Review

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Pharmacogenomics

Pharmacogenetics of childhood acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is the major pediatric cancer in developed countries. Although treatment outcome has improved owing to advances in chemotherapy, there is still a group of patients for which therapy fails while some patients experience severe toxicity. In the last few years, several pharmacogenetic studies have been performed to search for markers of outcome and toxicity in pediatric ALL. However, to date, *TPMT* is the only pharmacogenetic marker in ALL with clinical guidelines for drug dosing. In this article, we will provide an overview of the most important findings carried out in pharmacogenetics for pediatric ALL, such as the interest drawn by methotrexate transporters in the context of methotrexate treatment. Even if most of the studies are centered on coding genes, we will also point to new approaches focusing on noncoding regions and epigenetic variation that could be interesting for consideration in the near future.

Keywords: acute lymphoblastic leukemia • childhood • outcome • pharmacogenetics • toxicity • treatment

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Acute lymphoblastic leukemia (ALL) is the major pediatric cancer in developed countries, accounting for 30% of all malignancies in children [1]. Treatment outcome has improved substantially owing to advances in chemotherapy, with cure rates now exceeding 80% [2–4]. However, there is a group of patients that still remains refractory to therapy while, at the same time, there are patients who experience severe toxicity that can affect their quality of life during and after treatment. Consequently, there is a current interest in detecting markers to recognize in advance which patients are going to be resistant to treatment or suffer from the adverse effects of therapy and so that treatment can be adjusted from the beginning.

In this context, pharmacogenetic studies can be useful in childhood ALL for several reasons:

- Treatment protocols for ALL are standardized and well-established, which allows for the inclusion of large and homogeneously treated groups of patients in the studies. As a result, larger and homogeneous samples increase the statistical power of the studies and strengthen the reliability of the results.
- Chemotherapeutic drugs used in ALL treatment, such as methotrexate (MTX) or 6-mercaptopurine (6-MP), have a very narrow therapeutic range. This means that there is a small difference between the effective dose and the dose that causes toxicity [5,6]. Consequently, toxicity prevention is challenging beacuse it is difficult to predict and dose reduction is dangerous since under-dosage is usually associated with decreased survival.
- Genes involved in the metabolic pathways of the drugs used in ALL treatment are highly variable [7,8]. In fact, several genetic variants have been described that lead to interindividual variability in protein levels or activity in those pathways. Those

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variations can lead to changes in drug exposure or activity and impact treatment response.

Several authors have carried out studies in order to search for pharmacogenetic markers of outcome and toxicity in pediatric ALL. However, the results are controversial for most of the analyzed polymorphisms. The lack of replication could be due, on the one hand, to the characteristics of the samples included (small or mixed patients populations) or toxicity criteria studied (different among studies) or to differences among treatment protocols. On the other hand, most studies are centered on variations in candidate coding regions and very few of them have analyzed the role of variation in noncoding regulatory regions or epigenetic markers, which could be relevant. For example, miRNAs are a class of small noncoding RNA molecules that regulate gene expression at the post-transcriptional level by binding to the 3′-UTR of their target genes. As they have the potential to regulate the expression of genes involved in drug pathways, they are starting to provide promising results.

In this article, we will provide an outline of the most important findings in pharmacogenetics for pediatric ALL in the last few years. We will review state-of-the-art pharmacogenetic markers for the different drugs included in pediatric ALL treatment and we will overview new approaches that could be interesting for consideration in the near future.

Treatment protocols for pediatric ALL

Most treatment protocols for pediatric ALL are typically composed of three differentiated phases: induction, consolidation and maintenance [9].

The induction phase is applied to restore normal bone marrow function by reducing the number of leukemic cells. During induction, patients are generally given a glucocorticoid such as prednisone, vincristine, which interferes with microtubules of the mitotic spindle, and L-asparaginase, which catalyzes the hydrolysis of asparagine to aspartic acid and interferes with the synthesis of proteins. The treatment may be completed with anthracyclines and cyclophosphamide. Tyrosine kinase inhibitors, such as imatinib, are also included in the treatment of patients with *BCR–ABL1*-positive disease.

In the consolidation phase, the treatment is enhanced to prevent the onset of therapy-resistant clones. At this stage, 6-MP and MTX are used. Furthermore, the treatment is usually completed with cytarabine, a cytosine analog that inhibits DNA polymerase.

Finally, the maintenance phase, which can be extended for approximately 2 years, is intended to maintain remission. It starts with reinductions and continues with MTX and 6-MP, which can be administered in combination with other drugs. In high-risk groups, the therapy must be intensified and patients who respond worse to treatment may undergo hematopoietic cell transplantation.

In addition, control of CNS disease is needed. In order to prevent secondary effects, cranial irradiation has been progressively replaced by triple intrathecal chemotherapy (MTX, hydrocortisone and cytarabine) in most treatment protocols.

Despite the clinical success of these treatment protocols, all these chemotherapeutic drugs have the potential to produce adverse reactions. For most of them, pharmacogenetic studies have been performed in order to search for markers to adjust the doses from the first moment to improve the outcome and avoid toxicity. The most important findings will be reviewed in the following sections.

