmacodynamics data to aid dosing guidelines. Standardization of clinical outcome data and toxicity definitions within electronic health records combined with the increased availability of genomic sequences techniques in clinical practice will help to validate these models in larger populations.

AUTHOR CONTRIBUTIONS

EB developed the search codes (with libraries from Utrecht University Library) for the databases and collected the data. All authors reviewed the results and contributed to the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.01184/full#supplementary-material

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Association of Apelin and Apelin Receptor Polymorphisms With the Risk of Comorbid Depression and Anxiety in Coronary Heart Disease Patients

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The Apelin (APLN)/apelin receptor (APLNR) signaling pathway is a newly identified regulator in various cardiovascular diseases, which is considered as a candidate pathway for the occurrence of coronary heart disease (CHD), depression, and anxiety. The goal of this study was to investigate the association between APLN/APLNR gene polymorphisms and the risk of depression and anxiety in CHD patients. To this end, a case-control study involving 269 CHD patients and 184 healthy control individuals was conducted. The 269 patients with CHD including 122 patients with and 147 patients without depression, and 56 patients with and 213 patients without anxiety Four single nucleotide polymorphisms were selected and successfully genotyped using Sanger sequencing. The APLN rs2235310T allele and APLNR rs9943582C allele were found to be associated with an increased risk of CHD after multiple test correction (*P*-adjust < 0.05). The patients with CHD who carried the rs9943582C allele had a higher risk of depression, after adjusting for alcohol drinking habits, insomnia, hypertension, and stroke history, with the Bonferroni correction (*P*-adjust = 0.018). The APLNR rs2282623 T allele was associated with an increased risk of anxiety in CHD patients after adjusting for related disease complications, with the Bonferroni correction (*P*-adjust = 0.022). We reported for the first time that the APLN rs2235310 and APLNR rs2282623 polymorphisms are associated with the risks of psychiatric disorders in CHD patients and may serve as novel biomarkers for therapy.

Keywords: coronary heart disease, depression, gene polymorphisms, apelin, anxiety

INTRODUCTION

Coronary heart disease (CHD) is one of the most common chronic diseases, having a serious effect on human health and quality of life (Pezzella, 2010). Epidemiological studies show that CHD patients are more likely to suffer from psychiatric disorders, including depression and anxiety (Chauvet-Gélinier et al., 2013). Over the past few decades, an ever-expanding number of

prospective reports and meta-analyses have provided evidence that depression and anxiety are risk factors for the morbidity and mortality in patients with and without established CHD (Lichtman et al., 2008; Carney and Freedland, 2017; Hohls et al., 2020). Patients with CHD, who also have depression or anxiety, have worse outcomes than do those without depression and anxiety (Hare et al., 2014). Researchers have suggested that the risks of mortality and cardiovascular events are directly correlated with the severity of depression, the more severe depression associated with more severe cardiac events (Rugulies, 2002). Meanwhile, cognitive-behavioral treatment and positive psychology intervention have been showed to improve several psychological symptoms and reduced cardiac mortality for patients with CHD or coronary artery disease (CAD; Richards et al., 2018; Magan et al., 2020). Hence, it's quite important to disclose the relationship between CHD and psychical disorders.

Reasons for the relationship between CHD and psychical disorders are complicated, mainly including biological and behavioral mechanisms. An increasing number of studies show that CHD and psychiatric disorders share common biological mechanisms, including endothelial and platelet dysfunction, inflammation, autonomic dysfunction, and hypothalamus–pituitary–adrenocortical axis dysfunction (Martinez-Quintana et al., 2020). The behavioral factors, including poor adherence to medical treatment and physical inactivity, associate with increased risk of both CHD and depression (Kamphuis et al., 2007). In recent decade, numbers studies have suggested that genetic factors are important determinants of CHD and psychiatric disorders (Han et al., 2019).

The APLN/APLNR signaling pathway is involved in various pathological processes and physiological functions, including cardiovascular disease, angiogenesis, energy metabolism, and central nervous system disease (Lv et al., 2020). A previous meta-analysis has shown that the circulating apelin level is a prominent athero-protective marker against the development of CAD, while the APLNR rs9943582 polymorphism in the APLNR promoter, is associated with an increased risk of CAD (Chen et al., 2017). Furthermore, increased levels of serum apelin have been shown to be significant independent predictors of the development of depression and anxiety in patients on peritoneal dialysis (Gok Oguz et al., 2016).

Thus, the APLN/APLNR signaling pathway is considered a candidate pathway for the occurrence of CHD, depression, and anxiety. Therefore, the present study aimed to evaluate the association between APLN and APLNR gene polymorphisms and the risk of comorbid depression and anxiety in Chinese patients with CHD.

MATERIALS AND METHODS

Subjects

A total of 269 patients with CHD and 184 matched healthy volunteers were enrolled in this study, which was conducted at the outpatient clinic of the Jining First People's Hospital in Shandong Province and at the Second Xiangya Hospital in Hunan Province to investigate the effects of genetic polymorphisms in patients with CHD combination with depression and anxiety. This study was conducted in compliance with the stipulations of the Declaration of Helsinki, and the study protocol was approved by the Ethics Committee of the Jining First People's Hospital. Written informed consent was received from each participant prior to the study, and the participants were identified by numbers throughout the study.

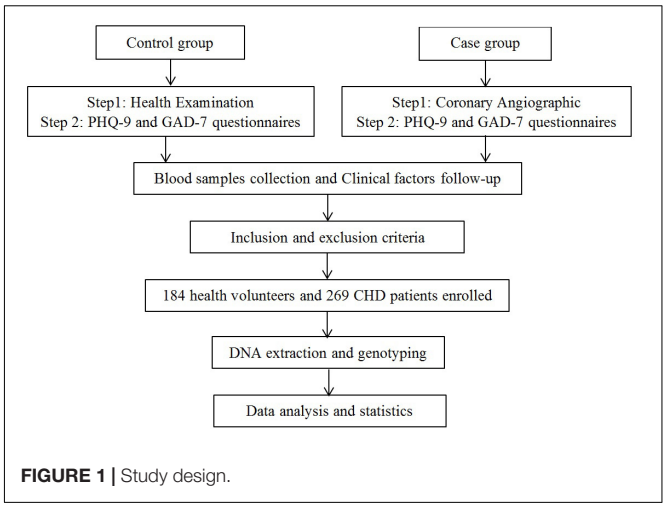

FIGURE 1 | Study design.

Study Design

The study design of this research is shown in **Figure 1**. The sample size for this research was calculated using Power and Sample Size Calculation version 3.1.2 (*Department of Biostatistics, Vanderbilt University, Nashville, TN, United States*) based on the following parameters: an independent case–control study; a type I error (α) of 0.05; a statistical power of 0.8; the probability of exposure in cases (p0) of 0.15; the probability of exposure in the control (p1) of 0.3; and the control to case ratio (m) of 0.8. Based on these parameters, the calculated minimum sample size was 137 experimental subjects and 110 control subjects.

Diagnosis of CHD

The diagnosis of CHD was independently made by two experienced cardiologists based on the coronary angiographic findings in the patients. The reference standard for CHD diagnosis was the presence of severe coronary artery stenosis (more than 50%) in at least one major coronary artery or major branches. Patients were excluded if they had other serious disease including severe autoimmune disease, valvular heart disease, severe liver and/or kidney disease, and cancer.

Symptoms of Depression and Anxiety

The presence of depression in the patients with CHD was evaluated by two experienced psychiatrists according to the criteria of the 5th Edition of the Diagnostic and Statistical Manual of Mental Disorders. The depression level was scored using the Patient Health Questionnaire-9 (PHQ-9), a nine-item questionnaire that is commonly used to screen for symptoms of depression in outpatients. To reduce the interference of environmental factors on

questionnaire performance, all patients were required to complete the questionnaire alone in a separate room, unless they required assistance with writing or reading. We set a threshold of the PHQ-9 scale >5 points to indicate the possibility of depression.

The seven-item Generalized Anxiety Disorder 7 (GAD-7) scale, which is widely used to measure anxiety symptoms in the general population, was used to assess symptoms of anxiety in the patients. The process of evaluating anxiety is the same as evaluating depression. A score of five or greater on the GAD-7 scale represented a cutoff point for identifying symptoms of anxiety.

Control Group

Healthy control individuals were selected among adults without CHD who had underwent several assessments including clinical physical examination, radiographic chest examination, electrocardiogram analysis, and evaluation of medical history. Moreover, all healthy volunteers completed questionnaires, including PHQ-9 and GAD-7 according to standard process. The presence of psychiatric disorders was evaluated by two independent psychiatrists based on the questionnaires. Finally, 184 age- and sex-matched healthy volunteers were enrolled in this study.

Data Collection

Data were collected by reviewing the outpatient medical records. Clinical factors were mainly obtained from the electronic medical records, including demographic variables (e.g., age, sex, height, weight, and smoking and drinking habits) and coexisting diseases (e.g., hypertension, diabetes mellitus, and chronic gastritis).

Single Nucleotide Polymorphism (SNP) Selection

We first systematically reviewed studies that reported genetic polymorphisms in the APLN/APLNR signaling pathway and selected previously investigated SNPs based on published studies found in PubMed. The SNP inclusion criteria were an association with a functional change, and a significantly associated with the risk of CHD and psychiatric disorders. The tag SNP strategy was further used to ensure a better detection rates, all the selected SNPs had a minor allele frequency >0.1 in Chinese or Asians. If several SNPs were in linkage disequilibrium (LD, $r^2 > 0.8$), only one representative SNP was selected, e.g., rs2235309 was in a high LD with rs2235310, and rs7119375 was in a high LD with rs9943582. The final candidate tag SNPs were as follows: rs3115757 and rs2235310 in APLN, and rs9543582 and rs2282623 in APLNR.

DNA Extraction and Genotyping

Peripheral venous blood (4 mL) was collected from each subject into an anticoagulation tube filled with sodium citrate. Genomic DNA was extracted using the TIANamp Blood DNA Kit (Tiangen, China) following the manufacturer's instructions. Primers for PCR amplification and sequencing are listed in **Supplementary Table S1**. The PCR reaction was performed in a total volume of 25 μl reaction containing 2.5 μl of 10 × PCR buffer (TaKaRa Bio Inc., Japan), 2 μl dNTP mixture (TaKaRa Bio Inc.), 2 μl genomic DNA, 0.2 μl Taq polymerase (TaKaRa Bio Inc.), 0.5 μl of each primer and 17.3 μl of water. The PCR products were sequenced by Sanger sequencing, with the assisted of Shanghai Majorbio Bio-pharm Technology, Co., Ltd., Company.

TABLE 1 | Demographic characteristics of the enrolled subjects.

Variables	CHD (*N* = 269)	Control (*N* = 184)	*P*-value
Age (years)	61.24 ± 12.08	59.82 ± 11.79	0.39
Gender (M/F)	144/127	45/39	0.78
Smoking	87 (32.34%)	54 (29.35%)	0.54
Drinking	90 (33.46%)	46 (25.00%)	0.06
BMI	23.69 ± 1.98	23.47 ± 2.13	0.67

CHD, coronary heart disease.

Statistical Analysis

All analyses were performed using SPSS Statistics version 19.0 (*SPSS, Inc., Chicago, IL, United States*). Data regarding the demographic, clinical, and genetic characteristics of the patients are presented as the mean ± standard deviation or counts (%) as appropriate. Differences in the demographic and clinical characteristics between the patients and healthy control individuals were evaluated using a *t*-test for continuous variables and χ^2-test for categorical variables. The Hardy–Weinberg equilibrium (HWE) for polymorphisms was assessed using the χ^2 test. Pairwise LD analyses were carried out using SHEsis. The genotype distributions and allele frequencies in the patients with CHD and controls were analyzed using the χ^2 test. The Bonferroni adjustment was applied to correct for multiple comparisons. Differences in the risks of CHD and psychiatric disorders between groups were calculated using multivariate analysis of variance followed by the Bonferroni correction for multiple comparisons. *P*-values were adjusted for complications, when needed. Odds ratios and 95% confidence intervals were also calculated.

RESULTS

Characteristics of the Study Participants

The demographic characteristics of the patients with CHD and healthy control individuals are provided in **Table 1**. No significant differences were observed between the patient and control groups ($P > 0.05$). The patients with CHD were further divided into the following subgroups, depending on whether comorbid depression (D) or anxiety (A) was present: CHD+D, CHD-D, CHD+A, and CHD-A. The demographic and clinical characteristics of the patients from these subgroups are shown in **Table 2**. Approximately 50% of the patients with CHD had depression or anxiety. Moreover, 51 of the 56 patients (91.7%) with anxiety had comorbid depression.

The patients with CHD who had depression had a markedly lower rate of alcohol intake than did those without depression (28.69% vs. 44.22%, respectively; $P = 0.011$). Furthermore, the patients with comorbid depression or anxiety had an increased rate of insomnia relative to those without these psychiatric disorders (47.54% vs. 24.49% and 50.00% vs. 30.98%, respectively; $P < 0.05$). We also found that patients with CHD who had depression had markedly lower rates of comorbid hypertension and a stroke history than did those without depression (50.82% vs. 99.32% and 7.38% vs. 15.65%, respectively; $P < 0.001$). Similarly, the patients with anxiety symptoms had lower rates of comorbid hypertension, diabetes mellitus, and a stroke history than did those without anxiety symptoms (57.14% vs. 82.63%, 16.07% vs. 20.19%, and 10.71% vs. 12.20%, respectively; $P < 0.001$).

Hardy–Weinberg Equilibrium Analysis

The following candidate tag SNPs were selected for analysis: rs3115757 and rs2235310 in APLN gene and rs9543582, and rs2282623 in APLNR gene. The genotypes of the four SNPs in the CHD and control groups were in HWE based on the χ^2 test results, suggesting that the cases enrolled in this study were representative of the population as shown in **Table 3**. The LD analysis indicated that the SNPs studied were in a low LD with each other ($r^2 < 0.8$), as shown in **Supplementary Figure S1**.

Association Between Gene Polymorphisms and CHD Risk

The frequency distributions of the genotypes and alleles of the four selected SNPs between the patients with CHD and controls are shown in **Table 4**. The data showed that the C allele of the APLN rs2235310 polymorphism occurred less frequently in the former group (19.96% vs. 30.53%, respectively; $P = 2.38E\text{-}4$) and, thus, might be a protective factor against the development of CHD. After the Bonferroni correction, the *P*-value for the rs2235310 polymorphism and the risk of CHD was less than 0.01. By contrast, the CC genotype and C allele of the APLNR rs9943582 polymorphism were associated with an increased risk of CHD ($P = 0.04$ and $P = 0.027$, respectively). And the APLNR rs9943582 C allele was still significantly associated with the incidence of CHD after multiple test correction ($P = 0.014$). However, no significant relationships between the other two SNPs and the risk of CHD were observed.

TABLE 2 | Demographic and clinical characteristics among different coronary heart disease (CHD) groups.

Variables	CHD (*N* = 269)	CHD+D (*N* = 122)	CHD-D (*N* = 147)	P_1-value	CHD+A (*N* = 56)	CHD-A (*N* = 213)	P_2-value
Age (years)	61.24 ± 12.08	61.62 ± 13.71	60.92 ± 10.60	0.64	61.00 ± 11.77	61.30 ± 12.18	0.87
Gender (M/F)	144/127	70/52	74/75	0.22	32/24	112/103	0.55
Smoking	87 (32.34%)	36 (29.51%)	51 (34.69)	0.43	16 (28.57%)	71 (33.33%)	0.53
Drinking	90 (33.46%)	35 (28.69%)	65 (44.22%)	**0.011**	17 (30.36%)	73 (34.27%)	0.64
BMI	23.69 ± 1.98	24.17 ± 2.05	23.86 ± 2.45	0.34	24.13 ± 2.48	24.85 ± 2.96	0.26
Insomnia	94 (34.94%)	58 (47.54%)	36 (24.49%)	**0.006**	28 (50.00%)	66 (30.98%)	**0.011**
Hypertension	208 (77.3%)	62 (50.82%)	146 (99.32%)	**6.77E-15**	32 (57.14%)	176 (82.63%)	**4.96E-62**
Diabetes mellitus	52 (19.33%)	22 (18.03%)	30 (20.41%)	0.645	9 (16.07%)	43 (20.19%)	**7.11E-57**
Stroke	32 (11.90%)	9 (7.38%)	23 (15.65%)	**0.039**	6 (10.71%)	26 (12.20%)	**3.18E-42**

CHD+D, CHD patients with depression; CHD-D, CHD patients without depression; CHD+A, CHD patients with anxiety; CHD-A, CHD patients without anxiety. P_1-value: CHD+D versus CHD-D; P_2-value: CHD+A versus CHD-A. Bold values means: $p < 0.05$.

TABLE 3 | Hardy–Weinberg equilibrium analysis of studied single nucleotide polymorphisms (SNPs) in coronary heart disease (CHD) patients and controls.

SNP-ID	Genotype	CHD			Control		
		Number	X_1^2	P_1	Number	X_2^2	P_2
APLN: rs3115757	GG	134	0.29	0.59	83	0.17	0.68
	GC	107			76		
	CC	25			15		
APLN: rs2235310	TT	167	1.29	0.26	115	0.16	0.69
	TC	79			59		
	CC	14			9		
APLNR: rs9943582	TT	122	1.37	0.24	94	2.18	0.14
	TC	107			73		
	CC	38			12		
APLNR: rs2282623	CC	121	3.08	0.079	89	0.28	0.59
	CT	112			73		
	TT	35			18		

APLNR, apelin receptor.

TABLE 4 | Association between *APLNR/APLNR* gene mutations and patients with CHD.

Gene	SNP-ID	CHD (*N* = 269)	Control (*N* = 184)	*P*-value	*P*-adjust
APLN	rs3115757				
	GG	134 (50.38%)	83 (47.70%)	0.77	1.00
	GC	107 (40.22%)	76 (43.68%)		
	CC	25 (9.39%)	15 (8.62)		
	G	375 (70.67%)	242 (69.54%)	0.76	1.00
	C	157 (29.33%)	106 (30.46%)		
	rs2235310				
	TT	167 (64.23%)	115 (62.84%)	0.91	1.00
	CT	79 (30.38%)	59 (31.24%)		
	CC	14 (5.39)	9 (5.12%)		
	T	413 (80.04%)	289 (69.47%)	**2.38E-4**	**<0.01**
	C	103 (19.96%)	127 (30.53%)		
APLNR	rs9943582				
	TT	122 (45.69%)	94 (52.51%)	**0.040**	0.06
	TC	107 (40.07%)	73 (40.78%)		
	CC	38 (14.34%)	12 (6.71%)		
	T	351 (65.73%)	261 (72.90%)	**0.027**	**0.014**
	C	183 (34.26%)	97 (27.10%)		
	rs2282623				
	CC	121 (45.15%)	89 (49.44%)	0.52	0.32
	CT	112 (41.79%)	73 (40.56%)		
	TT	35 (13.06%)	18 (10.00%)		
	C	374 (69.78%)	251 (69.72%)	0.35	0.21
	T	188 (30.22%)	109 (30.28%)		

P-adjust means P-value adjusted with Bonferroni test. APLNR, apelin receptor; CHD, coronary heart disease; SNP, Single nucleotide polymorphism. Bold values means: p < 0.05.

Influence of Gene Polymorphisms on the Risk of Depression in CHD Patients

The results of the association analysis between the selected polymorphisms and the risk of depression in patients with CHD are shown in **Table 5**. We found that only the rs9943582 data reached statistical significance at the $P < 0.05$ level. Both the CC genotype and C allele were associated with a higher risk of depression in patients with CHD (21.00% vs. 8.78% and 40.76% vs. 29.06%; $P = 0.013$ and $P = 0.006$, respectively). Furthermore, the association between rs9943582 and the risk of depression remained significant after adjusting for alcohol drinking habits, insomnia, hypertension, and the stroke history, with the Bonferroni correction (P-adjust = 0.042 and P-adjust = 0.018, respectively). We also examined the association of rs9943582 with the severity of depressive symptoms and found that the PHQ-9 scores were markedly higher in the patients with CC genotype than in those with the other genotypes ($P < 0.001$; **Figure 2**).

Influence of Gene Polymorphisms on the Risk of Anxiety in CHD Patients

The results of the association analysis between the studied mutations and the risk of anxiety are shown in **Table 6**. The C allele of the rs9943582 polymorphism was associated with a lower risk of anxiety in patients with CHD (25.46% vs. 36.73%, respectively; $P = 0.032$). However, the association did not remain statistically significant after adjusting for insomnia, hypertension, diabetes mellitus, and the stroke history, with the Bonferroni correction (P-adjust = 0.081). The TT genotype and T allele of the APLNR rs2282623 polymorphism were associated with a higher risk of anxiety in patients with CHD (19.64% vs. 11.32% and 47.32% vs. 30.42%; $P = 0.003$ and $P = 0.001$, respectively). These associations remained statistically significant after adjusting for complications (P-adjust = 0.017 and P-adjust = 0.022, respectively). The rs2282623 polymorphism was also associated with the severity symptoms of anxiety, with the GAD-7 scores being higher in the CC genotype carriers than in the TT + TC genotype carriers ($P = 0.013$; **Figure 3**).

DISCUSSION

In this study, we investigated the associations of four promising polymorphisms in the APLN/APLNR pathway with the risks of depression and anxiety in patients with CHD. Our principal findings demonstrated that genetic polymorphisms in the APLN/APLNR pathway might result in a potential risk for depression and anxiety in patients with CHD. To the best of our knowledge, this is the first pilot study exploring the genetic contribution of the APLN/APLNR pathway to the susceptibility to depression and anxiety in Chinese patients with CHD.

We first found that patients with CHD who experienced insomnia had a high risk of comorbid depression and anxiety, which was consistent with previous findings, demonstrating that insomnia is frequently co-morbid with depression and anxiety (Gebara et al., 2018). Additionally, patients with CHD who has alcohol drinking habits were less likely to have

TABLE 5 | Genotype distribution of polymorphisms between CHD+D and CHD-D.

SNP	CHD+D (*N* = 121)	CHD-D (*N* = 148)	*P*-value	*P*-adjust*
rs3115757				
GG	61 (51.69%)	73 (49.32%)	0.80	1.00
GC	45 (38.13%)	62 (41.89)		
CC	12 (10.28%)	13 (8.79%)		
G	167 (70.76%)	208 (70.27%)	0.92	1.00
C	69 (29.24%)	88 (29.73%)		
rs2235310				
TT	78 (68.42%)	89 (60.96%)	0.45	0.62
CT	31 (27.19%)	48 (32.88%)		
CC	5 (4.39%)	9 (6.16%)		
T	187 (82.02%)	226 (77.40%)	0.23	0.58
C	41 (17.98%)	66 (22.60%)		
rs9943582				
TT	47 (39.50%)	75 (50.68%)	**0.013**	**0.042**
TC	47 (39.50%)	60 (40.54%)		
CC	25 (21.00%)	13 (8.78%)		
T	141 (59.24%)	210 (70.94%)	**0.006**	**0.018**
C	97 (40.76%)	86 (29.06%)		
rs2282623				
CC	61 (50.83%)	63 (43.45%)	0.42	0.74
CT	43 35.83)	63 (43.45%)		
TT	16 (13.16%)	19 (13.10%)		
C	165 (68.75%)	189 (65.17%)	0.40	0.98
T	75 (31.25%)	101 (34.83%)		

**P-value adjustment for drinking habit, insomnia, hypertension, and stroke history, multiple comparisons with Bonferroni test. CHD, coronary heart disease. Bold values means: $p < 0.05$.*

TABLE 6 | Genotype distribution of polymorphisms between CHD+A and CHD-A.

SNP	CHD+A (*N* = 56)	CHD-A (*N* = 213)	*P*-value	*P*-adjust*
rs3115757				
GG	28 (50.0%)	106 (50.48%)	0.98	0.95
GC	23 (41.07%)	84 (40.09%)	1.00	1.00
CC	5 (8.93%)	20 (9.52%)		
G	79 (70.54%)	296 (70.48%)		
C	33 (29.46%)	124 (29.52%)		
rs2235310				
TT	33 (58.93%)	134 (65.69%)	0.61	0.53
CT	20 (35.71%)	59 (28.92%)	0.43	0.47
CC	3 (5.36%)	11 (5.39%)		
T	86 (76.78%)	327 (80.15%)		
C	26 (23.22%)	81 (19.85%)		
rs9943582				
TT	32 (57.14%)	90 (42.65%)	0.088	0.24
TC	20 (35.71%)	87 (41.23%)	**0.032**	0.11
CC	4 (7.15%)	34 (16.12%)		
T	82 (74.54%)	267 (63.27%)		
C	28 (25.46%)	155 (36.73%)		
rs2282623				
CC	14 (25.00%)	107 (50.47%)	**0.003**	**0.017**
CT	31 (55.36%)	81 (38.21%)	**0.001**	**0.022**
TT	11 (19.64%)	24 (11.32%)		
C	59 (52.68%)	295 (69.58%)		
T	53 (47.32%)	129 (30.42%)		

**P-value adjustment for drinking habit, insomnia, hypertension, and stroke history, multiple comparisons with Bonferroni test. Bold values means: $p < 0.05$.*

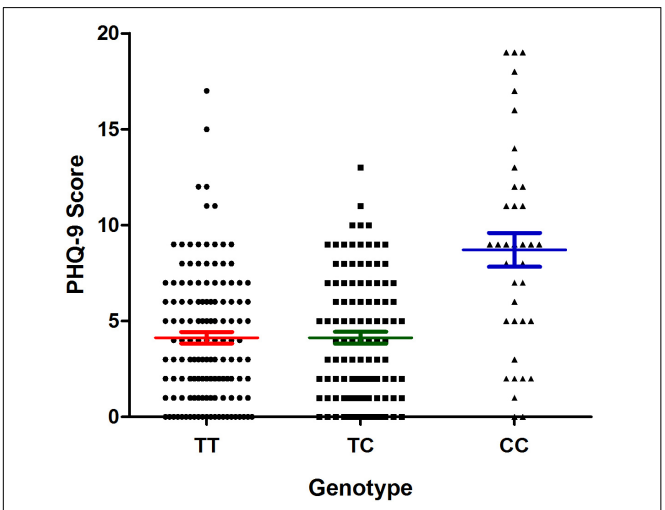

FIGURE 2 | Association of apelin receptor (APLNR) rs9943582 polymorphism with PHQ-9 scores in coronary heart disease (CHD) patients with comorbid depression. The filled circle means TT genotype carriers, the filled square means TC genotype carriers and the filled triangle means the CC genotype carriers. The red lines mean the mean ± SD of PHQ-9 score of TT group, the green lines mean the mean ± SD of PHQ-9 score of the TC group, and the blue lines mean the mean ± SD of the PHQ-9 score of the CC group.

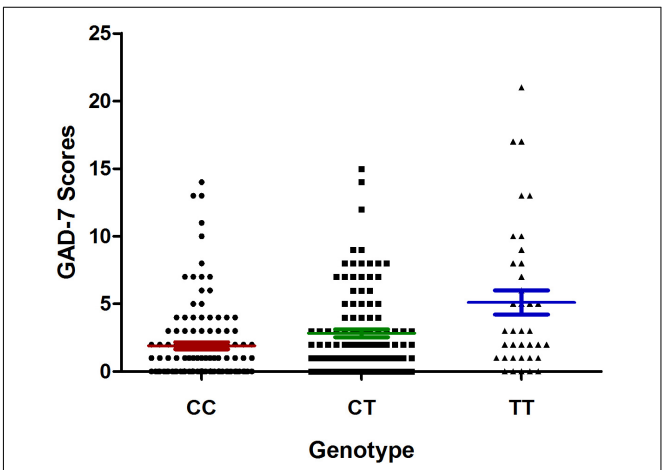

FIGURE 3 | Association of apelin receptor (APLNR) rs2282623 polymorphism with generalized anxiety disorder 7 (GAD-7) scores in coronary heart disease (CHD) patients with comorbid anxiety. The filled circle means CC genotype carriers, the filled square means CT genotype carriers and the filled triangle means the TT genotype carriers. The red lines mean the mean ± SD of GAD-7 score of CC group, the green lines mean the mean ± SD of GAD-7 score of the CT group, and the blue lines mean the mean ± SD of the GAD-7 score of the TT group.

depression than were non-drinkers. This finding, however, deviates from previously published results, which suggested a causal relationship between alcohol use disorders and major depression (Agabio et al., 2018). Moreover, patients with CHD who had more complications were more likely to have depression or anxiety, which may be explained by the additional psychological stress that these patients experience.

Apelin is an endogenous peptide capable of binding to the apelin receptor. Both apelin and its receptor are widely distributed in various tissues, including the central nervous and cardiovascular systems, and play a dominant role in cardiovascular homeostasis and disease (Chen et al., 2015). The beneficial effects of the apelin/apelin receptor pathway are well established. Apelin treatment has been proven beneficial for conditions as diverse as hypertension, atherosclerosis, myocardial infarction, and other cardiovascular diseases (Zhou et al., 2016). Numerous studies have shown that APLN/APLNR polymorphisms are associated with the risk of several diseases such as hypertension, CAD, and diabetes mellitus (Nowzari et al., 2018; Wu et al., 2018; Zhang et al., 2019). Our results demonstrated that the T allele of the rs2235310 polymorphism and the C allele of the rs9943582 polymorphism were risk factors in the development of CHD.

The association between rs9943582, a functional variant in the 5' flanking region (-154G/A) of APLNR, and the incidence of cardiovascular disease has been extensively studied; however, the results have been contradictory. One study showed that the rs9943582 polymorphism is associated with the expression levels of APLNR. In particular, C allele carriers had lower expression level of the APLNR because of a lower binding affinity of a transcription factor to the promoter of APLNR (Hata et al., 2007). A published meta-analysis showed that the T allele of rs9943582 is associated with an increased risk of CAD ($P = 0.100$) relative to that of the wild-type C allele (Chen et al., 2017). Additionally, a previous genome-wide association study conducted in a Japanese population, showed a significant association between rs9943582 and the risk of stroke (Hata et al., 2007), whereas no allelic or genotypic associations were found between rs9943582 and ischemic stroke in a Chinese Han population (Wang et al., 2017; Zhang et al., 2017). The APLN rs2234306 polymorphism was in a strong LD with the APLN rs3115757 polymorphism in the Chinese population from this study ($r^2 > 0.9$), and both SNPs were located in APLN introns. Previous studies showed that the rs3115757 polymorphism is associated with the expression level of APLN (Liao et al., 2011) and the incidence of diabetes and hypertension (Huang et al., 2016; Zheng et al., 2016). Our findings support a moderate contribution of the APLN/APLNR pathway polymorphisms to the development of CHD in Chinese patients.

The APLN/APLNR pathway may also serve as a promising therapeutic target for the treatment of psychosis and neuropathy (Lv et al., 2020). We further investigated the influence of APLN/APLNR pathway polymorphisms on the risks of depression and anxiety in patients with CHD. Among the patients with CHD, the rs9943582 C-allele carriers had an increased risk of depression, whereas rs2282623 T-allele carriers had a higher risk of anxiety. This is the first pharmacogenomics study to examine the associations between polymorphisms located in genes of the APLN/APLNR pathway and the susceptibility to depression and anxiety in patients with CHD.

Apelin-13 has exhibited antidepressant and anxiolytic effects in different animal models (Telegdy and Jaszberenyi, 2014; Dai et al., 2018). A functional study of the rs2282623 SNP, located in the 3′UTR of APLNR, showed that the rs2282623 T-allele is associated with the decreased levels of apelin-13 and nitrite (Mishra et al., 2015). Moreover, another study demonstrated that the rs2282623 mutation is associated with diastolic blood pressure and the mean arterial pressure response to a low-sodium intervention (Zhao et al., 2010). In the current study, we showed that the SNPs associated with low apelin expression levels were also associated with an increased risk of depression or anxiety in patients with CHD.

However, other studies have shown controversial roles of APLN in psychosis. For example, patients on peritoneal dialysis, who had depression and anxiety, had higher serum apelin levels than those without depression and anxiety (Gok Oguz et al., 2016). Furthermore, apelin-13 exhibited depression-promoting effects in both forced swimming and tail suspension tests (Lv et al., 2012). Hence, additional pharmacogenomics studies with a larger sample size should be conducted to further validate the results of this study.

This study has several limitations. First, the candidate SNP approach was used to identify emotional disorder associated polymorphisms in patients with CHD. This approach might have missed mutations that are truly associated with the studied phenotypes. Second, although the final sample size was larger than the minimum calculated sample size, the results of this study should be verified using a larger sample size. More large-sample-size, high-quality, multicenter clinical trials are urgently needed to validate the association of gene polymorphisms in the APLN/APLNR pathway on the risk of emotional disorders in patients with CHD.

CONCLUSION

The present study supports the hypothesis that APLN/APLNR polymorphisms contribute to the susceptibility to depression and anxiety in Chinese patients with CHD. Replication studies with larger samples are required to verify the role of these polymorphisms in patients with CHD, who comorbid depression and anxiety.

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 Jining First People's Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZL and PJ designed the experiments and drafted the manuscript. YW and WL accomplished works of the data collection, DNA extraction, and genotyping. YX and HY helped to enroll the all of the patients. FW guided to conduct the statistic works. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Beyond Single Nucleotide Polymorphisms: *CYP3A5*3*6*7* Composite and *ABCB1* Haplotype Associations to Tacrolimus Pharmacokinetics in Black and White Renal Transplant Recipients

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Interpatient variability in tacrolimus pharmacokinetics is attributed to metabolism by cytochrome P-450 3A5 (CYP3A5) isoenzymes and membrane transport by P-glycoprotein. Interpatient pharmacokinetic variability has been associated with genotypic variants for both *CYP3A5* or *ABCB1*. Tacrolimus pharmacokinetics was investigated in 65 stable Black and Caucasian post-renal transplant patients by assessing the effects of multiple alleles in both *CYP3A5* and *ABCB1.* A metabolic composite based upon the *CYP3A5* polymorphisms: **3*(rs776746), **6*(10264272), *and *7(41303343*), each independently responsible for loss of protein expression was used to classify patients as extensive, intermediate and poor metabolizers. In addition, the role of *ABCB1* on tacrolimus pharmacokinetics was assessed using haplotype analysis encompassing the single nucleotide polymorphisms: *1236C > T (rs1128503), 2677G > T/A(rs2032582), and 3435C > T(rs1045642)*. Finally, a combined analysis using both *CYP3A5* and *ABCB1* polymorphisms was developed to assess their inter-related influence on tacrolimus pharmacokinetics. Extensive metabolizers identified as homozygous wild type at all three *CYP3A5* loci were found in 7 Blacks and required twice the tacrolimus dose (5.6 ± 1.6 mg) compared to Poor metabolizers [2.5 ± 1.1 mg ($P < 0.001$)]; who were primarily Whites. These extensive metabolizers had 2-fold faster clearance ($P < 0.001$) with 50% lower AUC* ($P < 0.001$) than Poor metabolizers. No differences in $C_{12\ h}$ were found due to therapeutic drug monitoring. The majority of blacks (81%) were classified as either Extensive or Intermediate Metabolizers requiring higher tacrolimus doses to accommodate the more rapid

clearance. Blacks who were homozygous for one or more loss of function SNPS were associated with lower tacrolimus doses and slower clearance. These values are comparable to Whites, 82% of who were in the Poor metabolic composite group. The *ABCB1* haplotype analysis detected significant associations of the wildtype *1236T-2677T-3435T* haplotype to tacrolimus dose (P = 0.03), CL (P = 0.023), CL/LBW (P = 0.022), and AUC* (P = 0.078). Finally, analysis combining *CYP3A5* and *ABCB1* genotypes indicated that the presence of the *ABCB1* 3435 T allele significantly reduced tacrolimus clearance for all three CPY3A5 metabolic composite groups. Genotypic associations of tacrolimus pharmacokinetics can be improved by using the novel composite *CYP3A5*3*4*5 and ABCB1* haplotypes. Consideration of multiple alleles using *CYP3A5* metabolic composites and drug transporter ABCB1 haplotypes provides a more comprehensive appraisal of genetic factors contributing to interpatient variability in tacrolimus pharmacokinetics among Whites and Blacks.

