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TESE

FARMACOGENÉTICA DA HIDROXIUREIA EM CRIANÇAS COM ANEMIA FALCIFORME

SETONDJI COCOU MODESTE ALEXANDRE YAHOUEDEHOU

Salvador – Bahia

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RESUMO

INTRODUÇÃO: a hidroxiuréia (HU) é o tratamento de escolha para os indivíduos com anemia falciforme (AF) que apresentam o quadro clínico grave. Ela foi aprovada em 1998 pela FDA, sendo conhecida por aumentar as concentrações de hemoglobina fetal (HbF) e reduzir a polimerização da hemoglobina variante S (HbS), com melhoria do quadro clínico e de alguns parâmetros laboratoriais desses indivíduos. Entretanto, apesar desses efeitos benéficos, observa-se variabilidade na resposta ao tratamento, desde ausência de melhoria até ocorrência de toxicidade. **OBJETIVO**: o presente estudo buscou investigar a farmacogenética da HU em crianças com AF. **MÉTODO**: inicialmente, foi realizada a revisão de literatura para identificar os prováveis fatores genéticos que podem influenciar na resposta à HU, com foco nos polimorfismos em genes de enzimas metabolizadores de drogas (EMD) e proteínas transportadoras de solutos. Além disso, foram realizados dois tipos de estudo, um longitudinal e outro transversal. O estudo longitudinal envolveu 22 crianças com AF e as amostras foram coletadas antes de iniciar o tratamento com HU e durante o tratamento. O estudo de corte transversal envolveu uma casuística composta por 90 crianças com AF em uso ou não de HU. As amostras foram utilizadas para as determinações laboratoriais e a investigação dos polimorfismos em genes de EMD bem como da α-talassemia e dos haplótipos ligados ao grupo de genes da globina β^S. **RESULTADOS**: inicialmente, a revisão de literatura resultou na identificação de vários polimorfismos em genes relacionados à expressão da HbF (*HBG2*, *BCL11A*, *HMIP*, *OR51B5/6*, *MAP3K5*, *FLT1*, *KLF10*), bem como ao metabolismo de drogas e proteínas transportadoras (*CYP450*, *CAT*, *SLC*), os quais estão associados a variabilidade na resposta à HU. Além disso, foi possível propor o modelo de metabolismo diferencial da HU, que em conjunto com os dois modelos (modelo de níveis basais de HbF diferencial e modelo de suscetibilidade diferencial), já descritos na literatura, poderiam explicar a variabilidade interindividual na resposta à HU. A análise dos parâmetros laboratoriais antes e durante o tratamento pela HU demonstrou a associação da HU com o aumento da HbF em algumas crianças bem como o aumento da glicose, HDL-C, proteína total e albumina e, redução da AST. Além disso, os alelos variantes 1934A, -21T, -262T e A dos polimorfismos *CYP2D6* 1934G>A, *CAT* -21A>T, *CAT* -262C>T e *SLC14A1* G>A rs2298720, respectivamente, estiveram associados ao aumento dos efeitos da HU. **CONCLUSÃO**: o presente estudo demonstra a importância de se avaliar o perfil metabólico durante a terapia com a HU, além dos parâmetros clássicos investigados, visto que o medicamento parece possuir amplo espectro de ação. Os resultados de associação entre os polimorfismos em genes de EMD e proteínas transportadoras de solutos corroboram o modelo proposto (modelo de metabolismo diferencial da HU) e demonstram a necessidade de se identificar genes candidatos envolvidos no metabolismo da HU.

Palavras-chave: Anemia falciforme, Hidroxiuréia, Resposta, Variabilidade, Farmacogenética.

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ABSTRACT

INTRODUCTION: hydroxyurea (HU) is the treatment of choice for individuals with sickle cell anemia (SCA) who have a severe clinical profile. Approved in 1998 by the FDA, HU is known to increase fetal hemoglobin (HbF) concentrations and reduce polymerization of variant hemoglobin S (HbS), with improvement in the clinical profile, as well as some laboratory parameters. However, despite these benefits, there is variation in response to treatment, from non-improvement to toxicity. **OBJECTIVE**: the present study aimed to investigate the pharmacogenetics of HU in children with SCA. **METHOD**: first, we performed a literature review for identifying the candidate genetic factors, which may influence HU response, focusing on polymorphisms in genes encoding enzymes metabolizing drugs (EMD) and solute carriers. In addition, we performed 02 types of study: longitudinal and cross-sectional. The longitudinal study involved 22 children with SCA, whose blood samples were collected before and during HU use. The cross-sectional study involved 90 children with SCA with or without HU use. Blood samples collected were used for laboratory determinations and investigation of polymorphisms in genes encoding EMD, α-talassemia and β^S haplotypes. **RESULTS**: the literature review resulted in the identification of several polymorphisms in genes related to HbF expression (*HBG2*, *BCL11A*, *HMIP*, *OR51B5/6*, *MAP3K5*, *FLT1*, *KLF10*), as well as drug metabolism and solute carriers (*CYP450*, *CAT*, *SLC*), which are associated with variations in HU response. Furthermore, we proposed the differential HU metabolism model, which together with the two models (differential baseline HbF model and differential susceptibility model), previously described in the literature, could explain the inter-individual variability in HU response. The laboratory parameters analysis, before and during HU treatment, demonstrated the association of HU with increase in HbF in a fraction of the children as well as increases in glucose, HDL-C, protein and albumin and, reduction in AST. In addition, variants alleles 1934A, -21T, -262T and A of polymorphisms *CYP2D6* 1934G>A, *CAT* -21A>T, *CAT* -262C>T and *SLC14A1* G>A rs2298720, respectively, were associated with increase in HU effects. **CONCLUSION**: the present study demonstrates the importance of evaluate metabolic profile during the HU therapy, in addition to classical parameters investigated, since the drug seems possess large spectra of action. Moreover, the results of the association between the polymorphisms in genes encoding DME and solute carriers corroborate our model (differential HU metabolism model) and demonstrate the necessity to identify genes candidates involved in HU metabolism.