Pharmacogenetics of 6-MP

The thiopurine antimetabolite 6-MP is an analog of purine that interferes with nucleic acid biosynthesis. 6-MP is very important in childhood ALL treatment protocols and is typically used during consolidation and maintenance phases [8]. However, long-term usage can cause hematologic and hepatic toxicity in some patients, leading to hospital admissions and treatment withdrawal.

6-MP enters the cell via nucleoside transporters such as SLC28A2, SLC28A3, SLC29A1 and SLC29A2 and, then, it can follow different metabolic pathways. On the one hand, it can be metabolized by HPRT1, leading to the synthesis of active cytotoxic metabolites that will incorporate into DNA or RNA, resulting in cell death (Figure 1) [8]. On the other hand, 6-MP can be inactivated if it is methylated to methyl-MP by TPMT [10]. In fact, TPMT is the main regulator of the balance between active and inactive 6-MP metabolites [8].

It has been shown that TPMT activity is highly variable among individuals and those differences have been associated with genetic polymorphisms in the *TPMT* gene. 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 (although more than 20 less active TPMT variants have been described) [8,11]. These polymorphisms do not change expression at the mRNA level but the protein becomes more susceptible to degradation by the proteasome [12], leading to lower drug inactivation. Approximately 90% of the population has two active *TPMT* alleles (*TPMT*1*) and normal protein activity; 5–10% people have one nonfunctional *TPMT* allele and intermediate activity; and only one in 300 people is TPMT-deficient with two nonfunctional alleles [13].

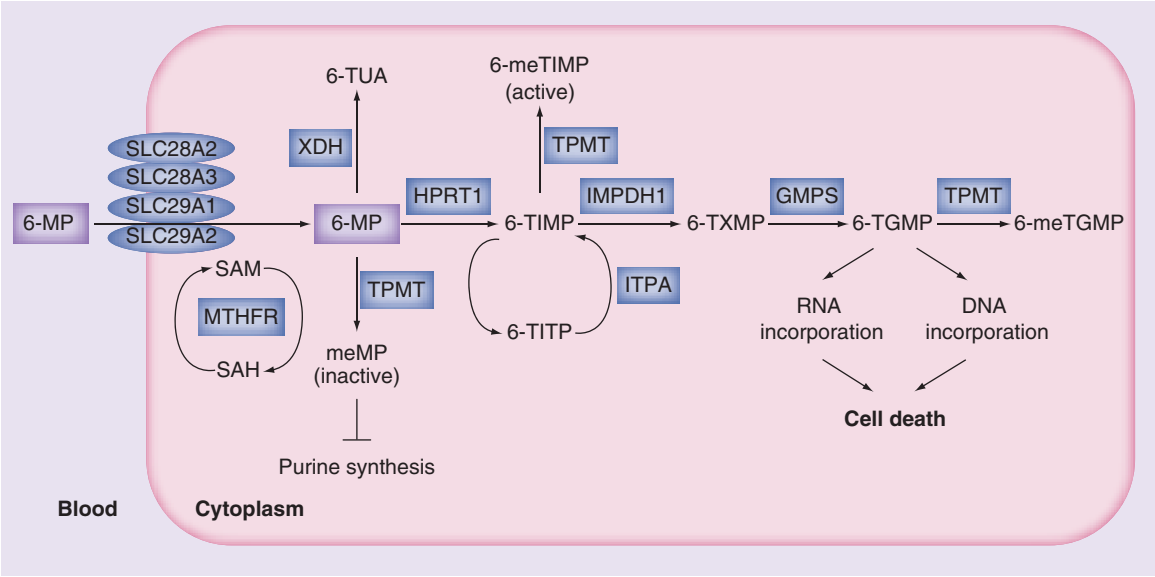

Figure 1. 6-mercaptopurine metabolic pathway. The relevant steps of 6-MP transport, metabolism and effects are represented. Important proteins/genes are represented inside blue squares and ovals.
6-meTGMP: 6-methyl thioguanylic acid; 6-meTIMP: 6-methyl thioinosinic acid; 6-MP: 6-mercaptopurine; 6-TGMP: 6-thioguanylic acid; 6-TIMP: 6-thioinosinic acid; 6-TITP: 6-thioinosinetriphosphate; 6-TUA: 6-thiouric acid; 6-TXMP: 6-thioxanthylic acid; meMP: Methyl-mercaptopurine; SAH: S-adenosyl-L-homocysteine; SAM: S-adenosylmethionine.
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Numerous studies have shown that *TPMT* genotype is associated with susceptibility to hematopoietic toxicity after 6-MP therapy [8,14]. TPMT-deficient patients have a higher risk of severe toxicity due to the accumulation of excessive intracellular active 6-MP metabolites [15,16]. As a result, a reduction of more than 90% of the baseline dose of 6-MP is needed in TPMT-deficient patients from the beginning of treatment to avoid complications [8].

Whether ALL patients with intermediate TPMT activity would need dose reduction is less clear. Some authors found an association between *TPMT* heterozygosis and increased toxicity [16–21] while others found no association [22–28]. It has been proposed that heterozygous *TPMT* patients treated with higher doses (75 mg/m^2 per day) are more likely to benefit from dose reduction because of hematopoietic toxicity [16], while those treated with lower doses [23] may not have higher risk of hematologic toxicity and might not need a dose reduction [29].