Keywords: renal transplantation, immunosuppression, CYP3A5 genotypes, tacrolimus, race, tacrolimus pharmacokinetics, pharmacogenomics, ABCB1 haplotypes

INTRODUCTION

The combination of tacrolimus and mycophenolic acid is the mainstay of maintenance immunosuppressive regimens to prevent renal allograft rejection (Hart et al., 2017; Jouve et al., 2018). Tacrolimus exhibits variable pharmacokinetics and clinical response, necessitating the use of therapeutic drug monitoring (TDM) (Schiff et al., 2007; de Jonge et al., 2009; Shuker et al., 2015, 2016b; Vanhove et al., 2016). However, trough tacrolimus concentration versus effect relationships for clinical responses and adverse drug effects are not well defined in sub-populations stratified by sex or race (Bouamar et al., 2013; Vanhove et al., 2016). Tacrolimus pharmacokinetic and pharmacodynamic variability is attributed, in part to variation in both cytochrome P-450 3A5 isoenzymes and P-glycoprotein (P-gp) (Fredericks et al., 2006; Wang et al., 2006; Staatz et al., 2010a).

The duration of chronic renal allograft survival in Blacks is significantly shorter compared to other races receiving similar immunosuppression (Young and Gaston, 2000, 2002, 2005; Young and Kew, 2005; Eckhoff et al., 2007; Fan et al., 2010; Andrews et al., 2016). Contributing factors include socioeconomics, genomic variants, medication adherence, pharmacokinetic and pharmacodynamic variability, donor-recipient mismatches, time on dialysis and racial variation in immunodynamic responses (Young and Gaston, 2005; Young and Kew, 2005; Eckhoff et al., 2007; Page et al., 2012). Interestingly, Blacks require higher tacrolimus doses compared to Whites to achieve similar allograft outcomes (Neylan, 1998; Young and Gaston, 2005; Vadivel et al., 2007). Tacrolimus bioavailability is reduced in healthy Blacks (Fitzsimmons et al., 1998; Mancinelli et al., 2001). Though not examined here, sex is another recognized factor influencing the activity of CYP3A4/5 isoenzymes and P-glycoprotein (Cummins et al., 2002; Scandlyn et al., 2008; Hu and Zhao, 2010; Soldin et al., 2011; Momper et al., 2017).

Pharmacogenomic testing has been incorporated into clinical studies of calcineurin inhibitors (Barbarino et al., 2013; Hesselink et al., 2014; Bruckmueller et al., 2015; Andrews et al., 2016; Tang et al., 2016; Meng et al., 2018) and may be responsible for the differences among races. For example, the wild-type variant, *CYP3A5*1*, is common in Blacks as compared to *CYP3A5*3* (rs776746) the major variant in Whites which is associated with loss of protein expression. This variant (*CYP3A5*3*) contributes to interpatient tacrolimus pharmacokinetic variability as reflected in dose-normalized trough, area under the concentration vs. time curve (AUC) and clearance (MacPhee et al., 2004; Dai et al., 2006; Thervet et al., 2010; de Jonge et al., 2012; Andrews et al., 2016; Oetting et al., 2016; Shuker et al., 2016a). However, in Blacks, other *CYP3A5* variants including *CYP3A5*6* (rs10264272) and *CYP3A5*7* (rs41303343), are associated with loss of function, and may also contribute to interracial variability in tacrolimus pharmacokinetics (Birdwell et al., 2015; Oetting et al., 2016; Sanghavi et al., 2017). Recent studies in Black recipients with *CYP3A5*1* alleles require higher daily tacrolimus doses than Whites with the variant *CYP3A5*3*, or Blacks exhibiting variants *CYP3A5*3*, *CYP3A5*6*, and/or *CYP3A5*7* to achieve comparable troughs (Birdwell et al., 2015; Oetting et al., 2016; Sanghavi et al., 2017). Our group has recently reported the *CYP3A5*3*6*7* metabolic composite to provide a comprehensive phenotypic representation (Campagne et al., 2018).

Pharmacokinetic estimates in these studies were limited to trough concentrations which has limitations in describing accurate tacrolimus exposures. Variable correlations between tacrolimus troughs and area under the concentration curve

Abbreviations: *ABCB1,* ATP binding cassette gene subfamily B member 1; AUC 0-12, Area under the concentration vs. time curve from 0 to 12 h; AUC*, dose normalized AUC 0 to 12 h; AUC 0–4 hr, Area under the concentration vs. time curve from 0 to 4 h; AUC* 0–4 hr, dose normalized AUC 0–4 hr; BMI, Body mass index; CNI, Calcineurin inhibitors; *CYP3A5,* Cytochrome P-450 3A5 isoenzymes; *CYP3A5*3*6*7* metabolic composite: genotypic variants *CYP3A5*3*, *CYP3A5*6*, *CYP3A5*7*$C_{12\ h}$: 12 h trough concentration; C 2 h, 2 h concentration; C max, Maximum concentration; CLss, steady state oral clearance; ECMPS, enteric Coated Mycophenolate Sodium; MPA, Mycophenolic Acid; N, Number of patients; P-gp, P glycoprotein; LBW, Lean body Weight; SNP, Single Nucleotide Polymorphism; Std, Standard Deviation; T_{max}, Time to maximum concentration; TBW, Total Body Weight.

(AUC) have been reported contributing to interpatient pharmacokinetic variability (Schiff et al., 2007; Staatz et al., 2010b). Therefore, an alternative study design that uses intensive sampling to characterize tacrolimus AUC or drug exposure in African American and Caucasian recipients is needed to guide further development of dosing strategies in relation to wild-type *CYP3A5*1* and variant *CYP3A5*3*6*7* genotypes (Birdwell et al., 2015).

In contrast to the relationship of tacrolimus pharmacokinetics to CYP3A5*1/*3 variants, the relationship of the ATP binding cassette gene subfamily B member 1*(ABCB1)* variants, as surrogate markers for P-gp has an unclear relationship to calcineurin inhibitor pharmacokinetics and pharmacodynamics (Staatz et al., 2010b; Knops et al., 2013; Hesselink et al., 2014). P-glycoprotein serves as an adenosine triphosphate (ATP)-dependent efflux pump for substrates, such as calcineurin inhibitors(CNI), resulting in reduction of systemic exposure and lower intracellular drug accumulation (Huang et al., 2010; Cascorbi, 2011; Hodges et al., 2011; Barbarino et al., 2013). Extensive P-gp tissue distribution, reinforces its functional contribution in the development of adverse effects (Haufroid et al., 2006; Provenzani et al., 2011; Knops et al., 2013; Hesselink et al., 2014; Stefanovic et al., 2015; Venuto et al., 2015). Alterations in P-gp expression or function have been attributed to genetic polymorphisms, race, sex, environment, or endogenous inhibitors (Cattaneo et al., 2009; Huang et al., 2010; Hodges et al., 2011; Barbarino et al., 2013; Hesselink et al., 2014). Reports regarding the influence of common ABCB1 single nucleotide polymorphisms (SNPs): *1236C > T (rs1128503), 2677G > T/A (rs2032582), and 3435C > T (rs1045642)* have focused on tacrolimus pharmacokinetics or renal pharmacodynamics including acute rejection and nephrotoxicity (Staatz et al., 2010b; Hesselink et al., 2014). Conflicting reports have examined individual SNPs, an approach that may not include the effect of multiple *ABCB1* polymorphisms and their interrelationship to selected tacrolimus pharmacokinetics or associated adverse effects (Staatz et al., 2010b; Knops et al., 2013). These commonly evaluated *ABCB1* SNPs are inherited as a haplotype with distinct racial frequencies (Kim et al., 2001; Kimchi-Sarfaty et al., 2007a; Hodges et al., 2011). Due to linkage disequilibrium, the *1236T-2677T-3435T (TTT)* haplotype is the most prevalent variant, and is associated with significant reductions in P-gp activity compared to wild type (Kimchi-Sarfaty et al., 2007b). This haplotype variant is postulated to decrease P-gp activity and subsequently impact systemic tacrolimus exposure and increase intracellular drug exposure with the potential for increased adverse effects (Staatz et al., 2010b; Picard and Marquet, 2011; Hesselink et al., 2014). Different frequencies of *ABCB1* SNPs and haplotypes between Blacks and Whites have been described and should be considered in pharmacogenomic analysis (Kim et al., 2001; Keskitalo et al., 2008; Woodahl et al., 2008; Kassogue et al., 2013). The inclusion of *ABCB1* haplotypes may provide more insightful associations to pharmacokinetic and adverse effects phenotypes during tacrolimus immunosuppression (Kimchi-Sarfaty et al., 2007a; Liu et al., 2008, 2009; Staatz et al., 2010b). Most studies including *ABCB1* SNPs or haplotypes have investigated either tacrolimus dose-normalized troughs or daily doses and acute rejection with no evaluation of the important non-renal adverse effects (Staatz et al., 2010b; Hesselink et al., 2014).

The objectives of this study were to assess: (1) the influence of CYP*3A5* metabolic composite genotypes combining three common loss of function SNPs, *CYP3A5*3* (rs776746), *CYP3A5*6* (rs10264272), and *CYP3A5*7* (rs41303343) on tacrolimus pharmacokinetic phenotypes; (2) to assess tacrolimus pharmacokinetics in association to *ABCB1* haplotypes; and (3) an integrated analysis combining the novel CYP*3A5* metabolic composite and *ABCB1 3435 (rs1045642*) to assess the combined role of the two loci to tacrolimus pharmacokinetic parameters.

MATERIALS AND METHODS

Study Population

Sixty-five (33 Black and 32 White) stable male and female renal transplant recipients receiving tacrolimus (*Prograf)* and mycophenolic acid as enteric-coated mycophenolate sodium (ECMPS; *(Myfortic)* for ≥6 months participated in a 12-h pharmacokinetics-pharmacogenomic study. Patients were recruited by a nephrologist during their transplant clinic visit if they demonstrated clinical stability in renal function, clinical laboratory tests and concurrent disorders. Physical exams, comprehensive metabolic panels including liver and renal function tests, electrolytes, glucose, albumin and protein concentrations with complete blood counts and differentials were used to confirm clinical stability. Tacrolimus doses were adjusted to 4–9 ng/ml troughs based upon time post-transplant and clinical response using a program-specific minimization protocol. ECMPS was dose adjusted based upon clinical response. Estimated glomerular filtration rate (e-GFR) was calculated using the four-factor MDRD equation (Levey et al., 1999). Medication adherence was verified by transplant nurse clinician and medication adherence assessment by transplant pharmacist at enrollment. Ethnicity for two previous generations was verified prior to study.

Inclusion criteria were: (1) ≥6 months post-renal transplant; (2) age 25–70 years; (3) first or second deceased-donor or living allograft recipient; (4) same immunosuppressive doses for ≥7 days; (5) Serum creatinine ≤3.25 mg/dl with no change >0.25 mg/dl during prior 2 visits; (6) leukocyte count ≥ 3000/mm^3 and hemoglobin ≥8.0 g/dl. Exclusion criteria were: (1) infection or acute rejection within 2 weeks; (2) drugs interfering with tacrolimus or MPA absorption; (3) cytochrome P4503A4/3A5 or P-glycoprotein inhibitors or inducers within 4 weeks; (5) significant medical or psychiatric diseases that would limit participation.

Study Procedure

This was a cross-sectional, open-label pharmacokinetic-pharmacogenomic study in stable male and female Black and White recipients conducted at the University at Buffalo (UB) Renal Research Center at the Erie County Medical Center (ECMC). The UB Health Sciences Institutional Review Board approved the study (IRB# PHP0599703-4) which was conducted

in accordance with the ethical standards for human subjects and the 1964 Helsinki Declaration. Upon enrollment, patients provided written consent after review of the study purpose, risks and benefits.

All patients were at steady-state conditions for both tacrolimus and ECMPS. Patients were enrolled only if they had received the same dose of tacrolimus and ECMPS for ≥7 days prior to study. This was assumed to be sufficient to approach steady-state plasma concentrations. Proton pump inhibiters, H_2 antagonists and antacids were discontinued at least 36 h prior to study. Patients took immunosuppressives between 5:30 to 6:30 PM prior to study, fasted and abstained from caffeine and alcohol for 12 h prior to study. At 6:00 AM, patients were admitted, vital signs documented and an intravenous angiocatheter inserted. A 0 h sample (∼15 ml) was collected prior to immunosuppressives for drug troughs and laboratory tests (ECMC Clinical Chemistry Laboratory). Oral study medications [(single lot of tacrolimus *(Prograf) and* ECMPS *(Myfortic)*] were administered at 7:00 AM. Patients remained upright throughout the study. Standardized low fat meals were provided after 4 h. Antihypertensives were administered after 1.5 h and non-immunosuppressives after 4 h. Blood samples (7 ml) were collected at 0 h and 1, 2, 3, 4, 6, 8, 10, and 12 h after drug administration. Whole blood samples were aliquoted within 30 minutes and stored at −70°C until analysis.

Blood was collected in cell preparation tubes (CPT- BD Vacationer) with sodium citrate pre-dose for separation of peripheral blood mononuclear cells (PBMCs) according to processing protocol at 25°C. Plasma was aspirated with PBMC harvested; immediately frozen in liquid nitrogen and stored at −70°C until genotype analysis.

Genetic Analysis

All blood samples provided viable DNA for genotyping and were analyzed at the University of New England Genomics Research Core. Genomic DNA was isolated from 600 μl of PBMCs per manufacturers' protocol (Wizard[§] Genomic DNA Purification. Promega Madison, WI). Personnel with no knowledge of clinical data assayed for *CYP3A5* variants, *CYP3A5*3* (rs776746), *CYP3A5*6* (rs10264272), and *CYP3A5*7* (rs41303343) and *ABCB1* SNPs: *1236C > T(rs1128503), 2677G > T/A (rs2032582), and 3435C > T (rs1045642)*. Ten ng of patient genomic DNA was used to characterize each single nucleotide polymorphism (SNPs) using validated *TaqMan* allelic discrimination assays (Thermo Fisher Scientific, Applied Biosystems, Foster City, CA) with Bio-Rad Laboratories CFX96 Real-Time Polymerase Chain Reaction Detection System (Hercules, CA). For each SNP assay, duplicate samples were analyzed. All protocols and sample handling were in accordance with published guidelines. Allele frequencies for all SNPs were confirmed to be in Hardy-Weinberg equilibrium when adjusted for race.

Given the known linkage among all three *ABCB1* SNPs, haplotype analysis was conducted. Haplotype analysis provides greater power to detect potential unknown functional variants than SNPs alone (Venuto et al., 2015). *ABCB1* haplotype estimation was determined using the THESIAS program (Tregouet et al., 2004; Tregouet and Garelle, 2007). THESIAS uses a maximum likelihood algorithm for the simultaneous estimation of haplotype frequencies and their association to tacrolimus pharmacokinetic parameters. Significant associations for tacrolimus pharmacokinetics as phenotypic means with confidence intervals for each haplotype on a single chromosome were reported.

Metabolic Composite *CYP3A5*3*6*7* Analysis

The variants, *CYP3A5*3*, *CYP3A5*6,* and *CYP3A5*7*, all result in loss of protein gene expression (Birdwell et al., 2015). Loss of protein function due to any one of these variants can occur independent of allelic status at the other two loci; thus assessment of any single SNP may be misleading as an indicator of enzyme function. Patients were assigned a metabolic composite designation as described earlier (Campagne et al., 2018) based upon the combined allelic status at all three independent loci (**Figure 1**). The Extensive Metabolizer phenotype was assigned to individuals with functional genes on both chromosomes. Patients were assigned the Poor Metabolizer phenotype if they were homozygous for the variant allele at any one of the three SNPs and thus CYP3A5 genes on both chromosomes are non-functional (**Figure 1C**). Patients were designated as Intermediate Metabolizer if they were heterozygous for one loss of function SNP at any of the three loci (**Figure 1B**). Finally, for patients who were heterozygous at two or more of the SNPs responsible for loss of function, the level of enzyme is dependent upon the arrangement of the variant alleles on each chromosome (**Figure 1D**). For example, if all of the loss of function SNP's are located on the same chromosome, this individual would have one "functional" gene similar to the single heterozygote. For this analysis double heterozygotes (**Figure 1D**) were conservatively assigned as an Intermediate Metabolizer since individuals could either be poor or intermediate metabolizers.

Combined CYP3A5 Metabolic Composite and ABCB1 and Tacrolimus Pharmacokinetics

To assess the role of both *CYP3A5* and *ABCB1* variants on tacrolimus pharmacokinetics the *CYP3A5* metabolic composite groups and the *ABCB1 3435C > T* (rs1045642) were included in a single analysis. The *ABCB1 3435C > T* variant was used as a proxy for wildtype *ABCB1* haplotype in this combined analysis since the haplotype assignment algorithm, THESIAS, does not assign individual haplotype scores to individuals. Significant effects of both *CYP3A5* and *ABCB1* variants on tacrolimus pharmacokinetics was assessed using multivariate analysis of variance.

Assay Methodology for Tacrolimus

Tacrolimus troughs and pharmacokinetic concentrations were analyzed within 24 h at the ECMC Clinical Laboratory using the *ARCHITECT* tacrolimus assay (Abbott, Abbott Park, IL), a chemiluminescent microparticle immunoassay. The lower limit of detection was 0.5 ng/ml and intraday assay variability was <7%. The calibration standard curve ranged from 1 to 30 ng/ml and quality controls (QC) were 3.0, 12.0, and 25 ng/mL (Bio-Rad, Hercules, CA, United States). The interday coefficient of variation (CV) for each QC was <4% and intra-day CV was

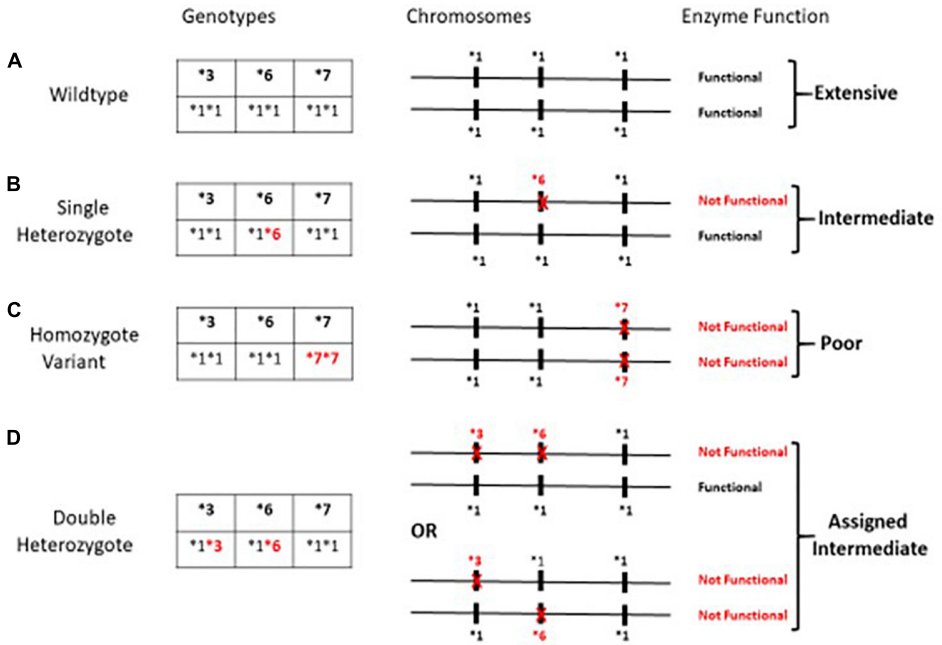

FIGURE 1 | Metabolic Composite scoring algorithm for CYP3A5*3*6*7 SNPs. Metabolic Composite Score for hypothetical patients based upon the combined allelic status from each chromosome is summarized in **(A–D)** above. **(A–D)** illustrates a few of the possible arrangements of the three alleles on the chromosomes and not specific allelic arrangements that have been described to date. **(A)** depicts an Extensive Metabolizer with two completely functional genes. **(C)** depicts individuals who carry a loss of function allele on both chromosomes and are Poor Metabolizers. **(B,D)** represents Intermediate Metabolizers due to at least one loss of function allele (Campagne et al., 2018).

<5%. Selected troughs and peaks ($N = 40$ samples) were analyzed using a validated LCMSMS assay by a CLIA certified external analytical laboratory and compared to the results generated from the *ARCHITECT* tacrolimus assay with excellent agreement ($R^2 = 0.98$). For the tacrolimus LCMSMS assay, the interday and intraday CV were <5% at the low and high concentration QC.

Pharmacokinetic Analysis

Pharmacokinetic parameters included area under the concentration versus time curve 0 to 12 h ($AUC_{0-12\ h}$), dose-normalized AUC_{0-12} (AUC*); 12-h trough ($C_{12\ h}$) and peak concentration (C_{max}) with dose normalization and time to peak (T max). Oral clearance of tacrolimus was the ratio of dose to $AUC_{0-12\ h}$. Tacrolimus clearances were adjusted for TBW and LBW. AUC_{0-12} was determined by the linear trapezoidal rule using non-compartmental methods (*Phoenix WINNONLIN* Version 6.3. *Pharsight Corp, Mountain View, Calif*). $C_{12\ h}$, C_{max}, $AUC_{0-4\ h}$, and AUC_{0-12} were dose-normalized to 1 mg dose equivalent.

Statistical Analysis

All patient demographics and tacrolimus pharmacokinetic parameters were summarized by metabolic composite score for *CYP3A5*3*6*7* using the mean and standard deviation for continuous variables. The potential trend between metabolic composite genotypes and association to tacrolimus pharmacokinetics were evaluated using the two-sided Jonckheere-Terpstra test for trends (Jonckheere, 1983). *Post hoc* pairwise comparisons were made using Holm-Bonferroni adjusted Wilcoxon rank sum tests (*version 9.3, SAS Institute, Cary, NC*). Significant combined effects reflecting composite metabolic *CYP3A5* and *ABCB1* variants on tacrolimus pharmacokinetics was assessed using multivariate analysis of variance (Littell et al., 2006). Significant effects are shown in bold.

RESULTS

Patients

Sixty-five recipients completed the study with no statistical differences in age or time post-transplant albumin, liver function tests and hematologic parameters were within normal range for patients with no group differences (**Table 1**). Mean MPA doses were not different among groups.

*CYP3A5*3*6*7* Genotype Associations With Tacrolimus Pharmacokinetics

As previously described, the CYP3A5 metabolic composite (Campagne et al., 2018; **Figure 1**), was used to assess the role of multiple genotypes on tacrolimus pharmacokinetics. There were no significant differences among metabolic composite groups for age, gender and time post-transplant as well as clinical measures (**Table 1**). Frequency differences among metabolic composite groups between races were significant.

TABLE 1 | Patient demographics clinical characteristics adjusted for *CYP3A5*3*6*7* metabolic composite groups.

		***CYP3A5*3*6*7* Metabolic Groups**				
		Poor	**Intermediate**	**Extensive**	**Overall**	**JT Trend *P*-value**
	N (%)	35 (53.8)	23 (35.4)	7 (10.8)	65 (100%)	
Age (yrs)	Mean/Std/N	49.8/12.6/35	48.5/11.0/23	45.4/9.0/7	48.9/11.6/65	0.426
Gender	Male	16 (45.7%)	9 (39.1%)	4 (57.1%)	29 (44.6%)	
	Female	19 (54.3%)	14 (60.9%)	3 (42.9%)	36 (55.4%)	0.690
Race	Black	6 (17.1%)	20 (87.0%)	7 (100.0%)	33 (50.8%)	
	White	29 (82.9%)	3 (13.0%)		32 (49.2%)	**<0.001**
Time Post-Transplant (yrs)	Mean/Std/N	2.9/3.0/35	3.4/2.0/23	2.5/1.9/7	3.0/2.6/65	
						***P*-value**
Serum Creatinine (mg/dl)		1.3 (0.3)	1.6 (0.40	1.7 (0.4)	1.4 (0.4)	**0.001**
Estimated Glomerular Filtration Rate ADJ Black Female (ml/min/1.73 m^2)		58.4 (14.3)	54.0 (17.4)	48.4 (13.1)	55.8 (15.5)	0.237
Glucose (mg/dl)		114.7 (68.8)	122.8 (79.3)	93.0 (20.8)	115.3 (69.2)	0.449
Total White Blood Cells (x10^3 cells/mm^3)		5.4 (2.1)	4.9 (1.7)	5.9 (1.9)	5.3 (1.9)	0.361
Platelets (cells x 10^6)		196.4 (46.5)	195.6 (60.6)	213.0 (45.4)	197.9 (51.3)	0.472
Hemoglobin (g/dl)		12.4 (1.4)	12.2 (1.4)	12.2 (1.2)	12.3 (1.4)	0.870
Body Mass Index (kg/m^2)		29.8 (5.5)	30.1 (6.9)	31.6 (6.6)	30.1 (6.1)	0.758
Albumin (g/dl)		4.1 (0.3)	4.1 (0.30	4.1 (0.3)	4.1 (0.3)	0.874
Prednisone N(%)		5 (14.3%)	6 (26.1%)	2 (28.6%)	13 (20.0%)	0.457
MPA trough at 12 h (mcg/dl)		3.2 (1.7)	4.1 (2.2)	4.2 (2.9)	3.7 (2.0)	0.305

P-values from two-sided Jonckheere–Terpstra Trend (JT) test.

TABLE 2 | *CYP3A5*3*6*7* metabolic composite groups and associations to tacrolimus pharmacokinetics.

	Composite *CYP3A5*3*6*7* Metabolic Groups				**Group Pairwise Comparison***		
Tacrolimus pharmacokinetic parameters	**Poor (1)**	**Intermediate (2)**	**Extensive (3)**	**JT Trend *P*-value**	**1 vs. 2**	**1 vs.3**	**2 vs. 3**
	Mean/Std/N	Mean/Std/N	Mean/Std/N				
Study dose (mg)	2.46/1.06/35	4.07/1.57/23	5.64/1.60/7	**<0.001**	**<0.001**	**<0.001**	**0.039**
Study dose/TBW (mg/kg)	0.03/0.02/35	0.05/0.02/23	0.07/0.02/7	**<0.001**	**0.004**	**0.004**	0.062
C_{12h} (ng/ml)	6.99/1.83/35	7.70/1.88/23	6.77/1.67/7	0.550	0.737	0.808	0.808
C_{12}/dose (ng/ml/mg)	3.32/1.64/35	2.13/0.81/23	1.31/0.63/7	**<0.001**	**0.005**	**<0.001**	**0.011**
Cmax (ng/ml)	16.89/6.90/35	20.41/8.14/23	20.81/13.48/7	0.129	0.249	1.000	1.000
Cmax/Dose (ng/ml/mg)	7.69/3.33/35	5.49/2.23/23	4.00/2.69/7	**<0.001**	**0.029**	**0.016**	0.155
T max (hr)	1.81/0.81/35	2.17/1.50/23	1.52/0.54/7	0.954	0.729	0.729	0.580
AUC $_{0-12}$ (ng.hr/ml)	119.66/28.73/35	135.75/35.14/23	125.66/31.30/7	0.261	0.515	1.000	1.000
AUC* (ng.hr/ml/mg)	56.03/24.69/35	37.06/12.83/23	24.26/10.80/7	**<0.001**	**0.005**	**0.002**	**0.019**
CL_F (L/hr)	21.14/8.02/35	30.79/12.19/23	47.43/17.30/7	**<0.001**	0.008	**0.002**	**0.019**
CL/LBW (L/hr/kg)	0.38/0.16/35	0.55/0.27/23	0.91/0.40/7	**<0.001**	**0.036**	**0.003**	**0.044**
AUC $_{0-4}$ (ng.hr/ml/mg)	51.20/15.22/35	60.77/20.07/23	56.89/19.24/7	0.153	0.285	1.000	1.000
AUC* $_{0-4}$ hr (ng.hr/ml/mg)	23.77/10.53/35	16.46/6.22/23	11.02/5.24/7	**<0.001**	**0.016**	**0.004**	0.056

*P-values from two-sided Jonckheere–Terpstra Trend (JT) test. *Post hoc pairwise comparisons using Holm-Bonferroni adjusted Wilcoxon rank sum tests.*

For the metabolic composite *CYP3A5*3*6*7* frequencies Extensive Metabolizers were identified in 7 Blacks with no Extensive Metabolizers among White patients. Twenty-three patients were genotyped as Intermediate Metabolizers with 20 Blacks and 3 Whites. Poor Metabolizers consisted of 29 Whites and 6 Blacks.

Significant associations of tacrolimus pharmacokinetics with *CYP3A5*3*6*7* metabolic composite are summarized in **Table 2**. Extensive Metabolizers exhibited a 2-fold greater tacrolimus dose ($P < 0.001$) and Dose/TBW ($P < 0.001$) compared to Poor Metabolizers. Although no difference was noted with troughs between the 3 metabolic composite groups (**Figure 2C**), a 2.5-fold greater dose normalized trough (Cp_{12hr}/dose) ($P = 0.0016$) was found in Poor compared to Extensive Metabolizers (**Table 2**). Tacrolimus clearance ($P < 0.001$) was twice as rapid in Extensive Metabolizers compared to Poor Metabolizers (**Figure 2A**). The dose-adjusted $AUC_{0-12\ h}$ ($P < 0.001$) was 2 fold higher in Poor than Extensive metabolizers (**Figure 2B**), though no difference between groups was found with $AUC_{0-12\ h}$ (**Figure 2D**).

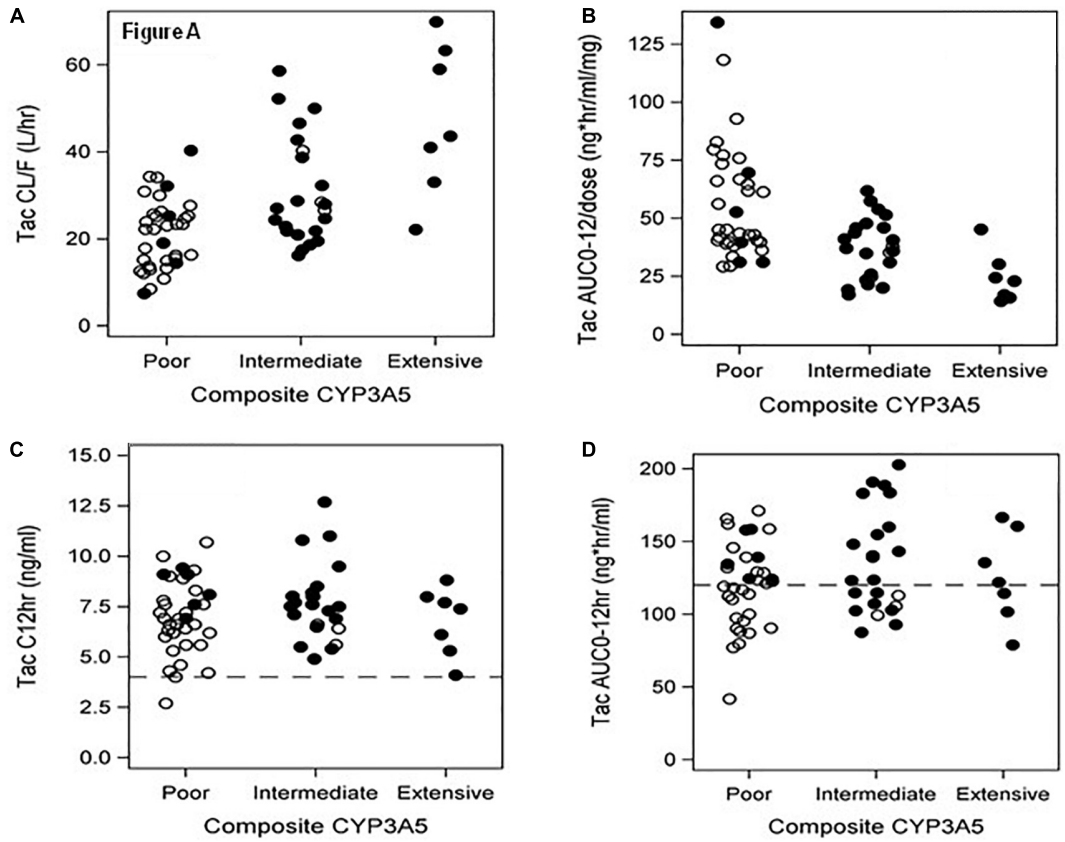

FIGURE 2 | (A–D) Metabolic Composite for CYP3A5*3*6*7 and associations to tacrolimus pharmacokinetic parameters – **(A)** represents tacrolimus clearance classified by 3 metabolic composite groups for CYP3A5*3*6*7. The Extensive Metabolizers are all Blacks with more rapid clearance than Poor Metabolizers ($P < 0.001$), who were primarily Whites. **(B)** depicts dose normalized AUC $_{0-12}$ with Poor Metabolizers with twice the dose normalized tacrolimus exposure compared to Extensive Metabolizers ($P < 0.001$); **(C)** presents tacrolimus troughs divided by metabolic composite groups using the target range of >4 ng/ml and <15 ng/ml for our study. No difference was found between these groups. Note that 64 of 65 patients are within the therapeutic trough range. **(D)** depicts $AUC_{0-12\ h}$ graphs of the metabolic composite groups using the tacrolimus target of >120 and ≤200 ng.hr/ml (Wallemacq et al., 2009). Note that 17/32(53%) of Whites and 10/33(30%) of Blacks had 12-h tacrolimus exposures <120 ng.hr/ml distributed across all groups in spite of the therapeutic troughs **(C)**. OPEN Circle = Whites; CLOSED Circle = Blacks.

ABCB1 Variants and Associations With Tacrolimus Pharmacokinetic Parameters

The *ABCB1* SNPs:*1236C > T(rs1128503), 2677G > T/A (rs2032582), and 3435C > T (rs1045642)* were assessed using validated TaqMan allelic discrimination assays. Hardy-Weinberg equilibrium was confirmed for allele frequencies at each position. Linkage disequilibrium (LD) among the three *ABCB1* SNPs was found to be significant and ranged from 0.89 (*ABCB1* 2677–3435) to 0.72 (*ABCB1* 1236–3435). Estimated *ABCB1* haplotype frequencies ($n = 65$) are summarized in **Figure 3** and do not vary significantly compared to previously reported estimated frequencies (Venuto et al., 2015). The most frequent variant haplotype (TTT) displayed significantly different frequencies between Whites and Blacks compared to the wild-type CGC (Venuto et al., 2015).

The significant associations of tacrolimus pharmacokinetic parameters with *ABCB1* variants are summarized in **Table 3**. A significant association of the TTT variant was found with maximum concentration/dose (Cmax/Dose) ($p = 0.050$) with no sex or race association. With sex-adjusted Thesias analysis, significant associations of TTT haplotypes were found with tacrolimus study dose ($P = 0.03$), oral clearance at steady state ($P = 0.023$), CL/LBW ($P = 0.022$) with lower doses and slower clearances in Whites. With race adjusted Thesias analysis, an association of TTT was noted with AUC_{0-4} ($P = 0.041$) and a trend noted with AUC* ($p = 0.078$) with lowest exposures found in White males.