Keywords: Sickle cell anemia, Hydroxyurea, Response, Variability, Pharmacogenetics.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

AF:	Anemia falciforme
ATP:	Adenosina trifosfato
AVC:	Acidente vascular cerebral
BEN:	Haplótipo do grupo de genes da globina beta do tipo Benin
CAM:	Haplótipo do grupo de genes da globina beta do tipo Camarões
CAR:	Haplótipo do grupo de genes da globina beta do tipo Bantu
CAT:	Catalase
CEP:	Comitê de ética em pesquisa
cGMP:	Guanilato monofosfato cíclico
CNS:	Conselho Nacional de Saúde
CYP2D6:	Citocromo P450 2D6
CYP3A4:	Citocromo P450 3A4
DF:	Doença falciforme
DMT:	Dose máxima tolerada
DNA:	Ácido dexosirribonucleotídeo
2,3-DPG:	2,3-difosfoglicerato
EMD:	Enzima metabolizadora de drogas
EROs:	Espécies reativas de oxigênio
EUA:	Estados Unidos da América
GST:	Glutationa S transferase
FDA:	Agência de administração de droga e alimentos
Hb:	Hemoglobina
HbA:	Hemoglobina A
HBB:	Gene da globina beta
HbF:	Hemoglobina Fetal
HbS:	Hemoglobina S
HEMOBA:	Fundação de Hematologia e Hemoterapia da Bahia
HUPES:	Hospital Universitário Professor Edgar Santos
HU:	Hidroxiuréia
HSR:	Hospital São Rafael
H_2O:	Água
H_2O_2:	Peroxido de hidrogênio

LDH:	Lactato desidrogenase
MI:	Metabolizador intermediário
ML:	Metabolizador lento
MR:	Metabolizador rápido
MS:	Ministério da Saúde
MU:	Metabolizador ultrarrápido
NO:	Óxido nítrico
OCTN1:	Transportadora de cátion orgânico
OMS:	Organização Mundial da Saúde
PNTN:	Programa Nacional de Triagem Neonatal
QTL:	Lócus traço quantitativo
SEN:	Haplótipo do grupo de genes da globina beta do tipo Senegal
SLC:	Transportadora de soluto
SNP:	Polimorfismo de um único nucleotídeo
STA:	Síndrome Torácica Aguda
SULT:	Sulfotransferase
sGC:	Guanilato ciclase solúvel
TCLE:	Termo de consentimento livre e esclarecido
UGT:	UDP glucurunosiltransferase
UTB:	Transportadora de ureia B

LISTA DE SÍMBOLOS

α:	alfa
β:	beta
γ:	gama

SUMÁRIO

1. INTRODUÇÃO

1.1. A ANEMIA FALCIFORME: CARACTERIZAÇÃO E FISIOPATOLOGIA

A doença falciforme (DF) é uma doença monogênica, autossômica recessiva, caracterizada pela presença da hemoglobina variante S (HbS) em homozigose ou em associação com outra variante (HbC, HbD, HbE, etc.) ou talassemias. A forma mais grave da doença é a anemia falciforme (AF), caracterizada pela homozigose do alelo beta S e, consequentemente, da HbS (HbSS) (KATO et al., 2018). A HbS é decorrente da substituição de adenina por timina (G**A**G>G**T**G) no sexto códon do gene da globina beta (*HBB*), localizado no cromossomo 11 (AL-SUBAIE et al., 2009; FERNANDES et al., 2010; FRANCESCHI; CAPPELLINI; OLIVIERI, 2011; HATZIPANTELIS et al., 2013). Essa substituição leva à troca do ácido glutâmico (aminoácido polar) pela valina (aminoácido não polar) na sexta posição da cadeia polipeptídica beta (CALDAS et al., 2010; CHIKEZIE; CHIKEZIE; AMARAGBULEM, 2011).

Em condição de hipóxia, a cadeia lateral hidrofóbica da valina acopla-se na dobra hidrofóbica formada a partir da leucina β88 e da fenilalanina β85 adjacente (FOLASHADE; OMOREGIE, 2013), favorecendo a polimerização da HbS. Esse fenômeno altera a conformação bicôncava das hemácias, que adquirem o formato de foice ou drepanócito (Figura 1) (CALDAS et al., 2010; FELIX; SOUZA; RIBEIRO, 2010; PROENÇA-FERREIRA et al., 2014).

A HbS polimerizada interfere na bomba de Ca^{2+}, responsável pela manutenção da integridade da membrana, diminuindo a sua eficiência, favorecendo o envelhecimento precoce das hemácias e leva à rigidez da sua membrana. Para manter a integridade celular, faz-se necessário o aumento de adenosina trifosfato (ATP), que resulta no acúmulo do 2,3-difosfoglicerato (2,3-DPG) nas hemácias, que devido a sua afinidade pela hemoglobina se liga a essa protéina, facilitando a liberação rápida do oxigênio, com ocorrência de hipóxia parcial na hemácia. A hipóxia e a absorção subsequente do dióxido de carbono (CO_2) são responsáveis pela acidose local, falcização precoce, aumento da viscosidade sanguínea, diminuição do fluxo sanguíneo e hipercoagulação (FOLASHADE; OMOREGIE, 2013).