As a result, 6-MP is the only antileukemic agent used in ALL treatment for which the US FDA recommends the determination of germline genetic variation in order to adjust the treatment. In addition, based on all the published results, the Clinical Pharmacogenetics Implementation Consortium (CPIC) has developed pharmacogenetic-based guidelines to provide dosing recommendations for 6-MP according to *TPMT* genotype in patients with ALL [30,31].

However, there are still severe toxicities associated with 6-MP treatment that cannot be explained by those *TPMT* polymorphisms. Other environmental and genetic factors may have a role in the determination of these adverse events [32]. Therefore, there is a current interest in the study of other polymorphisms in *TPMT* and other genes that could be used as markers of 6-MP toxicity.

For instance, in the promoter of *TPMT*, variable number of tandem repeats have been described to affect the level of expression of the *TPMT* gene. A preliminary study in a group of 26 ALL patients treated with 6-MP revealed that the patients with the variable number of tandem repeat combinations that lead to lower *TPMT* expression were more sensible to 6-MP therapy. This was reflected in a tendency to more frequent and longer 6-MP treatment discontinuation [33].

On the other hand, a polymorphism (rs1127354) has been studied in the *ITPA* gene, which encodes a cytosolic enzyme involved in 6-MP metabolism (Figure 1). rs1127354 leads to an amino acid change in ITPA that causes a dramatic decrease in enzyme activity [34]. The variant allele is more frequent in the Asian populations (0.11%) than in Europeans or Africans (0.08 and 0.03, respectively). In a group of 244 ALL patients with mixed ethnicities (77% white, 18% black, 8% other), those who had inherited an *ITPA* rs1127354A variant allele had a higher risk of severe febrile neutropenia

during maintenance therapy with 6-MP [35]. In Asian populations, it has also been associated with fever and with liver [22] and hematological toxicity [36].

With the aim to identify *trans*-acting genes whose expression and/or SNPs are related to TPMT activity, a genome-wide analysis was performed in a panel of human HapMap cell lines. The *PACSIN2* gene, which plays a role in the formation of normal caveolae at the cell membrane, was discovered to be the highest correlated gene [37]. The subsequent validation in ALL patients showed that rs2413739 CC genotype, the most significant SNP in *PACSIN2*, was associated with higher TPMT activity in comparison to the TT genotype, independently from the *TPMT* genotype. Moreover, *PACSIN2* SNP rs2413739 also showed a significant association with gastrointestinal toxicity during consolidation therapy. The effects of *PACSIN2* polymorphism on severe mucositis during consolidation therapy were further confirmed in another cohort of patients [37,38].

In addition, interactions have been shown between TPMT activity and polymorphisms in genes of the folate pathway such as the folate transporter *SLC19A1*, *TYMS* and *FOLH1*. *FOLH1* C1561T polymorphism even showed independent association with 6-MP-mediated hematologic toxicity [39]. Further replication would be needed to assess the role of this polymorphism on 6-MP toxicity.

Furthermore, other genes involved in 6-MP transport and metabolism (Figure 1) have been suggested as possible candidate genes for pharmacogenetic studies such as *XDH, GMPS*, *HPRT1* and the transporters *SLC28A2, SLC28A3, SLC29A1* and *SLC29A2* [40]. However, association studies have not been performed yet. In addition, the role of epigenetic variations that could affect the regulation of key genes in the 6-MP pathway, such as DNA methylation, miRNAs or histone modifications, has not been properly addressed.

In summary, genetic polymorphisms in *TPMT* play an important role in the processes of toxicity induced by 6-MP in children with ALL. Therefore, clinical guidelines have been developed to adjust the drug therapy based on the genotype of this gene. The application of genetic analysis to identify additional polymorphisms or epigenetic variations in other genes of the intracellular pathway of 6-MP, in addition to *TPMT*, and the study of their interactions, could be helpful to better adjust the 6-MP doses in ALL patients.

Pharmacogenetics of MTX

MTX is a folate analog that, once inside the cell, directly or through its derivatives, inhibits nucleic acid and protein synthesis. MTX is an important component of almost all treatment protocols for pediatric ALL. High-dose MTX is usually included in consolidation and low-dose oral MTX in the maintenance phase [8].

MTX enters the cell primarily via active transport by SLC19A1 or by passive diffusion [41,42] and once inside the cell, MTX is metabolized into MTX polyglutamates (MTXPGs). Both MTX and MTXPGs inhibit DHFR, responsible for the convertion of dihydrofolates to active tetrahydrofolates, causing depletion of intracellular tetrahydrofolate. On the other hand, MTXPGs target other folate-dependent enzymes such as TYMS, leading to nucleic acid and protein synthesis inhibition and consequent cell death, particularly in rapidly dividing cells [43]. Finally, the role of membrane transporter proteins is essential for MTX elimination from the cell and from the organism through bile and urine efflux [44]. These proteins include ATP-binding cassette transporters, such as ABCB1 [45], and organic anion transporters, such as SLCO1B1 [46,47] (Figure 2).