Combined Role of *CYP3A5* and *ABCB1* on Tacrolimus Pharmacokinetics

The combined role of both *CYP3A5* and *ABCB1* variants on tacrolimus pharmacokinetics was assessed by including both the *CYP3A5* metabolic composite groups and the *ABCB1 3435C > T (rs1045642)* in a single analysis. As observed in the individual CYP3A5 composite analysis above, significant associations of tacrolimus pharmacokinetics with *CYP3A5*3*6*7* metabolic composite were again observed even with the inclusion of *ABCB1* variants (**Table 4**). Significantly, for apparent clearance (Tac

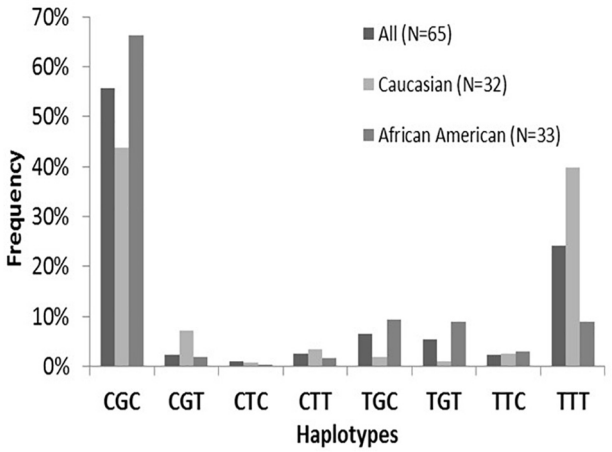

FIGURE 3 | *ABCB1* haplotype frequencies by Race. Note the distributions of ABCB1 wild-type CGC compared to variants. The frequency of the major variant is TTT is depicted with a frequency of 39.8% in Whites compared to 8.9% in Blacks. There were significant overall differences ($p < 0.001$) for haplotype frequencies between Whites and Blacks attributed to CGC and TTT (brackets). This finding is consistent with previously evaluated populations (29–31). No differences in haplotype frequencies are found between sexes. Figure from Venuto et al. (2015).

CL_F L_hr) both the *ABCB1* variant and the CYP*3A5* metabolic composite had a significant effect. Across all 3 metabolic composite groups, the presence of an *ABCB1 3435 T* allele significantly reduced tacrolimus clearance beyond that accounted for by CYP3A5 variants alone (**Figure 4**).

DISCUSSION

P-glycoprotein interacts with gastrointestinal and hepatic cytochrome P450 3A isoenzymes to modulate tacrolimus pharmacokinetics which impacts systemic and cellular drug distribution (Fan et al., 2010; Hesselink et al., 2014; Shuker et al., 2016b). Thus both *ABCB1* and *CYP3A5* variants play an essential role in modulation of intracellular tacrolimus concentrations (Kim et al., 2001; Fredericks et al., 2006; Cattaneo et al., 2009; Hesselink et al., 2014) and overall systematic tacrolimus exposure. Therefore, assessments of the multiple polymorphisms in both genes may assist in understanding tacrolimus pharmacokinetics. We present a pharmacogenetic analysis employing a *CYP3A5*3*6*7* composite and *ABCB1* haplotypes to clinically identify rapid versus poor metabolizers of tacrolimus.

The majority of Blacks required higher daily doses and exhibited more rapid tacrolimus clearance attributed to *CYP3A5* in Extensive and Intermediate metabolic groups compared to Poor Metabolizers. The Clinical Pharmacogenetics Implementation Consortium (CPIC) has provided guidelines to incorporate these individual genotypic variants with tacrolimus dosing to account for inter-patient pharmacokinetic variability (Birdwell et al., 2015). We report the association of tacrolimus pharmacokinetics to the CYP3A5 metabolic composite. Approximately 17% of Blacks were identified as Poor Metabolizers due to the presence of CYP3A5*6/*7 variants. These individuals were similar to White Poor metabolizers whose reduced CYP3A5 function is due primarily to a different allele (*CYP3A5*3*). With increasing admixture among racial groups, the presence of multiple loss of function alleles will warrant the use of composite scoring. Studies investigating troughs as a surrogate marker for drug exposure in Black recipients have demonstrated associations with *CYP3A5*1* alleles with higher dose requirements to achieve therapeutic troughs comparable to *CYP3A5*3*6*7* (Oetting et al., 2016). While limited to troughs, these studies also did not include sex specific analysis or intensive sampling to accurately verify tacrolimus exposure over the 12-h dosing interval achieved during maintenance immunosuppression. Therefore, utility of these genotype-based dosing models may have limitations (Oetting et al., 2016; Sanghavi et al., 2017). Our study reinforces the interpatient variability in tacrolimus exposure with approximately 40% of patients exhibiting $AUC_{0-12\ h}$ below the recommended therapeutic range in spite of achieving target troughs (**Figure 2D**).

This is the first prospective study to incorporate *ABCB1* haplotype analysis with a CYP3A5 composite phenotype with

TABLE 3 | *ABCB1* haplotype associations with tacrolimus pharmacokinetics.

Tacrolimus pharmacokinetic parameter	Wild Type Haplotype[a] (CGC) Association to Phenotypic Mean[b] [95% CI]	Variant Haplotype[a] and Phenotypic Mean[b] (95% CI)		*P*-value[c]
		Variant haplotype	Phenotypic mean (95% CI)	
Study dose (mg)	2.45 [1.74 – 3.16]*	TTT	1.58 [0.71 – 2.45]*	**0.03**
CL_F (L/hr)	20.4 [14.5 – 26.3]*	TTT	13.99 [7.87 – 20.11]*	**0.023**
Clearance/LBW (L/hr/kg)	0.48 [0.37 – 0.60]*	TTT	0.36 [0.23 – 0.47]*	**0.022**
AUC* (ng.hr/ml/mg)	80.0[68.8 – 91.1]	TTT	92.2 [74.6 – 109.8]	0.078
AUC_{0-4} (ng.hr/ml/mg)	36.59 [30.2 – 42.9]	TTT	43.9 [33.4 – 54.4]	**0.041**
C max/Dose (ng/ml/mg)	3.01[2.42 – 3.59]	TTT	4.06[3.19 – 4.93]	**0.050**

The phenotypic means of the wildtype haplotype (CGC) vs. phenotypic means for variant haplotype (TTT). [a]*Haplotypes displayed at positions 1236-2677-3435. Wild-type is C-G-C.* [b]*Phenotypic mean estimated from maximum likelihood methods by THESIAS; Phenotype reported as contribution of single haplotype (one chromosome).* [c]*P value represents variant haplotype comparison to wild-type by THESIAS. *Sex-adjusted analysis; +race-adjusted analysis.*

TABLE 4 | Tacrolimus pharmacokinetics stratified by *CYP3A5*3*6*7* metabolic composite groups and *ABCB1 3435* genotypes.

	ABCB1 3534 CC			*ABCB1 3534 CT and TT*			Significance	
	***CYP3A5* Poor**	***CYP3A5* Intermediate**	***CYP3A5* Extensive**	***CYP3A5* Poor**	***CYP3A5* Intermediate**	***CYP3A5* Extensive**	***ABCB1 3435***	***CYP3A5* Composite**
N	13	10	6	20	10	6		
Study dose (mg)	2.46 (0.36)	4.00 (0.41)	5.67 (0.53)	2.35 (0.29)	3.55 (0.41)	5.08 (0.53)	0.281	**2.2E-08**
Study Dose/TBW (mg/kg)	0.03 (0.01)	0.05 (0.01)	0.07 (0.01)	0.03 (0.00)	0.04 (0.01)	0.06 (0.01)	0.846	**1.3E-05**
C_{12hr} (ng/ml)	6.94 (0.50)	7.55 (0.58)	7.40 (0.74)	6.81 (0.41)	8.37 (0.58)	6.58 (0.74)	0.930	0.107
C_{12hr}/dose (ng/ml/mg)	3.44 (0.37)	2.16 (0.43)	1.43 (0.55)	3.30 (0.30)	2.40 (0.43)	1.57 (0.55)	0.814	**2.3E-04**
Cmax (ng/ml)	15.68 (2.23)	18.62 (2.54)	16.97 (3.29)	16.96 (1.80)	23.33 (2.54)	23.67 (3.29)	0.057	0.099
Cmax/Dose (ng/ml)	7.20 (0.81)	5.21 (0.92)	3.36 (1.19)	8.09 (0.65)	6.61 (0.92)	4.95 (1.19)	0.105	**0.002**
Tmax (hr)	1.94 (0.31)	1.87 (0.35)	1.78 (0.46)	1.72 (0.25)	2.29 (0.35)	2.03 (0.46)	0.628	0.740
AUC* (ng.hr/ml)	116.68 (8.54)	130.75 (9.73)	121.82 (12.57)	117.75 (6.88)	150.30 (9.73)	129.45 (12.57)	0.263	**0.036**
AUC_{0-12} Dose (ng.hr/ml/mg)	56.39 (5.64)	36.76 (6.43)	23.95 (8.30)	56.80 (4.55)	42.94 (6.43)	28.97 (8.30)	0.485	**1.1E-04**
CL_F (L/hr)	22.61 (2.94)	31.30 (3.35)	48.23 (4.32)	20.09 (2.37)	24.49 (3.35)	39.53 (4.32)	**0.040**	**2.8E-07**
CL/LBW (L/hr/kg)	0.37 (0.06)	0.55 (0.07)	0.90 (0.09)	0.39 (0.05)	0.41 (0.07)	0.79 (0.09)	0.218	**7.2E-07**
$AUC_{0-4\ h}$ (ng.hr/mL/mg)	48.59 (4.75)	57.57 (5.41)	51.85 (6.99)	50.96 (3.83)	68.17 (5.41)	61.47 (6.99)	0.110	**0.032**

Univariate results from multivariate analysis of variance. Values are Mean (Std).

FIGURE 4 | Association of CYP3A5 metabolic composite groups and *ABCB1 T* allele with Tacrolimus clearance. There were significant differences in clearance among metabolic groups (overall $P < 0.00001$) and between individuals carrying at least one *ABCB1 T* allele ($P = 0.040$). Significant effects were determined using multivariate analysis. Corresponding N's for each group are given in **Table 4**.

intensive tacrolimus pharmacokinetics. Evaluation of *ABCB1* genotypes and in some cases, haplotypes, as indirect markers of cellular P-gp has been used to identify patients at higher risk for calcineurin inhibitor associated adverse effects including nephrotoxicity, gingival hyperplasia and neurotoxicity with tacrolimus dose normalized troughs or daily doses with conflicting results (Yamauchi et al., 2002; Drozdzik et al., 2004; Hauser et al., 2005; Kotrych et al., 2005; De Iudicibus et al., 2008; Garcia et al., 2013; Venuto et al., 2015). The validity of haplotype-based analysis has been widely accepted in association studies of unrelated individuals (Shuker et al., 2016a). Haplotype analyses using maximum likelihood methods combined with Stochastic Expectation-Maximization (SEM) algorithms have recently been used to assess candidate genes with specific phenotypes (Tregouet et al., 2004; Venuto et al., 2015). Haplotypic data provide greater power to detect associations compared to single genotypes especially when analyzed in conjunction with demographic and clinical covariates (Akey et al., 2001; Little et al., 2009). Inclusion of the 3 loci as *ABCB1* haplotypes improves detection probability relative to the pharmacokinetic parameters. The significant associations of TTT haplotypes to tacrolimus pharmacokinetics provides novel and important information that needs to be evaluated in a larger renal transplant population.

Although haplotype association studies provide a comprehensive view of genetic variants, limitations do exist. These limitations reflect small sample sizes, inconsistent use of haplotype analyses, and multiple testing. Due to our sample size, some of the rare haplotypes were poorly represented and may limit our ability to detect significant effects for those very rare haplotypes. Individual *ABCB1* SNPs: *rs1045642 (C3435T)* and *rs203582 (G2677A)* have variable outcomes as pharmacogenomic predictors with different drug substrates;

thus, the haplotype approach may improve detection ability for phenotypic differences (Chinn and Kroetz, 2007).

Given the role of both *CYP3A5* and *ABCB1* variants on tacrolimus pharmacokinetics, a combined analysis using both genotypes were examined. While the role of CYP3A5, as determined by the metabolic composite had the largest effect of tacrolimus clearance (Tac CL_F L _hr), the presence of the *ABCB1 3435 T* allele (here used as a proxy for the wildtype haplotype) was significantly associated with reduced tacrolimus clearance for all metabolic composite groups (**Figure 4**). Fredericks et al. (2006) found a minor effect due to *ABCB1* haplotypes on tacrolimus dose requirements. This effect was lost when the analysis further included patients who were classified as producers or non-producers of *CYP3A5* (based upon the presence of the *CYP3A5*3 allele*). Our significant finding may be due to (1) the inclusion of genotypic data for *CYP3A5*6 and *7* and both studies had Black patients where these alleles are common; (2) the use of a metabolic composite score; and (3) the comparison of different pharmacokinetic parameters.

The advantages of our study include the prospective enrollment of stable patients at steady state dosing conditions during intensive 12-h pharmacokinetic evaluation that quantitated actual drug exposure reflecting therapeutic drug monitoring of trough concentrations. All patients were enrolled using pre-determined Inclusion and Exclusion criteria which is an important advantage. Multivariate analysis also incorporated common clinical covariates to further identify patients at risk for adverse effects or variability in tacrolimus pharmacokinetics. Another advantage is the inclusion of *ABCB1* haplotype analysis to minimize the limitations of multiple allele test of individual variants and phenotypic endpoints. The use of intensive pharmacokinetic profiles in patients was combined with adherence assessment and tacrolimus concentrations measured in a single CLIA certified drug analysis laboratory provides an important study advantage. Using this approach provides consistency and accuracy for drug concentration analysis to use in determination of comprehensive tacrolimus pharmacokinetic parameters and systematic exposure. Finally, the inclusion of a metabolic composite to represent loss of function due to combinations of *CYP3A5*3*6*7* SNPs further identified factors that influence interpatient variability in tacrolimus pharmacokinetics in the groups. Our statistical model incorporated use of pair-wise group comparisons to substantiate differences in pharmacokinetics and pharmacogenomic associations that may improve our understanding of sub-population differences.

There are some limitations that should be considered from the findings of this report. Our assignment of CYP3A5 metabolic composites for the double heterozygotes is hampered by the fact that the use of individual genetic assays for each SNP do not provide information on the chromosomal arrangement of the variant alleles. Similarly, the use of Thesias to estimate *ABCB1* haplotype phenotypic effects is necessary since chromosomal arrangement of the three *ABCB1* SNPs are not known given the individual genetic assays used. Sequencing could solve this problem but would be prohibitively time consuming and expensive for even this study with this modest sample size.

This study provides support for a more comprehensive inclusion of multiple alleles either by utilizing composite metabolic scores for *CYP3A5* where multiple alleles such as *3, *6 and *7 likely have the loss of function phenotype and with the *ABCB1* haplotype analysis in situations where linkage combines alleles of unknown effects that may impact systemic distribution of tacrolimus. The combining of tacrolimus pharmacokinetics with a more inclusive representation of multiple alleles that reflect proteins that impact drug metabolism and distribution may provide more clinical utility in therapeutic drug monitoring of this immunosuppressive post-transplant.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available because access to de-identified patient data from this clinical study cannot be provided due to stipulations in the sponsor specific agreement from Astellas Scientific and Medical Affairs, Inc. Requests to access this data can be addressed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University at Buffalo Health Sciences Institutional Review Board (IRB# PHP0599703-4). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DB, RV, GW, and KT were involved in the study design of this project. RV, SC, AG, and KT were involved in recruitment and clinical evaluation of patients. DB, KA, GW, CM, JC, LC, and KT were involved in the data analysis of this project. DB, KA, GW, CM, JC, LC, RV, SC, AG, and KT were involved in writing this manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Pharmacogenetics in Psychiatry: An Update on Clinical Usability

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Using pharmacogenetics in guiding drug therapy experiences a steady increase in uptake, although still leads to discussions as to its clinical use. Psychiatry constitutes a field where pharmacogenomic testing might help in guiding drug therapy. To address current challenges, this minireview provides an update regarding genotyping (SNP analysis/arrays/NGS), structural variant detection (star-alleles/CNVs/hybrid alleles), genotype-to-phenotype translations, cost-effectiveness, and actionability of results (FDA/CPIC/PharmGKB) regarding clinical importance of pre-emptive pharmacogenomic testing for prescription of antidepressants and antipsychotics.

Keywords: pharmacogenetics, cytochrome P450, CYP2D6, CYP2C19, depression, psychiatry, antidepressant

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INTRODUCTION

Regarding DNA testing for guiding drug therapy, the value of *CYP2D6* and *CYP2C19* genotyping for optimizing drug treatment in psychiatry has been a focus point. Mental illness is a major health issues and has great individual and social-economical impact. In 2010, costs of mental disorders in US were USD$ 2.5 billion, and these are expected to increase considerably (Corponi et al., 2018). Rate of response to initial antidepressant treatment was only 49.6% (STAR*D trial (Rush et al., 2006)), and a systematic review showed that non-responders to one or more treatments have a 15% likelihood of suicide ideation compared to 6% of patients with treatment-responsive depression and 1% in the general population (Mrazek et al., 2014). Costs for managing nonresponders are USD $10,000/year/patient higher as compared to responsive patients (Mrazek et al., 2014). Currently, >200 drugs are available for treatment of psychiatric/neurologic patients (Hiemke et al., 2018). Use of this medication is hampered by side-effects and lack of effectivity, leaving therapeutic outcomes nonsatisfactory. Only 30% of patients suffering from major depressive disorder (MDD), bipolar disorder (BD), and schizophrenia remain compliant with medication and reach full and stable remission (Corponi et al., 2018), whereas 30–50% of patients with MDD do not respond to their first antidepressant (Rush et al., 2006). Remission rates for SSRI's are as low as 37% (Thase et al., 2010). Regarding side effects, 25,000 patients in US present to the emergency department each year due to antidepressant-induced adverse events (Hampton et al., 2014). A major determinant affecting side effects and lack of efficacy is the relation between dosage and systemic exposure to the drug.

Therapeutic drug monitoring can be used to guide antidepressant therapy. Most antidepressants/antipsychotics are being metabolized by CYP2D6, CYP2C19, and CYP3A4 enzymes in the liver (Hiemke et al., 2018). Because of the strong relation between genetic variants and enzymatic activity, analysis of *CYP2D6* and *CYP2C19* has been an early focus for the clinical use of pharmacogenetics in psychiatry. A summary of the relation between *CYP2D6*/*CYP2C19* genotypes and adjusted dose was published in 2013 (Stingl et al., 2013). Several evidence-based dosing guidelines for using pharmacogenetics for antidepressants/antipsychotics have been published (Swen et al., 2008; Swen et al., 2011; Hicks et al., 2013; Hicks et al., 2017). This mini-review addresses the latest developments in pharmacogenetics for psychiatry and discusses some challenges to be faced in the near future.

PROSPECTIVE RANDOMIZED CONTROLLED CLINICAL TRIALS FOR ANTIDEPRESSANTS

The relation between genotype and enzymatic activity is undisputed, as is the relationship between genotype and plasma concentration of a drug upon a specific dose. Yet, a major argument hampering clinical guidelines is evidence for improving clinical outcome. One of the first studies addressing clinical benefit of using pharmacogenetic information to guide drug therapy was published by Hall-Flavin et al. in 2013, showing a significant increase in responders after 8 weeks of antidepressant therapy when genetic information on *CYP2D6*, *CYP2C19*, *CYP1A2*, *SLC6A4*, and *HTR2A* was used to dose patients (n = 114) as compared to standard treatment (n = 113) (Hall-Flavin et al., 2013). In a recent meta-analysis, taking into account five prospective randomized-controlled trials on depressive symptom remission, published between 2013 and 2019 (Winner et al., 2013; Singh, 2015; Perez et al., 2017; Bradley et al., 2018; Greden et al., 2019), these initial findings were confirmed: in a total of 1,737 subjects, patients receiving pharmacogenetic-guided therapy (n = 887) were 1.71 times more likely to achieve symptom remission as compared to patients receiving usual treatment (p = 0.005) (Bousman C. A. et al., 2019). In a study on 2,066 patients, the CYP2C19 UMs and CYP2C19 PMs were more prone to switch escitalopram to another drug (Jukic et al., 2018). These studies indicate additional value of using genetics in guiding antidepressant therapy. However, it is important to realize that also negative results have been published. An excellent overview of positive and negative studies is summarized in a recent systematic review by Solomon et al. (2019), analyzing 16 studies published between 2013 and 2018. Some explanations mentioned for lack of positive associations were: non-randomization and underpowered studies, time of measuring the investigated endpoint, concomitant use of herbal remedies, unjustified exclusion of patients from the study, focus on particular ethnic groups, or more complex pharmacokinetics in relation to clinical outcome (i.e., venlafaxine metabolism).

RETROSPECTIVE, CONFIRMATORY COHORT-STUDY FOR ANTIPSYCHOTICS

A recent study on aripiprazole and risperidone (both CYP2D6 substrates) using data from 2005 to 2018 from Diakonhjemmet Hospital, Oslo, Norway, showed that, without prior knowledge of *CYP2D6* genotype at the time of treatment, clinicians reduced the daily risperidone dose for CYP2D6 poor metabolizers by an average of 19% (95% CI, 5–35; p = 0.010) and for aripiprazole by 15% (95% CI, 1–28; p = 0.033). The estimated dose reduction based on pharmacogenetic constitution of the patients would have been 40 and 35%, respectively (Jukic et al., 2019). The large number of patients (725 risperidone-treated and 890 aripiprazole-treated patients) makes this one of the larger studies in the field. The incidence of switching of risperidone to another antipsychotic was significantly higher in CYP2D6 ultra-rapid metabolizers (OR, 2.9; 95% CI, 1.4–6.0; p = 0.003) and for CYP2D6 poor metabolizers (OR, 1.9; 95% CI, 1.1–3.1; p = 0.015), indicating that, at least for risperidone, *CYP2D6* genotype status has a clinical impact.

FDA, CPIC, PHARMGKB, AND DPWG

Translating published evidence on pharmacogenetics into clinical actions is an important aspect needed for successful implementation. Both the Dutch Pharmacogenetics Working Group (DPWG; started in 2005) and the Clinical Pharmacogenetics Implementation Consortium (CPIC; started in 2009) use thorough review of the literature by a combination of experts in a transparent way. DPWG now has dosing advice for 94 drugs[1] and CPIC for 54 drugs[2]. DPWG published evidence-based dosing recommendations on *CYP2D6* and *CYP2C19* genotypes for antidepressants and antipsychotics in 2008, with an update in 2011 (Swen et al., 2008; Swen et al., 2011). These recommendations are currently used in the Netherlands by all pharmacists to advice patients and physicians on drug choice and drug dosing. The Clinical Pharmacogenetic Implementation Consortium (CPIC[3]) published guidance for using genetic information in drug therapy for Psychiatry (Hicks et al., 2013; Leckband et al., 2013; Hicks et al., 2015; Hicks et al., 2017; Phillips et al., 2018; Brown et al., 2019). Information on enzymes involved in drug metabolism is also present in the drug label of more than 160 drugs[4], but usually no specific dose recommendations are included. It must, however, be emphasized that recommendations on dosing based on genotype are not always the same between the different expert groups, and for some drugs to a great extent disparate from each other. In addition, the implementation of pharmacogenetic information into the product characteristic (SmPCs) is only found in about 50% of cases (Ingelman-Sundberg, 2020).

[1] www.pharmgkb.org – Clinical annotations (accessed June 1, 2020)
[2] www.pharmgkb.org – Clinical annotations (accessed June 1, 2020)
[3] https//cpicpgx.org (accessed June 1, 2020)
[4] www.fda.org – Table of Pharmacogenomic Biomarkers in Drug Labelling (accessed June 1, 2020)

The PharmGKB website hosts a huge amount of relevant information on pharmacogenetics, with at present 753 drug label annotations, 154 clinical guideline annotations, 149 curated pathways, and 700 annotated drugs. In 2018, the FDA issued a safety communication indicating a lack of clinical evidence supporting the utility of pharmacogenetic testing, specifically addressing the use of pharmacogenetics for antidepressants. This letter highlighted difference in opinion exist when judging published evidence, as stated in the recent perspective on antidepressant pharmacotherapy (Hicks et al., 2020). To create clarity, FDA published in 2020 a Table of Pharmacogenetic Associations[5], distinguishing three different categories: (a) pharmacogenetic associations for which data support therapeutic management recommendations, (b) pharmacogenetic associations for which data indicate a potential impact on safety or response, and c) pharmacogenetic associations for which data demonstrate a potential impact on pharmacokinetic properties only. Comparing this list with CPIC guidelines and DPWG recommendations (**Supplementary Table 1**), not all drugs are present in the FDA listing. Also, it can be seen in this table for which gene/drugs pairs there is agreement in proposed action, and where there is a difference in opinion. Although the FDA table is helpful in distinguishing which drugs could benefit from a pharmacogenetic test, it also shows difficulties in reaching a uniform guidance, even within the FDA. The FDA-statement from 2018 that "the relationship between DNA variations and the effectiveness of antidepressant medication has never been established"[6] seems to be a direct contradiction of the randomized-controlled clinical trials on antidepressants mentioned earlier in this paper. Also, the note from the FDA that "the relationship between *CYP2C19* genotype and drug response to escitalopram and sertraline is not established, and this relationship is not described in the FDA-approved labelling of the drug" seems to conflict with the FDA product label for escitalopram that mentions that "the exposure under supratherapeutic 30-mg dose is similar to the steady-state concentrations expected in CYP2C19 poor metabolizers following a therapeutic dose of 20 mg", as pointed out by Hicks et al. (2020). Also, for sertraline, which is not mentioned in the FDA table, there is substantial scientific evidence that indicates that CYP2C19 poor metabolizers have an approximately three-fold higher exposure to the drug as compared to normal metabolizers (Hicks et al., 2020). Indeed, plasma levels of antidepressants are associated with clinical outcome (Florio et al., 2017; De Donatis et al., 2019) and genetic pharmacokinetic variants showed a clinically relevant effect (Fabbri et al., 2018). Both CPIC and DPWG have adjusted dosing recommendations based on literature for sertraline and CYP2C19 PMs (**Supplementary Table 1**). Also interesting is that for tetrabenazine, for which genetic testing is required according to FDA, neither a PharmGKB clinical annotation nor a CPIC or DPWG guideline is available. It will be clear that the clinical field would benefit from clinical decision support tools, such as, for example, GeneSight, Translational Software, Corriel, PillCheck, OneOme, and Abomics. However, it should be clear which guidelines and which interpretations these dose recommendations originate from, to avoid conflicts in dosing advice. Again, harmonization would greatly help the field.

COST EFFECTIVENESS OF PHARMACOGENETIC TESTING

An important aspect of using pharmacogenetics, besides helping patients to reach therapeutic drug concentrations more quickly, are costs associated with this approach. One of the challenges is that pricing of health care costs as well as pharmacogenetic testing will differ between laboratories and across countries. This causes conflicting reports, as discussed by Rosenblat et al. (2017) and Peterson et al. (2017). A recent paper of Maciel et al. (2018), addressing cost savings of pharmacogenetic testing for depression in a real-world clinical setting, calculated a saving of USD$3,962 annually per patient, assuming a test cost of USD $ 2,000 (NeuroID genetix panel with 10 genes). Hornberger et al. (2015) calculated savings of USD $3,647 per patient using a USD $2,000 PGx testing panel. For comparison, cost of *CYP2D6*/*CYP2C19* genotyping in The Netherlands is between €100 and €300, thus much lower, strongly reducing expenses as compared to the US study. The impression is thus that indeed pharmacogenetic testing may be highly beneficial, also from an economical point of view. Developing countries can benefit from the knowledge obtained from developed countries, and in such implement pharmacogenetics into their healthcare system thus preventing adverse drug reactions and associated costs but also because a once in a lifetime genetic test can be more easily performed as compared to measuring drug concentrations. An approach to consider would be *CYP2B6* genotyping for efavirenz therapy, in the battle against HIV. Yet, costs, logistics, and knowledge about specific variants occurring in these countries are challenges to be addressed. The potential for implementation of pharmacogenetics in developing countries is reflected upon in several publications (Mitropoulos et al., 2011; Roederer et al., 2011; Mizzi et al., 2016; Mitropoulos et al., 2017).

In general, a more cost-effective approach might be to have for each patient a DNA passport for medication, covering most polymorphic genes involved in commonly prescribes drugs, and for which dosing recommendations are available. This would increase the benefit of pharmacogenetic tests, also beyond psychiatry, and would avoid that each separate clinical field would have to worry about cost effectiveness. In fact, the large European trial Ubiquitous PGx[7] is investigating this approach, monitoring both medical benefits and cost-effectiveness. The outcome of this study is expected in 2020/2021.

[5] https://www.fda.gov/medical-devices/precision-medicine/table-pharmacogenetic-associations

[6] https://www.fda.gov/medical-devices/safety-communications/fda-warns-against-use-many-genetic-tests-unapproved-claims-predict-patient-response-specific (accessed June 1, 2020)

[7] www.upgx.eu

GENOTYPING CHALLENGES

In laboratory settings, it is advocated that only tests are performed that are clinically actionable. For psychiatry, this holds true for *CYP2D6* and *CYP2C19*. The genotyping field has identified which variants per gene should be investigated, since the reliability of the predicted phenotype "Normal metabolizer" will depend on the number of variants investigated. The more variants analyzed (and found absent), the stronger the prediction "normal metabolizer" will be. Although there is a substantial agreement on this, each laboratory may have its own additional variants analyzed, usually depending on the genotyping platform used. It is therefore important that each laboratory also reports which SNPs were investigated. Clinical use of pharmacogenetics may benefit from consensus as to which variants should minimally be investigated. In 2018, the American Molecular Pathology (AMP) published a guideline for *CYP2C19* testing, giving as Tier 1 *CYP2C19*2, *3*, and **17* variant alleles and as Tier 2 *CYP2C19*4A-*4B*, **5*, **6*, **7*, **8*, **9*, **10* and **35* alleles (Pratt et al., 2018). Tier 1 variant alleles were defined as those having: (i) well-characterized alteration of CYP2C19 activity that has been shown to have an effect on drug response and for which the functional variant is known, (ii) appreciable minor allele frequencies in a patient population, and (iii) available reference materials. Tier 2 alleles were defined as alleles that meet at least one, but not all of the criteria for inclusion in Tier 1 and are considered optional for expanded clinical genotyping panels. These include normal function variant alleles, low frequency alleles and alleles without available reference materials. In their recommendations, the differences in allele frequencies in different populations are taken into account. A similar initiative from AMP is currently ongoing for *CYP2D6* genotyping, but has not yet been published. In a recent article by Bousman C. et al. (2019), it was suggested that, in addition to *CYP2C19* and *CYP2D6*, *CYP2C9* (for phenytoin) and *HLA-A/HLA-B* gene variants should be considered for a 'minimum, evidence-based genetic testing panel' (Bousman C. et al., 2019).

Important is the conversion of SNPs into variant alleles using star allele assignments, with **1* being a default value, encoding active enzyme. There are currently 131 variant *CYP2D6* alleles described, which can be divided into active, decreased activity and inactive variants (Nofziger et al., 2020). Although polymorphisms affecting mRNA or protein expression will be following such a general categorization, it is important to keep in mind that particular variants causing amino acid substitutions may also cause changes in enzyme activity that are substrate dependent. A way to fine tune predicted phenotypes is an activity score (AS) assignment, with values 0 for non-functional alleles, 0.25, 0.5, and 0.75 for decreased activity alleles to 1.0 for active alleles (Gaedigk et al., 2008). The total score will indicate whether an individual is poor metabolizer (AS = 0), intermediate metabolizer (AS = 0.25–1.25), normal metabolizer (AS = 1.5–2.25), or ultra-rapid metabolizer (AS > 2.25). The challenge here is whether this conversion to PM, IM, NM, and UM should be maintained, as it lowers the information grade. Yet, clinicians may be accustomed to working with these phenotyping groups. Therefore, it remains to be seen whether the AS system will be adopted in routine clinical practice. In addition, one need to consider that most variants are detected by SNP analysis, and these analyses focus on the frequent variants described in literature. Next generation sequencing (NGS) would be helpful is analyzing CYP alleles in detail, also detecting not yet described variants. However, especially the *CYP2D6* locus seems to be complex to analyze, partly because of high homology with *CYP2D7* and *CYP2D8* pseudogenes. Lauschke and Ingelman-Sundberg (2019) reported on the value of NGS, now also successfully used for *CYP2D6*, stressing the value of rare variants. Of course this poses another challenge, as to assign a clinical relevance to rare variants identified that have not previously been characterized. Recent evidence suggests that bioinformatic tools may successfully be applied to NGS (Fabbri et al., 2020a). Specific programs that can be used for this are Aldy, Astrolable, and Stargazer. The value of NGS is that also rare variants can be detected. A drawback, however, can be that in a clinical setting, a variant cannot be assigned to a specific predicted phenotype, complicating the actions from a prescriber point of view. Another challenge for *CYP2D6* is the occurrence of gene deletions, multiplications, and *CYP2D6/7* hybrid alleles, excellently documented in a recent PharmVar review on *CYP2D6* (Nofziger et al., 2020). Copy number variation in CYP2D6 can be investigated by using CNV assays investigating signal strength at exon 9 or by analysis of specific PCR products, as done by XL-PCR (i.e., Autogenomics or Luminex). Approaches using two probes for CNV, like for intron 2 and exon 9, or the VeriDose approach (Agena BioSciences) utilizing 13 *CYP2D6* probes can be useful to get detailed information on the existence of hybrid alleles. The technical complexities of *CYP2D6* genotyping highlight the need for harmonization.

FUTURE DIRECTIONS

It is challenging to harmonize the genotyping, since different platforms are in use, but it is clear that methods should be used that at least includes the AMP Tier 1 and 2 alleles that can detect hybrid alleles and that is FDA/CE-IVD approved. This then combined with clinical decision support software for conversion of genotyping to a specific dosing advice to help clinicians to better target their therapies. Of course, pharmacogenetics can be expanded from *CYP2D6* and *CYP2C19* genotypes to other genes, encoding enzymes, receptors, drug transporters, or other downstream molecules. From that point of view, there is still a lot to be discovered, with the challenge to see which genes do significantly improve therapeutic outcome. Implementation of *CYP2D6/CYP2C19* genotyping in psychiatry constitutes, in our opinion, an important first step in this.

CONCLUSION

Analyzing today's progress in clinical use of pharmacogenetics, we identify expert agreement on many aspects, but also still

differences in opinion. As indicated, this concerns genotyping itself (SNP analysis/arrays/NGS), structural variant detection (haplotypes/CNVs/hybrids), genotype-to-phenotype translation, cost-effectiveness, and actionability (FDA/CPIC/PharmGKB lists). Notably, this paper did not discuss pharmacodynamic gene variants, since the clinical relevance is still under investigation (Fabbri et al., 2020b). Despite the challenges described, there is an increase in uptake for clinical care, making harmonization and clinical guidelines important to bring this field further in facilitating effective and safe treatment of patients.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

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SYSTEMATIC REVIEW
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Diagnostic Test Criteria for HLA Genotyping to Prevent Drug Hypersensitivity Reactions: A Systematic Review of Actionable HLA Recommendations in CPIC and DPWG Guidelines

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Introduction: Certain HLA variants are associated with an increased risk of hypersensitivity reactions to specific drugs. Both the Clinical Pharmacogenetics Implementation Consortium (CPIC) and the Dutch Pharmacogenetics Working Group (DPWG) have issued actionable HLA gene – drug interaction guidelines but diagnostic test criteria remain largely unknown. We present an overview of the diagnostic test criteria of the actionable HLA – drug pairs.

Methods: A systematic literature search was conducted in PubMed, Embase, Web of Science and Cochrane Library. Original case-control and cohort studies were selected and sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and number needed to genotype (NNG) were calculated for the actionable HLA-drug pairs.

Results: In general, the HLA tests show high specificity and NPV for predicting hypersensitivity reactions. The sensitivity of HLA tests shows a wide range, from 0-33% for HLA-B*1502 testing to predict lamotrigine induced SJS/TEN up to 100% for HLA-B*5701 to predict immunologically confirmed abacavir hypersensitivity syndrome (ABC-HSR). PPV is low for all tests except for HLA-B*5701 and ABC-HSR which is approximately 50%. HLA-B*5701 to predict ABC-HSR shows the lowest NNG followed by HLA-B*5801 for allopurinol induced severe cutaneous adverse drug reactions and HLA-B*1502 for carbamazepine induced SJS/TEN.

Discussion: This is the first overview of diagnostic test criteria for actionable HLA-drug pairs. Studies researching HLA genes and hypersensitivity are scarce for some of the HLA-drug pairs in some populations and patient numbers in studies are small. Therefore, more research is necessary to calculate the diagnostic test criteria more accurately.