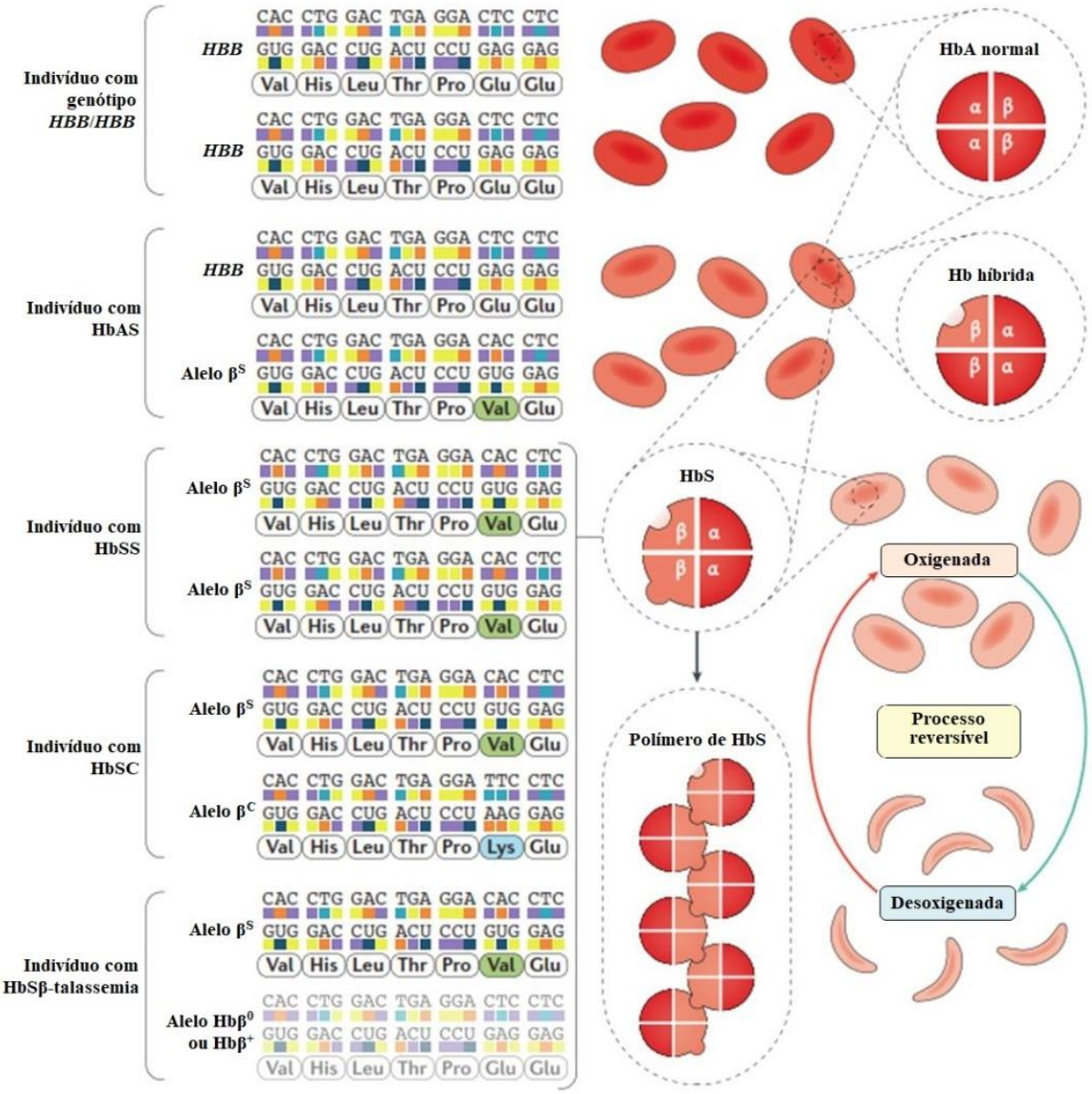


Figura 1: Representação esquemática da origem genética das hemoglobinas variantes S e C, bem como da presença dos heterozigotos e de diferentes genótipos da doença falciforme.
Os indivíduos com HbSS, HbSC, HbSβ-talassemia apresentam hemácia em formato de foice, devido a polimerização da HbS, em condição de desoxigenação. Já os indivíduos com HbAS apresentam hemácia normal, em formato bicôncavo, uma vez que possuem também Hb normal a qual impede a falcização, formando híbrido com a HbS. Adaptada de KATO et al., 2018.

A ocorrência de episódios vaso-oclusivos crônicos é responsável pelas crises dolorosas, síndrome torácica aguda (STA), acidente vascular cerebral (AVC), osteonecrose, úlceras de membros inferiores, sequestro esplênico, priapismo, retinopatia, insuficiência renal crônica e doença cardiovascular, apresentados pelos indivíduos com AF (BANDEIRA et al., 2004; FELIX; SOUZA; RIBEIRO, 2010; PROENÇA-FERREIRA et al., 2014). Observa-se também um quadro de hipertensão pulmonar (CHO et al., 2010).