Despite its clinical success, treatment with MTX can result in severe toxicities. Clinical manifestations of toxicity include gastrointestinal toxicity, mucositis, hepatic and renal toxicity, myelosuppression and neuropathy. In some patients, the toxic effects are so severe that the dose must be reduced or the treatment stopped. This means that, in addition to the problems related to toxicity, the associated treatment arrest can also have a negative impact on survival. Therefore, it would be really useful to identify predictors of the adverse effects of MTX treatment in order to make treatment adjustments [48]. In this context, polymorphisms in the genes involved in MTX pharmacokinetic and pharmacodynamic pathways have been studied in pediatric ALL patients.

For instance, a polymorphism in *SLC19A1* (G80A) that results in a less efficient transporter, has been associated with outcome [49–52], MTX plasma levels [49,53,54] and treatment interruption [24]. It has also been associated with gastrointestinal [55,56], hematologic [50,52,57] and hepatic toxicity [48,50,52]. Polymorphisms in *MTHFR* (C677T and A1298C) that reduce the activity of the protein have been associated with outcome [58–60] and several toxicities [24,48,61–68]. *MTRR* A66G and *SHMT1* C1420T polymorphisms have been associated with mucositis and hepatic toxicity, respectively [69], and *MTHFD1* 1958A allele with reduced hepatotoxicity [70]. Polymorphisms in *TYMS* (28 bp tandem repeat and 6 bp deletion) have been linked to differential TYMS expression and with treatment outcome [23,68,71–74], hematologic toxicity [62,70] and mucositis [58,75]. Several polymorphisms in *DHFR* (A-317G, C829T, C-1610G/T and C-680A) have been associated with outcome [76–78], *GGH* 401T with increased leukopenia and thrombocytopenia, and polymorphism