Keywords: HLA genes, pharmacogenomics, hypersensitivity, antiepileptic drugs, abacavir, allopurinol, flucloxacillin

INTRODUCTION

Adverse drug reactions (ADRs) are an important cause of hospitalization and mortality in modern healthcare. ADRs can be classified as type A or type B. Type A reactions are often common and can be predicted from the drug's pharmacological mechanism of action. Type B reactions, also known as idiosyncratic or hypersensitivity reactions, are usually much rarer and unpredictable and therefore pose a serious risk for patients as they can even be life-threatening.

The hypersensitivity reactions included in this review are: Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN), drug reaction with eosinophilia and systemic symptoms (DRESS), maculopapular exanthema (MPE), abacavir hypersensitivity syndrome (ABC-HSR) and drug-induced liver injury (DILI).

Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are the most serious types of severe cutaneous adverse drug reaction (SCAR). Both SJS and TEN are characterized by fever and mucocutaneous lesions leading to necrosis and sloughing of the epidermis. The two diseases are separated based on the percentage of body surface area detached: 1%–10% detachment defines SJS, 10%–30% detachment defines SJS/TEN overlap, and >30% detachment defines TEN. The mortality of SJS/TEN is estimated to be approximately 25%, ranging from 5%–10% for SJS to more than 30% for TEN (Sekula et al., 2013; Mahar et al., 2014; High, 2020; Mockenhaupt, 2020).

Drug reaction with eosinophilia and systemic symptoms (DRESS), also belonging to the term SCAR, describes a potentially life-threatening syndrome including a severe skin eruption, fever, hematologic abnormalities and involvement of internal organs. The mortality of DRESS is estimated to be about 5%–10% (Chen et al., 2010; Cacoub et al., 2011; Mockenhaupt, 2020). Another term used for DRESS is drug-induced hypersensitivity syndrome (DIHS).

Maculopapular exanthema (MPE) is a milder form of cutaneous ADR. It is the most common type of cutaneous ADR, occurring in approximately 2 percent of individuals exposed to drugs. MPE is characterized by erythematous macules and papules. Systemic symptoms include pruritus, low-grade fever, and mild eosinophilia. Usually the rash develops 5 to 14 days after starting treatment, but it may occur within one or two days. The rash usually improves within 2 weeks after withdrawal of the culprit drug.

Abacavir hypersensitivity (ABC-HSR) has several similar features as DRESS but does not have all the major criteria for DRESS. ABC-HSR is usually a combination of symptoms: Fever is almost always present and patients also often suffer from dizziness, headache, malaise and gastrointestinal symptoms. Respiratory symptoms and rash can be present as well. In the early use of abacavir, ABC-HSR became the main reason for drug discontinuation in approximately 8 percent of treated patients (Symonds et al., 2002; Young et al., 2008; Elizabeth J Phillips, 2020).

Drug-induced liver injury (DILI) is rare and has an estimated annual incidence between 10 and 15 per 10,000 to 100,000 persons exposed to prescription medications. Acute presentations of DILI include mild, asymptomatic liver test abnormalities but also liver failure. DILI accounts for approximately 10 percent of all cases of acute hepatitis. DILI is also a frequent reason for withdrawal of medications from the market (Larson, 2020).

The discovery of the first ADR-HLA genotype association, HLA-B*5701 associated with abacavir hypersensitivity, and its mandatory testing as obliged in the drug label initiated a whole new field of research leading to additional significant HLA-variant – ADR associations in the last decade.

Both the Clinical Pharmacogenetics Implementation Consortium (CPIC) and the Dutch Pharmacogenetics Working Group (DPWG) have issued actionable HLA gene – drug interaction guidelines. The HLA-drug pairs considered as actionable by CPIC and/or DPWG are: HLA-B*5701-abacavir, HLA-B*5701-flucloxacillin, HLA-B*5801-allopurinol, HLA-A*3101-carbamazepine, HLA-B*1511-carbamazepine, HLA-B*1502-carbamazepine, HLA-B*1502-oxcarbazepine, HLA-B*1502-lamotrigine and HLA-B*1502-phenytoin.

These guidelines are based upon different types of studies including case-control studies and cohort studies. While there is evidence for the association between HLA variants and the occurrence of ADRs, HLA testing is not yet being performed pre-emptively, except for HLA-B*5701 and abacavir and HLA-B*1502 and carbamazepine in some Asian populations. Pre-emptively testing HLA-A*3101 is recommended by the Canadian and Swiss drug label for some populations but it is not mandatory, We hypothesize that an important reason for a lack of implementation of pre-emptive HLA testing is the fact that diagnostic test criteria for the tests remain largely unknown. We have found one other review with an overview of diagnostic test criteria but this study has focused on antiepileptic drugs only and not on all drugs with actionable HLA gene – drug interactions (Mullan et al., 2019).

Therefore, we aim to present an overview of the diagnostic test criteria, namely the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and the number needed to genotype (NNG) for all actionable HLA – drug pairs.

METHODS

We conducted a systematic literature search in PubMed, Embase, Web of Science and the Cochrane Library for case-control studies and prospective and retrospective cohort studies that evaluated known HLA – ADR associations. Based on the CPIC and DPWG guidelines for actionable HLA-drug pairs we restricted our search to articles concerning abacavir, allopurinol, flucloxacillin, carbamazepine, oxcarbazepine, lamotrigine and phenytoin. Search terms consisted of "*drug name" AND drug hypersensitivity AND HLA* and included synonyms of these terms (see **Supplementary File 1**). Records were screened on title and abstract. Duplicates, comments, editorials, narrative reviews, letters without original data and publications in languages other than English were excluded. Papers reporting original data with a minimum of 40 patients of which at least 10 hypersensitivity cases in total were included. We selected only studies where the HLA variants were genotyped directly. Studies that used a variant in linkage with the causal HLA variant were excluded since linkage

disequilibrium is not known for all populations and shows considerable variation. We only selected studies where tolerant controls were available to calculate the diagnostic test criteria most accurately. All diagnostic test criteria were calculated by using the tolerant controls data while the population controls, if available, were used to calculate allele carrier frequencies in the general population. Calculation of sensitivity, specificity, PPV, NPV and NNG were done according to Tonk et al., and Steinberg et al. (Steinberg et al., 2009; Tonk et al., 2017) using the data described in the original articles. Considered endpoints included SCAR, SJS/TEN, DRESS, MPE, ABC-HSR and DILI. The endpoints used in the calculation of the diagnostic test criteria are similar to the endpoints mentioned in the specific CPIC and DPWG guidelines. For instance when the CPIC and/or DPWG guidelines only mention an association with SJS/TEN, other endpoints are not included in the results section.

To calculate the NPV, PPV and NNG, the incidence of the ADR is required. However, the incidence is not always available. Therefore we extracted the incidence from literature in one of the following ways and in this order:

i. Directly from the included article itself when the article is a cohort study
ii. From the DPWG or CPIC guideline based on a review, meta-analysis or original article
iii. Derived from an original article in a similar population

In the tables in the result section, the source used for the incidence is mentioned.

RESULTS

Study Selection

Figure 1 shows the result of the study selection. Initially, 1,383 publications were identified. The publications were first screened by title and abstract and then the full-text articles were assessed for eligibility. In total, 69 studies matched the inclusion criteria for analysis in this systematic review. Of these 69 studies, only the 56 studies investigating the same endpoints as the CPIC and/or DPWG guidelines and having the data needed for calculating the diagnostic test criteria were used in the *Results* section of this systematic review.

Abacavir

Abacavir is a nucleoside analog reverse-transcriptase inhibitor (NRTI) used to treat and prevent HIV and is always used in combination with other antiretroviral drugs. To date, it is the only drug for which a pre-emptive HLA test is mandatory according to the drug label. Abacavir is contraindicated for patients who have been tested positive for the HLA-B*5701 variant or for patients who have not been tested because a positive test result is strongly associated with abacavir hypersensitivity syndrome (ABC-HSR) (Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie, 2020a). ABC-HSR occurs in 4%–8% of patients receiving abacavir prior to implementation of pre-emptive HLA-B*5701 testing (Hetherington et al., 2001; Mallal et al., 2008). The incidence of ABC-HSR seems to be much higher in Caucasian populations due to a higher frequency of the HLA-B*5701 allele than in non-Caucasian populations (To et al., 2013; Zhang H. et al., 2015; Youssoufi et al., 2017; Agbaji et al., 2019).

We have identified nine articles that met our inclusion criteria, all but one only in Caucasians. The results are summarized in **Table 1** for clinically diagnosed ABC-HSR and in **Table 2** for immunologically confirmed ABC-HSR.

Almost all the included studies were performed in Caucasians but the reported incidence of abacavir hypersensitivity differs between the studies. Therefore different incidences (1.4%–9.0% for Caucasian populations) are used for calculating the PPV, NPV, and NNG.

The specificity of the HLA-B*5701 test is high in all studies: 90-100%, as is the NPV: 95%–100% in clinically diagnosed and 100% in immunologically confirmed subjects. The sensitivity of the HLA-B*5701 test for abacavir HSR differs greatly between the studies and is between 31% and 90% for clinically diagnosed Caucasian patients. However, for immunologically confirmed HSR the sensitivity increases to 100%. The PPV is around 50%. The number of new abacavir users needed to genotype to prevent one case of ABC-HSR is 14–90 in Caucasians but ~10 times higher in Blacks.

Allopurinol

Allopurinol is the most commonly used drug for the treatment of gout and hyperuricemia. However, a great safety concern of allopurinol is the risk of SCAR which is estimated to be 0.1%–0.4% among new users. (Hershfield et al., 2013) It has been proven that HLA-B*58:01 is associated with an increased risk for allopurinol induced SCAR. Nineteen studies are identified using the inclusion criteria described in the methods. Of these 19 studies, 14 are included in the results tables whereas the others investigated other endpoints or did not genotype cases and tolerant controls. The results of the HLA-B*5801 test criteria are shown in **Table 3** for SCAR, **Table 4** for DRESS, and **Table 5** for SJS-TEN.

The incidence of SCAR is 0.1%–0.4% according to the CPIC guideline (Hershfield et al., 2013). Based on the results of a PubMed search the incidence of SJS/TEN is estimated to be 0.16% for SJS/TEN and 0.05% for DRESS (Sunicha Limkobpaiboon and Naruemon Dhana, 2010; Saokaew et al., 2014; Chong et al., 2018). Thus we use an incidence of 0.21% for SCAR (Min et al., 2015; Ke et al., 2019; Lin et al., 2019).

HLA-B*5801 testing in Asian populations shows high sensitivity and high specificity for allopurinol induced SCAR: 88%–100% and 82%–94% respectively. The only identified study in a non-Asian population, a Portuguese population, shows a lower sensitivity of 64% but a high specificity of 94%. The PPV is low (1.1% - 3.0%) while the NPV is approximately 1. The frequency of carriers of the HLA-B*5801 allele is lower in the Portuguese population than in Asian populations (4% versus 10%-20% respectively). If HLA-B*5801 is the only SNP associated with allopurinol induced SJS/TEN, the incidence of SCAR in this population is expected to be lower, leading to a higher NNG. In the Portuguese population also other HLA variants may be of importance. The calculated NNG for SCAR is 476-540 in Asian populations and is assumed to be much higher in the Portuguese population and other populations with a lower frequency of the HLA-B*5801 allele.

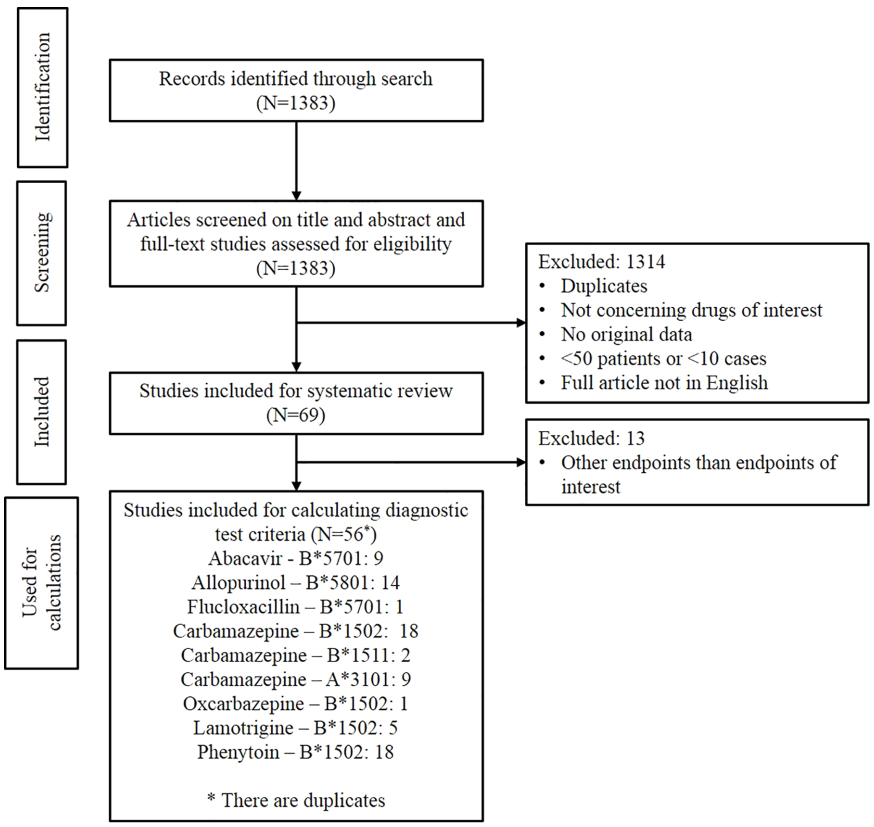

FIGURE 1 | Study flow diagram of the systematic review inclusion.

The high sensitivity and specificity are seen for both SJS/TEN and DRESS, as shown in **Table 4** (DRESS) and **Table 5** (SJS/TEN).

Sensitivity and specificity for the HLA-B*5801 test for allopurinol induced DRESS and SJS/TEN are comparable to the numbers mentioned above for SCAR. Sensitivity for allopurinol induced DRESS and SJS/TEN is 91.7%–100% and 80.0%–100% respectively in Asians. Specificity is between 82.1% and 96.0%. Due to the low incidence of allopurinol induced DRESS the NNG to prevent one case of DRESS is 2,000–2,182 in Asian populations. The NNG for allopurinol induced SJS/TEN is 625–781 in Asian populations.

Flucloxacillin

Flucloxacillin is a penicillin antibiotic used for treating infections caused by Gram-positive bacteria such as staphylococci or streptococci. Although extremely rare, flucloxacillin has been associated with drug-induced liver injury (DILI). DILI is a collective term of different liver injuries as adverse drug reactions (ADRs) due to various drugs. The drug label of flucloxacillin mentions a clear correlation between HLA-B*5701 and flucloxacillin-induced liver damage but does not advise routine pre-emptive testing due to the rarity of DILI and the low PPV of 0.12% (Aurobindo Pharma, 2019). The incidence of DILI is estimated to be about 8.5/100,000 in new flucloxacillin users (Russmann et al., 2005). The DPWG has issued recommendations for HLA-B*5701 and flucloxacillin induced DILI. The recommendations indicate to monitor liver function more regularly and switch to an alternative when liver enzymes or bilirubin increase (Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie, 2020b). The CPIC however has not defined an actionable guideline about this drug-gene interaction.

Only one article is identified meeting our inclusion criteria. The results are shown in **Table 6**.

The HLA-B*5701 test has relatively high sensitivity and specificity (84.3% and 93.8% respectively) for flucloxacillin induced DILI. However, due to the rarity of flucloxacillin induced DILI, the test has a very low PPV of 0.11% and high NPV (99.99%). The low incidence of DILI results in a high NNG for DILI of 13,953.

Antiepileptic Drugs

Epilepsy is one of the most common chronic neurological disorders affecting millions of people worldwide. Many epileptic people use antiepileptic drugs (AEDs) to treat their condition. But these AEDs, especially carbamazepine, oxcarbazepine, phenytoin and lamotrigine are, along with allopurinol, the most common cause of cutaneous adverse drug reactions (cADRs) including the previously mentioned severe SJS/TEN and DRESS but also a milder form of cADR called macopapular exanthema (MPE). Carbamazepine, oxcarbazepine, lamotrigine and phenytoin induced cADRs are associated with HLA-B*1502 (Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie, 2020c;

TABLE 1 | HLA-B*5701 and clinically diagnosed abacavir hypersensitivity reaction.

Article	Population	Description	Sensitivity	Specificity	PPV	NPV	Incidence	NNG	Frequency carriers in population (%)
Berka et al. (2012)	Canada	Prospective cohort study. N = 489	0.90	1	1	0.996	0.037 [i]	27	4.1 (Berka et al., 2012)
Hetherington et al. (2002)	North America	Case-control study. 85 cases + 115 controls	0.554	0.988	0.666	0.980	0.043[ii] (Hetherington et al., 2001)	42	7.2 (USA NMDP European Caucasian) (McCabe et al., 2020)
Hughes et al. (2004)	UK	Case-control study. 13 cases + 51 controls	0.462	0.902	0.153	0.978	0.037[ii] (Symonds et al., 2002)	59	9.0 (England Blood Donors of Mixed Ethnicity) (McCabe et al., 2020)
Mallal et al. (2002)	West Australia	Cohort study. N = 200. 18 cases.	0.778	0.976	0.778	0.976	0.090 [i]	14	8.7 (Mallal et al., 2002)
Martin et al. (2004)	West Australia	Cohort study. N = 248. Includes Mallal et al. (2002) patients. Mallal's cases were reassessed using updated diagnostic criteria	0.947	0.983	0.818	0.996	0.073[i]	14	8.5 (Martin et al., 2004)
Mallal et al. (2008)	19 countries. Mostly white.	Randomized clinical trial. 980 prospectively genotyped + 976 control group retrospectively genotyped	0.455	0.976	0.612	0.955	0.078 [i]	29	5.6 (Mallal et al., 2008)
Rauch et al. (2008)	Switzerland	prospective cohort study. N= 1,877 of which 149 suspected cases (of which 27 likely). 140 controls. Clinically suspected	0.309	0.986	0.244	0.990	0.0794[i]	41	6.0 (Rauch et al., 2008)
		Clinically likely	0.778	0.986	0.827	0.981	0.0144[i]	89	
Rodriguez et al. (2007)	Spain	case-control study. 26 cases + 27 controls.	0.423	0.963	0.491	0.952	0.078[ii] (Mallal et al., 2008)	30	4.7 (Spain (Catalunya, Navarra, Extremadura, Aaragón, Cantabria),(McCabe et al., 2020)
Saag et al. (2008)	US White	case-control study. 130 white cases + 202 controls	0.442	0.960	0.492	0.952	0.080[ii] (Brothers C et al., 2006)	28	7.2 (USA NMDP European Caucasian) (McCabe et al., 2020)
Saag et al. (2008)	US Black	case-control study. 69 black cases + 206 controls	0.145	0.990	0.358	0.969	0.036[ii] (Brothers C et al., 2006)	192	1.4 (USA NMDP African American pop 2) (McCabe et al., 2020)

Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie, 2020d; Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie, 2020e; Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie, 2020f). For carbamazepine induced cADRs also associations with HLA-B*1511 and HLA-A*3101 have been shown.

There is evidence of cross-sensitivity among the AEDs carbamazepine, oxcarbazepine, lamotrigine and phenytoin (Bloch et al., 2014). In a Norwegian retrospective study of medical records it was found that phenytoin, carbamazepine and oxcarbazepine caused rashes in 27%–35% of patients with a history of another AED related rash (Alvestad et al., 2008). Lamotrigine was with 17% less involved in cross-sensitivity than carbamazepine, oxcarbazepine and phenytoin. A retrospective study of medical records in China also found high cross-sensitivity rates between the four AEDs, especially when carbamazepine and phenytoin were involved (Wang et al., 2010). There was a highly significant mutual risk for cross-sensitivity for CBZ and PHT, and OXC, and LTG. The substantial evidence for cross-sensitivity means caution is needed when prescribing these AEDs, especially when switching from one of these AEDs to another due to an cADR.

Carbamazepine

Carbamazepine is a widely used drug approved for the treatment of epilepsy, bipolar disorder and neuropathic pain. It is however known to be able to cause cADRs. Associations have been shown between HLA-B*1502 in Asian populations and SJS/TEN (Leckband et al., 2013; Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie, 2020c). Also in some populations HLA-B*1511 has been shown to be associated with SJS/TEN (Kim et al., 2011; Shi et al., 2012). In contrast, in European and Japanese populations not HLA-B alleles but HLA-A*3101 has been shown to be associated with SJS/TEN. This variant has also been shown to be associated with DRESS and MPE. The DPWG does not give an actionable advice on MPE, but the CPIC guideline on the other hand does state that an HLA-A*3101 positive carbamazepine user has a higher chance of MPE as well as SJS/TEN and DRESS. In the drug label HLA-B*1502 and HLA-A*3101 testing is advised in high risk patients. Patients are deemed to be at risk when they come from countries with a high prevalence of the HLA-B*1502 allele such as Hong Kong, Thailand, Taiwan, Malaysia, and parts of the Philippines. The drug label states the use of carbamazepine and other AEDs associated with SJS/TEN should be avoided in patients who test positive for the HLA-A*3101 or HLA-B*1502 alleles (ULC MP, 2015). The CPIC's advice is the same whereas the DPWG guideline also advises to consider an alternative in HLA-B*1511 positive patients (Hershfield et al., 2013; Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie, 2020c). The Swiss and Canadian drug label strongly recommend testing HLA-A*3101 in high risk populations. The Swiss label considers Japanese, Caucasians, American indigenous population and patients of Spanish, Portuguese, South Indian and Arabic ancestry to be at high risk.

The incidence of SJS/TEN is 0.005% in European Caucasian populations (Genin et al., 2014; Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie, 2020c) and in Chinese and other some Asian populations it is 0.25% (Genin et al., 2014; Koninklijke Nederlandse Maatschappij ter bevordering

TABLE 2 | HLA-B*5701 and immunologically confirmed abacavir hypersensitivity reaction.

Article	Population	Description	Sensitivity	Specificity	PPV	NPV	Incidence	NNG	Frequency carriers in population (%)
Mallal et al. (2008)	19 countries. Mostly white.	Randomized clinical trial. 980 prospectively genotyped. 976 control group (retrospectively genotyped)	1	0.969	0.479	1	0.027 [i]	37	5.6 (Mallal et al., 2008)
Saag et al. (2008)	US White	Case control study. 130 white cases + 202 controls	1	0.960	0.406	1	0.026[i,iii] (Brothers C et al., 2006)	38	7.2 (USA NMDP European Caucasian) (McCabe et al., 2020)
Saag et al. (2008)	US. Black	Case-control study. 69 black cases + 206 controls	1	0.990	0.206	1	0.0025 [i,iii] (Brothers C et al., 2006)	397	1.4 (USA NMDP African American pop 2) (McCabe et al., 2020)

der Pharmacie, 2020c). To be able to calculate NPV, PPV and NNG for other Asian populations such as Vietnamese and Malaysian, an incidence of 0.25% is assumed. The incidence of SJS/TEN in non-European Caucasians is assumed to be 0.005%.

The results of studies studying the association of HLA-B*1502 and carbamazepine induced SJS/TEN are summarized in **Table 7**.

In general, studies find a relatively high sensitivity (67-100%) and specificity (73-100%) for HLA-B*1502 and carbamazepine induced SJS/TEN. However in some populations such as Korean, Canadians of diverse ancestry, Indonesian, North east Chinese and Indians in Malaysia lower sensitivities of 14-57% are found (Kim et al., 2011; Amstutz et al., 2013; He et al., 2013; Khor et al., 2017; Khosama et al., 2017). In these populations, the allele frequency is lower than in the other Asian populations which may indicate that in these populations also other HLA variants may be of importance, for instance the HLA-A*3101 or HLA-B*1511 variants. In the study from Genin et al. the sensitivity in Europeans is zero and the specificity is 100% since there were no Europeans positive for HLA-B*1502 in this study.

Due to the low incidence, the PPV is low (0.14%–7.8%) while the NPV is high (99.2%–100%). The NNG is 400–2,800 in Asian populations of which most studies lead to a NNG of 400–700. In Canadians, the NNG is 60,000.

Studies about the association of carbamazepine induced SJS/TEN and HLA-B*1511 are scarce. Only two studies meet our inclusion criteria. See **Table 8**.

In southern Han Chinese, the HLA-B*1511 test shows very low sensitivity (7.1%) for carbamazepine induced SJS/TEN. In a Korean population the sensitivity is higher but still low (42.9%). The low sensitivity in southern Han Chinese can be explained by the low allele frequency of HLA-B*1511 in this population (0.4%). The low sensitivity indicates that testing only HLA-B*1511 in carbamazepine initiators might be insufficient to predict SJS/TEN, because of the influence of other HLA-genes such as HLA-B*1502. Specificity in both studies is high and so is the NPV. The PPV is low. The NNG in the study by Kim et al. is 933 while in the study among Southern Han Chinese from Shi et al. it is 5,600 (Kim et al., 2011; Shi et al., 2012).

The results of carbamazepine induced SJS/TEN and HLA-A*3101 are summarized in **Table 9**.

HLA-A*3101 testing shows high specificity (86.0%–96.7%) but mostly low sensitivity (0.0%–83.3%) for carbamazepine induced SJS/TEN. Only in Japanese, Korean and Indian Malaysian relatively high sensitivities are found (42.9%–83.3%). The PPV is 0%–1.6% while the NPV is close to 100.0% in all studies. The NNG shows a high variability and is between 480 and 133,333.

The results of carbamazepine induced DRESS and HLA-A*3101 are summarized in **Table 10**. The incidence of DRESS is assumed to be 0.05% in both Asian and Caucasian populations (Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie, 2020c).

Sensitivity for HLA-A*3101 and carbamazepine induced DRESS is about 50% in several populations (30.4%–70.0%) while specificity is high (86%–97%). The exception is the study by Ihtisham et al. with a sensitivity of 0. However, this study only had two DRESS cases which both did not carry the HLA-A*3101 allele Ihtisham et al. (2019). The NNG is high in all studies (2,857–6,571).

The results of HLA-A*3101 and carbamazepine induced MPE are summarized in **Table 11**. The incidence of MPE is assumed to be 10% in Caucasian users and 4.4% in Chinese and other Asian users (Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie, 2020c).

Sensitivity for HLA-A*3101 and carbamazepine induced MPE is low (1%–23%). The sensitivity is higher in Europeans, Canadians of mixed ethnicities and Indian populations than in Chinese populations. Specificity is high in all populations (94%–99%). PPV is low (3%–44%) while NPV is consistently high (91%–98%). Due to the high incidence of MPE, the NNG is low, especially in Canadians and Europeans (Locharernkul et al., 2008; GlaxoSmithKline, 2009; US Food and Drug Administration, 2009; He et al., 2012; Shi et al., 2012; Amstutz et al., 2013; He et al., 2013; Lv et al., 2013; Caudle et al., 2014; Genin et al., 2014; ULC MP, 2015; Moon et al., 2016; Pfizer, 2016; Chen et al., 2017; Khor et al., 2017; Khosama et al., 2017; Deng et al., 2018; Fowler et al., 2019). But NNG is also low in North India (An et al., 2010) due to a relatively high sensitivity (22.2%). In the other study populations NNG is 166-1818.

Oxcarbazepine

Oxcarbazepine is an AED structurally related to carbamazepine. Therefore it might not be surprising that there is also evidence of an association of HLA-B*1502 and oxcarbazepine induced SJS/TEN leading to CPIC and DPWG guidelines about this association (Leckband et al., 2013; Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie, 2020e). The drug label of oxcarbazepine warns to only use the drug in HLA-B*1502 positive patients when the benefits clearly outweigh the risk (US Food and Drug Administration, 2009).

TABLE 3 | HLA-B*5801 and allopurinol induced severe cutaneous adverse drug reaction (SCAR).

Article	Population	Description	Sensitivity	Specificity	PPV	NPV	Incidence[ii,iii]	NNG	Frequency carriers in population (%)
Chen et al. (2015)	Eastern China	17 SCAR cases + 31 tolerant controls +120 population controls	0.882	0.935	0.0280	0.9997	0.0021	540	14.2 (Chen et al., 2015)
Cheng et al. (2015)	Han Chinese	92 SCAR cases + 75 tolerant controls + 99 population controls	0.946	0.880	0.0163	0.9999	0.0021	504	10.1 (Cheng et al., 2015)
Zhang X. et al. (2015)	Han Chinese	48 SCAR cases + 133 controls + 280 population controls	0.938	0.925	0.0256	0.9999	0.0021	508	12.1 (Zhang X. et al., 2015)
Chiu et al. (2012)	Hong Kong Han Chinese	19 SCAR cases + 30 controls	1	0.867	0.0155	1	0.0021	476	14.2 (Hong Kong Chinese) (McCabe et al., 2020)
Jung et al. 2011)	Korea	Retrospective cohort study. N = 448. 9 cases.	1	0.905	0.0217	1	0.0021	476	12.2 (Jung et al., 2011)
Kang et al. (2011)	Korea	26 SCAR cases + 57 controls	0.923	0.895	0.0181	0.9998	0.0021	516	11.8 (South Korea pop 10) (McCabe et al., 2020)
Gonçalo et al. (2013)	Portugal	25 SCAR cases + 23 controls	0.640	0.957	0.0300	0.9992	0.0021	744	4.0 (Portugal Center) (McCabe et al., 2020)
Cao et al. (2012)	Southern Han Chinese	16 SCAR cases + 63 controls	1	0.889	0.0186	1	0.0021	476	14.0 (Cao et al., 2012)
Chung et al. (2015)	Taiwan	48 cases + 138 controls	0.958	0.826	0.0115	0.9999	0.0021	497	20.0 (Taiwan Han Chinese) (McCabe et al., 2020)
Hung et al. (2005)	Taiwan	51 cases + 135 tolerant controls + 93 population controls	1	0.852	0.0140	1	0.0021	476	20.4 (Hung et al., 2005)
Ng et al. (2016)	Taiwan	106 cases + 285 controls	0.906	0.821	0.0105	0.9998	0.0021	526	20.0 (Taiwan Han Chinese) (McCabe et al., 2020)
Saksit et al. (2017)	Thailand	86 cases + 182 controls	0.965	0.885	0.0173	0.9999	0.0021	493	14.8 (Thailand) (McCabe et al., 2020)

TABLE 4 | HLA-B*5801 and allopurinol induced drug reaction with eosinophilia and systemic symptoms (DRESS).

Article	Population	Description	Sensitivity	Specificity	PPV	NPV	Incidence[ii,iii]	NNG	Frequency carriers in population (%)
Kang et al. (2011)	Korea	21 DIHS cases + 57 controls	0.952	0.895	0.0045	1.0000	0.0005	2100	11.8 (South Korea pop 10) (McCabe et al., 2020)
Gonçalo et al. (2013)	Portugal	19 DRESS cases + 23 controls	0.632	0.957	0.0072	0.9998	0.0005	3167	4.0 (Portugal Center) (McCabe et al., 2020)
Cao et al. (2012)	Southern Han Chinese	3 DRESS cases + 63 tolerant controls + 572 population controls	1	0.889	0.0045	1	0.0005	2000	14.0 (Cao et al., 2012)
Chung et al. (2015)	Taiwan	22 DRESS cases + 138 controls	1	0.826	0.0029	1	0.0005	2000	20.0 (Taiwan Han Chinese) (McCabe et al., 2020)
Ng et al. (2016)	Taiwan	60 DRESS cases + 285 controls	0.917	0.821	0.0026	0.9999	0.0005	2182	20.0 (Taiwan Han Chinese) (McCabe et al., 2020)
Sukasem et al. (2016)	Thailand	6 DRESS cases + 100 tolerant controls +1095 population controls	1	0.960	0.0124	1	0.0005	2000	10.1 (Sukasem et al., 2016)
Saksit et al. (2017)	Thailand	19 DRESS cases + 182 controls	1	0.885	0.0043	1	0.0005	2000	14.8 (Thailand) (McCabe et al., 2020)

TABLE 5 | HLA-B*5801 and allopurinol induced Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN).

Article	Population	Description	Sensitivity	Specificity	PPV	NPV	Incidence[i,iii]	NNG	Frequency carriers in population (%)
Kang et al. (2011)	Korea	5 SJS/TEN cases + 57 controls	0.800	0.895	0.0120	0.9996	0.0016	781	11.8 (South Korea pop 10) (McCabe et al., 2020)
Gonçalo et al. (2013)	Portugal	6 SJS/TEN cases + 23 controls	0.667	0.957	0.0240	0.9994	0.0016	938	4.0 (Portugal Center) (McCabe et al., 2020)
Cao et al. (2012)	Southern Han Chinese	13 SJS/TEN cases + 63 tolerant controls + 572 population controls	1	0.889	0.0142	1	0.0016	625	14.0 (Cao et al., 2012)
Chung et al. (2015)	Taiwan	26 SJS/TEN cases + 138 controls	0.923	0.826	0.0084	0.9999	0.0016	677	20.0 (Taiwan Han Chinese) (McCabe et al., 2020)
Ng et al. (2016)	Taiwan	46 SJS/TEN cases + 285 controls	0.891	0.821	0.0079	0.9998	0.0016	701	20.0 (Taiwan Han Chinese) (McCabe et al., 2020)
Sukasem et al. (2016)	Thailand	13 SJS-TEN cases + 100 tolerant controls + 1095 population controls	1	0.96	0.0385	1	0.0016	625	10.1 (Sukasem et al., 2016)
Tassaneeyakul et al. (2009)	Thailand	27 SJS/TEN cases + 54 controls	1	0.870	0.0122	1	0.0016	625	14.8 (Thailand) (McCabe et al., 2020)
Saksit et al. (2017)	Thailand	67 SJS/TEN cases + 182 controls	0.955	0.885	0.0131	0.9999	0.0016	654	14.8 (Thailand) (2020)

TABLE 6 | HLA-B*5701 and flucloxacillin induced drug-induced liver injury (DILI).

Article	Population	Description	Sensitivity	Specificity	PPV	NPV	Incidence[iii]	NNG
Daly et al. (2009)	White European	51 DILI cases + 64 tolerant controls	0.843	0.938	0.0011	0.9999	0.000085	13953

However, only four studies for oxcarbazepine and hypersensitivity are identified. Three of these four studies were studying oxcarbazepine induced MPE and only one study investigated oxcarbazepine induced SJS/TEN (He et al., 2012; Lv et al., 2013; Moon et al., 2016; Chen et al., 2017). This may be due to the fact oxcarbazepine-induced SJS/TEN is less common than carbamazepine-induced SJS/TEN. Results of the study can be found in **Table 12**. The incidence used for calculations is 0.0826% (Chen et al., 2017; Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie, 2020e).

Chen et al. investigated the association of HLA-B*1502 and oxcarbazepine induced cutaneous adverse drug reactions including SJS/TEN in Taiwanese and Thai Han Chinese. However, because there were only three Thai cases and no tolerant controls, we do not report this data. Sensitivity for Taiwanese Han Chinese is 70.6% and specificity is 92.1%. The NNG for Taiwanese Han Chinese 1715.

Lamotrigine

Lamotrigine is approved for epilepsy and for bipolar disorders. There is evidence HLA-B*1502 is associated with lamotrigine induced SJS/TEN. The drug label of lamotrigine does give a warning about serious skin rashes including SJS usually occurring within 2–8 weeks of initiation of lamotrigine. The incidence is higher in pediatric patients than adults. Interestingly, HLA-B*1502 is not mentioned in the drug label at all (GlaxoSmithKline, 2009).

We have identified nine articles about lamotrigine and hypersensitivity that meet our inclusion criteria. Of these only five articles report information about the association of HLA-B*1502 and lamotrigine induced SJS/TEN, the other studies investigate other endpoints such as DRESS or MPE. The results of the studies investigating SJS/TEN can be found in **Table 13**.

The incidence of lamotrigine induced SJS/TEN is assumed to be 0.1% (Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie, 2020d).

The HLA-B*1502 test shows low sensitivity (0%–33.3%) but high specificity (81.4%–100%) in various populations. the NNG is with 3,000–4,400 much higher than for carbamazepine and oxcarbazepine.