As manifestações clínicas são baseadas em três mecanismos inter-relacionados: 1) adesão de eritrócitos, granulócitos, monócitos e plaquetas ao endotélio vascular; 2) fenômenos inflamatórios crônicos; 3) produção de citocinas pró-inflamatórias e alterações no metabolismo do óxido nítrico (NO) (Figura 2) (ZAGO; PINTO, 2007). Essas características clínicas aparecem, principalmente, após os seis (06) primeiros meses de vida, quando a concentração de HbF é reduzida a níveis normais (≤ 2,0 %) (ZAGO; PINTO, 2007). Sabe-se que a HbF possui afinidade maior pelo oxigênio e isso está relacionada à diminuição na troca de oxigênio entre a vascultura e os órgãos, aumento da biodisponibilidade do oxigênio e redução da polimerização da HbS, mesmo em condição de desoxigenação (KOZBERG; HILLMAN, 2016; KAUFMAN; LAPPIN, 2020).

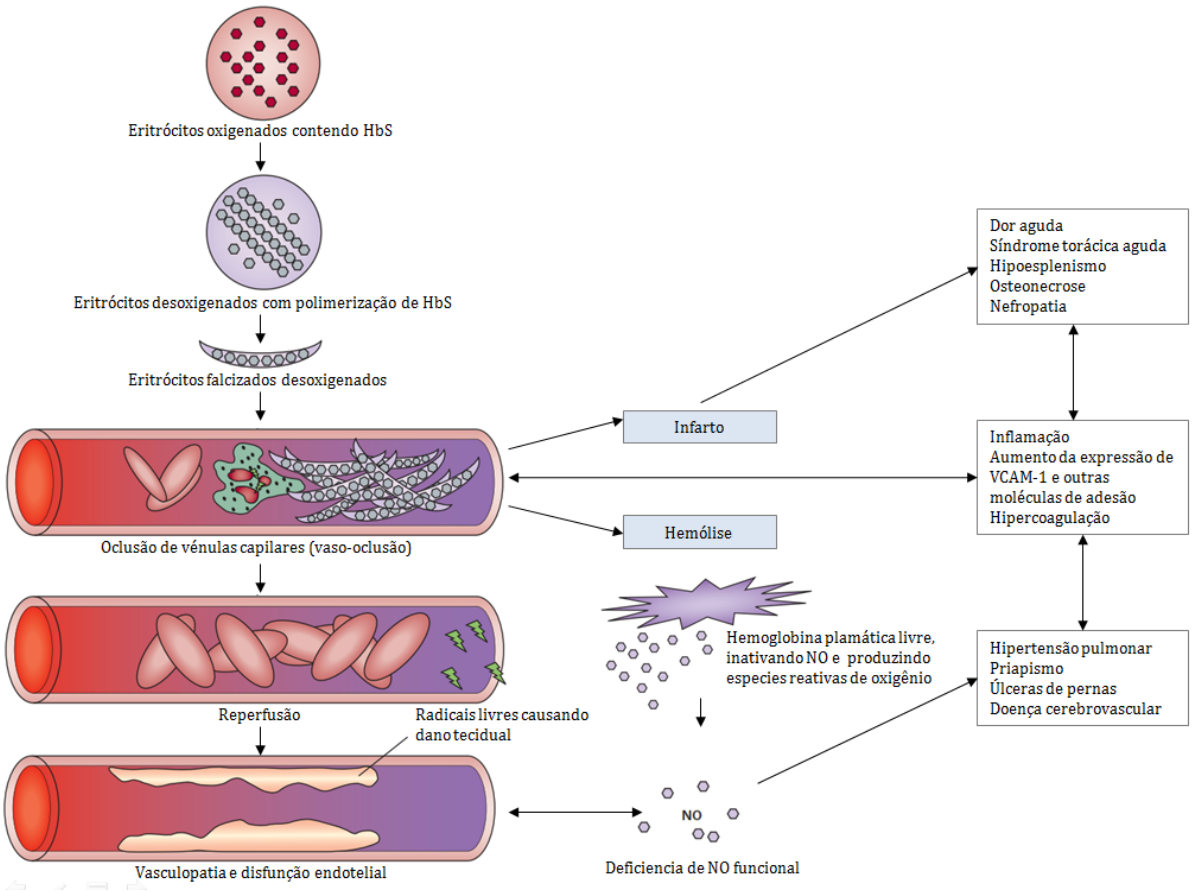


Figura 2: Fisiopatologia da anemia falciforme, principais etapas do processo de vasculopatia e disfunção endotelial.
A hemácia falcizada sofre hemólise levando à redução da biodisponibilidade do óxido nítrico (NO). Essas hemácias interagem também com o endotélio vascular e outras células dificultando a reperfusão sanguínea. Dessa forma, os indivíduos com anemia falciforme passam a apresentar um quadro clínico heterogêneo, caracterizada por síndrome torácica aguda, crise de dor, priapismo, úlcera maleolar, acidente vascular cerebral, etc. Adaptada de REES et al., 2010.

1.2. FATORES MODULADORES DA ANEMIA FALCIFORME

Vários fatores genéticos foram descritos como moduladores da AF, tais como o tipo de haplótipos ligados ao grupo de genes da globina β^S. Esses haplótipos são caracterizados pela associação não casual de sítios de clivagem por endonucleases de restrição em torno do grupo de genes da globina β^S e foram denominados de acordo com as regiões geográficas e origem étnica dos indivíduos investigados (ADORNO et al., 2008; SHIMAUTI et al., 2015). Foram descritos cinco haplótipos associados à globina β^S: Benin (BEN), República Centro-Africana (CAR) ou Bantu, Senegal (SEN), Camarões (CAM) e Árabe-indiano (Figura 3) (STUART; NAGEL, 2004; OKUMURA et al., 2016; ALELUIA et al., 2017). Dentre eles destacam-se o haplótipo CAR, que está associado a concentrações de HbF<5% e, consequentemente, a sintomas mais graves da doença, bem como o haplótipo SEN, que está associado a concentrações de HbF>15% e ao quadro clínico menos grave. O haplótipo BEN está associado a concentrações de HbF entre 5% e 15% e a uma clínica intermediária (ADORNO et al., 2008; CAJADO et al., 2011; LAURENTINO et al., 2014).