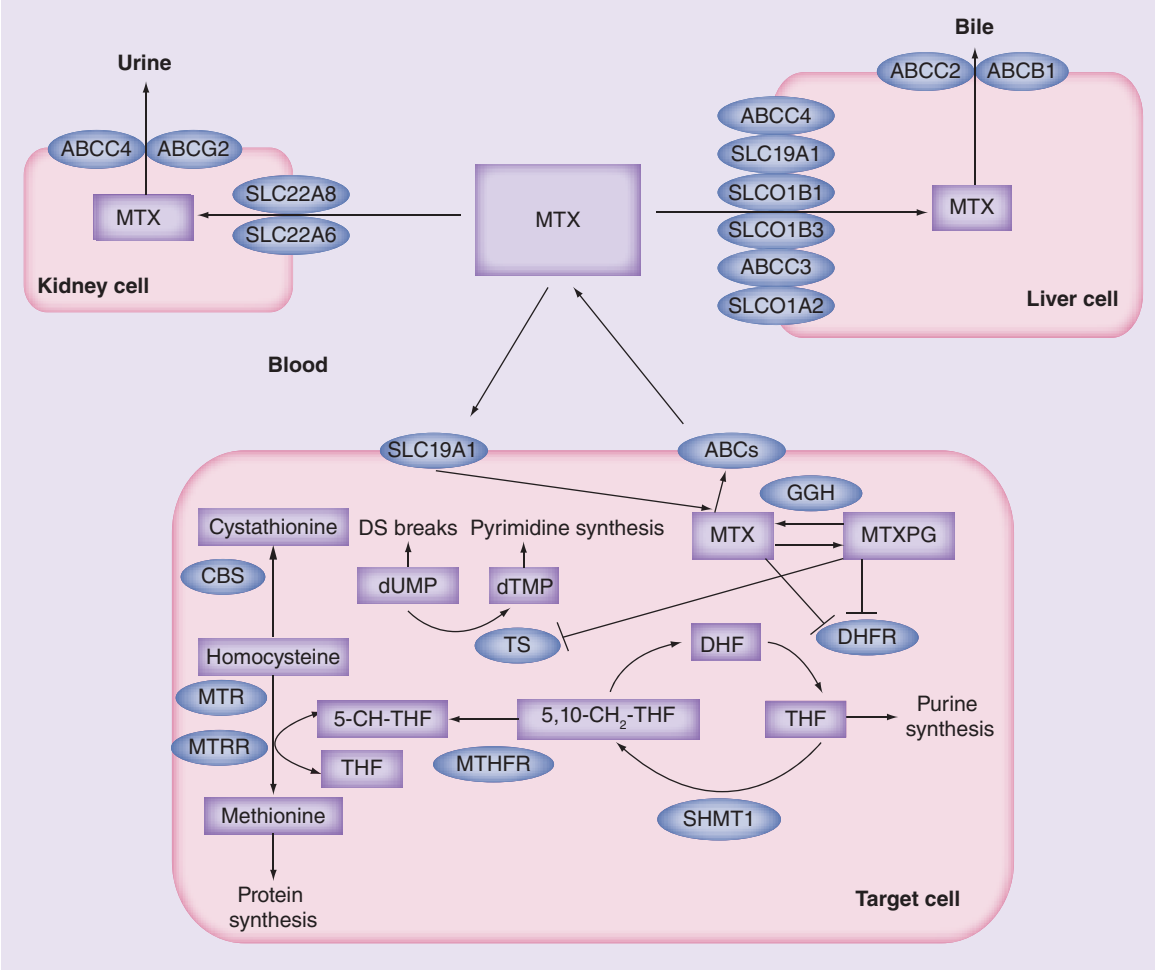

Figure 2. Methotrexate pharmacodynamic/pharmacokinetic pathway. The relevant steps of MTX transport, action, metabolism and elimination are represented. Drug and organic metabolites are in purple boxes and relevant proteins/genes are represented by blue ovals.
5-CH-THF: 5-methyltetrahydrofolate; 5,10-CH_2-THF: 5,10-methylenetetrahydrofolate; ABC: ATP-binding cassette transporter; DHF: Dihydrofolate; DS breaks: Double-strand breaks; MTX: Methotrexate; MTXPG: Methotrexate polyglutamated form; THF: Tetrahydrofolate.
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A870G in *CCND1*, a gene involved in cell cycle regulation, with outcome and hepatic and hematologic toxicity [79]. In addition, the polymorphisms C3435T and C421A in the ATP-binding cassette transporters *ABCB1* and *ABCG2* have been associated with outcome [80–83], neuropathy and infections [84,85].

However, the reported associations between polymorphisms and treatment response are not usually confirmed. *MTHFR* polymorphisms (C677T and A1298C) are a paradigmatic example of this problem. For instance, the 677T variant has been associated with increased toxicity in some studies [24,48,61–68] but other authors have not found any association [41,55,56,58,60,69,70,86–92] and others even found a protective effect associated with this variant [79,93,94]. It has been suggested that these contradictory results could be due to low statistical power due to small or non-homogeneous populations, or to differences among treatment protocols, or to the use of different criteria for toxicity. In a recently published meta-analysis, an extensive analysis was performed for each toxicity criterion in order to assess the relevance of *MTHFR* SNPs on MTX toxicity in pediatric ALL [95]. In this study, 24 articles were taken into consideration and, using the available data, no association was found between *MTHFR* C677T or A1298C polymorphisms and any individual toxicity criterion, except for some protective effect of the *MTHFR* 1298CC genotype against leukopenia. These results lead to the conclusion that there is no evidence to support the use of either the *MTHFR* C677T or the A1298C SNP as MTX toxicity markers. By contrast, a previous meta-analysis performed with 14 studies found associations between *MTHFR* C677T polymorphism and liver toxicity, hematologic

toxicity, mucositis, gastrointestinal and skin toxicity and between *MTHFR* A1298C polymorphism and skin toxicity [96]. In this case, the study included adults and children and the inclusion of adult patients may have led to differences in results. All these conflicting results contribute to increase the controversy. Additionally, similar inconclusive results are obtained for the other SNPs previously mentioned in the genes of the MTX pathway.

Recently, using a genome-wide approach, several polymorphisms in the *SLCO1B1* gene, especially rs4149081 and rs11045879, were strongly associated for the first time with MTX clearance and gastrointestinal toxicity [46]. SLCO1B1 is localized at the membrane of hepatocytes and transports substrates, including MTX, from blood to the liver, which results in their elimination via bile [46,97,98]. After that first report, association between *SLCO1B1* polymorphisms and MTX pharmacokinetics and toxicity was confirmed in subsequent studies [58,91,99]. *In vitro* analyses have also demonstrated that *SLCO1B1* nonsynonymous SNPs predicted to be functionally damaging could be associated with reduced MTX transport capacity [100].

As a result, other studies have focused their interest on the analysis of hepatic and renal transporters (Figure 2). In this context, an interesting study found that SNPs in enhancer sequences of hepatic transporter genes, especially in the organic anion transporter *SLCO1A2* gene, could be important for drug response and toxicity, regulating gene expression *in vitro* and MTX clearance *in vivo* [101].

Additionally, polymorphisms in other transporters of the ATP-binding cassette family, rs9516519 in *ABCC4* and rs3740065 in *ABCC2*, have been associated with MTX plasma levels in pediatric ALL patients [102]. The SNP rs3740065 in *ABCC2* had been previously associated with gastrointestinal MTX toxicity in rheumatoid arthritis patients [103]. Although another SNP in *ABCC2*, rs717620, located in the 5′-UTR region, has been associated with MTX clearance and a wide range of hematologic and nonhematologic toxicities [104,105], other studies have not confirmed those associations [106,107]. However, what was more remarkable was that the SNP rs9516519 in *ABCC4*, a gene that had been previously associated with MTX plasma levels in ALL patients [108], is located in a putative miRNA binding site. The G allele, associated with lower MTX plasma levels, disrupts the putative binding site for miR-367. Consequently, the loss of a miRNA binding site could explain an increased ABCC4 function and the decrease in MTX plasma levels. This was not the first time that miRNA-related SNPs were associated with MTX response. In fact, *in vitro* response to MTX had been previously associated with the SNP C829T, located near the miR-24 binding site in the 3′-UTR of *DHFR*, that caused increased DHFR expression [109]. These results point to a mechanism based on miRNA regulation, which could be and interesting field in ALL pharmacogenetics [110–114].

In conclusion, although there is no pharmacogenetic marker for MTX in use in the clinic at present, polymorphisms in *SLCO1B1* have an important role in MTX pharmacokinetics and toxicity in pediatric ALL patients, and show the most consistent and promising results. Other MTX transporters could also be interesting but further studies are needed.