Phenytoin

Phenytoin is an AED but is in some countries also approved as a class 1b antiarrhythmic. There is evidence HLA-B*1502 is associated with phenytoin induced SJS/TEN as mentioned in the DPWG and CPIC guidelines (Caudle et al., 2014; Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie, 2020f). Also the drug label mentions this association but states that the evidence is weak. The label mentions consideration should be given to avoid phenytoin as an alternative for carbamazepine in patients positive for HLA-B*1502 (Pfizer, 2016).

We have identified 10 articles about phenytoin and hypersensitivity that meet our inclusion criteria. Of these 10 articles, eight articles report information about the association of HLA-B*1502 and phenytoin induced SJS/TEN. The results of these studies can be found in **Table 14**.

The incidence of phenytoin induced SJS/TEN is assumed to be 0.24% in Asians and 0.069% in Caucasians (Fowler et al., 2019; Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie, 2020f).

The sensitivity of HLA-B*1502 for phenytoin induced SJS/TEN in the included studies differs from 12.8% to 100%. Of note, the study of Locharernkul et al. (2008) reporting a sensitivity of 100% has only four SJS cases. The second highest sensitivity found is only 61.5%. The specificity is 77.5%–94.9% which is quite low compared

TABLE 7 | HLA-B*1502 and carbamazepine induced Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN).

Article	Population	Description	Sensitivity	Specificity	PPV	NPV	Incidence	NNG	Frequency carriers in population (%)
Amstutz et al. (2013)	Canada	42 cases (9 SJS/TEN, 6 HSS, 26 MPE, 1 AGEP) + 92 controls	0.333	0.989	0.0014	0.99996	0.00005	60000	No data available of Canadians or North Americans of mixed ethnicity. (McCabe et al., 2020)
Wu et al. (2010)	Central China	36 cases (8 SJS/TEN + 28 MPE) + 50 tolerant controls + 71 population controls	1	0.92	0.0303	1	0.0025	400	8.5 (Wu et al., 2010)
He et al. (2013)	China Northeast Han Chinese	35 SJS/TEN cases + 125 controls	0.229	0.984	0.0346	0.9980	0.0025	1750	3.8 (China North Han) (McCabe et al., 2020)
Shi et al. (2012)	China Southern Han Chinese	18 SJS/TEN cases + 93 controls	0.722	0.886	0.0160	0.992	0.0025	554	13.7 (China South Han) (McCabe et al., 2020)
Shi et al. (2017)	China Southern Han Chinese	56 SJS/TEN cases + 180 controls	0.696	0.844	0.0110	0.9991	0.0025	574	13.7 (China South Han) (McCabe et al., 2020)
Cheung et al. (2013)	Hong Kong Han Chinese	26 SJS/TEN cases + 135 controls	0.923	0.881	0.0191	0.9998	0.0025	433	17.9 (Hong Kong Chinese BMDR) (McCabe et al., 2020)
Khosama et al. (2017)	Indonesia	14 SJS/TEN cases + 53 controls	0.571	0.736	0.0054	0.9985	0.0025	700	22.9 (Indonesia Java Western) (McCabe et al., 2020)
Kim et al. (2011)	Korea	24 SCAR cases (7 SJS, 17 HSS) + 50 tolerant controls + 485 population controls	0.143	1	1	0.9979	0.0025	2800	0.4 (Kim et al., 2011)
Khor et al. (2017)	Malaysia	28 SJS/TEN cases + 227 controls	0.714	0.899	0.0174	0.9992	0.0025	560	
	Malaysia Chinese	6 cases + 106 controls	0.667	0.877	0.0134	0.9990	0.0025	600	11.3 (Malaysia Peninsular Chinese) (McCabe et al., 2020)
	Malaysia Indian	6 cases + 57 controls	0.333	0.965	0.0233	0.9983	0.0025	1200	5.2. (Malaysia Peninsular Indian) (McCabe et al., 2020)
	Malaysia Malaysian	16 cases + 64 controls	0.875	0.875	0.0172	0.9996	0.0025	457	22.3 (Malaysia Peninsular Malay) (McCabe et al., 2020)
Wang et al. (2011)	Southern Han Chinese	48 cases (9 SJS/TEN, 39 MPE) + 80 tolerant controls + 62 population controls	1	0.863	0.0179	1	0.0025	400	17.7 (Wang et al., 2011)
Chung et al. (2004)	Taiwan Han Chinese	44 SJS cases + 101 tolerant controls + 93 population controls	1	0.970	0.0781	1	0.0025	400	8.6 (Chung et al., 2004)
Hsiao et al. (2014)	Taiwan Han Chinese	194 cases (51 MPE, 112 SJS/TEN, 8 other) + 152 controls	0.884	0.928	0.0297	0.9997	0.0025	453	8.8 (Taiwan Han Chinese) (McCabe et al., 2020)
Kulkantrakorn et al. (2012)	Thailand	34 SJS/TEN cases + 40 controls	0.941	0.825	0.0133	0.9998	0.0025	425	16.1 (Thailand Northeast pop 2) (McCabe et al., 2020)
Locharernkul et al. (2008)	Thailand	15 cases (6 SJS, 9 MPE) + 42 controls	1	0.810	0.0130	1	0.0025	400	16.1 (Thailand Northeast pop 2) (McCabe et al., 2020)
Sukasem et al. (2018)	Thailand	38 cases (17 MPE, 16 SJS/TEN, 5 DRESS) + 271 tolerant controls + 470 population controls	0.75	0.959	0.0443	0.993	0.0025	533	15.1 (Sukasem et al., 2018)
Tassaneeyakul et al. (2010)	Thailand	42 SJS/TEN cases and 42 controls	0.881	0.881	0.0182	0.9997	0.0025	454	16.1 (Thailand Northeast pop 2) (McCabe et al., 2020)
Nguyen et al. (2015)	Vietnam	38 cases (20 SJS, 7 TEN, 8 SJS-TEN, 3 DRESS) + 25 controls	0.914	0.760	0.0095	0.997	0.0025	438	25,2 (Vietnam Hanoi Kinh pop 2) (McCabe et al., 2020)
Genin et al. (2014)	European	20 SJS/TEN cases, 10 DRESS cases + 43 tolerant controls from other study + 8862 population controls	0	1	–	1	0.00005	–	0.05 (Genin et al., 2014)
Genin et al. (2014)	Chinese from Taiwan	53 SJS/TEN cases, 10 DRESS cases + 72 tolerant controls + 710 population controls	0.774	0.44	0.033	0.994	0.0025	517	8.5 (Genin et al., 2014)

TABLE 8 | HLA-B*1511 and carbamazepine induced Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN).

Article	Population	Description	Sensitivity	Specificity	PPV	NPV	Incidence	NNG	Frequency carriers in population (%)
Kim et al. (2011)	Korea	24 SCAR cases (7 SJS, 17 HSS) + 50 tolerant controls + 485 population controls	0.429	0.96	0.0262	0.9985	0.0025	933	3.9 (Kim et al., 2011)
Shi et al. (2012)	China Southern Han Chinese	56 SJS/TEN cases + 180 controls	0.071	0.843	0.0011	0.9972	0.0025	5600	0.4 (China South Han) (McCabe et al., 2020)

TABLE 9 | HLA-A*3101 and carbamazine induced Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN).

Article	Population	Description	Sensitivity	Specificity	PPV	NPV	Incidence	NNG	Frequency carriers in population (%)
Amstutz et al. (2013)	Canada	42 cases (9 SJS/TEN, 6 HSS, 26 MPE, 1 AGEP) + 92 controls	0	0.967	0	0.9999	0.00005	–	No data available of Canadians or North Americans of mixed ethnicity.
Hsiao et al. (2014)	Han Chinese from Taiwan	194 cases (51 MPE, 112 SJS/TEN, 8 other) + 152 controls (investigates associations)	0.018	0.967	0.0014	0.9974	0.0025	22400	5.5 (Taiwan Han Chinese) (McCabe et al., 2020)
Ozeki et al. (2011)	Japan	77 cADR cases (36 DIHS, 6 SJS/TEN, 35 other) + 420 controls	0.833	0.871	0.0160	0.9995	0.0025	480	16.1 (Japan pop 16) (McCabe et al., 2020)
Kim et al. (2011)	Korea	24 SCAR cases (7 SJS, 17 HSS) + 50 tolerant controls + 485 population controls	0.429	0.86	0.0076	0.9983	0.0025	933	10.3 (Kim et al., 2011)
Khor et al. (2017)	Malaysia	28 SJS/TEN cases + 227 controls	0.107	0.947	0.0051	0.9976	0.0025	3733	
	Malaysia Chinese	6 cases + 106 controls	0	0.972	0	0.9974	0.0025	–	2.6 (Malaysia Peninsular Chinese) (McCabe et al., 2020)
	Malaysia Indian	6 cases + 57 controls	0.500	0.912	0.0141	0.9986	0.0025	800	4.1 (Malaysia Peninsular Indian) (McCabe et al., 2020)
	Malaysia Malaysian	16 cases + 64 controls	0	0.938	0	0.9973	0.0025	–	0.8 (Malaysia Peninsular Malay) (McCabe et al., 2020)
Ihtisham et al. (2019)	North India	35 cases (27 MPE/6 SJS-TEN/2 DRESS) +70 controls	0	0.957	0	0.9974	0.0025	–	3.8 (India North pop 2) (McCabe et al., 2020)
Genin et al. (2014)	European	20 SJS/TEN cases, 10 DRESS cases + 257 tolerant controls + 8862 population controls	0.150	0.961	0.0002	0.99996	0.00005	133333	4.5 (Genin et al., 2014)
Genin et al. (2014)	Chinese from Taiwan	53 SJS/TEN cases, 10 DRESS cases + 72 tolerant controls + 710 population controls	0.019	0.958	0.0011	0,9974	0.0025	21200	3.7 (Genin et al., 2014)

to the other drug-gene interactions in this review. The PPV is low (0.2%–1.4%) meaning false positives are common. The NNG to prevent one case of phenytoin induced SJS/TEN is 417–3,250.

DISCUSSION

In this review we provide a systematic overview of the diagnostic test criteria for HLA- genotyping to prevent drug hypersensitivity reactions. We have focused on the seven drugs for which actionable CPIC and/or DPWG guidelines are available. In general, specificity of all included HLA tests for drug hypersensitivity is high (80%–100%). Sensitivity shows a larger variability ranging from 100% for HLA-B*5701 testing and immunologically confirmed ABC-HSR to less than 30% for HLA-B*5701 testing and lamotrigine induced SJS/TEN. For allopurinol induced SCAR and HLA-B*5801 and flucloxacillin induced DILI and HLA-B*5701 sensitivity is high. For the other drugs and associated HLA variants, a wide range of sensitivities are found. Due to the rarity of some of the included hypersensitivity reactions, the NNG is very high for some drugs, especially for flucloxacillin and lamotrigine. Taking into consideration the low NNG of around 40 in combination with the severity of the side effect, it may not be surprising that HLA-B*1502 testing is mandatory for abacavir. Pre-emptively testing HLA-B*1502 for Asian carbamazepine initiators and HLA-B*5801 for high-risk allopurinol initiators could also be worthwhile.

TABLE 10 | HLA-B*3101 and carbamazepine induced drug reaction with eosinophilia and systemic symptoms (DRESS).

Article	Population	Description	Sensitivity	Specificity	PPV	NPV	Incidence	NNG	Frequency carriers in population (%)
Amstutz et al. (2013)	Canada	42 cases (9 SJS/TEN, 6 HSS, 26 MPE, 1 AGEP) + 92 controls	0.5	0.967	0.0075	0.9997	0.0005	4000	No data available of Canadians or North Americans of mixed ethnicity.
Genin et al. (2014)	Chinese from Taiwan	53 SJS/TEN cases, 10 DRESS cases + 72 tolerant controls + 710 population controls	0.5	0.958	0.0060	0.9997	0.0005	4000	3.7 (Genin et al., 2014)
Genin et al. (2014)	European	20 SJS/TEN cases, 10 DRESS cases + 257 tolerant controls from other study + 8,862 population controls	0.7	0.961	0,0089	0.9998	0.0005	2857	4.5 (Genin et al., 2014)
Hsiao et al. (2014)	Han Chinese from Taiwan	194 cases (51 MPE, 112 SJS/TEN, 8 other) + 152 controls	0.304	0.967	0.0046	0.9996	0.0005	6571	5.5 (Taiwan Han Chinese) (McCabe et al., 2020)
Ozeki et al. (2011)	Japan	77 cADR cases (36 DIHS, 6 SJS/TEN, 35 other) + 420 controls	0.583	0.871	0.0023	0.9998	0.0005	3429	16.1 (Japan pop 16) (McCabe et al., 2020)
Kim et al. (2011)	Korea	24 SCAR cases (7 SJS, 17 HSS) + 50 tolerant controls + 485 population controls	0.588	0.86	0.0021	0.0098	0.0005	3400	10.3 (Kim et al., 2011)
Itisham et al. (2019)	North India	35 cases (27 MPE/6 SJS-TEN/2 DRESS) +70 controls	0	0.957	0	0.9995	0.0005	–	3.8 (India North pop 2) (McCabe et al., 2020)

TABLE 11 | HLA-B*3101 and carbamazepine induced maculopapular exanthema (MPE).

Article	Population	Description	Sensitivity	Specificity	PPV	NPV	Incidence	NNG	Frequency carriers in population (%)
Amstutz et al. (2013)	Canada with diverse ethnic background	42 cases (9 SJS/TEN, 6 HSS, 26 MPE, 1 AGEP) + 92 controls	0.231	0.967	0.438	0.919	0.1	43	No data available of Canadians or North Americans of mixed ethnicity.
Li et al. (2013)	China Han Chinese	40 MPE cases + 52 controls + 72 population controls (allele frequency instead of carrier frequency)	0.013	0.990	0.056	0.956	0.044	1818	3.0 (China Sichuan HIV negative (McCabe et al., 2020)
McCormack et al. (2018)	European descended	95 MPE cases + 869 controls	0.168	0.969	0.376	0.913	0.1	59	4.6 (Poland BMR) (McCabe et al., 2020)
McCormack et al. (2018)	Han Chinese descended	85 MPE cases + 197 controls	0.043	0.940	0.032	0.955	0.044	523	12.2 (China Han HIV negative) (McCabe et al., 2020)
Hsiao et al. (2014)	Han Chinese from Taiwan	194 cases (51 MPE, 112 SJS/TEN, 8 other) + 152 controls (investigates associations)	0.137	0.967	0.161	0.961	0.044	166	5.5 (Taiwan Han Chinese) (McCabe et al., 2020)
Itisham et al. (2019)	North India	35 cases (27 MPE/6 SJS-TEN/2 DRESS) +70 controls	0.222	0.957	0.193	0.964	0.044	102	3.8 (India North pop 2) (McCabe et al., 2020)

TABLE 12 | HLA-B*1502 and oxcarbazepine induced Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN).

Article	Population	Description	Sensitivity	Specificity	PPV	NPV	Incidence[i,iii]	NNG	Frequency carriers in population (%)
Chen et al. (2017)	Taiwan Han Chinese	50 cADR cases (20 SJS-TEN, 6 DRESS, 22 MPE, 2 BDFE) + 101 controls	0.706	0.921	0.0073	0.9997	0.000826	1715	8.8 (Taiwan Han Chinese) (McCabe et al., 2020)

To our knowledge, this is the first overview of the diagnostic test criteria of drug-HLA interactions for the actionable CPIC and/or DPWG guidelines. A review by Mullan et al. (2019) provides diagnostic test criteria but is limited to four AEDs. A strength of our study is that we calculated the diagnostic test criteria using the original data of genotyping results in cases and controls instead of only reporting diagnostic criteria if they were reported in the original studies. Also, some of the original studies did not take into account the incidence of the endpoint in the general population for calculating the NPV and PPV when this correction should be performed in case of a case-control design (Steinberg et al., 2009; Tonk et al., 2017).

A limitation of the available data is that most of the included studies have a limited sample size with mostly positive results, indicating a high likelihood of publication bias. Therefore presented results may be inflated and true effects may be lower than reported in this review. We included original studies which did not find a significant association of the variant but appeared significant in meta-analyses. This was particularly the case for lamotrigine where none of the selected original studies show a significant association of HLA-B*1502 and lamotrigine-induced SJS/TEN. However, in two meta-analyses, a significant association has been shown (Zeng et al., 2015; Deng et al., 2018). It must be noted however that also in these meta-analyses, the total number of patients was still low. The first meta-analysis had only 12 cases and 128 controls where the other meta-analysis had 54 cases and 313 controls. Besides the sample size of the studies, the number of studies found may also be a limitation. For flucloxacillin and oxcarbazepine results from only one study are available. Nevertheless, CPIC and/or DWPG released actionable guidelines for these gene-drug interactions and therefore the studies are included in this review. To better estimate the sensitivity, specificity, NPV, PPV and NNG, more and larger studies are warranted.

It should be noted that in the studies investigating HLA gene-drug interactions the studied endpoint is often a clinically diagnosed endpoint and the criteria of these endpoints may differ between studies. For example, the symptoms of ABC-HSR are nonspecific and may be difficult to objectify. In a study by Martin et al. (2004) data of some of the patients of the study by Mallal et al. (2002) are re-analyzed with stricter criteria for abacavir hypersensitivity. As a result Martin et al. reports a higher sensitivity of 94.7% instead of 77.8% in the original study. Also the difference of sensitivity between clinically diagnosed and immunologically diagnosed abacavir hypersensitivity further confirms the challenges related to the use of a clinical diagnosis as endpoint. Obviously, this has major influence on the estimated parameters such as sensitivity and specificity. When ABC-HSR is immunologically confirmed the sensitivity is 100%, however when clinically diagnosed, the sensitivity is much lower: 31%–90%. Also with conditions such as DRESS and SJS/TEN, the clinically diagnosed endpoint may differ between studies resulting in both over- or underestimation. The fact that clinically diagnosing hypersensitivity reactions is difficult is also exemplified by lamotrigine induced SJS. The drug label of lamotrigine states when 14 cases of serious rash associated with hospitalization were reviewed by three expert dermatologists, one dermatologist considered 7 of the 14 cases as SJS, while another dermatologist considered none of the cases to be SJS (GlaxoSmithKline, 2009). In many studies, SCAR is considered to be one endpoint even though it is a composite endpoint combining DRESS and SJS/TEN which are potentially related to two different biomarkers. Because of this, incidence rates of the separate conditions were sometimes hard to find. In this review we report numbers for allopurinol induced-SCAR but we also looked at DRESS and SJS/TEN separately. Sensitivity is high for both SJS/TEN and DRESS suggesting HLA-B*5801 is an appropriate biomarker for both endpoints.

For our analysis to calculate NPV, PPV and NNG for case-control studies, data on the incidence of the hypersensitivity reaction is needed. The incidence of drug hypersensitivity differs greatly between populations which can be explained by the differences in allele carrier frequencies. For example, the carrier frequency of HLA-B*5801 in the Portuguese population is approximately 4% while this is lower in most other European populations. In an Irish population and a German population for instance the carrier frequency of HLA-B*5801 is only 1%. Also in a Spanish population, geographically close to Portugal, the carrier frequency is already lower with 2.2%. This exemplifies that diagnostic test criteria should not be extrapolated. Since incidences of ADRs in specific populations are sometimes unknown, the best we could do is to assume these incidence figures from the general population. This assumption may influence the calculated NPV, PPV and NNG with the highest potential impact on the latter. The effect on the NPV and PPV will probably be low since NPV is already close to 1 and PPV close to 0. In these cases, we have made a best estimate based on results of incidence rates in similar populations. We used a systematic three-step approach. First, if available, we used the incidence mentioned in the original article. Next, we would take the incidence from the DPWG or CPIC guideline. Lastly, if no incidence or only a wide range was described in the guidelines, we used the incidence from a similar population derived from an original article. Therefore, we believe our study uses the most accurate incidence figures available for calculating NPV, PPV, and NNG.

Theoretically, in case of rare ADRs such as investigated here, population controls could be an alternative for the methodologically preferred tolerant controls. However, our search revealed the availability of tolerant controls for most gene-drug pairs and therefore we consistently chose using tolerant controls as to avoid confounding since mostly it is unknown if the ADR is also associated with disease susceptibility.

TABLE 13 | HLA-B*1502 and lamotrigine induced Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN).

Article	Population	Description	Sensitivity	Specificity	PPV	NPV	Incidence[i]	NNG	Frequency carriers in population (%)
Kazeem et al. (2009)	European	22 cases (10 SJS, 12 HSR) + 43 controls	0	1	–	0.9990	0.001	–	0.0 (Bulgaria, Croatia, Czech Republic, Germany, Ireland Northern, Netherlands, Poland DKMS) (McCabe et al., 2020)
An et al. (2010)	Han Chinese	25 cases (3 SJS/TEN, 22 MPE) + 21 tolerant controls + 71 population controls	0.333	0.952	0.0070	0.9993	0.001	3000	8.5 (Koomdee et al., 2017)
Shi et al. (2011)	Han Chinese	14 cADR cases (2 SJS/TEN + 12 MPE) + 28 tolerant controls + 264 population controls	0	0.931	0	0.9989	0.001	–	14.1 (Shi et al., 2011)
Shi et al. (2017)	Southern Han Chinese	22 SJS cases + 102 controls	0.227	0.814	0.0012	0.9991	0.001	4400	13.7 (China South Han) (McCabe et al., 2020)
Koomdee et al. (2017)	Thailand	15 cADR cases (4 SJS, 1 dress, 10 MPE) + 50 tolerant controls + 986 population controls	0.25	0.88	0.0021	0.9991	0.001	4000	15.5 (Koomdee et al., 2017)

TABLE 14 | HLA-B*1502 and phenytoin induced Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN).

Article	Population	Description	Sensitivity	Specificity	PPV	NPV	Incidence	NNG	Frequency carriers in population (%)
Chang et al. (2017)	Malaysia	16 SCAR cases (13 SJS/TEN, 3 DRESS) + 32 tolerant controls + 300 population controls	0.615	0.781	0.0067	0.9988	0.0024	677	15.7 (Chang et al., 2017)
Cheung et al. (2013)	Hong Kong Han Chinese	15 SJS/TEN cases + 75 controls	0.467	0.8	0.0056	0.9984	0.0024	893	17.9 (Hong Kong Chinese BMDR) (McCabe et al., 2020)
Hung et al. (2010)	Taiwan Han Chinese	26 SJS/TEN cases + 113 tolerant controls + 93 population controls	0.308	0.920	0.0092	0.9982	0.0024	1354	7.5 (Hung et al., 2010)
Locharernkul et al. (2008)	Thailand	16 SJS or MPE cases (4 SJS, 12 MPE) + 45 controls	1	0.822	0.0134	1	0.0024	417	16.1 (Thailand Northeast pop 2) (McCabe et al., 2020)
Shi et al. (2017)	China, Southern Han Chinese	13 SJS/TEN cases + 40 controls	0.462	0.775	0.0049	0.9983	0.0024	903	13.7 (China South Han) (McCabe et al., 2020)
Su et al. (2019)	Taiwan	128 SCAR (65 SJS/TEN, 63 DRESS) cases + 107 MPE cases + 376 controls	0.308	0.949	0.0144	0.9982	0.0024	1354	10.1 (Taiwan pop 2) (McCabe et al., 2020)
Tassaneeyakul et al. (2016)	Thailand	60 cases (39 SJS/TEN + 21 DRESS)+ 92 controls	0.128	0.859	0.0022	0.9976	0.0024	3250	16.1 (Thailand Northeast pop 2) (McCabe et al., 2020)
Yampayon et al. (2017)	Thailand	36 cases (15 SJS + 21 DRESS) + 100 tolerant controls + 758 population controls	0.333	0.820	0.0044	0.9980	0.0024	1250	14.2

For a predictive HLA test to be implemented for pre-emptive testing, the NNG should be low, the clinical endpoint to be prevented should be of high severity and/or mortality and alternative treatments are available. The posterchild example of HLA testing is pre-emptive HLA-B*5701 testing for abacavir. The HLA-B*5701 test has a low NNG (~40). Besides, due to the high incidence of ABC-HSR, the test has a relatively high PPV of about 50%. Therefore when testing positive, there is a 50% chance the patient will develop abacavir hypersensitivity. The first symptoms of ABC-HSR are relatively mild composing of rash, fever, gastrointestinal disturbances and non-specific complaints as malaise, dizziness and headache. However, re-exposure is potentially fatal. Among patients who received abacavir in clinical trials, the mortality rate was 0.03 percent while the mortality among patients experiencing hypersensitivity symptoms after a re-challenge was around 5% (Hetherington et al., 2001). Also the mandatory testing as mentioned in the abacavir drug label has greatly stimulated HLA-B*5701 testing.

By pre-emptively testing patients who are at increased risk of developing hypersensitivity, a lower NNG can be reached. Therefore, based on our results we recommend to consider pre-emptively testing HLA-B*5801 in high risk allopurinol initiators and HLA-B*1502 in Asian carbamazepine initiators. The NNG for HLA-B*5801 and allopurinol induced SCAR is relatively low, approximately 500. However, due to the rarity of SCAR, the PPV is low. Therefore, allopurinol should only be withheld after a positive test result when alternative drugs for treatment are available. The NNG can be reduced 5–10 folds when testing only high risk patients such as patients with chronic renal insufficiency. Two studies find an incidence of allopurinol induced SCAR of 1%–2% in patients with chronic renal insufficiency, which is 5–10 times higher than the incidence in the overall allopurinol initiators (Jung et al., 2011; Park et al., 2019). By pre-emptively testing only patients with chronic renal insufficiency who are at higher risk of developing SCAR, the NNG would decrease to 50–100 patients.

For Asian carbamazepine initiators we consider the NNG of 500 to be low enough for pre-emptively testing. In this situation, the availability of alternative drugs for treatment is of major importance. When a patient is HLA-*1502 positive, the CPIC and/or DPWG recommendations advise to avoid the use of carbamazepine and to also avoid lamotrigine, phenytoin and oxcarbazepine when possible. Due to the rarity of the outcome, the positive predictive value is lower than 1% for HLA-B*1502 and SJS/TEN so false positives are common. This means for every 100 patients who switch to another AED, only one case of SJS/TEN is prevented while the alternative therapy may be suboptimal for the particular patient. The NNG can be lower than 500 when testing only in patients who are at higher risk such as patients with an Human immunodeficiency virus (HIV) infection. Patients with HIV infection have been reported to have a 100-fold higher risk of SJS/TEN than the general population. Also patients with active malignancy have an increased risk of SJS/TEN (High, 2020).

The aim of our review is to give an overview of diagnostic test criteria and this may help clinicians to decide which HLA tests could be implemented. The DPWG introduced the clinical implication score to assist this decision (Swen et al., 2018). The clinical implication score takes into account the clinical effect of the drug-gene interaction, the level of evidence, the NNG and pharmacogenetics information included in the drug label. Drug-gene pairs can be scored as essential, beneficial and potentially beneficial. When scored as essential, the DPWG concludes genotyping must be performed before initiation of drug therapy. For beneficial drug-gene interactions the DPWG recommends genotyping the patients before or directly after initiation of drug therapy. When scored as potentially beneficial, the DPWG states genotyping should be considered on an individual patient basis, but when genetic information is available, they recommend adhering to the guideline. The DPWG considers HLA-B*5701 testing essential for abacavir. HLA-B*1502 testing for oxcarbazepine and lamotrigine were scored to be beneficial. Other HLA-drug interactions have not yet been given a clinical implication score.

In this review we provide a systematic overview of the diagnostic test criteria for actionable drug-HLA gene interactions. In general, specificity and NPV of the HLA tests to predict drug hypersensitivity reactions are high whereas sensitivity shows a wide range across the different tests, ranging from 0-33% for HLA-B*1502 testing to predict lamotrigine induced SJS/TEN up to 100% for HLA-B*5701 to predict immunologically confirmed ABC-HSR. PPV is low for all tests where HLA-B*5701 testing for abacavir has the highest PPV of approximately 50%. The NNG is low for HLA-B testing for flucloxacillin and lamotrigine. HLA-B*5701 testing to predict ABC-HSR shows the lowest NNG followed by HLA-B*5801 for allopurinol induced SCAR and HLA-B*1502 for carbamazepine induced SJS/TEN.

AUTHOR CONTRIBUTIONS

LM performed the literature review and systemic analysis and contributed to writing the manuscript. JS and H-JG contributed to writing the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Genetic Variants in DNA Repair Pathways as Potential Biomarkers in Predicting Treatment Outcome of Intraperitoneal Chemotherapy in Patients With Colorectal Peritoneal Metastasis: A Systematic Review

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Hulshof EC, Lim L, de Hingh IHJT, Gelderblom H, Guchelaar H-J and Deenen MJ (2020) Genetic Variants in DNA Repair Pathways as Potential Biomarkers in Predicting Treatment Outcome of Intraperitoneal Chemotherapy in Patients With Colorectal Peritoneal Metastasis: A Systematic Review. Front. Pharmacol. 11:577968. doi: 10.3389/fphar.2020.577968

Background: The introduction of cytoreductive surgery (CRS) followed by hyperthermic intraperitoneal chemotherapy (HIPEC) with either oxaliplatin or mitomycin C for patients with colorectal peritoneal metastasis (CPM) has resulted in a major increase in overall survival. Nonetheless, despite critical patient selection, the majority of patients will develop recurrent disease within one year following CRS + HIPEC. Therefore, improvement of patient and treatment selection is needed and may be achieved by the incorporation of genetic biomarkers. This systematic review aims to provide an overview of genetic biomarkers in the DNA repair pathway that are potentially predictive for treatment outcome of patients with colorectal peritoneal metastases treated with CRS + HIPEC with oxaliplatin or mitomycin C.

Methods: A systematic review was conducted according to the PRISMA guidelines. Given the limited number of genetic association studies of intraperitoneal mitomycin C and oxaliplatin in patients with CPM, we expanded the review and extrapolated the data from biomarker studies conducted in colorectal cancer patients treated with systemic mitomycin C– and oxaliplatin-based chemotherapy.

Results: In total, 43 papers were included in this review. No study reported potential pharmacogenomic biomarkers in patients with colorectal cancer undergoing mitomycin C–based chemotherapy. For oxaliplatin-based chemotherapy, a total of 26 genetic biomarkers within 14 genes were identified that were significantly associated with treatment outcome. The most promising genetic biomarkers were *ERCC1* rs11615, *XPC* rs1043953, *XPD* rs13181, *XPG* rs17655, *MNAT* rs3783819/rs973063/rs4151330, MMR status, ATM protein expression, *HIC1* tandem repeat D17S5, and *PIN1* rs2233678.

Conclusion: Several genetic biomarkers have proven predictive value for the treatment outcome of systemically administered oxaliplatin. By extrapolation, these genetic biomarkers may also be predictive for the efficacy of intraperitoneal oxaliplatin. This should be the subject of further investigation.

Keywords: biomarker, colorectal cancer, DNA repair, hyperthermic intraperitoneal chemotherapy, mitomycin C, oxaliplatin, treatment outcome

INTRODUCTION

Colorectal peritoneal metastasis (CPM) is associated with a poor prognosis and affects approximately 10–20% of colorectal cancer patients (Chu et al., 1989; Jayne et al., 2002; Verwaal et al., 2003; Lemmens et al., 2011). The introduction of cytoreductive surgery (CRS) followed by hyperthermic intraperitoneal chemotherapy (HIPEC) with either oxaliplatin or mitomycin C for patients with isolated CPM has led to a major increase in overall survival and even cure in up to 15% of patients (Sugarbaker, 1995; Goere et al., 2013). Therefore, CRS + HIPEC is at present considered standard of care for patients with limited peritoneal metastases. Currently, patient selection for CRS + HIPEC is mainly based on the peritoneal carcinomatosis index (PCI) and performance status (Froysnes et al., 2016; Kwakman et al., 2016; Kusamura et al., 2016). In addition, several clinical and pathological prognostic biomarkers have been identified, including completeness of cytoreduction, locoregional lymph node status and signet ring cell differentiation (Simkens et al., 2017). Nonetheless, despite critical patient selection, the majority of patients will develop recurrent disease within one year following CRS + HIPEC (Konigsrainer et al., 2013; Braam et al., 2014). In addition, post-operative surgical complications following CRS + HIPEC are frequent, including mortality in about 1–2% of patients (Chua et al., 2009).

Knowledge of genetic biomarkers that are predictive or prognostic for treatment outcome may be of additional value in patient and treatment selection, allowing further improvement of treatment outcome for the individual patient. In contrast to thousands of pharmacogenetic association studies that have been conducted in cancer patients treated with systemic chemotherapy, almost no data exist of genetic biomarkers in patients treated with intraperitoneal chemotherapy. Following intraperitoneal administration, oxaliplatin and mitomycin exert their anti-tumor effect locally at the tumor site. Both drugs share a comparable mechanism of action in that they both interfere with DNA synthesis and repair. Thereby, genetic variation in genes involved in DNA repair may reduce the functional activity of certain DNA repair genes, making tumor cells more susceptible for drug-induced DNA damage and hence increased drug efficacy (Kweekel et al., 2005; D'Andrea, 2014). The DNA repair system is divided into six major DNA repair pathways, i.e. base-excision repair (BER), nucleotide-excision repair (NER), mismatch repair (MMR), homologous recombination (HR), nonhomologous end joining (NHEJ), and translesion DNA synthesis (TLS). In addition, pathways on damage response and DNA synthesis exist (D'Andrea, 2014).

Notwithstanding the in general increasingly applied knowledge of genetic biomarkers in cancer therapy as a proven tool for patient and treatment selection, almost no predictive or prognostic data of genetic biomarkers for treatment outcome exist in patients with CPM treated with intraperitoneal chemotherapy. Therefore, we conducted a systematic review to provide an overview of genetic biomarkers in the DNA repair pathway that are potentially predictive for treatment outcome of patients with colorectal peritoneal metastases treated with CRS + HIPEC with oxaliplatin or mitomycin C.

METHODS

A systematic literature review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al., 2009).

Of the studies on the use of mitomycin C and oxaliplatin in HIPEC treatment, only two studies were found that have reported biomarkers related to DNA repair (Massalou et al., 2017; Shannon et al., 2019). Data obtained from genetic association studies conducted in other than CPM patients treated with oxaliplatin or mitomycin C may potentially be extrapolated to patients with CPM. Therefore, we expanded this review with studies investigating the association between genetic biomarkers related to DNA repair and treatment outcome in patients with colorectal cancer undergoing mitomycin C– and oxaliplatin-based chemotherapy.

We searched PubMed until February 2020 without any limitations on publication year using the following search terms: "biomarker," "oxaliplatin," "mitomycin C," "colorectal cancer," and "treatment outcome." The full search string is provided in the **Supplementary Material**. In addition, reference lists in original articles and review articles were manually searched to identify additional potentially relevant publications. Literature was reviewed by two independent reviewers (LL and EH). In case of inconsistencies, results were discussed with a third reviewer (MD).

All publications were screened on title and abstract. Only studies that included patients with colorectal cancer were included, and studies that were retracted and studies that did not provide original data or case reports were excluded. The remaining publications were assessed based on screening of the full text. Only studies that reported on the association between genetic biomarkers related to DNA repair and treatment outcome undergoing mitomycin C– and oxaliplatin-based chemotherapy were included. To provide a total overview of

the available evidence, we included studies on various types of genetic biomarkers including genetic polymorphism, mRNA expression, and protein expression. Treatment outcome had to be reported as overall survival (OS), progression-free survival (PFS), or disease-free survival (DFS).

Risk of bias assessment was performed and adapted from the Q-genie tool and was based on the following bias items: clear phenotype and outcome definition and correct nomenclature of genotype. We decided not to exclude studies because of small sample size, ethnic differences, differences in treatment regimens or type of biomarker, or no correction for covariates affecting treatment outcome due to scarcity of data.

All identified genetic biomarkers were subdivided into either one of the six described major DNA-repair pathways (Mendelsohn et al., 2015), i.e., NER, BER, MMR, HR, NHEJ, TLS, or otherwise into a category of genes involved in DNA damage response and DNA synthesis (D'Andrea, 2014). Results were summarized and presented per gene including a mechanistic background for the drug-gene interaction. The following information per study or genetic biomarker was reported: sample size, CRC type, treatment schedule, biomarker, type of sample, type of assay, rs number (if applicable), reference group and comparator group, and treatment outcome. Treatment outcomes were expressed as hazard ratios, relative risks, or differences in median survival with 95% confidence intervals and p-values, whichever was available.