Outro fator modulador do quadro clínico dos indivíduos com AF é a co-herança da alfa (α)-talassemia, caracterizada pela ausência (α^0-talassemia) ou redução (α^+-talassemia) na síntese da globina α, cujo gene está localizado no cromossomo 16 (CANÇADO, 2007; GONÇALVES, 2014; LAURENTINO et al., 2014; TAHER; WEATHERALL; CAPPELLINI, 2018). A α-talassemia está associada à redução da concentração de HbS, hemólise e aumento do hematócrito. Além disso, os indivíduos com AF e α-talassemia apresentam sobrevida mais elevada das hemácias e redução na ocorrência de úlcera maleolar. Entretanto, observa-se o aumento no número de crises dolorosas, devido ao aumento na viscosidade sanguínea, retinopatia e osteonecrose (GONÇALVES, 2014). Foi também descrita a participação de loci traço quantitativos (QTLs), como *HBG2*, *BCL11A* e *HBS1L-MYB,* na modulação da AF (ALELUIA et al., 2017).

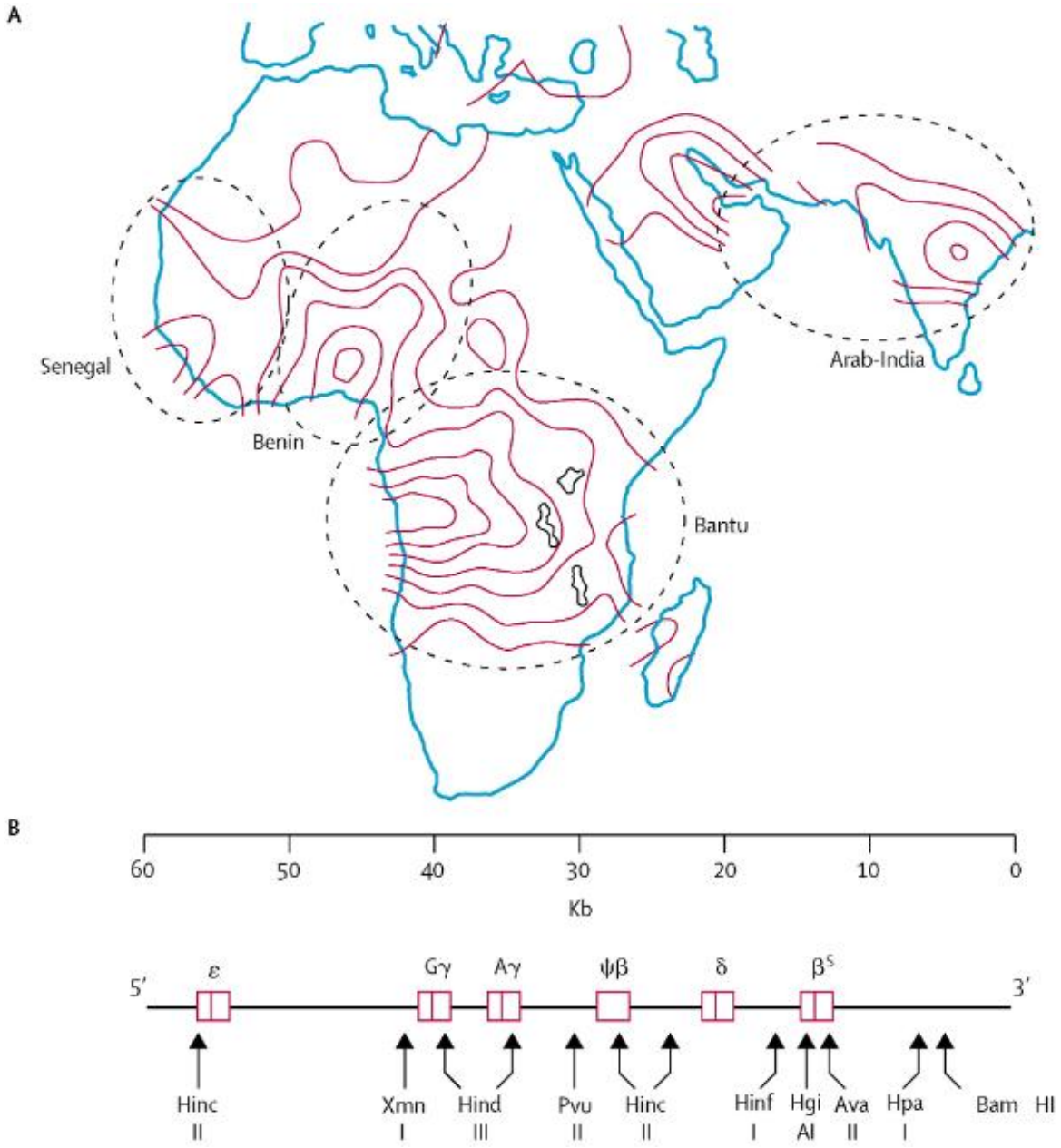


Figura 3: Representação esquemática dos haplótipos ligados ao grupo de genes da globina beta S e sua distribuição geográfica. Adaptada de STUART; NAGEL, 2004.