Pharmacogenetics of vincristine

Vincristine is an antimitotic agent that acts by inhibiting microtubule assembly through binding to tubulin. Despite its extended use in different phases of treatment, vincristine can cause hematologic toxicity and dose-dependent neurotoxicity, its most important adverse effect [115].

To date, there have been few studies that have analyzed the association between genetic polymorphisms and vincristine toxicity in childhood ALL. Four studies have studied polymorphisms in *CYP3A5* [116–119], responsible for metabolic clearance of vincristine in ALL patients. Two of them found association between *CYP3A5*3*, **6* and **7* polymorphisms and vincristine-induced neuropathy [116,117]. However, the other two studies did not find any association [118,119]. One study analyzed *MAPT* and *ABCB1*, a transporter involved in vincristine elimination from the cells, and another study analyzed *ABCB1* only. These studies included a total of six SNPs in *MAPT* (A68504G, A80659G, C90092T, T96360C, T96214C and T102787C) and three in *ABCB1* (C3435T, C1236T and G2677A/T), without finding any association with toxicity [119,120].

The limited number of performed studies and the conflicting results have failed to define toxicity markers for vincristine. This could be due in part to the relatively small sample sizes, without sufficient power to detect small effects, and also to the fact that these studies are based on few candidate genes and few SNPs within each gene, and/or to differences in treatment guidelines. So it is difficult to draw firm conclusions.

On the other hand, some authors have shown interest in the role of miRNA expression in vincristine resistance. In a recent study, leukemic cells were collected from 61 patients with B-cell ALL and *in vitro* resistance to vincristine was analyzed. When they compared the miRNA expression profile at diagnosis of the patients whose leukemic cells were resistant to vincristine versus those that were sensitive, ten miRNAs out of 397 analyzed were differentially expressed. The most remark-

able result was the 14- to 25-fold upregulation of miR-125b, miR99a and miR-100 in resistant patients [121]. In a subsequent study, coexpression of two or three of those miRNAs in Reh cells was shown to increase *in vitro* resistance to vincristine [122]. These data suggest that changes in miRNA expression or function could have an effect on vincristine response.

In brief, studies with a sufficient set of patients following standard treatment and with a larger number of genes and polymorphisms are needed to find good markers of vincristine-induced neurotoxicity. Further miRNA studies may also reveal interesting markers.

Pharmacogenetics of asparaginase

Asparaginase is an enzyme that metabolizes extracellular asparagine into aspartic acid. Its antileukemic effect is based on relative inability of leukemic cells to synthesize asparagine, as opposed to normal cells. The depletion of asparagine diminishes protein synthesis, leading to leukemic cell death. However, some patients are resistant to asparagine and the basis for interindividual differences in asparaginase sensitivity remains unclear [123]. In addition, allergy reactions including localized pain, fever, skin rash, urticaria, respiratory distress and anaphylaxis are common. In those cases, discontinuation of treatment is usually needed and antibody production may also attenuate asparaginase activity and antileukemic effect [124].

In order to search for SNPs associated with asparaginase sensitivity, a genome-wide study was performed in 87 lymphoblastoid cell lines from HapMap and samples from 54 patients with ALL. The most significant pathway associated with *in vitro* asparaginase sensitivity was alanine/aspartate metabolism, with the two highly ranked genes being *ADSL* and *DARS*, involved in aspartate metabolism [123,125]. Recently, a 14 bp tandem repeat in *ASNS*, another gene of the aspartate metabolism, was associated with treatment outcome in a group of 264 children with ALL. In fact, carriers of the R3 genotype and with a poor response at day 15 had a lower probability of event-free survival, suggesting an interaction between the *ASNS* 14 bp genotype and early response to treatment [126].

Regarding asparaginase allergy, in a genome-wide study, SNPs in the *GRIA1* gene involved in the transmition of glutamatergic signals were among the most significantly associated in 322 ALL patients. In fact, five SNPs in this gene were also associated with allergy in the validation cohort of 163 ALL patients [124].

On the other hand, miRNA expression has also been analyzed in resistant patients versus those sensitive to asparaginase. In this case, one out of 397 analyzed miRNAs, miR-454, was found to be downregulated in resistant cases [121].

In conclusion, although the bibliography on pharmacogenetic markers of asparaginase response in pediatric ALL is limited, the results obtained seem promising. While aspartate metabolism seems to be the most important pathway, miRNA studies could also lead to interesting results.

Pharmacogenetics of glucocorticoids

Because of their lympholytic actions, glucocorticoids are included in many therapeutic regimens for the treatment of various forms of leukemia. The most common glucocorticoids in ALL treatment protocols are prednisone and dexamethasone. Although a significant number of acute lymphoblastic leukemia patients respond well to glucocorticoid treatment during initial phases, prolonged treatments sometimes results in steroid resistance [127]. In addition, adverse reactions are common, including sepsis, osteonecrosis, diabetes, myopathy, hypertension or behavioral changes [128].