The most promising genetic biomarkers were extracted from the results and summarized in a table. Evidence for these biomarkers had to meet the following 2 criteria: (1) no or almost none conflicting data and (2) an association with treatment outcome was reported in at least two studies or in one study with sufficient power (arbitrarily defined in this review as a minimum number of 300 patients) or the study included a control group with non-oxaliplatin based–chemotherapy in which no association or an association in the opposite direction was seen compared to the group with oxaliplatin-based chemotherapy.

RESULTS

Study Selection

The search string in the PubMed database resulted in a total of 346 identified articles. **Figure 1** provides the selection procedure of relevant articles. An additional 17 studies were added that were identified from meta-analyses. After screening the title and abstract, 122 studies were excluded leaving 241 articles for further evaluation. After reviewing the full-text, 198 articles were excluded, resulting in a total of 43 studies that were included in this systematic review. The percent agreement between the two reviewers was 97%, and Cohen's kappa was 0.87.

Main Results

The identified potential genetic biomarkers for treatment outcome of oxaliplatin-based chemotherapy could be divided over four out of the six major DNA-repair pathways, i.e., NER, BER, MMR, and HR or were involved in DNA damage response or DNA synthesis, respectively. No studies were identified that reported on the association between genetic biomarkers and treatment outcome of mitomycin C–based chemotherapy in CRC patients. From all eligible studies, a total of 26 genetic biomarkers within 14 genes were identified in which at least one study had reported a significant association with treatment outcome. The most promising genetic biomarkers belonged to the NER, MMR, or DNA damage response pathway and are summarized in **Table 1** and explained in more detail below; in contrast to biomarkers that belong to the BER, HR, or DNA synthesis pathway, which seem less promising due to lack of evidence or conflicting results. The results from all included studies are summarized in **Figure 2**, discussed per gene below, and reported in detail in the **Supplementary Material—Tables S1–S10**.

NER Pathway

ERCC1

Oxaliplatin DNA adducts are mainly removed by the NER pathway (Shirota et al., 2001). Excision repair cross-complementation group 1 (ERCC1) is a key protein in the NER pathway that is encoded by the *ERCC1* gene. Together with xeroderma pigmentosum complementation group F (XPF), ERCC1 forms a heterodimer complex that can incise damaged DNA strands at the 5' side of the lesion (Sijbers et al., 1996). In addition to their involvement in the NER pathway, the XPF/ERCC1 complex is also involved in double strand break repair (DSBR) (Ahmad et al., 2008). Therefore, the expression of *ERCC1* is potentially associated with treatment outcome of oxaliplatin in CRC patients.

In two preclinical studies, elevated ERCC1 protein level was suggested to correlate with oxaliplatin-resistance in cells (Boyer et al., 2004; Lin et al., 2012). Alteration in single nucleotide polymorphisms (SNPs) is expected to have an effect in gene expression level and function. Several *ERCC1* SNPs have been evaluated for their association with treatment outcome of oxaliplatin in CRC patients (**Supplementary Material—Table S1**). The most commonly investigated nucleotide polymorphism is rs11615 (Stoehlmacher et al., 2004; Ruzzo et al., 2007; Liang et al., 2008; Martinez-Balibrea et al., 2008; Pare et al., 2008; Chang et al., 2009; Chua et al., 2009; Chen et al., 2010; Liang et al., 2010; Huang et al., 2011; Lamas et al., 2011; Farina Sarasqueta et al., 2011; Li et al., 2012; Kumamoto et al., 2013; Nishina et al., 2013; van Huis-Tanja et al., 2014; Zaanan et al., 2014; Rao et al., 2019). A total of 10 studies showed a significant association between this polymorphism and treatment outcome (Stoehlmacher et al., 2004; Ruzzo et al., 2007; Martinez-Balibrea et al., 2008; Pare et al., 2008; Chang et al., 2009; Chua et al., 2009; Chen et al., 2010; Huang et al., 2011; Li et al., 2012; Rao et al., 2019). Most studies, six out of 10, reported the mutant CC genotype to be the favorable genotype, with significantly better DFS, PFS, and OS (Stoehlmacher et al., 2004; Chang et al., 2009; Chua et al., 2009; Chen et al., 2010; Huang et al., 2011; Li et al., 2012). However, a few studies showed contradictory results. Three studies (Martinez-Balibrea et al., 2008; Pare et al., 2008;

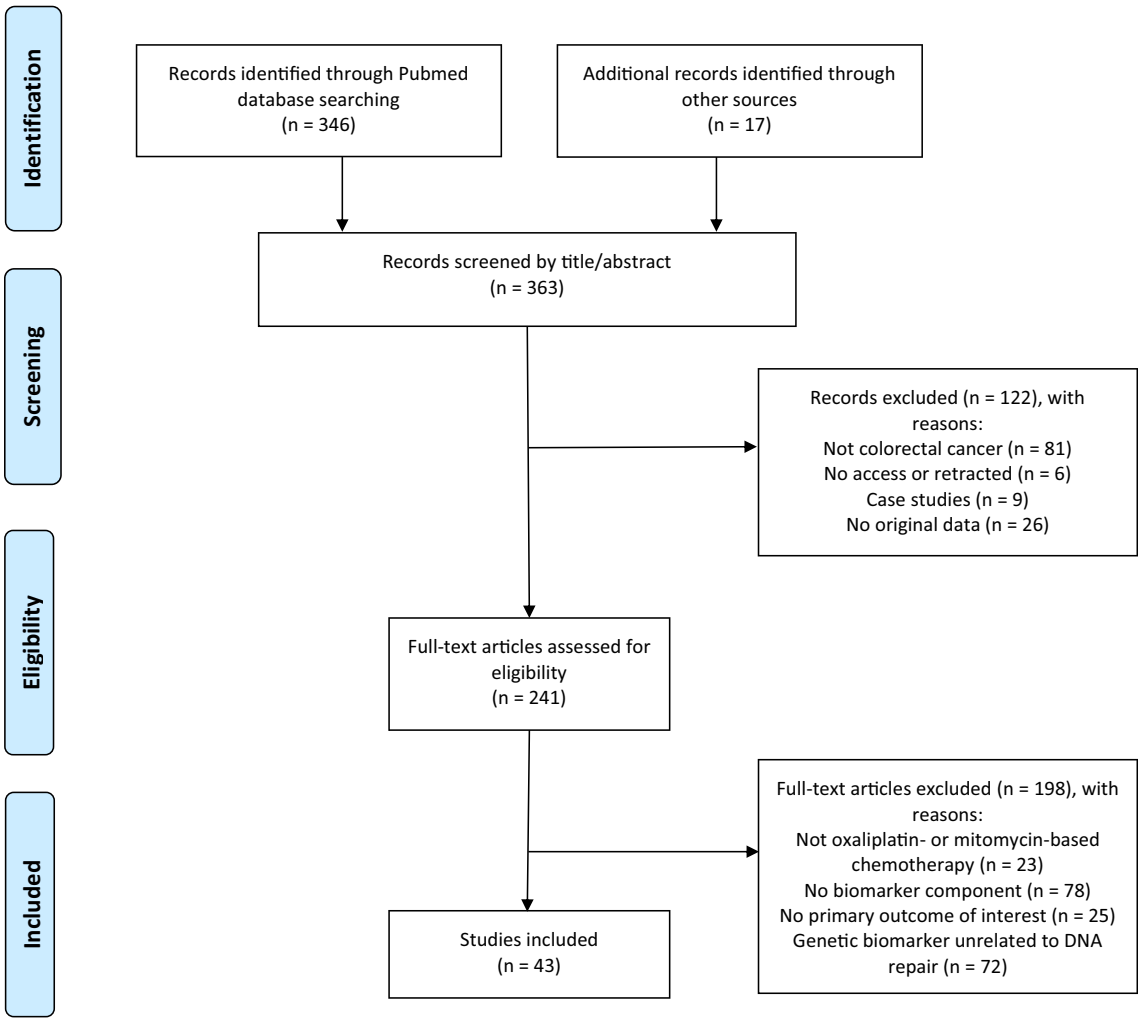

FIGURE 1 | Flow chart of selection procedure literature.

TABLE 1 | Overview of most promising genetic biomarkers within DNA repair for treatment outcome of hyperthermic intraperitoneal chemotherapy in colorectal cancer patients.

Biomarker	Location	Pathway	Favorable genotype/expression
ERCC1 c.354T>C[A]	rs11615	NER	CC
*XPC c.*463A>G*	rs1043953	NER	GG
XPD c.2251A>C[B]	rs13181	NER	AA
XPG c.3310G>C	rs17655	NER	GG
MNAT1 c.688-30168A>G[C]	rs3783819	NER	GG
MNAT1 c.562-88A>G[C]	rs973063	NER	GG
MNAT1 c.809+24992A>G[C]	rs4151330	NER	GG
MMR status	n.a.	MMR	MMR deficient
ATM protein expression	n.a.	DNA damage response	Loss of ATM expression
HIC1 tandem repeat	D17S5	DNA damage response	≤4 tandem repeats
PIN1 NC_000019.9:g.9945179G>C	rs2233678	DNA damage response	GG

[A]*Six studies reported that the CC genotype was favorable, and three studies reported that the TT genotype was favorable.*
[B]*Eight studies reported that the AA genotype was favorable, three studies reported that the CC genotype was favorable.*
[C]*SNPs are in high linkage disequilibrium.*

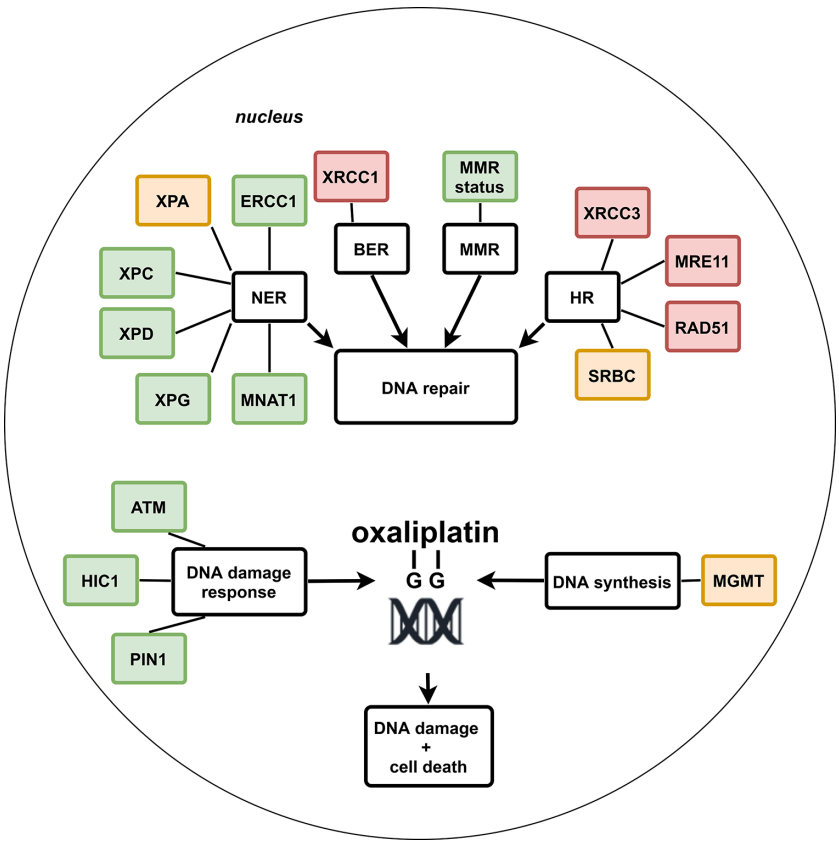

FIGURE 2 | Schematic overview of potential genetic biomarkers within DNA repair pathways for treatment outcome of systemic oxaliplatin in colorectal cancer patients. Green: no or almost none conflicting results and significant association with treatment outcome in ≥2 studies, or in one study with a sample size of ≥300, or inclusion of a non-oxaliplatin–based chemotherapy control group in which no association or an association in the opposite direction was seen compared to the group with oxaliplatin-based chemotherapy. Orange: significant association with treatment outcome in one study. Red: conflicting results or no significant association with treatment outcome.

Rao et al., 2019) reported that patients with the CC genotype had a worse treatment outcome in terms of PFS and OS. Another contradicting result was reported by Ruzzo et al. (2007) where the rs11615 TT genotype was associated with prolonged PFS in univariate analysis and shorter PFS in multivariate analyses.

Two other reported polymorphisms of *ERCC1* are at codon 259 and 504 (Monzo et al., 2007; Nishina et al., 2013). Both polymorphisms showed no significant association with treatment outcome. Moreover, two (Kassem et al., 2017; Rao et al., 2019) out of five (Basso et al., 2013; Li et al., 2014; Sfakianaki et al., 2019) studies based on mRNA or protein expression level of ERCC1 showed a significant association between low ERCC1 expression and prolonged PFS and OS.

XPC

Xeroderma pigmentosum group C (*XPC*), located at chromosome 3p25, encodes for another important protein in the early steps of the NER pathway. XPC binds to RAD23B to form the heterodimeric complex, which is the first NER factor to facilitate the recognition of DNA damage and the initiation of DNA repair (Sugasawa et al., 1998). As DNA damage recognition is the rate-limiting step in the NER pathway, the XPC protein plays a critical role in proper DNA repair. Therefore, genetic biomarkers in *XPC* may have potential value in predicting response for oxaliplatin-based chemotherapy.

In **Table S3** (**Supplementary Material**), three SNPs in the *XPC* gene that are potentially predictive of treatment response to oxaliplatin-based therapy in CRC patients are reported (Liu et al., 2012; Kap et al., 2015; Hu et al., 2019). Only one SNP was significantly associated with survival. In the study by Kap et al. (2015), patients carrying the variant allele rs1043953 had a longer OS after treatment with oxaliplatin-based chemotherapy compared to non-carriers after adjusting for multiple testing, while the opposite association was found in patients who were treated with non-oxaliplatin based–chemotherapy.

XPD/ERCC2

The xeroderma pigmentosum group D (*XPD*), or *excision repair cross complementation group 2* (*ERCC2*) gene, encodes for a helicase protein of 761 amino acids located on chromosome 19q13.3 (Weber et al., 1990). The XPD protein is a part of the general transcription factor IIH complex, which is involved in the NER pathway by opening DNA double helix after damage recognition by XPC-RAD23B (Oksenych and Coin, 2010). SNPs

in *XPD* gene can alter the efficiency of DNA repair capacity and could thus be used as a predictive factor for oxaliplatin-based chemotherapy.

SNPs affecting codons 156, 312, and 751 (rs238406, rs1799793, and rs13181, respectively) proved to be extensively studied for their predictive value in CRC treatment (**Supplementary Material—Table S4**). *XPD* rs238406 SNP was significantly associated with treatment outcome in one (Kjersem et al., 2016) out of three studies (Park et al., 2001; Stoehlmacher et al., 2004). The second SNP, rs1799793, was also significantly associated with treatment outcome in one (Liu et al., 2019) out of three studies (Park et al., 2001; Ruzzo et al., 2007). The wild type GG genotype seemed to be the favorable genotype. Sixteen studies assessed the predictive value of *XPD* rs13181polymorphism. In most studies a worse treatment outcome was observed in C allele carriers (Park et al., 2001; Stoehlmacher et al., 2004; Le Morvan et al., 2007; Ruzzo et al., 2007; Pare et al., 2008; Lai et al., 2009; Chen et al., 2010; Kumamoto et al., 2013). Le Morvan et al. compared oxaliplatin treatment with irinotecan treatment and reported that the CC genotype was associated with a lower OS in patients treated with oxaliplatin, in contrast this was not observed in the same patient category treated with irinotecan (Le Morvan et al., 2007). However, the opposite association was observed in three studies (Lamas et al., 2011; Gan et al., 2012; Li et al., 2012), and five studies did not find significant associations with treatment outcome (Monzo et al., 2007; Martinez-Balibrea et al., 2008; Etienne-Grimaldi et al., 2010; Farina Sarasqueta et al., 2011; Huang et al., 2011). Lastly, one study assessed mRNA expression level of *XPD* for its association with treatment outcome, and no significant association was observed (Kassem et al., 2017).

XPG/ERCC5

The *xeroderma pigmentosum group G* (*XPG*) gene, also known as *ERCC5* (*excision repair cross complementation group 5*), is one of the eight core functional genes in the NER pathway. The *XPG* gene, located at chromosome 13q32-33, encodes for a structure specific endonuclease protein that cleaves the 3' side of the damaged nucleotide during NER (Aboussekhra et al., 1995). The low expression level of *XPG* has been shown to be associated with response to platinum-based chemotherapy in ovarian cancer (Stevens et al., 2008; Walsh et al., 2008).

Four studies reported on the association between four different SNPs in the *XPG* gene and treatment outcome of oxaliplatin-based chemotherapy in CRC patients (**Supplementary Material—Table S5**). The -763A>G and +25A>G polymorphisms in the promoter region of *ERCC5* were significantly associated with PFS and OS in patients treated with oxaliplatin (Chen et al., 2016). Also, SNPs in rs1047768 and rs17655 were significantly associated with treatment outcome (Monzo et al., 2007; Kweekel et al., 2009; Liu et al., 2012).

MNAT1

The *MNAT1* gene encodes for the ménage à trois-1 (MAT1) enzyme that is involved in the assembly of the cyclin dependent kinase-activating kinase (CAK) complex. Together with XPD and other subunits, the CAK-complex forms the TFIIH complex that is involved in the NER pathway (Marinoni et al., 1997).

Kap et al. (2015) found three predictive SNPs, rs3783819, rs973063, and rs4151330 of the *MNAT1* gene for OS in CRC patients treated with oxaliplatin-based chemotherapy compared to CRC patients with non-oxaliplatin–based chemotherapy (**Supplementary Material—Table S6**). All three SNPs are in high linkage disequilibrium, and p-values were corrected for multiple testing. Compared to non-carriers, carriership of these genetic variants was associated with longer OS, but not in patients who received non-oxaliplatin–based chemotherapy.

MMR Pathway

MMR Status

The DNA mismatch repair (MMR) system recognizes and repairs genetic mismatches that occur during DNA replication and DNA damage. MMR status is defined as deficient (dMMR) when one or more MMR protein (*MLH1*, *MSH2*, *PMS2*, and *MSH6*) expression is lost (Ionov et al., 1993). Germline mutations in MMR genes were found to be the driving mechanism for Lynch syndrome, also known as hereditary nonpolyposis colorectal cancer (HNPCC) (Pino et al., 2009). A defective MMR system will result in DNA replication errors, particularly in the short tandem repeat of DNA sequences of the genome referred to as microsatellites, which may lead to microsatellite instability (MSI). It has been suggested that MSI positively affects the clinical outcome of CRC. Mechanistically, oxaliplatin treatment is expected to be more effective in patients with defective MMR protein status as platinum adducts formed by oxaliplatin cannot be repaired.

A total of three studies, evaluating the predictive ability of MMR status in relation to oxaliplatin-based treatment, are included in **Table S9** (**Supplementary Material**). In two out of three studies, OS was significantly higher in multivariate analysis in dMMR patients treated with oxaliplatin-based therapy (Gallois et al., 2018; Sfakianaki et al., 2019). In contrast, Kim et al. did not find an association between dMMR and treatment outcome of oxaliplatin-based chemotherapy (Kim et al., 2010).

DNA Damage Response

ATM

Ataxia telangiectasia mutated (ATM) is a serine/threonine protein kinase that is recruited and activated by the MRN complex during DNA DSBR (Uziel et al., 2003). The activation of the *ATM* gene leads to the phosphorylation of several key proteins that mediates the effect of ATM protein on DNA repair, cell cycle arrest, or apoptosis (Shiloh, 2003). Loss of *ATM* in preclinical models seems to increase sensitivity to DNA damaging agents, including platinum-based chemotherapy and ATM inhibitors (Reaper et al., 2011).

Two studies reported a significant association of *ATM* with treatment outcome of oxaliplatin in CRC patients (**Supplementary Material—Table S10**) (Kweekel et al., 2009; Sundar et al., 2018). Sundar et al. (2018) reported that loss of ATM protein expression in CRC resulted in favorable OS when treated with first line oxaliplatin chemotherapy (49 vs. 32 months; HR: 2.52 [1.00–6.37]). It is important to note that loss of ATM expression did not result in favorable OS among patients

treated with first line irinotecan-based therapy (24 vs. 33 months; HR: 0.72 [0.28–1.84]). In addition, the explorative study by Kweekel et al. (2009) found a significantly shorter PFS for homozygous carriers of the *ATM* rs1801516 SNP, for OS no differences were found.

HIC1

The hypermethylated in cancer 1 (HIC1) protein plays an important role in the DNA repair through its direct binding to the Sirtuin 1 (SIRT1) promoter, thereby suppressing its transcription. SIRT1 is a deacetylase of XPA protein, a component of the NER pathway (Fan and Luo, 2010). Since the variable number of tandem repeats near the promoter lesion of HIC1, which is associated with *HIC1* gene expression, there is a potential value of *HIC1* as a predictive biomarker for oxaliplatin efficacy.

In a study by Okazaki et al. (2017), shown in **Table S10** (**Supplementary Material**), patients treated with oxaliplatin-based chemotherapy with at least five tandem repeats of *HIC1*, in both alleles of the *HIC1* promoter region, had a significantly shorter PFS. In a control group who received irinotecan-based chemotherapy this difference in PFS was not seen. However, no significant association with OS was found.

PIN1

Peptidyl-prolyl cis/trans isomerase NIMA-interacting 1 (PIN1) is an enzyme encoded by the *PIN1* gene. It interacts with prominent DSBR factors and is involved in the regulation of HR and non-homologous end-joining (NHEJ) of DNA DSBR. Previous study showed that the overexpression of PIN1 suppresses HR and its depletion reduces NHEJ by promoting CtIP polyubiquitylation and subsequent proteasomal degradation (Steger et al., 2013).

A study by Suenaga et al. (2018), shown in **Table S10** (**Supplementary Material**), reported that genetic polymorphism in *PIN1* was associated with treatment outcome of oxaliplatin. Patients treated with oxaliplatin-based chemotherapy carrying the *PIN1* rs2233678 C allele had a shorter PFS and OS compared to wild type patients. For OS this was replicated in a validation cohort. In contrast, in a control group treated with non-oxaliplatin-based chemotherapy patients with a C allele had longer median PFS than wild type patients.

Miscellaneous

Following our selection criteria, for XPA in the NER pathway (Stoehlmacher et al., 2004; Monzo et al., 2007; Hu et al., 2019), SRBC in the HR pathway (Moutinho et al., 2014) and MGMT in the DNA synthesis pathway (Park et al., 2010) results remain inconclusive because the observed associations have not yet been replicated and the studies itself were relatively small (<300 patients).

For XRCC1 in the BER pathway a total of nine studies were identified that assessed the association between the *XRCC1* gene and treatment outcome of oxaliplatin-based chemotherapy in CRC patients, and showed conflicting results (Suh et al., 2006; Martinez-Balibrea et al., 2008; Chua et al., 2009; Liang et al., 2010; Huang et al., 2011; Lamas et al., 2011; Gan et al., 2012; Zaanan et al., 2014). All nine studies investigated the *1196A>G* polymorphism, and three studies showed a significant association (Suh et al., 2006; Huang et al., 2011; Gan et al., 2012). However, two out of three studies (Suh et al., 2006; Huang et al., 2011) found a significantly longer OS for the GG genotype, whereas the other study (Gan et al., 2012) a longer OS for the AA genotype.

For XRCC3 (Ruzzo et al., 2007; Martinez-Balibrea et al., 2008), MRE11 (Ihara et al., 2016), and RAD51 (Ihara et al., 2016) in the HR pathway, no significant associations with treatment outcome were reported.

DISCUSSION

The majority of patients with peritoneal metastases of colorectal cancer treated with CRS + HIPEC will develop recurrent disease despite critical patient selection. Therefore, improvement of patient and treatment selection is needed and further investigation of genetic biomarkers that are predictive or prognostic for treatment outcome may be of aid herein. We conducted a systematic review to provide an overview of genetic biomarkers in the DNA repair pathway that are potentially predictive for treatment outcome of patients with colorectal peritoneal metastases treated with CRS + HIPEC with oxaliplatin or mitomycin C.

We expanded our review with studies investigating the association between genetic biomarkers related to DNA repair and treatment outcome in patients with colorectal cancer undergoing systemic chemotherapy, because only two studies could be retrieved that investigated the association of biomarkers related to DNA repair and intraperitoneally administered mitomycin C or oxaliplatin. The most promising genetic biomarkers were *ERCC1* rs11615, *XPC* rs1043953, *XPD* rs13181, *XPG* rs17655, *MNAT* rs3783819/rs973063/rs4151330, MMR status, ATM protein expression, *HIC1* tandem repeat D17S5 and *PIN1* rs2233678. Combination studies of two DNA repair genes have also been studied and showed significant associations with treatment outcome.

Our findings for *ERCC1* rs11615 and *XPD* rs13181 are supported in four meta-analyses (Yin et al., 2011; Qian et al., 2014; Ma et al., 2015; Shahnam et al., 2016). The other biomarkers have not been studied as extensively. To our knowledge the current review is the first to summarize the available evidence for these markers.

Our results showed that genetic biomarkers in the DNA repair pathway seem of added value in predicting oxaliplatin treatment outcome in colorectal cancer patients. Since the mechanism of action of oxaliplatin is irrespective of the route of administration, it is assumed very reasonable to extrapolate these associations to patients with colorectal peritoneal metastases treated with CRS + HIPEC. In our opinion, single genetic biomarkers within DNA repair should be incorporated into a polygenic risk profile because the effect of a single gene polymorphism may be partially overcome by compensation mechanisms. Comparable to the study by Kap et al., in which the predictive value of the model significantly improved by

including more genetic variants (Kap et al., 2015). Moreover, besides DNA repair, other pathways may also be of relevance in predicting treatment outcome, such as genetic variation in pharmacokinetic genes (Hulshof et al., 2020).

For some genetic biomarkers conflicting results were reported. This might partially be explained by ethnic discrepancy as has been suggested (Yin et al., 2011; Ma et al., 2015). In addition, studies with small sample sizes and differences in treatment regimens between studies may also attribute to these conflicting results. However, for the selection of the most promising genetic biomarkers, we only selected biomarkers for which no or almost none conflicting data existed and results had to be replicated in at least two studies or in one study with sufficient power (>300 patients) or the study had to have a control group with non-oxaliplatin based chemotherapy.

Moreover, genetic variants in the DNA repair pathway seem to affect cancer susceptibility, prognosis and treatment outcome (Dai et al., 2015). Therefore, it is difficult to distinguish between prognostic effects of these genetic variants or predictive effects on treatment outcome of oxaliplatin. To differentiate between these prognostic effects and predictive effects, a control group consisting of a patient cohort treated with non-oxaliplatin based chemotherapy should be added. Most of the studies that were included had no control group. Nonetheless, the studies that did include a control group with non-oxaliplatin based-chemotherapy did find differences in the association between the genetic biomarker (*XPC* rs1043953, *XPD* rs13181, *MNAT* rs3783819/rs973063/rs4151330, ATM protein expression, *HIC1* tandem repeat D17S5, and *PIN1* rs2233678) and treatment outcome of oxaliplatin-based chemotherapy and non-oxaliplatin based–chemotherapy, thereby suggesting these biomarkers to be more likely predictive than prognostic.

In addition, we included various types of biomarkers such as genetic polymorphism, mRNA expression and protein expression, these are quite different assays and normally we would not pile together these various types of biomarkers. However, our aim was to give a complete overview of all genetic biomarkers in order to provide a selection of potential promising genetic biomarkers for further research.

As data scarcity and sparsity were encountered, we decided to expand our search from intraperitoneal chemotherapy to systemic chemotherapy. No formal search in other databases than PubMed was conducted, since it was assumed that the majority of relevant literature was identified using this database. However, this might be considered a limitation of our study. Moreover, the addition of gray literature could have been of added value in terms of data scarcity and publication bias. Nonetheless, gray literature is mostly not peer-reviewed and not always traceable. In addition, the quality of data could potentially be improved by applying a standardized tool for the risk of bias assessment. However, as described in the methods section, a customized assessment of bias was performed which was mainly based on the Q-genie tool.

Lastly, not all studies corrected for additional covariates affecting treatment outcome such as clinical, molecular, and pathological patient and tumor characteristics. This might have influenced the effect of the genetic biomarkers on treatment outcome. Therefore, additional prospective research including a multivariate analysis is needed, especially in patients with colorectal peritoneal metastases treated with CRS + HIPEC as literature is scarce in this population.

In this review, several genetic biomarkers in the DNA repair pathway were identified that showed promise for predicting outcome in colorectal cancer patients treated with oxaliplatin. These findings might be extrapolated to patients with colorectal peritoneal metastases treated with CRS + HIPEC and should be the subject of further investigation.

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

Study design: EH and MD. Literature search, data interpretation, and data analysis: EH, LL, and MD. Manuscript writing: EH, LL, and MD, Critical revision of data presentation and manuscript: IH, HG, and HJG. Approval final version of manuscript: EH, LL, IH, HG, HJG, and MD.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.577968/full#supplementary-material

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Risperidone-Induced Obesity in Children and Adolescents With Autism Spectrum Disorder: Genetic and Clinical Risk Factors

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Aims: Obesity is a significant problem for patients taking atypical antipsychotics. There were two aims of our study. The first aim was to compare the prevalence of overweight and obesity between children and adolescents with autism spectrum disorder (ASD) treated with risperidone with the general pediatric population. The second aim was to investigate the association of the *HTR2C -759C>T, ABCB1 1236C>T, ABCB1 2677G>T/A,* and *ABCB1 3435C>T* polymorphisms with risperidone-induced overweight and obesity in children and adolescents with ASD.

Methods: Body weight and height were measured in 134 subjects. Overweight and obesity in children and adolescents were classified using the International Obesity Task Force (IOTF) criteria. Genotyping was performed by TaqMan real-time polymerase chain reaction (PCR).

Results: Our study found that the prevalence of overweight and obesity was significantly higher in children and adolescents with ASD treated with risperidone compared with healthy individuals (p = 0.01 and p = 0.002). The genetic polymorphisms of *HTR2C –759C>T, ABCB1 1236C>T, ABCB1 2677G>T/A,* and *ABCB1 3435C>T* were not associated with overweight/obesity in children and adolescents with ASD treated with risperidone after adjustment for multiple comparisons by the method of Bonferroni. Additionally, haplotype analysis revealed that there was no significant association between *ABCB1 3435T-2677T/A-1236T* haplotype and overweight/obesity. In multivariate logistic regression, after adjustment by the Bonferroni correction, there was only the duration of risperidone treatment that was significantly associated with overweight/obesity in children and adolescents with ASD.

Conclusions: The findings suggest that children and adolescents with ASD treated with risperidone are at a higher risk of obesity, especially patients with extended treatment with risperidone. For the pharmacogenetic factors, *–759C>T* polymorphism of *HTR2C* gene

and *1236C>T, 2677G>T/A*, and *3435C>T* polymorphisms of *ABCB1* gene were not likely to be associated with the susceptibility to overweight/obesity in children and adolescents treated with risperidone. Due to the small sample size, further studies with a larger independent group are needed to confirm these findings.

Keywords: obesity, children and adolescents, autism spectrum disorder, risperidone, *ABCB1*, *HTR2C*

INTRODUCTION

The use of atypical antipsychotics (AAPs) in children and adolescents has increased sharply over the last decade (Comer et al., 2010; Olfson et al., 2010). Risperidone is an AAP approved by the U.S. Food and Drug Administration (FDA) for treating behavioral disturbances in children and adolescents with autism spectrum disorder (ASD) (Hsia et al., 2014; LeClerc and Easley, 2015). A particularly concerning side effect of AAPs is weight gain (Calarge et al., 2009; Correll et al., 2009) as childhood obesity is one of the most significant public health concerns (Chia and Boston, 2006; Güngör, 2014). Weight gain and obesity pose health risks in children and adolescents including sleep-disordered breathing (Bixler et al., 2009), hypertension (Friedemann et al., 2012), dyslipidemia (Friedemann et al., 2012), type 2 diabetes (Goran et al., 2003), cardiovascular disease (Umer et al., 2017), cancer (Weihrauch-Blüher et al., 2019), and reduced lifespan regardless of adult weight status (Must et al., 2012). Obesity also presents a major economic burden (Trasande and Chatterjee, 2009; Wang and Dietz, 2002). Therefore, the prevention and management of overweight and obesity are necessary for children and adolescents undergoing treatment with AAPs.

There is significant inter-individual susceptibility to AAP-induced weight gain in childhood (Correll et al., 2009), yet the pharmacogenomics underlying this relationship is still poorly understood. Several studies have examined the relationship between genetic factors and AAP-induced weight gain. Genetic variations of AAP target receptors have been widely studied for their potential use as genetic markers to predict side effects such as weight gain and obesity. Risperidone is a potent antagonist of the serotonin receptor 5-hydroxytryptamine receptor 2C (HTR2C) (Di Matteo et al., 2002), a proposed target for AAP-induced weight gain (Tecott et al., 1995). Studies in adult patients have shown that genetic polymorphisms in the *HTR2C* gene are associated with inter-individual differences in antipsychotic-induced weight gain. Reynolds and colleagues found that the *HTR2C –759T>C* allele is protective against weight gain in patients with schizophrenia treated with antipsychotics (Reynolds et al., 2002). However, previous studies have failed to establish a consistent effect of the *–759C>T* polymorphism in AAP-induced weight gain (Ellingrod et al., 2005; Park et al., 2008; Tsai et al., 2002).

In addition to target receptors, the efflux transporter P-glycoprotein (P-gp), encoded by the *ABCB1* gene, plays an essential role in antipsychotic drug response. P-gp regulates drug bioavailability by controlling transport across the blood-brain barrier (McCaffrey and Davis, 2012), kidney (Masereeuw and Russel, 2012), and other organs. Risperidone has a strong affinity for P-gp *in vitro* (Boulton et al., 2002), supporting a mechanism by which *ABCB1* gene polymorphisms might influence risperidone pharmacokinetics and weight gain. Interestingly, the *ABCB1* gene is highly polymorphic, with more than 1,200 identified single nucleotide polymorphisms (Fung and Gottesman, 2009). The three most common *ABCB1* polymorphisms include two silent polymorphisms, *c.1236C>T* (exon 12, p.Glu412Glu) and *c.3435C>T* (exon 26, p.Ile1145Ile), and the missense variant *c.2677G>T/A* (exon 21, p.Ala893Ser/Thr), each of which has been shown to significantly minimize P-gp functionality *in vitro* (Salama et al., 2006). Additionally, *2677G>T/A* and *3435C>T* result in decreased intestinal *ABCB1* expression (Hoffmeyer et al., 2000). Moreover, *ABCB1 2677G>T/A* and *3435C>T* polymorphisms were shown to influence risperidone-induced weight gain in patients with schizophrenia (Kuzman et al., 2008).

The aims of this study were 1) to compare the prevalence of overweight and obesity between children and adolescents with ASD treated with risperidone with the general pediatric population and 2) to investigate the association of genetic polymorphisms of the target receptor gene *HTR2C –759C>T* (rs3813929) and efflux transporter gene *ABCB1 1236C>T* (rs1128503), *2677G>T/A* (rs2032582) and *3435C>T* (rs1045642) with overweight/obesity in children and adolescents with ASD treated with risperidone. We hypothesized that children with ASD might be vulnerable to obesity, compared to the general pediatric population. Moreover, considering the role of target receptor HTR2C and efflux transporter P-gp in risperidone pharmacokinetics, we hypothesized that polymorphisms in the *HTR2C* and *ABCB1* genes might impact obesity in children and adolescents treated with risperidone.