O estresse oxidativo também tem sido considerado fator modulador da AF, uma vez que aumenta a adesão de hemácias, leucócitos e plaquetas ao endotélio vascular, com aumento do processo de vaso-oclusão (CHAVES; LEONART; DO NASCIMENTO, 2008). Sabe-se que indivíduos com hemoglobinopatias apresentam níveis elevados de espécies reativas de oxigênio (EROs) quando comparados aos indivíduos com o genótipo normal HbAA, e isso contribui para a peroxidação lipídica (TORRES et al., 2012).

1.3. TRATAMENTO

As estratégias para o tratamento da AF são baseadas, principalmente, em medidas profiláticas que têm como objetivos: reduzir as crises dolorosas, com a utilização de analgésicos, antipiréticos, hidratação intravenosas e o uso de terapia de transfusão bem como reduzir a ocorrência de infecções, através do uso de antibióticos orais como a penicilina. Além disso, é recomendado o uso profilático do ácido fólico que auxilia na recuperação celular (MCGORON et al., 2000; STOCKER, 2003). As transfusões sanguíneas e a suplementação com ácido fólico são os tratamentos mais comumente utilizados no acompanhamento dos indivíduos com AF (TORRES et al., 2012). O uso da eritropoietina e de inibidores da lactato desidrogenase (LDH) mostraram-se também eficazes no monitoramento da AF (OKPUZOR et al., 2008). O tratamento de cura atualmente disponível é o transplante de medula óssea ou células hematopoiéticas. No entanto, esse tratamento apresenta custo e relação risco/benefício elevados (FOLASHADE; OMOREGIE, 2013). Além disso, tem sido desenvolvida a terapia gênica com o objetivo de curar os indivíduos com AF, sendo que os estudos realizados demonstraram resultados preliminares encorajadores (WARE et al., 2017).

Várias abordagens terapêuticas têm sido adaptadas visando a descoberta de agentes inibidores da polimerização da HbS e, em consequência, prevenção ou redução na ocorrência dos eventos clínicos como crise de dor, STA, priapismo, úlcera maleolar, AVC, bem como de uso de hemoderivado e número de internação (IYAMU; TURNER; ASAKURA, 2002). Neste contexto, o oxigênio, o monóxido de carbono e o nitrito de sódio têm sido utilizados para reduzir a quantidade de desoxihemoglobina (FOLASHADE; OMOREGIE, 2013), assim como os indutores de HbF, tais como butiratos, decitabina e hidroxiuréia (HU) (MCGORON et al., 2000; STOCKER, 2003). Entre esses, a HU era o único medicamento liberado pela *United State of America Food and Drug Administration* (*USA FDA*) para tratar os indivíduos com AF que apresentam quadro clínico mais grave, até a aprovação recente da L-Arginina (NIIHARA et al., 2018).

1.4. HIDROXIUREIA

1.4.1. Uso, efeitos e mecanismos de ação

A HU ou hidroxicarbamida (Figura 4) foi sintetizada pela primeira vez por DRESSLER e STEIN em 1869 e começou a ser utilizada no tratamento de indivíduos com doenças neoplásicas, leucemia mielóide crônica, psoríase e policitemia vera após sua aprovação pela FDA em 1967 (BANDEIRA et al., 2004; KOVACIC, 2011). O uso da HU para o tratamento do câncer explica-se pela sua capacidade de inibir a ribonucleotídeo redutase, bloqueando a

síntese de DNA e mantendo as células na fase S do ciclo celular (BANDEIRA et al., 2004). Após demonstrar seu potencial antifalcizante, ela começou a ser utilizada, na década de 80, nos protocolos de tratamento de adultos com AF e, posteriormente, em crianças, mantendo a mesma eficácia (BANDEIRA et al., 2004). O primeiro estudo randomizado e multicêntrico foi realizado na década de 1990. Esse estudo comprovou a eficácia do uso da HU em indivíduos com AF e teve grande impacto sobre o monitoramento da doença (SILVA-PINTO et al., 2013). Em 1998, a HU foi aprovada pela FDA para o tratamento de indivíduos adultos com AF, clinicamente graves, que apresentavam três ou mais episódios dolorosos graves nos últimos 12 meses, história de STA, Hb<6g/dL, ou qualquer outra complicação vaso-oclusiva grave como priapismo e hipertensão pulmonar (MEHANNA, 2001; KOVACIC, 2011). A HU está disponível em cápsulas de gel sólido contendo 500mg do princípio ativo. O tratamento começa com uma dose única diária de 10-15mg/kg considerando o peso real ou ideal, seguida de um aumento de 5mg a cada 4 a 6 semanas até atingir a dose máxima tolerada (DMT) que não deve ultrapassar 35mg/kg/dia (CANÇADO et al., 2009).

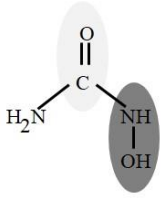


Figura 4: Estrutura química da hidroxiuréia.
Fonte: YAHOUEDEHOU et al., 2018.

Na AF, o principal efeito da HU é o aumento nas concentrações de HbF (BANDEIRA et al., 2004; TORRES et al., 2012; NOTTAGE et al., 2013). Entretanto, o mecanismo envolvido nesse processo não está completamente elucidado (SILVA-PINTO et al., 2013). Sabe-se que, a HU reduz os produtos oxidados pelo grupo heme e estimula a produção de NO (GLADWIN; SCHECHTER, 2001; CHO et al., 2010), o que leva à ativação da guanilato ciclase solúvel (sGC), aumento na produção de guanilato monofosfato cíclico (cGMP) e reativação/transcrição do gene gama (γ) da HbF (Figura 5) (IKUTA; AUSENDA; CAPPELLINI, 2001; COKIC et al., 2003). Além disso, foi relatado que a HU pode induzir a produção da HbF através da ativação de mecanismos de regulação epigenética (WALKER et al., 2011).