The first study to report associations between genetic polymorphisms and osteonecrosis in children with ALL included *VDR Fok*I CC genotype and the TYMS low activity 2R2R 14 bp enhancer repeat genotype as risk factors of osteonecrosis [129]. More recently, in another candidate gene approach based on putative mechanisms underlying osteonecrosis risk, a polymorphism (rs6092) in the *SERPINE1*, which acts as a major control point in the regulation of fybrinolysis, was associated with risk of osteonecrosis [130]. However, these result were not replicated in later studies [131,132]. Finally, in a recent genome-wide study, polymorphisms in *ACP1* were linked to osteonecrosis for the first time [132].

Hypertension is another complication associated with glucocorticoid treatment. In a candidate gene study analyzing genes previously linked to hypertension or to the glucocorticoid pathway, 12 SNPs in eight genes (*CNTNAP2*, *LEPR*, *CRHR1*, *NTAN1*, *SLC12A3*, *ALPL*, *BGLAP* and *APOB*) were found to be significantly associated with hypertension. Several of those genes interacted with the hypothalamus–pituitary–adrenal axis [133].

Recently, the 1088G allele of the glucocorticoid receptor (*NR3C1*) has been associated with increased risk of hepatotoxicity and glucose metabolism abnormalities. The 1088G carriers were also more prone to have a combination of toxicities. However, it should be taken into consideration that all 1088G carriers, despite their increased risk of toxicity, had the advantage of being good prednisone responders and had significantly better 5-year event-free survival rates [134].

Polymorphisms in GST genes, responsible for the inactivation of xenobiotics, have also been associated with glucocorticoid response. This will be described in more detail in the following section.

Finally, recent studies have explored the role of miRNAs in glucocorticoid resistance. For instance, downregulation of miR-708 has been related to poor reduction in leukemic cells after prednisone treatment *in vivo* and overall survival in a group of 103 pediatric B-cell ALL patients [135]. In another study, low expression of miR-100 and miR-99a was correlated with a decrease in survival in 111 ALL patients. Subsequent *in vitro* experiments revealed that restoration of those two miRNAs increased dexamethasone-induced apoptosis [136]. Another plausible candidate for the regulation of response to glucocorticoids is miR-335. It is less expressed in patients with poor outcome and, *in vitro*, its overexpression sensitizes the cells to prednisolone (but not to other drugs such as vincristine) [137]. Something similar happens with miR-128b and miR-221, which are usually downregulated in ALL patients with *MLL* translocations, whose overexpression *in vitro* sensitizes MLL-AF4 cell lines to glucocorticoids [138,139]. By contrast, none of the 397 miRNAs analyzed by Schotte *et al.* were differentially expressed between patients that were resistant and sensitive to prednisolone [121].

In conclusion, several interesting results have been obtained for different asparaginase side effects but further validation is needed.

GST genes

As ALL patients are treated with complex multidrug regimens, polymorphisms affecting the clearance of several drugs would be expected to influence the risk of treatment failures [140]. Theglutathione S-transferase (GST) family of enzymes is responsible for the inactivation of xenobiotics through conjugation with glutathione. Therefore they are responsible for inactivating a wide range of drugs used in childhood ALL therapy, such as glucocorticoids, vincristine, anthracyclines and cyclophosphamide. Among the important pharmacogenetic polymorphisms described in ALL are deletions of *GSTM1* and *GSTT1* genes and the A313G substitution in the *GSTP1* gene (*GSTP1*B*).

The frequency of the homozygous deletion of the *GSTM1* gene is approximately 50% in Caucasian populations. A possible outcome of this deletion has been described in patients with ALL in several studies with controversial results [23,140–145]. It has also been related to decreased hepatotoxicity [48,68], but in other studies no association with this parameter was found [55,92]. Moreover, the same genotype has been associated with increased hyperbilirubinemia [86] and severe infections [146], associations which were not confirmed in another study [68].

Regarding the *GSTT1* gene, the homozygous deletion, found in 25% of Caucasian individuals, is associated with early response to prednisone and outcome with contradictory results [140–142,144,145]. A possible association between this polymorphism and increased hyperbilirubinemia has also been proposed [68] although this association was not found in another study that reported an association with increased gastrointestinal toxicity [55].

Finally, the *GSTP1*B* allele codes for a low-activity enzyme. A possible association between this variant and relapse has been described with inconclusive results [68,80,144,145,147,148]. The *GSTP1* 313GG genotype has also been associated with CNS toxicity [55] but this result has not been confirmed in another pediatric ALL cohort [68].

In short, the relevance of polymorphisms in GST enzymes in ALL is not clear. It might be dependent on the multidrug therapeutic strategy applied.

Conclusion & future perspective

Although a wide range of studies have been performed in the context of ALL pharmacogenetics, lack of replication has failed to produce consistent results. As a result, at the moment, *TPMT* polymorphisms are the only pharmacogenetic markers for which the evidence is sufficient to be implemented in clinical practice.

Lately, interesting results have been obtained in regards to the *SLCO1B1* gene and MTX toxicity. Polymorphisms in *SLCO1B1* arise as new potential predictors of MTX clearance and toxicity. Other transporters could also be interesting but further studies are required.