MATERIALS AND METHODS

Participants

Between 2012 and 2013, 134 children and adolescents with autism spectrum disorder (ASD) were recruited from Yuwaprasart Waithayopathum Child Psychiatric Hospital, Samut Prakan, Thailand. Patient compliance was confirmed by the nursing staff. All patients 1) were diagnosed with ASD according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria, 2) were medicated with risperidone for more than 3 months, and 3) had a complete record of duration and dose of risperidone treatment. We excluded patients 1) who changed medication or were unable to take medicine regularly, 2) with a known history of serious physical conditions, and 3) who took valproic acid, carbamazepine, and lithium. All patients

signed an informed consent document before enrolling in the project. Bodyweight and height were measured cross-sectionally with light clothing in the morning. Age, gender, duration of risperidone treatment, the dose of risperidone, and concomitant medication were recorded. The study was approved by the Ethics Committee of the Faculty of Medicine, Ramathibodi Hospital, Mahidol University (MURA2011/541). After the clinician and researcher described the protocol to the patients, the parents of all children involved in the study gave informed written consent to participate. The authors confirm that all research was performed following relevant guidelines and regulations.

Blood Collection and Genotyping Analysis

Blood samples were obtained after overnight fasting and were collected by venipuncture into EDTA. Whole blood was used for genotype analysis. DNA was isolated using the MagNA Pure automated extraction system (Roche Applied Science, Penzberg, Germany). Genotyping was performed by allele-specific TaqMan MGB probe 5′ nuclease assay with real-time polymerase chain reaction ViiA 7 system (Applied Biosystems, Life Technologies). TaqMan-based analysis was performed with indicated primers: *HTR2C −759C>T* (rs3813929; assay ID: C__27488117_10), *ABCB1 1236C>T* (rs1128503; assay ID: C___7586662_10), *ABCB1 2677G>T/A* (rs2032582; assay ID: C_11711720C_30), *ABCB1 3435C>T* (rs1045642; assay ID: C___7586657_20). Each 20 μl PCR mixture contained 4 μl 5 ng/μl genomic DNA, 10 μl TaqMan Genotyping Mastermix, 1 μl allele-specific TaqMan MGB probe and sequence-specific primer kit, and 5 μl DNase-free H_2O. The thermal cycler program was set up as follows: 95°C for 10 min followed by 50 repeated cycles at 92°C for 15 s and 60°C for 90 s. The allelic discrimination plot was generated by ViiA 7 software (Applied Biosystems, Life Technologies). In this study, we selected single-nucleotide polymorphisms (SNPs) from the HCB (Han Chinese in Beijing, China) database in the International Hap-Map Project (http://hapmap.ncbi.nlm.nih.gov). The SNPs with minor allele frequencies >10% for each gene were selected for this study.

Classification of Overweight and Obesity

Overweight and obesity were classified using the International Obesity Task Force (IOTF) criteria, which were established using data sets from six different countries (Cole et al., 2000). According to the IOTF guidelines, the international cut-off points for children and adolescents between 2 and 18 years are a body mass index (BMI) of 25 and 30 kg/m^2 for overweight and obesity, respectively. We compared the prevalence of overweight and obesity in children and adolescents with ASD treated with risperidone with age-matched participants derived from the general child population (Jitnarin et al., 2011).

Statistical Analyses

Statistical analyses were performed using the SPSS program. Descriptive analysis was used to summarize the clinical characteristics of patients. The Kolmogorov–Smirnov test was used to test for normal distribution with a 95% confidence level (95% CI). Data were conveyed as a median and interquartile range (IQR) due to a non-normal distribution and compared with the non-parametric Mann–Whitney *U* test. Genetic polymorphisms were assessed for concordance with Hardy–Weinberg equilibrium (HWE). Chi-squared analysis or Fisher's exact test was used to determine the association between categorical measures, including allele and the presence/absence of obesity. Multiple logistic regression analysis was used to evaluate factors significantly associated with risperidone-induced obesity. Analysis of the *ABCB1* haplotype associated with overweight/obesity was performed using the Haploview program v4.2 (Broad Institute, Cambridge, MA, United States). Statistical significance was set at p-value < 0.05. Bonferroni's correction was applied to adjust for multiple comparisons. According to Bonferroni's procedure, corrected p-values (Pc) were calculated by p-values multiplied for the following numbers: for genotypes and alleles = 4, for haplotypes = 6, for the multiple regressions model = 9. Then the significance of Pc value was set to be 0.05.

RESULTS

Comparison of the Prevalence of Overweight and Obesity Between Children and Adolescents With Autism Spectrum Disorder Treated With Risperidone and the General Pediatric Population Using International Obesity Task Force Criteria

Among the general pediatric population (Jitnarin et al., 2011), 9.2% of children were overweight and 6.5% were obese, whereas 21.6% of children with ASD treated with risperidone from the current study were overweight and 21.6% were obese (**Table 1**). When classified by age group, no overweight or obesity was observed in children with ASD under the age of 6 years old. Differences in overweight and obesity among children with ASD treated with risperidone compared with the general population were present from 6 years onward. The prevalence of overweight and obesity between children treated with risperidone and the general pediatric population are summarized in **Table 1**.

Impact of Clinical Characteristics on the Presence of Overweight/Obesity in Children and Adolescents With Autism Spectrum Disorder

A total of 134 children and adolescents with ASD (121 males, 90.3%; 13 females, 9.7%) with a median age of 10.00 years (IQR = 8.58–12.95) were included in this analysis (**Table 2**). The prevalence of overweight/obesity varied for each age group. The median duration of risperidone treatment was 65.10 months (IQR = 42.10–84.67) and the median daily dose of risperidone was 1.00 mg/day (IQR = 0.50–1.50). Sixty-seven patients (50.0%) received risperidone monotherapy; the remaining 67 (50.0%) received another drug in conjunction with risperidone. We found that age, duration of risperidone treatment, and the dose of risperidone had a statistically significant association with the presence of overweight/obesity.

TABLE 1 | Comparison of the prevalence of overweight and obesity in children with ASD treated with risperidone and the general pediatric population using IOTF criteria (n = 134).

	Overweight				Obesity			
	Healthy children (n = 855 from 9,287) (Jitnarin et al., 2011)	Children with ASD (n = 29 from 134)	Odds ratio (95%confidence intervals)	p-value	Healthy children (n = 604 from 9,287) (Jitnarin et al., 2011)	Children with ASD (n = 29 from 134)	Odds ratio (95%confidence intervals)	p-value
Total	855 (9.20%)	29 (21.60%)	2.85 (1.24–6.56)	0.01*	604 (6.50%)	29 (21.60%)	3.79 (1.53–9.33)	0.002*
Age group								
3–5 years	242 (2.60%)	0 (0.00%)	0.32 (0.03–3.16)	0.37[a]	269 (2.90%)	0 (0.00%)	0.32 (0.03–3.16)	0.3[a]
6–11 years	390 (4.20%)	15 (11.20%)	2.97 (0.91–9.66)	0.06	251 (2.70%)	20 (14.90%)	5.70 (1.59–20.38)	0.01*
12–18 years	223 (2.40%)	14 (10.40%)	5.44 (1.16–25.52)	0.02*	84 (0.90%)	9 (6.70%)	7.45 (0.90–61.73)	0.03*

Data analyzed with the chi-square test.
[a]*Data analyzed with Fisher's exact test.*
**Statistical significance of* p*-value < 0.05.*

The details of the clinical characteristics and their association with overweight/obesity are described in **Table 2**.

Association of *HTR2C –759C>T*, *ABCB1 1236C>T*, *ABCB1 2677G>T/A*, and *ABCB1 3435C>T* Polymorphisms With Risperidone-Induced Overweight/Obesity in Children and Adolescents With Autism Spectrum Disorder

This study investigated the relation of overweight/obesity with *HTR2C –759C>T*, *ABCB1 1236C>T, ABCB1 2677G>T/A*, and *ABCB1 3435C>T* polymorphisms in children and adolescents with ASD treated with risperidone. There was no association of overweight/obesity with *HTR2C –759C>T* polymorphism (Bonferroni corrected p-value = 0.48) (**Table 3**) or with *ABCB1 1236C>T*, *ABCB1 2677G>T/A*, and *ABCB1 3435C>T* polymorphisms (Bonferroni corrected p-value = 0.08, 0.08, and 0.36, respectively) (**Table 4**). Moreover, for gender analysis, *ABCB1* and *HTR2C* genes polymorphisms were not related to overweight/obesity in both males and females (**Tables 3**, **4**).

The most frequently observed *ABCB1* haplotypes for *1236C>T, 2677G>T/A,* and *3435C>T* polymorphisms were identified in patients enrolled in the current study (**Table 5**). There was no association between the *ABCB1* haplotypes and overweight/obesity in children and adolescents with ASD treated with risperidone (**Table 5**).

Multivariate Logistic Regression Analysis of Predictive Factors for Overweight/Obesity in Children and Adolescents With Autism Spectrum Disorder

A multivariate logistic regression analysis was applied to analyze the association of risperidone-induced overweight/obesity in children and adolescents with genetic variables and non-genetic variables. The results demonstrated that the *ABCB1 1236C>T* and duration of risperidone treatment associated with overweight/obesity. However, after Bonferroni correction, only the duration of risperidone treatment was significantly related to overweight/obesity in children and adolescents with ASD (OR = 1.02, 95% CI [1.01, 1.04], Bonferroni corrected p-value = 0.009) (**Table 6**).

DISCUSSION

The pandemic of obesity and its long-term consequences on healthspan and longevity is a major global challenge. The growing prevalence of obesity in childhood, especially, portends a staggering burden of disease in individuals and healthcare systems in the decades to come (Hruby and Hu, 2015; Skinner et al., 2018). Several studies indicate that risperidone is an AAP that causes weight gain (Calarge et al., 2009; Correll et al., 2009). Moreover, weight gain is a cause of non-adherence with risperidone medication in

TABLE 2 | Impact of clinical characteristics on the presence of overweight/obesity in children and adolescents with ASD treated with risperidone (*n* = 134).

	Outcome			
	Total	**Healthy weight (*n* = 76)**	**Overweight/obesity (*n* = 58)**	***p*-value**
Gender				
Male, *n* (%)	121 (90.30%)	67 (88.20%)	54 (93.10%)	0.19[a]
Female, *n* (%)	13 (9.70%)	9 (11.80%)	4 (6.90%)	
Age, years (IQR)	10.00 (8.58–12.95)	9.40 (7.55–11.53)	10.95 (9.48–13.90)	0.001[b,c,*]
Age group				
3–5 years, *n* (%)	7 (5.20%)	7 (9.20%)	0 (0.00%)	0.01[a]
6–11 years, *n* (%)	88 (65.70%)	53 (69.70%)	35 (60.30%)	
12–18 years, *n* (%)	39 (29.10%)	16 (21.10%)	23 (39.70%)	
Duration of risperidone, months	65.10 (42.10–84.67)	55.45 (32.08–79.14)	68.57 (59.92–93.83)	0.01[b,c,*]
Dose of risperidone, mg/day	1.00 (0.50–1.50)	0.78 (0.50–1.00)	1.00 (0.50–2.00)	0.03[b,c,*]
Drug regimen				
Risperidone single regimen, *n* (%)	67 (50.00%)	39 (51.30%)	28 (48.30%)	0.69[a]
Concomitant medication, *n* (%)	67 (50.00%)	37 (48.70%)	30 (51.70%)	
Aripiprazole	1 (1.49%)	1 (2.70%)	0 (0.00%)	
Diphenhydramine	1 (1.49%)	0 (0.00%)	1 (3.33%)	
Methylphenidate	52 (77.61%)	30 (81.09%)	22 (73.33%)	
Fluoxetine	6 (8.96%)	2 (5.41%)	4 (13.34%)	
Folic acid	1 (1.49%)	1 (2.70%)	0 (0.00%)	
Atomoxetine	2 (2.99%)	1 (2.70%)	1 (3.33%)	
Sertraline	3 (4.48%)	1 (2.70%)	2 (6.67%)	
Topiramate	1 (1.49%)	1 (2.70%)	0 (0.00%)	

[a]*Data analyzed with the chi-square test.*
[b]*Data are shown as median (interquartile range).*
[c]*Data analyzed with the Mann–Whitney* U *test.*
**Statistical significance of* p*-value < 0.05.*

children and adolescents (Ceylan et al., 2017). Thus, it is critical to mitigating weight gain as a consequence of risperidone treatment.

Our study indicates that the prevalence of overweight and obesity was significantly higher among children and adolescents with ASD treated with risperidone compared with the general pediatric population (9.2% vs. 21.6%, p = 0.01 and 6.5% vs. 21.6%, p = 0.002, respectively; **Table 1**), consistent with a previous study (Hill et al., 2015). These results indicate that the different trajectories of weight gain in these patient populations may start in early childhood. Although risk factors for obesity are similar in children with ASD and the general child population (Kipping et al., 2008; Kirk et al., 2010), children with ASD might be susceptible to other risks such as food selectivity (Schreck et al., 2004; Sharp et al., 2013) and a sedentary lifestyle (Bandini et al., 2013; Must et al., 2014). Our results also support a previous study that demonstrated risperidone was related to an average weight gain in children compared with placebo (McCracken et al., 2002). Taken together, the increased prevalence of obesity observed in patients with ASD may be the result of the lifestyle and/or risperidone medication (Calarge et al., 2009; Scahill et al., 2016) or additional undefined factors.

This study assessed the contribution of non-genetic variables on overweight/obesity in patients with ASD, revealing that the age of patients, a higher dose of

TABLE 3 | Association between *HTR2C −759C>T* polymorphism and overweight/obesity in children and adolescents with ASD treated with risperidone (*n* = 134).

	HTR2C −759C>T				
	Absence of the T allele (*n* = 108)	**Presence of the T allele (*n* = 26)**	**Odds ratio (95% CI)**	***p*-value**	**Corrected *p*-value[a]**
All (*n* = 134)					
Healthy weight (*n* = 76)	64 (84.20%)	12 (15.80%)	1.69 (0.87–3.30)	0.12	0.48
Overweight/obesity (*n* = 58)	44 (75.90%)	14 (24.10%)			
Male (*n* = 121)					
Healthy weight (*n* = 67)	58 (86.60%)	9 (13.40%)	2.25 (0.89–5.71)	0.08	0.32
Overweight/obesity (*n* = 54)	40 (74.10%)	14 (25.90%)			
Female (*n* = 13)					
Healthy weight (*n* = 9)	6 (66.70%)	3 (33.30%)	0.05 (0.28–0.88)	0.23[b]	0.92
Overweight/obesity (*n* = 4)	4 (100.00%)	0 (0.00%)			

Data analyzed with the chi-square test.
[a]*p-values after Bonferroni's correction for multiple tests.*
[b]*Data analyzed with the Fisher's exact test.*

TABLE 4 | Association between *ABCB1 1236C>T, 2677G>T/A,* and *3435C>T* polymorphisms and overweight/obesity in children and adolescents with ASD treated with risperidone (*n* = 134).

	ABCB1 1236C>T					***ABCB1 2677G>T/A***					***ABCB1 3435C>T***				
	Absence of the T allele (*n* = 23)	**Presence of the T allele (*n* = 111)**	**Odds ratio (95% CI)**	***p*-value**	**Corrected *p*-value[a]**	**Absence of the T/A allele (*n* = 36)**	**Presence of the T/A allele (*n* = 98)**	**Odds ratio (95% CI)**	***p*-value**	**Corrected *p*-value[a]**	**Absence of the T allele (*n* = 49)**	**Presence of the T allele (*n* = 85)**	**Odd ratio (95% CI)**	***p*-value**	**Corrected *p*-value[a]**
All (*n* = 134)															
Healthy weight (*n* = 76)	18 (23.70%)	58 (76.30%)	3.29 (1.18–9.17)	0.02*	0.08	26 (34.20%)	50 (65.80%)	2.50 (1.14–5.45)	0.02*	0.08	32 (42.10%)	44 (57.90%)	1.75 (0.91–3.40)	0.09	0.36
Overweight/ obesity (*n* = 58)	5 (8.60%)	53 (91.40%)				10 (17.20%)	48 (82.80%)				17 (29.30%)	41 (70.70%)			
Males (*n* = 121)															
Healthy weight (*n* = 67)	15 (22.40%)	52 (77.60%)	2.83 (0.99–8.07)	0.04*	0.16	23 (34.30%)	44 (65.70%)	2.30 (0.98–5.39)	0.052	0.21	27 (40.30%)	40 (59.70%)	1.60 (0.75–3.43)	0.22	0.88
Overweight/ obesity (*n* = 54)	5 (9.30%)	49 (90.70%)				10 (18.50%)	44 (81.50%)				16 (29.60%)	38 (70.40%)			
Females (*n* = 13)															
Healthy weight (*n* = 9)	3 (33.30%)	6 (66.70%)	0.80 (0.62–1.03)	0.53[b]	2.12	3 (33.30%)	6 (66.70%)	0.80 (0.62–1.03)	0.53[b]	2.12	5 (55.60%)	4 (44.40%)	3.75 (0.34–41.08)	0.35[b]	1.4
Overweight/ obesity (*n* = 4)	0 (0.00%)	4 (100.00%)				0 (0.00%)	4 (100.00%)				1 (25.00%)	3 (75.00%)			

Data analyzed with the chi-square test.

[a]*p-values after Bonferroni's correction for multiple tests.*

[b]*Data analyzed with the Fisher's exact test.*

**Statistical significance of p-value < 0.05.*

TABLE 5 | Frequency of *ABCB1* haplotypes in children and adolescents with ASD treated with risperidone with overweight/obesity and with healthy weight (*n* = 134).

Haplotypes C3435T-G2677T/A-C1236T	Haplotype frequencies	Clinical outcome in children with ASD treated with risperidone		x^2	*p*-value	Corrected *p*-value[a]
		Overweight/obesity	Healthy weight			
TTT	0.350	0.409	0.306	3.042	0.0811	0.4866
CGC	0.261	0.235	0.281	0.700	0.4029	2.4174
CGT	0.193	0.175	0.207	0.418	0.5177	3.1062
CTC	0.075	0.061	0.085	0.521	0.4702	2.8212
CTT	0.072	0.089	0.060	0.823	0.3644	2.1864
TGC	0.042	0.021	0.059	2.380	0.1229	0.7374

Haplotype order: C3435T-G2677T/A-C1236T.
[a]p-*values after Bonferroni's correction for multiple tests.*

risperidone, and/or a longer treatment time are related to overweight/obesity in children and adolescents treated with risperidone (**Table 2**). However, after adjustment for multivariate regression analysis, only the duration of risperidone treatment was found to correlate with overweight/obesity in children and adolescents (OR = 1.02, 95% CI [1.01, 1.04], Bonferroni corrected *p*-value = 0.009; **Table 6**). Our finding is consistent with previous studies in children and adolescents treated with risperidone (Calarge et al., 2012; Martin et al., 2004). Since the pathophysiology of risperidone-associated obesity remains poorly understood, the involvement of non-genetic factors on the risk of obesity in children and adolescents with ASD requires further evaluation.

To the best of our knowledge, this is the first study to examine the pharmacogenetic impact of *HTR2C −759C>T*, *ABCB1 1236C>T, ABCB1 2677G>T/A,* and *ABCB1 3435C>T* polymorphisms on overweight/obesity in Thai children and adolescents with ASD treated with risperidone. The role of the serotonergic system on appetite control has been known for decades (Lee and Clifton, 2010; Feijo et al., 2011). Notably, the HTR2C receptor can regulate satiety and food intake (Lee and Clifton, 2010). Tecott et al. (1995) revealed that HTR2C receptor-deficient mice are overweight as a result of abnormal control of feeding behavior. Moreover, HTR2C receptor antagonists have been found to delay or prevent the onset of satiety, thereby increasing the size of the meal and weight gain (Balt et al., 2011). The risperidone is an antagonist against the HTR2C receptor (Di Matteo et al., 2002), suggesting that *HTR2C* gene polymorphisms might inform risperidone pharmacogenetics. Antipsychotics downregulate mRNA levels of *HTR2C* in rodents (Buckland et al., 1997). In the context of the *HTR2C −759C>T* gene polymorphism, the *−759T* allele is more abundantly expressed than *−759C* (Buckland et al., 2005). Many genetic studies have reported an association of the *HTR2C −759C>T* gene polymorphism with antipsychotic-induced weight gain. Reynolds et al. (2002) found that Chinese patients with schizophrenia with *−759T* allele experienced were significantly protected from weight gain after treatment with antipsychotics for 10 weeks. The protective potential of the *−759T* allele has also been established in a small trial in youths with ASD treated with risperidone for eight weeks (Hoekstra et al., 2010). However, in our study, the *HTR2C −759C>T* polymorphism was not associated with obesity in children and adolescents with ASD treated with risperidone (**Tables 3, 6**). Several factors could contribute to the discrepancy, such as inconsistencies in study and treatment duration (Hoekstra et al., 2010; Reynolds et al., 2002), ethnicity, study design, and sample size (; Sicard et al., 2010; Gregoor et al., 2011; Lett et al., 2012; Daray et al., 2017). Importantly, subjects in our study were treated with risperidone for at least one year, yielding results consistent with a prior study of children and adolescents with long-term risperidone treatment (Del Castillo et al., 2013).

Risperidone has a strong affinity to P-gp *in vitro* (Boulton et al., 2002) and it is extensively localized in cerebral capillaries forming the blood-brain barrier (Cordon-Cardo et al., 1989). Moreover, an animal study indicated that P-gp in the blood-brain barrier significantly affects the brain concentrations of risperidone and 9-OH-risperidone by limiting their CNS access (Wang et al., 2004). P-gp is encoded by *ABCB1*, a highly polymorphic gene (Ito et al., 2001; Kroetz et al., 2003). *ABCB1 1236C>T*, *2677G>T/A*, and *3435C>T* polymorphisms significantly minimize P-gp functionality *in vitro* (Salama et al., 2006). Since brain penetration of risperidone and 9-OH-risperidone is limited by P-gp (Wang et al., 2004), patients with

TABLE 6 | Multivariate logistic regression analysis of predictive factors for risperidone-induced overweight/obesity in children and adolescents with ASD treated with risperidone (*n* = 134).

Predictive factors	β (SE)	Odd ratio (95%confidence intervals)	*p*-value	Corrected *p*-value[a]
ABCB1 (1236C>T)	1.51 (0.59)	4.52 (1.44–14.23)	0.01	0.09
Duration of treatment	0.21 (0.01)	1.02 (1.01–1.04)	0.001*	0.009*

Data are from logistic regression analyses; backward stepwise method. Variables entered on method: HTR2C −759C>T, ABCB1 1236C>T, ABCB1 2677G>T/A, ABCB1 3435C>T, age, gender, duration of treatment, risperidone dose, and co-medication. β, *regression coefficient; SE, standard error.*
[a]p-*values after Bonferroni's correction for multiple tests.*
**Statistical significance of* p < 0.05.

1236C>T, *2677G>T/A*, and *3435C>T* polymorphisms would likely have decreased P-gp functionality in the blood-brain barrier, a lower efflux function for risperidone, and consequent accumulation of risperidone in the brain. Moreover, the variants of these three coding SNPs, at nucleotides 1236, 2677, and 3435 are in high linkage disequilibrium (Fung and Gottesman, 2009; Hoffmeyer et al., 2000). The previous study showed that patients with *ABCB1 1236T-2677T-3435T* haplotype affected the circulating levels of 9-hydroxyrisperidone and the active moiety (Gunes et al., 2008). Therefore, *ABCB1* gene polymorphisms might affect risperidone access to the brain and consequent adverse drug effects such as weight gain. In the present work, analysis of the *1236C>T*, *2677G>T/A*, and *3435C>T* polymorphisms in the *ABCB1* gene have shown that *1236C>T* and *2677T>A* polymorphisms seem to be associated with overweight/obesity in children and adolescents with ASD treated with risperidone. However, after Bonferroni correction for multiple comparisons, none of the variants exceeded a significant threshold (**Table 4**). Consistently, our haplotype analysis demonstrated that there were no statistically significant correlations for the *ABCB1* haplotypes with overweight/obesity in children and adolescents with ASD treated with risperidone (**Table 5**). In a multivariate logistic regression model, *ABCB1 1236C>T* SNP seems to be associated with risperidone-induced overweight/obesity in children and adolescents (**Table 6**). However, the relation of *ABCB1 1236C>T* with overweight/obesity did not survive Bonferroni correction (**Table 6**). The current study supports prior reports that did not find associations between *ABCB1 1236C>T, 2677T>A*, and *3435C>T* polymorphisms with the risperidone response (Nuntamool et al., 2017), with metabolic abnormality/insulin resistance (Sukasem et al., 2018), or with steady-state plasma concentrations of risperidone or 9-hydroxyrisperidone (Yasui-Furukori et al., 2004). However, here further discrepancies are apparent in the literature, where the *ABCB1 1236C>T* polymorphism showed a significant association with response to risperidone (Xing et al., 2006) and an observed effect of the *ABCB1 2677G>T* and *3435C>T* polymorphisms on risperidone-induced weight gain in patients with schizophrenia (Kuzman et al., 2008). Future studies using larger samples are required to establish unequivocally whether or not *ABCB1* genotypes are related to the development of obesity in children and adolescents treated with risperidone.

Our study has several limitations. First, it was a retrospective, cross-sectional study. Observations in drug-naive patients are required to establish an association of risperidone treatment with weight gain. Second, environmental factors such as food intake and physical activity that affect body weight were not considered in our study design. Third, the size of our sample was relatively small (n = 134), since the highest power of sample size that was calculated by *HTR2C −759C>T (rs3813929)* polymorphism was n = 590 (**Supplementary Table S1**). However, our result demonstrated that there was a borderline significant trend of the association between *ABCB1 (1236C>T)* polymorphism and obesity in children and adolescents with ASD (Bonferroni corrected p-value = 0.09) (**Table 6**). The power of sample size that was calculated by *ABCB1 (1236C>T)* polymorphism was n = 145 (**Supplementary Table S1**. Therefore, our results need to be validated with a larger independent sample of patients.

CONCLUSION

In summary, our study suggested that children and adolescents with ASD treated with risperidone are at a higher risk of overweight/obese compared to the general pediatric population. The duration of risperidone treatment was a non-genetic factor found to associate with overweight/obesity after adjustment for multivariate regression analysis and Bonferroni correction. For the pharmacogenetic factors, after Bonferroni correction for multiple comparisons, no effect of the *HTR2C −759C>T* and *ABCB1 1236C>T*, *2677G>T/A*, and *3435C>T* polymorphisms on risperidone-induced overweight/obesity was observed. Since our study had a relatively small number of subjects, our results need to be replicated in prospective trials in a larger independent group with the cautious characterization of confounding factors such as food intake and energy expenditure.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of the Faculty of Medicine, Ramathibodi Hospital, Mahidol University (MURA2011/541). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

CS, NV, NaN, and PL designed the research study. PL and NaN diagnosed and recruited the patients with ASD treated with risperidone. NV, NoN, AP, YH, and RS collected the clinical data, performed the experiments, and evaluated the results. NV analyzed the data. NV wrote the manuscript. CS contributed to the discussion and reviewed/edited the manuscript. All authors are accountable for all aspects of the work. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.565074/full#supplementary-material

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REVIEW
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Pharmacogenetics Guidelines: Overview and Comparison of the DPWG, CPIC, CPNDS, and RNPGx Guidelines

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Many studies have shown that the efficacy and risk of side effects of drug treatment is influenced by genetic variants. Evidence based guidelines are essential for implementing pharmacogenetic knowledge in daily clinical practice to optimize pharmacotherapy of individual patients. A literature search was performed to select committees developing guidelines with recommendations being published in English. The Dutch Pharmacogenetics Working Group (DPWG), the Clinical Pharmacogenetics Implementation Consortium (CPIC), the Canadian Pharmacogenomics Network for Drug Safety (CPNDS), and the French National Network (Réseau) of Pharmacogenetics (RNPGx) were selected. Their guidelines were compared with regard to the methodology of development, translation of genotypes to predicted phenotypes, pharmacotherapeutic recommendations and recommendations on genotyping. A detailed overview of all recommendations for gene-drug combinations is given. The committees have similar methodologies of guideline development. However, the objectives differed at the start of their projects, which have led to unique profiles and strengths of their guidelines. DPWG and CPIC have a main focus on pharmacotherapeutic recommendations for a large number of drugs in combination with a patient's genotype or predicted phenotype. DPWG, CPNDS and RNPGx also recommend on performing genetic testing in daily clinical practice, with RNPGx even describing specific clinical settings or medical conditions for which genotyping is recommended. Discordances exist, however committees also initiated harmonizing projects. The outcome of a consensus project was to rename "extensive metabolizer (EM)" to "normal metabolizer (NM)". It was decided to translate a CYP2D6 genotype with one nonfunctional allele (activity score 1.0) into the predicted phenotype of intermediate metabolizer (IM). Differences in recommendations are the result of the methodologies used, such as assessment of dose adjustments of tricyclic antidepressants. In some cases, indication or dose specific recommendations are given for example for clopidogrel, codeine, irinotecan. The following drugs have recommendations on genetic testing with the highest level: abacavir (HLA), clopidogrel (CYP2C19), fluoropyrimidines (DPYD), thiopurines (TPMT), irinotecan (UGT1A1), codeine

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(CYP2D6), and cisplatin (TPMT). The guidelines cover many drugs and genes, genotypes, or predicted phenotypes. Because of this and their unique features, considering the totality of guidelines are of added value. In conclusion, many evidence based pharmacogenetics guidelines with clear recommendations are available for clinical decision making by healthcare professionals, patients and other stakeholders.

Keywords: pharmacogenetics, guidelines, pharmacogenomics, DPWG, CPIC, CPNDS, RNPGx, recommendations

INTRODUCTION

The effects of drugs in terms of the beneficial outcomes of drug treatment, development of side effects, and toxicity are influenced by genetic variants. Many studies have shown that the pharmacokinetics and effects of drugs differ among patients with specific genetic profiles. Sufficient evidence is available for a clinically relevant impact of patients' genotype on the balance between the benefits and risk of a substantial number of drugs. Evidence-based guidelines with pharmacotherapeutic recommendations for combinations of specific drugs and genotypes or predicted phenotypes are essential for implementing acquired pharmacogenetics knowledge in daily clinical practice. The Dutch Pharmacogenetics Working Group (DPWG) and the Clinical Pharmacogenetics Implementation Consortium (CPIC) have been developing guidelines for more than a decade (Swen et al., 2008; Swen et al., 2011a; Caudle et al., 2017). Recommendations are preferably made available at the time of drug prescribing and dispensing for a patient with a genotype that requires an action, such as a dose reduction (Swen et al., 2008; Swen et al., 2011a; Deneer and van Schaik, 2013).

Additional sources of information regarding the pharmacogenetics of drugs are the Summary of Product Characteristics (SmPC) approved by the European Medicines Agency (EMA) and other agencies, as well as drug labels approved by the US Federal Drug Agency (FDA). The number of drugs with pharmacogenetics information in their SmPCs or labels has increased over the years because of regulatory guidelines and policy (TanKoi et al., 2018). In addition, the PharmGKB website is an open pharmacogenetics support tool with curated and graded evidence of combinations of drugs and genes (Barbarino et al., 2018).

Several genes encoding cytochrome P450 enzymes are highly polymorphic. This requires a translation of genotypes into predicted phenotypes, such as the metabolizer status of an intermediate metabolizer (IM). However, it has been recognized that translation methods differ among researchers, pharmacogenetic laboratories, and groups of professionals developing guidelines (Van Schaik et al., 2008; Deneer V.H.M., 2013).

Clinical decision making by healthcare professionals is primarily based on multidisciplinary pharmacogenetic guidelines, in daily clinical practice. Therefore, one should become familiar with the process of guideline development and differences between guidelines. In this article, we compare the guidelines with regard to the methodology of development, translation of genotypes to predicted phenotypes, pharmacotherapeutic recommendations and recommendations on genotyping.

METHODS OF THE LITERATURE SEARCH

A systematic literature search was performed on June 19, 2020 in the PubMed database to select committees, consortia or working groups that aim to develop pharmacogenetics guidelines and to select their published guidelines. The search was performed with "pharmacogenetics or pharmacogenomics and guidelines." The labels' languages (English), species (humans), and text availability (full text) were also added to the search criteria. The selection criterion was as follows: guidelines published in the English language developed by committees having the intention to recommend on several drugs irrespective of indication, disease, or medical specialty.

HA screened the results of the PubMed search for relevance by reading the title and abstract of the articles. VD also screened the included articles by reading the title and abstract. Additionally, if HA had doubts about any article, she read the article and discussed it with VD.

The pharmacogenetics guidelines by the DPWG, the CPIC, the Canadian Pharmacogenomics Network for Drug Safety (CPNDS), and the French National Network (Réseau) of Pharmacogenetics (RNPGx) were found. An overview and comparison of these guidelines were performed. Articles from and about these pharmacogenetics groups from the aforementioned PubMed search were used. Moreover, additional searches were performed on the websites of the DPWG (www.knmp.nl) and CPIC (https://cpicpgx.org/guidelines/), as well as that of PharmGKB (www.pharmgkb.org), a worldwide resource for pharmacogenomic information, to obtain an overview of the existing guidelines (Barbarino et al., 2018).

RESULTS OF THE LITERATURE SEARCH

Pharmacogenetics guidelines of the DPWG, CPIC, CPNDS and RNPGx were found through performing the PubMed search. **Supplementary Table S1** presents an overview of the recommendations (Hicks et al., 2015; Hicks et al., 2016; Swen et al., 2011b; Hershfield et al., 2012; Martin et al., 2012; Madadi et al., 2013; Scott et al., 2013; Amstutz et al., 2014; Caudle et al., 2014b; Clancy et al., 2014; Crews et al., 2014; Muir et al., 2014; Ramsey et al., 2014; Relling et al., 2014; Birdwell et al., 2015;

Gammal et al., 2015; Shaw et al., 2015; Aminkeng et al., 2016; Lee et al., 2016; Bell et al., 2017; Hicks et al., 2015; Hicks et al., 2016; Johnson et al., 2017; Lamoureux and Duflot, 2017; Moriyama et al., 2017; Quaranta et al., 2017; Quaranta and Thomas, 2017; Woillard et al., 2017; Amstutz et al., 2018; Goetz et al., 2018; Phillips et al., 2018; Maagdenberg et al., 2018; Brown et al., 2019; Desta et al., 2019; Drögemöller et al., 2019; Gonsalves et al., 2019; Relling et al., 2019; Theken et al., 2020;). The DPWG and CPIC guidelines were compared by Bank et al. (2018) based on the guidelines published until March 1, 2017. Therefore, in this article, we describe the changes that have been made since this comparison was published. Also the CPNDS and RNPGx are included in this comparison in terms of the process of literature assessment, the process of guideline development, and the content of their recommendations.

As of July 1, 2020, the DPWG had reviewed more than 100 gene-drug pairs. 60 gene-drug pairs are considered by the DPWG as gene-drug interaction requiring action such as adjustment of the dose or monitoring of adverse side effects. The remaining gene-drug pairs do not require additional action or monitoring due to pharmacogenetics. 18 gene-drug pairs are classified as gene-drug interaction for which no action is needed, and 29 gene-drug pairs considered as no gene-drug interaction and no actions needed to be made (The Dutch Pharmacogenomic Working Group, 2020a). The CPIC has reviewed more than 400 gene-drug pairs and has published recommendations on 106 gene-drug pairs with sufficient evidence for at least one prescribing action in 24 published guidelines, the CPNDS recommend on 13 gene-drug pairs, and the RNPGx on 8 gene-drug pairs. The DPWG publishes their recommendations on the website www.knmp.nl (search for pharmacogenetic recommendations) and also as of recently in the *European Journal of Human Genetics*. Previously an overview was given in two publications in *Clinical Pharmacology & Therapeutics* (Swen et al., 2008; Swen et al., 2011a). The CPIC publishes their guidelines on their website https://cpicpgx.org/guidelines/as well as in *Clinical Pharmacology & Therapeutics*. The CPNDS publishes their guidelines in several journals, while the guidelines of the RNPGx are published in *Thérapie*. All the guidelines can also be found on the website of PharmGKB: www.pharmgkb.org (Ross et al., 2010; Picard et al., 2017; CPIC, 2020; PharmGKB, 2020). Furthermore, each group has its own method of grouping genes and/or drugs or drug classes; for example, some guidelines include one gene and one drug, whereas others include two to three genes and one or more drugs (Amstutz et al., 2014; Picard et al., 2017; CPIC, 2020; The Dutch Pharmacogenomic Working Group, 2020a).