O aumento nas concentrações de HbF leva à redução da polimerização da HbS e da densidade eritrocitária, com inibição da falcização das hemácias (SILVA-PINTO et al., 2013), o que está associado à redução na frequência de episódios vaso-oclusivos, no número de crises de dor, STA, AVC, internações e transfusões sanguíneas, bem como da taxa de mortalidade (BANDEIRA et al., 2004; TORRES et al., 2012; NOTTAGE et al., 2013). No entanto, em

alguns casos, a melhora clínica ocorre antes do aumento da HbF, sugerindo a existência de outros mecanismos envolvidos. Entre outros efeitos observados, pode-se incluir: (a) redução no número de leucócitos, plaquetas e reticulócitos; (b) redução na capacidade de adesão dos drepanócitos, com redução na expressão de moléculas de adesão de superfície; (c) indução na produção de NO; e (d) aumento do volume celular dos drepanócitos (BANDEIRA et al., 2004; SILVA-PINTO et al., 2013).

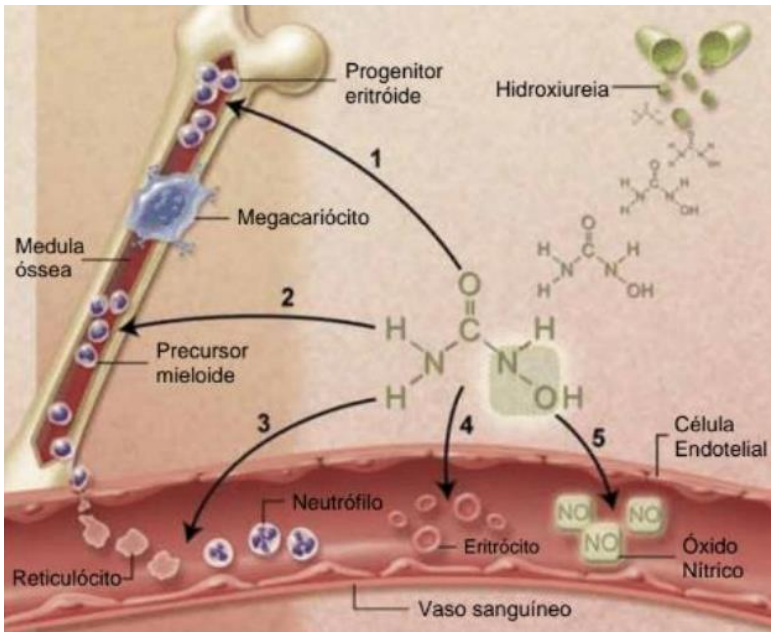


Figura 5: Principais mecanismos de ação da hidroxiuréia.
1) Indução da produção de HbF através da ativação da guanilate ciclase solúvel; 2) Redução de neutrófilos e reticulócitos através da inibição da ribonucleotídeo redutase e citotoxicidade; 3) Diminuição da adesão dos neutrófilos e reticulócitos ao endotélio vascular; 4) Redução da falcização e da hemólise; 5) Aumento do óxido nítrico. Adaptada de WARE, 2010.

O aumento do volume corpuscular médio (VCM) tem sido observado após quatro a seis semanas do início do tratamento com a HU, sendo um parâmetro hematológico que tem sido correlacionado à redução dos episódios vaso-oclusivos (SILVA-PINTO et al., 2013). Embora a HU apresente eficácia elevada para a maioria dos indivíduos com AF, observa-se variabilidade na resposta terapêutica (WARE et al., 2002; WARE, 2010).

1.4.2. Farmacocinética e toxicidade

Estudos de farmacocinética em humanos demonstraram que a HU possui um volume de distribuição aparente aproximadamente igual à da água corporal, devido a sua propriedade hidrossolúvel, com concentrações séricas máximas atingidas entre 3 e 6h (STEINBERG, 2002;

SASSI et al., 2010). Após a administração oral em pacientes com câncer, recebendo doses de 20-30 mg/kg, 79% da dose é encontrada disponível na circulação sistêmica (SASSI et al., 2010). A biodisponibilidade oral absoluta média da HU foi estimada em 108%, com variabilidade interindividual baixa em pacientes com tumores sólidos avançados (RODRIGUEZ et al., 1998). Cerca de 80% da HU disponível é encontrada ligada a proteínas séricas e atravessa a barreira celular por difusão passiva (SASSI et al., 2010). Sabe-se que 35% da dose administrada por via oral em pacientes com função renal normal é diretamente excretada na urina sem ser metabolizada (YAN et al., 2005), enquanto 30 a 50% da dose administrada por via intraperitoneal é excretada na urina como uréia (SASSI et al., 2010). Além disso, 0,1 a 0,8% do medicamento é recuperado nas fezes, após administração por via intraperitoneal (SASSI et al., 2010). Estudos experimentais em ratos demonstraram que em presença do nucleotídeo piridina reduzida (NADH ou NADPH), a HU é biotransformada em uréia no fígado e rins como redução direta, catalisada pelas enzimas hepáticas. Tem sido relatado que além da via da uréia, a HU pode ser metabolizada pelo sistema de citocromo P-450 dependente de monooxigenase, gerando produtos genotóxicos (STEINBERG, 2002).