Most of the pharmacogenetic studies carried out until now have focused in coding regions. Nevertheless, these regions correspond only to approximately 1.5% of the entire genome. Consequently, a major landmark in recent studies is the analysis of regions that do not code for proteins but may have a regulatory function, such as miRNAs. Published data described in this review suggests that changes in miRNA expression or function could have an effect on treatment response.

Variations in miRNA expression and function may occur through genetic polymorphisms. Consequently, miRNA-related SNPs interfering with miRNA levels or function may lead to drug resistance or to drug sensitivity. This field of miRNA pharmacogenomics is very promising; especially if we consider that miRNA expression could be exogenously controlled by blocking the expression of upregulated miRNAs or by restoring the expression of downregulated miRNAs.

In addition, other epigenetic regulators, such as DNA methylation, histone modifications and long noncoding RNAs could also have a role in the regulation of treatment response in pediatric ALL. This developing field of pharmacoepigenetics has started to produce promising results and epigenetic variants have great potential to be used as biomarkers for personal-

ized therapy. For example, several pharmacogenes such as *ABCB1* or *CYP3A4* have been shown to be regulated by epigenetic mechanisms *in vitro* and methylation of *SLC19A1* is associated with MTX resistance in primary CNS lymphomas [149,150]. In the context of ALL, it has been reported that methylation signatures can predict relapse-free survival. In addition, the epigenetic regulation of certain genes has been linked with drug response. For instance, methylation of *CYP1B1*, which is involved in drug metabolism and steroid synthesis, was associated with its expression and with decreased survival in 33 adolescent and young adult patients [151]. Re-expression of *TWIST2*, inactivated in more than 50% of cases of ALL through promoter methylation, resulted in increased sensitivity to the chemotherapeutic agents etoposide, daunorubicin and dexamethasone [152]. On the other hand, a study reported epigenetic repression of the *BCL2L11* gene in dexamethasone-resistant pediatric ALL xenografts and in leukemic blasts from prednisolone poor responders. In this case, epigenetic repression of this gene involved in apoptosis was not due to DNA methylation but to decreased association of acetylated histone H3, which could be reversed with vorinostat treatment [153]. Along the same line, high *HDAC4* expression has been associated with prednisone poor response [154]. Its role could be due to histone modifications regulating other drug-related genes. Further studies in pediatric ALL patients are needed in order to reveal the relevance of pharmacoepigenetic markers in the context of this disease.

Executive summary

- New markers of treatment resistance or toxicity are needed in pediatric acute lymphoblastic leukemia (ALL).
- Pharmacogenetic studies can be useful for childhood ALL because treatment protocols are standardized and well-established, which makes obtaining statistically valid conclusions easier. In addition, drugs used to treat ALL have a narrow therapeutic range. This means that small changes in the genes involved in their pathways can have a great impact and, in fact, these genes are highly variable.

Pharmacogenetics of 6-mercaptopurine

- 6-mercaptopurine (6-MP) is the only drug for which the US FDA recommends adjustment of dose based on a pharmacogenetic marker (*TPMT* genotype). Clinical guidelines for this have also been developed.
- New polymorphisms in *ITPA*, *FOLH1*, *PACSIN2* and other genes of the 6-MP pathway are being considered.

Pharmacogenetics of methotrexate

- Despite the number of studies performed, there is no confirmed pharmacogenetic marker for methotrexate (MTX) that can be used in the clinic at present.
- Polymorphisms in *SLCO1B1* and other MTX transporters could be new markers of MTX pharmacokinetics and toxicity in pediatric ALL patients.

Pharmacogenetics of vincristine

- The limited number of studies performed and the conflicting results do not allow for any firm conclusions to be drawn regarding vincristine pharmacogenetics.

Pharmacogenetics of asparaginase

- There is a limited number of studies in pediatric ALL regarding asparaginase and, as a consequence, there are no established markers. Nevertheless, according to the existing results, aspartate metabolism seems to be the most important pathway.

Pharmacogenetics of glucocorticoids

- Again, the limited number of studies does not allow for firm conclusions to be drawn regarding glucocorticoid pharmacogenetics.
- Changes in miRNA expression have been related to resistance to glucocorticoids in pediatric B-cell ALL.

GST genes

- Polymorphisms in GST genes have been associated with toxicity and outcome in ALL but the results are not clear and it has been suggested that it might be dependent on the multidrug therapeutic strategy applied.

Conclusion & future perspective

- Most of the pharmacogenetic studies carried out until now have focused in coding regions, which correspond to only approximately 1.5% of the entire genome. A major landmark is the analysis of regions that do not code for proteins but may have a regulatory function.
- Changes in miRNA expression or function and other noncoding RNAs could have an effect on treatment response.
- Additional epigenetic regulators, such as DNA methylation or histone modifications could also have a role in the regulation of treatment response in pediatric ALL.
- Prospective studies in large, well-characterized, homogeneously treated populations with objective and well-collected outcome parameters are needed to grant conclusive results. In this context, international collaborative projects would be helpful.

In the next few years great effort should be put into performing large prospective studies in well-characterized, homogeneous populations with objective and well-collected outcome parameters. In this context, international collaborative projects are needed to grant conclusive results. This will be essential in order to confirm interesting results and every effort should be made to translate new pharmacogenetic markers to the clinic.

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