METHODOLOGY FOR GUIDELINE DEVELOPMENT

The objectives of the DPWG and CPIC and their multidisciplinary processes of guideline development have been described and compared previously (Swen et al., 2008; Caudle et al., 2014a; Bank et al., 2018). Bank et al. compared the DPWG and CPIC based on the guidelines published until March 1, 2017. Bank et al. compared 30 gene-drug pairs, and since then, the DPWG has updated 24 of these gene-drug pairs, and the CPIC has updated 22 gene-drug pairs (Bank et al., 2018; CPIC, 2020; The Dutch Pharmacogenomic Working Group, 2020a).

The CPNDS, founded in 2004, aims to uncover the genetic and mechanistic basis of drug response phenotypes and improve the safety and efficacy of medications used in children and adults (Amstutz et al., 2014). The approaches of the DPWG, CPIC, and CPNDS are generally similar. An additional strength of the CPNDS is the involvement of patients and other stakeholders. The guideline development group is multidisciplinary, and patients and healthcare policy makers also participate. In short, guideline development consists of 1) a systematic literature search; 2) critical appraisal of the retrieved evidence; 3) development of clinical practice recommendations during a workshop meeting for guideline development group members; 4) internal review of draft guidelines by the guideline development group members; and 5) external review by content experts and members of the intended target audience (Amstutz et al., 2014).

The RNPGx has approximately 30 members across France as well as other French-speaking nations (i.e., Belgium and more recently Switzerland and Canada). The RNPGx board was elected by the members. Active RNPGx members are professionals with hospital activities in pharmacogenetics. The guidelines are elaborated by working groups of active members with specific expertise in the domain concerned, and are subsequently validated by the RNPGx board.

Methodologies for grading scientific evidence and the strength of the recommendation differ among the four groups of guideline developers (Amstutz et al., 2014; Picard et al., 2017; CPIC, 2020; The Dutch Pharmacogenomic Working Group, 2020a), see **Supplementary Table S2**. Furthermore, the DPWG, CPNDS, and RNPGx provide directions or recommendations regarding pharmacogenetic testing in daily clinical practice for a specific gene-drug combination.

Dutch Pharmacogenetics Working Group

The methodology used by the DPWG for scoring the level of evidence and clinical impact is applied to all medication surveillance functionalities—such as drug interactions—of the drug database incorporated in drug prescribing and dispensing software in The Netherlands. Healthcare professionals have been familiar with this methodology for many years (Van Roon et al., 2005). A five-point-scale scoring system is used for the level of evidence (0–4) and a seven-point scale ($AA^{\#}$–F) is used for clinical relevance or impact, to which AA# has been added for statistically significant, positive clinical effects. For every scientific publication, both scores are assigned to each combination of genotype or predicted phenotype and a specific drug. Finally, the overall score of each combination is the highest level of evidence and the highest level of relevance assigned to any of the articles included in the assessment (Swen et al., 2008; Deneer and van Schaik, 2013). Adjustments of the starting dose are calculated based on the pharmacokinetic data in patients with a specific genotype or predicted phenotype. The method has previously been described

in detail (Swen et al., 2008; Deneer and van Schaik, 2013). Recently, the DPWG has developed the clinical implementation score to advise and direct healthcare professionals on ordering pharmacogenetic testing before starting treatment with a specific drug or during treatment (Swen et al., 2018). The criteria for this score are as follows: 1) clinical effect, 2) level of evidence, 3) number needed to genotype, and 4) pharmacogenetics information in SmPCs. The total score is translated to three levels of pharmacogenetic testing in clinical practice, namely potentially beneficial, beneficial, and essential (**Supplementary Table S3**) (Swen et al., 2018).

Clinical Pharmacogenetics Implementation Consortium

The CPIC guidelines are established according to a standardized format. The CPIC has a standard system for grading levels of evidence linking genotype to phenotype using three levels namely high, moderate, and weak. They use a system with three categories for their recommendations namely strong, moderate, and optional. For gene-drug combinations in the categories strong and moderate guidelines are being developed and published. "Strong recommendation" means "The evidence is high quality and the desirable effects clearly outweigh the undesirable effects" and a recommendation classified as "Moderate" means "There is a close or uncertain balance as to whether the evidence is high quality and the desirable clearly outweigh the undesirable effects" (CPIC, 2020).

Canadian Pharmacogenomics Network for Drug Safety

Two grading schemes are used by the CPNDS. The quality of individual studies selected from the literature search are assessed. The grading scheme for the totality of evidence is based on the quality criteria of the Appraisal of Guidelines Research and Evaluation Enterprise (AGREE) and consists of four grades (+ to ++++) varying from the lowest grade +, meaning "Inconsistent or insufficient quantity/quality, discouraging" (further described as follows: "No conclusions can be drawn or conclusions are likely to change based on future studies, and current evidence is discouraging") to the highest grade ++++, meaning "Consistent, generalizable" (further described as follows: "Strong general conclusions can be drawn that are unlikely to change based on further research"). The grading scheme for clinical practice recommendations (about the genotype guided treatment) has three levels (i.e., A: strong, B: moderate, and C: optional) varying from "Based on strong scientific evidence; benefits clearly outweigh risks (A)" to "Based mainly on expert opinion, for use with evidence development in a research context (C)." Furthermore, preferences of patients are taken into account when developing the recommendations. A strong recommendation (level A) is expected to be chosen by a majority of informed healthcare providers and patients. A recommendation in the category "Moderate" is expected to require individualized informed decision making by patients and healthcare providers (Ross et al., 2010; Amstutz et al., 2014; Tanoshima et al., 2019).

French National Network (Réseau) of Pharmacogenetics

The focus and aim of the RNPGx differ from the others and concern recommending pharmacogenetic testing. First, the level of evidence for the functionality of a variant of a pharmacogene is considered and assigned to a three-level scale: demonstrated, probable, or potential functionality. Only variants with demonstrated or probable functionality are considered for a recommendation of testing. Depending on the total evidence the network has issued, three levels of recommendation for pharmacogenetic tests namely essential test, advisable test, or possibly helpful test. These vary from "Demonstrated impact on a major clinical phenotype i.e. efficacy or toxicity (essential test)" to "Probable impact that remains to be demonstrated having led to expert consensus in favor of testing (possibly helpful test)" (Picard et al., 2017).

Terminology of Predicted Phenotypes

In daily clinical practice, the term "extensive metabolizer" (EM) does not appear particularly intuitive and is often mistaken by healthcare professionals for a genotype predicted phenotype with increased enzyme function. The outcome of a consensus project initiated by CPIC was to rename it "normal metabolized" (NM). Additional terms for predicted phenotypes for drug metabolizing enzymes are "poor metabolizer" (PM), "intermediate metabolizer" (IM), "rapid metabolizer" (RM), and "ultrarapid metabolizer" (UM) (Caudle et al., 2017). The DPWG has adopted "normal metabolizer" (NM) for CYP2D6, CYP2C19, CYP3A4, CYP1A2, CYP2B6, TPMT, and NUDT15 (The Dutch Pharmacogenomic Working Group, 2020b). The CPNDS also used "normal metabolizer" in its guideline on tamoxifen (Tanoshima et al., 2019). However, for CYP3A5 the DPWG decided to maintain the phenotype terminology, "CYP3A5 non-expressor," for carriers of two nonfunctional alleles (CYP3A5*3/*3), "CYP3A5 heterozygous-expressor" and "CYP3A5 homozygous-expressor." In European populations, the CYP3A5*3/*3 prevalence is around 80% (Shuker et al., 2016). Furthermore, regulatory guidelines require clinical studies in European populations. This implies that drug dosages in SmPCs, namely standard dosages, are suitable for patients assigned to the CPIC's PM phenotype, and in some cases higher dosages might be required in patients assigned to the CPIC's NM phenotype. It was decided that this might increase prescribing errors in a European context because many drugs are CYP3A5 substrates, and healthcare professionals might intuitively reduce dosages for patients with a PM metabolizer phenotype.

The DPWG did not introduce RM as a metabolizer status, applicable to the CYP2C19 *1/*17 genotype, instead of NM. This would have no impact on the current guidelines, and the recommendations would remain unchanged. The guideline on antidepressants by the RNPGx shows that "slow metabolizer" is used as a predicted phenotype term instead of "poor metabolizer" for CYP2D6 and CYP2C19 (Quaranta et al., 2017).

For dihydropyrimidine dehydrogenase (DPD), encoded by the DPYD gene it is common to translate genetic test results into a

gene activity score (AS), which is the sum of activity scores of the alleles a patient carries. In the last update, the DPWG restricted DPYD phenotype terms to AS and decided to no longer use metabolizer status. Others also use AS which is also translated into metabolizer groups. Furthermore, patients with two fully dysfunctional alleles and those with one fully dysfunctional allele and one associated with reduced activity of the DPD enzyme were assigned PM as a predicted phenotype. However, in the updated guideline, recommendations differ for these two groups of patients (Lunenburg et al., 2020).

Genotype-To-Phenotype Translations

Translations from genotypes to predicted phenotypes differ among researchers. This has to be considered when assessing studies for guideline development. From the researchers' perspective it is logical because they mostly study a specific drug. The effect of a genetic variant is drug dependent. It is quite common that a specific genotype of a CYP enzyme has a large effect on the pharmacokinetics of one drug while having no effect on others, although both drugs are substrates of the CYP enzyme. The metabolic ratio of the CYP2D6 probe drug dextromethorphan is increased by a factor of 3.0 in carriers of one nonfunctional CYP2D6 allele compared with subjects without such an allele (Sachse et al., 1997). The area under the plasma concentration vs. the time curve of trimipramine in subjects with one nonfunctional allele is increased by a factor of 2.5 compared with those without such an allele (Kirchheiner et al., 2003). However, the clearance of haloperidol in both groups is similar with the ratio of those with one nonfunctional allele to those without being 0.9 (Brockmöller et al., 2002).

Furthermore, it has been recognized that translation methods differ among the DPWG and CPIC, and more specifically in the genotype-to-phenotype translation of CYP2D6, DPYD, and CYP2C19.

At the start of guideline development in 2005, the DPWG reached a consensus with Dutch laboratory specialists in the field of pharmacogenetics. They decided to translate a CYP2D6 genotype with one nonfunctional allele with AS 1.0, for example, CYP2D6 *1/*4, into a predicted phenotype of IM. The result of the consensus meeting on this topic was shared with professionals involved in genetic testing in The Netherlands (Van Schaik et al., 2008). In their guideline on tamoxifen, the CPNDS also classified AS 1.0 for CYP2D6 as IM.

By contrast, the CPIC and RNPGx classified such a genotype as EM (Quaranta et al., 2017). Furthermore, members of the CPIC and DPWG established an international expert panel and recently initiated a consensus procedure using a modified Delphi method. The procedures and results have been described extensively (Caudle et al., 2020). Moreover, the decision was made to downgrade the value of the CYP2D6*10 allele to 0.25 for AS instead of 0.5. Furthermore, the consensus definitions are as follows: PM (AS 0), IM (AS 0.25–1.0), NM (AS 1.25–2.25), and UM (AS > 2.25).

Since the previous comparison by Bank et al. (2018), the guidelines of both the DPWG and CPIC have been updated regarding DPYD genetic variants, the translation of genotypes into activity scores of DPD, other phenotype categories such as PM and IM, and therapeutic recommendations for capecitabine among others. The DPWG translates genotypes into activity scores unless two variants associated with the reduced functionality of DPYD, such as c.1236G>A/c.2846A>T, are detected, or in case one variant is associated with fully dysfunctional DPD activity and one with reduced functionality, such as *2A/c.1236G>A. It was decided that DPD enzyme activity cannot be predicted correctly because compound heterozygosity is uncertain. In such cases, an additional phenotyping test is required to determine the DPD enzyme activity. Currently, measuring DPD enzyme activity in peripheral blood mononuclear cells is the most frequently used test in The Netherlands (Meulendijks et al., 2016; Lunenburg et al., 2020). The starting doses of capecitabine and 5-fluorouracil, for example, should be selected based on the totality of genotyping and phenotyping test results. The CPIC, on the other hand, assigns AS of 0 and 0.5 to PM and of 1 and 1.5 to IM (Amstutz et al. 2018). In the RNPGx guideline on the pharmacogenetics of anticancer drugs, only DPYD alleles and genotypes are mentioned without them being translated into predicted phenotypes. The DPWG also recommends performing additional phenotyping in patients with an AS of 0.

Pharmacotherapeutic Recommendations

A detailed overview of the recommendations of the DPWG, CPIC, CPNDS, and RNPGx are included in **Supplementary Table S1**. Gene-drug combinations for which the DPWG concluded that no gene-drug interaction exists for example CYP1A2-clozapine, as well as for example SLC01B1-atorvastatin classified by CPIC as no gene-drug interaction, are not summarized. A selection of the discordances are discussed. Main differences from a clinical perspective are shown in **Supplementary Table S4**.

Discordances Fluoropyrimidines and Irinotecan

The committees have published advises on several anti-cancer drugs. Pharmacotherapeutic recommendations of the DPWG and the CPIC guidelines on fluoropyrimidines are different in some aspects. The DPWG advises to initiate fluorouracil or capecitabine in patients with DPD AS of 1.0 or 1.5 at a starting dose of 50%, while the CPIC also recommends 50% followed by dose titration based on clinical judgement of the healthcare professional and TDM. One indicates that the drugs should be avoided or are contraindicated in patients with DPYD AS 0, while the DPWG adds that if this is not possible the residual DPD activity in mononuclear cells from peripheral blood should be determined, and the initial dose should be adjusted accordingly. The DPWG guideline also includes tegafur and cutaneous fluorouracil (CPIC, 2020; Lunenburg et al., 2020). The RNPGx refers to dose and pharmacotherapeutic recommendations of others (Quaranta and Thomas, 2017).

In addition, the DPWG recommends giving 70% of the irinotecan dose in patients with UGT1A1 PM predicted phenotype and increasing it if the patient can tolerate said dosage, which must be guided by the neutrophil count. The

RNPGx recommends a 25–30% dose reduction in UGT1A1 *28/*28 patients for irinotecan 180–230 mg/m^2 spaced by 2–3 weeks intervals. The RNPGx marks irinotecan at a higher dose (240 mg/m^2 or higher spaced by 2–3 weeks intervals) as contraindicated for patients with the UGT1A1*28/*28 genotype (Quaranta and Thomas, 2017).

Discordances Clopidogrel, Warfarin, Statins

Within the field of cardiovascular diseases, the pharmacogenetics of clopidogrel has been extensively studied. The DPWG, CPIC, and RNPGx have guidelines for the CYP2C19-clopidogrel pair (Scott et al., 2013; Lamoureux and Duflot, 2017; The Dutch Pharmacogenomic Working Group, 2020a). The DPWG recommends patients with the CYP2C19 IM phenotype being treated with an alternative to clopidogrel for the indications of percutaneous coronary intervention, stroke, or Transient Ischemic Attack (TIA), or that they double the daily dosage to 150 mg. They also recommend considering an alternative for CYP2C19 PM in case of percutaneous coronary intervention, stroke, or TIA. Moreover, the DPWG recommends no action in case of other indications for CYP2C19 IM patients, while one suggests measuring platelet function testing and selecting an alternative in case of high on treatment reactivity in PM patients. The CPIC and RNPGx recommend an alternative to clopidogrel in CYP2C19 IM and PM patients. The CIPC's recommendation is only applicable for patients with an acute coronary syndrome undergoing percutaneous coronary intervention.

All the four committees developed guidelines for the CYP2C9-VKORC1-warfarin pair in terms of dose adjustments or for calculating the dose using an algorithm; see **Supplementary Table S1** (Shaw et al., 2015; The Dutch Pharmacogenomic Working Group, 2016; Lamoureux and Duflot, 2017). The DPWG recommends using the EU-PACT algorithm (which includes maintenance dose and k = elimination rate constant per genotype) or dose reduction as shown in **Supplementary Table S1**. The CPIC and CPNDS recommend using www.WarfarinDosing.org to calculate the dosage by for example genetic information, co-medication, and target International Normalized Ratio (INR). The CPIC recommends also to use the Gage or the IWPC algorithms or both.

For patients with the SLCO1B1 *5/*5 and *1/*5 genotype, the RNPGx recommends avoiding high doses of statins and the concomitant use of OATP1B1 inhibitors and/or CYP3A4 inhibitors (such as amiodarone, verapamil, and diltiazem). They advise to lower the simvastatin dose to 20 mg per day or to select another statin (Lamoureux and Duflot, 2017; Picard et al., 2017). The DPWG recommends choosing an alternative for simvastatin in both SLCO1B1 521 CC and TC patients; see **Supplementary Table S1** for the recommendations (The Dutch Pharmacogenomic Working Group, 2020a). In SLCO1B1 TC patients simvastatin doses exceeding 40 mg/day should be avoided, if selecting an alternative is not an option. On the other hand, the CPIC recommends reducing the normal dose for patients with intermediate or low function of the transporter or choosing an alternative (e.g. rosuvastatin or pravastatin) (Ramsey et al., 2014). Furthermore, the CPIC has no recommendations for atorvastatin while the DPWG recommends an alternative for atorvastatin in patients with the SLCO1B1 521 CC and TC genotype, and additional risk factors for statin induced myopathy as for simvastatin (Ramsey et al., 2014; The Dutch Pharmacogenomic Working Group, 2020a).

Discordances Antidepressants

The dose recommendations from the CPIC and the RNPGx for CYP2D6-CYP2C19-tricyclic antidepressants (TCA) are mostly the same (Hicks et al., 2015; Hicks et al., 2016; Swen et al., 2011b; Quaranta and Thomas, 2017; The Dutch Pharmacogenomic Working Group, 2020a). They recommend avoiding high dose of tricyclic antidepressants in CYP2D6 UM and PM patients and CYP2C19 UM and PM patients, and also reducing the dose in patients with CYP2D6 IM (CPIC: −25% of the recommended dose, RNPGx: −50% of the recommended dose) in case depression is the indication; see **Supplementary Table S1** (Hicks et al., 2015; Hicks et al., 2016; Quaranta and Thomas, 2017). The recommendations for TCAs are based on amitriptyline literature and extrapolated to other tertiary amines (clomipramine, doxepin, imipramine, and trimipramine) because of comparable pharmacokinetic properties

The DPWG recommends increasing the dose of tricyclic antidepressants in CYP2D6 UM patients, and has specific calculated dose recommendations per gene-drug pair in CYP2D6 IM and PM patients (The Dutch Pharmacogenomic Working Group, 2020a). Patients with CYP2C19 UM metabolize amitriptyline in a greater extent into nortriptyline. According to the DPWG no additional action is required for CYP2C19 UM patients starting amitriptyline (The Dutch Pharmacogenomic Working Group, 2020a). On the other hand, the CPIC recommends starting nortriptyline instead of amitriptyline (Hicks et al., 2015; Hicks et al., 2016; CPIC, 2020). The CPIC mentions that nortriptyline and desipramine are secondary amines TCAs and CYP2D6 is the main gene for their metabolism. Another example is the DPWG recommending to avoid clomipramine in patients with CYP2C19 UM for the indications obsessive compulsive disorder (OCD) and anxiety disorder. In case of depression, the DPWG also recommends avoiding clomipramine in CYP2D6 PM (The Dutch Pharmacogenomic Working Group, 2020a).

There is no reason for any adjustment in patients with the CYP2D6 PM predicted phenotype according to the DPWG (The Dutch Pharmacogenomic Working Group, 2020a). However, the CPIC recommends choosing an alternative for paroxetine or considering a 50% reduction and a 25–50% reduction for fluvoxamine (Hicks et al., 2015; CPIC, 2020). In addition, the DPWG recommends adjusting the maximum dose instead of adjusting the starting dose of (es)citalopram because of the risk of Torsade de Pointes at high plasma concentrations (The Dutch Pharmacogenomic Working Group, 2020a).

Discordances Indication and Patient Population Specific Recommendations

Other differences between the recommendations is distinguishing between indications, as for example the DPWG has different

recommendations for CYP2D6-codeine for cough and pain while the CPIC and RNPGx mention no indication (Picard et al., 2017; CPIC, 2020; The Dutch Pharmacogenomic Working Group, 2020a). Furthermore, the CPIC has specific recommendations for adults and pediatrics with respect to voriconazole and atomoxetine (Krebs and Milani, 2019; CPIC, 2020).

Discordances Pharmacotherapeutic Recommendations Normal Metabolizers

Supplementary Table S1 shows that the CPIC, the RNPGx, and in some cases also the CPNDS give advices on the treatment of patients with a predicted phenotype of NM such as starting with standard dose, adjusting dose based on guidelines, and standard monitoring of treatment effect. In contrast to these committees, the DPWG does not recommend on drug treatment in NM patients. One exception is tacrolimus initiation in CYP3A5 homozygous expressors, named by CPIC as NM patients. Furthermore, the difference in the dose recommendations for CYP3A5-tacrolimus is as follows. The CPIC and the RNPGx recommend 1.5-2 times of the standard starting dose for both NM and IM as mentioned by the CPIC and *1/*1 and *1/*3 as mentioned by the RNPGx (Birdwell et al., 2015; Woillard et al., 2017). On the other hand, the DPWG recommends 1.5 times the normal dose for the CYP3A5 heterozygote expressor and 2.5 times for CYP3A5 homozygous expressor phenotype (The Dutch Pharmacogenomic Working Group, 2020a).

Genetic Testing Recommendations

The DPWG, CPNDS, and RNPGx provide recommendations regarding pharmacogenetic testing in daily clinical practice for specific gene-drug combinations (**Supplementary Table S1** and **Supplementary Table S5**). The grading schemes they use for these recommendations differ, but they all have three levels. The DPWG's highest level is "essential" meaning "PGx testing for this gene-drug pair is essential for drug safety or efficacy". Genotyping must be performed before drug therapy has been initiated to guide drug and dose selection (The Dutch Pharmacogenomic Working Group, 2020a). The grading scheme of the CPNDS has "strong" as highest level (A), defined as "based on strong scientific evidence; benefits clearly outweigh risks" (Amstutz et al., 2014), while for the RNPGx the highest classification is "essential test" with the following description "demonstrated impact on a major clinical phenotype [response (efficacy, resistance)/toxicity] for therapeutic management; difficult or impossible to predict with a non-genetic approach; having led to expert agreement in favor of systematic testing" (Picard et al., 2017).

Genetic Testing Anti-cancer Drugs

Among anti-cancer agents, both the DPWG and the RNPGx consider UGT1A1 genotyping essential before the start of the treatment with irinotecan and the RNPGx recommending genotyping more specifically for patients who will receive the intensified dose (>240 mg/m^2). The CPNDS strongly recommends (level A) genetic testing before cisplatin initiation for the associated functional TPMT variants (*3A, *3B, and*3C) in all patients, and the functionally inactive TPMT *2 variant in children receiving cisplatin to prevent cisplatin-induced hearing loss (Lee et al., 2016). Also, the CPNDS recommends genotyping (level B – moderate) for RARG rs2229774, SLC28A3 rs7853758 and UGT1A6*4 rs17863783 variants in all children with cancer that will initiate doxorubicin or daunorubicin to prevent antracycline-induced cardiotoxicity.

Genotype testing is not recommended in adults, also not in children receiving other anthracyclines (level C – optional) (Aminkeng et al., 2016). Furthermore on the anticancer drugs, the CPNDS recommendation including testing for CYP2D6 before initiation of adjuvant tamoxifen treatment, is classified as level B – moderate (Drögemöller et al., 2019).

The RNPGx prefers phenotyping by measuring the physiological dihydro-uracil/uracil (UH2/U) metabolic ratio in serum, over DPYD genotyping (Quaranta and Thomas, 2017; Loriot et al., 2018). If phenotyping is not available, genotyping should be performed pretreatment with dose reductions if genetic variants are detected (ref). They refer to the DPWG and CPIC guidelines for dose reductions. A phenotyping test in addition to genotyping should be performed, according to the DPWG, if two genetic variants are detected. In these rare cases the selection of the starting dose is at the discretion of the treating physician and other health care professionals involved, taking both genotyping and phenotyping test results into account.

Genetic Testing Clopidogrel and Warfarin

Also, the DPWG and the RNPGx consider CYP2C19 genotyping essential in patients who will be treated with clopidogrel because of a percutaneous coronary intervention. The DPWG also recommends testing in the case of stroke or TIA. Genotyping is advisable for CYP2C9-VKORC1-warfarin according to the RNPGx (Picard et al., 2017). The CPNDS recommends testing all warfarin-naïve patients for VKORC1 (−1639G>A), CYP2C9*2, and CYP2C9*3 before warfarin is started or within first 2 weeks of therapy (level B – moderate) (Shaw et al., 2015).

Genetic Testing Antidepressants

Moreover, the recommendations by the DPWG differ per gene-drug pair for tricyclic antidepressants, as shown in **Supplementary Table S1**. The DPWG classifies genotyping for tricyclic antidepressants as potentially beneficial meaning genotyping can be considered per patient. The RNPGx classifies genotyping for CYP2C19-CYP2D6-tricyclic antidepressants advisable (Quaranta and Thomas, 2017).

Genetic Testing Codeine

Guidelines regarding CYP2D6 genotype testing and codeine treatment are also available. This is considered essential according to the DPWG in case of planned doses of more than 20 mg every 6 h for adults and more than 10 mg every 6 h for children aged 12 years and older or in case of additional risk factors, such as comedication with CYP3A4 inhibitors and/or reduced kidney function. The CPNDS classifies genotyping as strong – level A in the treatment of young children and women with postpartum pain while breastfeeding meaning that they should be tested for CYP2D6 (Madadi et al., 2013). Furthermore, CPNDS assigned other levels of

recommendations for children and adults having pain despite high doses of codeine (level B – moderate recommendation), and patients who receive codeine for the first time to rule out non-responders and the ones who are susceptible to adverse side effects of codeine (level C – optional recommendation).

Genetic Testing Antiepileptics

As shown in **Supplementary Table S1**, the DPWG have published genotyping recommendations on the antiepileptics: phenytoine, lamotrigine, and oxcarbazepine in case of HLA-B*15:02. HLA-B*15:02 is common in patients of Asian descent, other than Japanese or Korean descent, and therefore is genotyping beneficial before (or directly after) starting the pharmacotherapy (The Dutch Pharmacogenomic Working Group, 2018a; The Dutch Pharmacogenomic Working Group, 2018b; The Dutch Pharmacogenomic Working Group, 2018c; The Dutch Pharmacogenomic Working Group, 2020a). Also the CPNDS recommends genotype testing for HLA-B*15:02 for all carbamazepine naïve patients prior to initiation of the pharmacotherapy (level A- strong) for those originating from populations where HLA-B*15:02 is common or its frequency is unknown or whose origin is unknown. The CPNDS classifies genotyping recommendation as optional (level C) in patients from populations where HLA-B*15:02 is rare. Testing for HLA-A*31:01 is classified as moderate (level B) by the CPNDS for all carbamazepine naïve patients before pharmacotherapy initiation (Amstutz et al., 2014).

Genetic Testing Several Drugs

Furthermore, the DPWG recommends to genotype for HLA-B-abacavir, TPMT and NUDT15-azathioprine/mercaptopurine/thioguanine, before initiation of the pharmacotherapy (classified as essential) (The Dutch Pharmacogenomic Working Group, 2020a). Genotyping is considered beneficial for VKORC1-acenocoumarol/phenprocoumon according to the DPWG. This means that genotyping the patient can be performed before (or directly after) pharmacotherapy therapy has been initiated (The Dutch Pharmacogenomic Working Group, 2020a). Genotyping is advisable for CYP3A5-tacrolimus according to the RNPGx (Picard et al., 2017).

DISCUSSION

Four committees develop pharmacogenetics guidelines regarding drugs in different drug classes, irrespective of indication, disease, or medical specialty. Their processes of guideline development have been described. They have published methodologies, guidelines, recommendations, and advice in scientific journals in English. In general, their recommendations add to the effective and safe use of drugs. However, the objectives differed at the start of each project, which have led to unique profiles and strengths of their approaches and guidelines.

The DPWG was first to start its project in 2005, and from the beginning all the pharmacotherapeutic recommendations were included in the drug database incorporated in electronic healthcare systems in The Netherlands. This implies that an alert is generated in case a drug is prescribed or dispensed to a patient with a genotype that requires an action. The system presents a short text addressing the pharmacotherapeutic advice, a summary of the underlying mechanism of the interaction between the gene and drug, and clinical and/or pharmacokinetic effects (Deneer and van Schaik, 2013). The methodology is equal to other medication surveillance functionalities, such as on drug interactions, within the electronic systems. This has facilitated healthcare professionals becoming familiar with pharmacogenetics.

The guidelines translating genetic laboratory test results into actionable prescribing decisions are essential for implementation in daily clinical practice. The CPIC guidelines are sent out in the review process to over 400 CPIC members. The CPIC has published all guidelines in a peer-reviewed scientific journal, and they are therefore reviewed by external experts. Detailed, and evidence-based information regarding allele definition, functionality, frequencies, and phenotype translations from genotype data are given. They have been the initiators of several projects to harmonize and reach a consensus on topics in the field of pharmacogenetics (Caudle et al., 2017; Caudle et al., 2020).

The CPNDS was established to focus on severe adverse drug reactions, which is also reflected by the drugs and genes they have selected for their guideline development. Participants enroll patients with serious adverse drug reaction into the CPNDS program. They aim to identify novel predictive genomic markers of severe adverse drug reactions and to provide clinical genetic information to the patient and healthcare professional (Ross et al., 2010). A broad panel of stakeholders, including patients and healthcare policy makers, are involved in guideline development.

The RNPGx has an interest in pharmacogenetic testing in daily clinical practice. They indicate which genetic variants should be included in a test offered in daily clinical practice to improve pharmacotherapy. The RNPGx also states whether genotype testing is recommended in daily clinical practice (Picard et al., 2017). They specifically recommend clinical indications and circumstances for genotyping either before the start of treatment or in case of problems when a patient is being treated with a specific drug (Lamoureux and Duflot, 2017).

The DPWG has assessed many gene-drug pairs with a relatively large number of pairs that require no action. The members of the DPWG select gene-drug combinations for further analysis. Even if the literature search shows that the genotype of the gene involved, does not influence the effect of the drug or to an extent that is not clinically relevant, a complete assessment report is prepared and made available by including it in the drug database. Although no action, such as adjustments of the dose and additional monitoring, is necessary, it appeared that the information and the report are of value for healthcare professionals. For example, healthcare professionals generally considered the CYP1A2 genotype to be relevant for clozapine. However, the assessment report summarized the results of the studies and concluded that CYP1A2 genotypes and the effect of clozapine or its blood concentrations are not associated with for example any adverse drug event, considering the totality of

available evidence (The Dutch Pharmacogenomic Working Group, 2020a).

As previously recognized by Bank et al., there are several other explanations for differences between the guidelines (Bank et al., 2018). Differences in methodology, in time points at which literature searches have been performed or guidelines have been updated, and differences in daily clinical practices between countries result in discordances in recommendations. The CPIC's dose recommendations are usually based on consensus of experts. They use published literature for dose. So if it is not clear what dose should be used and there are alternative therapies, they will most likely recommend another drug. On the other hand, the DPWG calculates adjustments of doses per genotype or predicted phenotype, based on pharmacokinetic data such as area under the concentration time curves (AUC), steady state concentrations found in published studies (Swen et al., 2008; Deneer and van Schaik, 2013). This explains differences between the recommended starting doses of tricyclic antidepressants. Furthermore, they only recommend on adjusting the dose if genotypes have a clinically relevant effect on for example blood concentrations, considering the therapeutic range of the drug or in case an association between the genotype and response of the drug in terms of efficacy or adverse drug events, has been observed. This explains for example the differences between the recommended starting doses of paroxetine and fluvoxamine in patients with a predicted PM phenotype for CYP2D6. According to the CPIC, the starting dose should be reduced. The DPWG does not consider this necessary since the drugs have a large therapeutic window.

The extent to which therapeutic drug monitoring (TDM), meaning measuring concentrations of a drug in blood or plasma to optimize treatment, is applied in daily clinical practice, differs among countries. For example, the DPWG indicates that endoxifen plasma concentrations should be measured to select an appropriate dose of tamoxifen in CYP2D6 IM and PM patients in contrast to the CPIC and the CPNDS. Clinical practices are also reflected in the DPYD-fluoropyrimidines. The UH2/U metabolic ratio test seems the preferred test to assess DPD enzyme activity, in France. Currently, the DPD enzyme activity measurement in peripheral blood mononuclear cells is the best developed and implemented test in routine patient care in The Netherlands. The CPIC mentions TDM in their guideline (Amstutz et al. 2018; Swen et al., 2011b; Picard et al., 2017; CPIC, 2020; The Dutch Pharmacogenomic Working Group, 2020a).

The DPWG, CPNDS, and RNPGx advise on genotyping. In general, they recommend to order genotyping tests in routine patient care if the clinical benefit for patients is considered relevant for example by lowering the risk of developing side effects or the risk of inefficacy of drug treatment. The committees assess the available data from published studies and also include the SmPC or drug label approved by EMA and FDA respectively. The number of drugs with pharmacogenetic information in these documents has increased over the years (TanKoi et al., 2018). The information is either included based on studies available before or after marketing authorization being granted or as a result of safety issues post approval. For example, in the SmPC of abacavir in the section regarding therapeutic indications it is stated that before starting the treatment every patient should be tested for carrying the HLA-*5701 allele (European Medicines Agency, 2020b). Several years ago, the FDA issued a black box warning on clopidogrel and the warnings section of the SmPC was updated with respect to reduced effectiveness of clopidogrel in patients with a CYP2C19 predicted phenotype of PM (TanKoi et al., 2018). Recently, EMA published a direct health care professional communication (DHPC) about the fact that patients with partial or complete DPD deficiency have an increased risk of developing toxicity when receiving fluoropyrimidines. It has been added that genotyping or phenotyping is recommended before the start of treatment with fluoropyrimidines such as capecitabine (European Medicines Agency, 2020a). Siponimod is a CYP2C9 substrate with plasma concentrations being influenced by the patient's CYP2C9 genotype. The posology of the SmPC indicates that CYP2C19 genotyping must be performed before starting the treatment. The CYP2C9 *3/*3 genotype is a contraindication and the maintenance dose for patients with the *2/*3 or *1/*3 genotype is lower as compared to all other genotypes (European Medicines Agency, 2020c).

Although it is evident that genotyping contributes to the appropriate and safe use of drugs, cost-effectiveness is often discussed in case of reimbursement issues. Several randomized controlled trials investigating a genotype guided strategy are available for some drugs in specific patient populations (Church, 2008; Claassens et al., 2019). It is not feasible to perform cost-effectiveness studies based on the results of randomized clinical trials or cost-effectiveness simulations for all drugs with a clinically relevant gene-drug interaction for all indications. However, the clinical implication score of the DPWG includes criteria that would determine the outcome of a cost-effectiveness study such as the clinical impact meaning the severity of the drug's side effect or the clinical impact of diminished efficacy, the number needed to genotype. It is worthwhile mentioning that currently the PREPARE study is investigating the (cost)-effectiveness and clinical utility of applying the DPWG guideline after a panel of genes being tested (van der Wouden et al., 2017; Van Der Wouden et al., 2020).

In conclusion, evidence based recommendations for many gene-drug pairs are available for supporting clinical decision making by healthcare professionals, patients and other stakeholders. Although there are many similarities in the methodologies the committees use, their guidelines have unique profiles and strengths. Therefore, considering the totality of guidelines are of added value.

AUTHOR CONTRIBUTIONS

HA-K and VD discussed and decided upon the method for literature search. HA-K performed the Pubmed search and

the search results were discussed by both of them. AK made a detailed overview of all the guidelines and recommendations. HA-K, VD, AK, and MN all had a major contribution in making the comparison between the guidelines and recommendations. HA-K and VD drafted the manuscript and all authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

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