Em relação à toxicidade, sabe-se que a HU induz a formação de radicais livres com potencial citotóxico e pode causar danos às hemácias e granulócitos (KOVACIC, 2011). Após tratamento durante longo prazo com a HU, os efeitos adversos comumente observados incluem a granulocitopenia e anemia, fadiga, dor de cabeça, náuseas, vômitos, diarreia ou febre (LATAGLIATA et al., 2012). Estudos experimentais realizados em animais tratados pela HU indicaram a ocorrência de hepatotoxicidade, além de lesão ao DNA entre outros efeitos adversos. Também foi sugerido que a HU pode afetar a fertilidade, reduzindo a quantidade de esperma, bem como alterando a mobilidade e a morfologia dos espermatozoides (KOVACIC, 2011). O uso de HU em indivíduos com AF, talassemia β maior (β^0) e intermediária (β^+) tem sido associado a efeitos colaterais dermatológicos, neurológicos, gastrointestinais e hematológicos, bem como aumento discreto nas concentrações de creatinina, sugerindo possível lesão renal (GHASEMI; KEIKHAEI; GHODSI, 2014).

1.5. FATORES MODULADORES DA RESPOSTA TERAPÊUTICA E METABOLISMO DE DROGAS

O alcance do alvo terapêutico no organismo é influenciado por vários fatores, tais como a idade, o estado fisiológico, a concentração do fármaco, a taxa de absorção, proporção de ligação a proteínas séricas, distribuição para os órgãos, transferência através da membrana plasmática, interação com receptores e organelas celulares, biotransformação e excreção

(AUDI; PUSSI, 2000). O metabolismo de drogas é realizado por proteínas cuja produção depende da transcrição e tradução de alguns genes nas diferentes etapas da farmacocinética (AUDI; PUSSI, 2000; CHO et al., 2014). Elas são classificadas em enzimas metabolizadoras de fase I (oxidação e hidrólise) e II (conjugação), e proteínas transportadoras, que estão envolvidas nos mecanismos de excreção/eliminação de drogas ou metabólitos (KELLY; PERENTESIS; CHILDREN'S ONCOLOGY GROUP, 2002; BOŽINA; BRADAMANTE; LOVRIĆ, 2009; ZUPA et al., 2009). As enzimas de fase I, principalmente a superfamília do citocromo P450 (CYP), seguida de oxidases, hidroxilases, epoxigenases, redutases, monooxigenases, peroxidases, desidrogenases e hidrolases, produzem grupos funcionais que servem de sítios de conjugação pelas enzimas de fase II. Essas, incluem as uridina glucuronosiltransferases (UGT), sulfotransferases (SULT), glutationa S transferases (GST), N-acetiltransferases (NAT), epoxidases, aciltransferases e transaminases (BOŽINA; BRADAMANTE; LOVRIĆ, 2009; ZUPA et al., 2009). As enzimas de faz II catalisam então as reações de biotransformação de produtos geralmente hidrfóbicos em produtos hidrofílicos, possibilitando a excressão adequada dos metabólitos intermediários gerados na fase I. Além disso, sabe-se que as proteínas transportadoras, tais como as proteínas membranares transportadoras de soluto, *solute carrier* (SLC) e *ATP-binding cassette transporter* (ABC *transporter*), expressas em vários tecidos, desempenham papel crucial na absorção, distribuição e excreção de xenobióticos (BOŽINA; BRADAMANTE; LOVRIĆ, 2009; BOCK, 2014).

1.6. POLIMORFISMOS EM GENES DE ENZIMAS METABOLIZADORAS DE DROGAS

A presença de polimorfismos em genes que codificam as enzimas metabolizadoras de drogas (EMD) é extremamente comum na população. Esses polimorfismos podem causar alterações no processo de desintoxicação de fase I e II (KELLY; PERENTESIS; CHILDREN'S ONCOLOGY GROUP, 2002; BU et al., 2004), além de afetar a farmacocinética e farmacodinâmica das drogas (BELLE; SINGH, 2008). Tem sido demonstrado que polimorfismos em gnese de EMD são responsáveis pelas variações interindividuais comumente observadas na biotransformação de drogas bem como na resposta ao tratamento por determinados fármacos (AUDI; PUSSI, 2000; ZUPA et al., 2009). Dessa forma, eles podem contribuir para desfechos inesperados pós-administração, em função da concentração plasmática anormal da droga, variando de ausência de resposta terapêutica até toxicidade (AUDI; PUSSI, 2000).

De acordo com o perfil metabólico, os indivíduos podem ser classificados em metabolizadores lentos (ML), intermediários (MI), rápidos (MR) e ultrarrápidos (MU) (AUDI;

PUSSI, 2000). A perda do alelo ativo leva ao fenótipo ML, o que torna o indivíduo suscetível a mais eventos adversos em doses habituais, com redução na concentração plasmática do metabólito ativo da droga, podendo não responder ao tratamento (AUDI; PUSSI, 2000). O fenótipo MI resulta da homozigose ou heterozigose para o alelo variante, enquanto o indivíduo que apresenta o fenótipo MR possui os dois alelos totalmente ativos e apresenta resposta adequada à dose de referência ou padrão (BOŽINA; BRADAMANTE; LOVRIĆ, 2009). Os indivíduos com o fenótipo MU têm mais de duas cópias do gene ativo e, devido ao aumento no metabolismo, podem necessitar de doses medicamentosas excessivamente maiores do que as usuais para alcançar o efeito terapêutico desejado (AUDI; PUSSI, 2000; BOŽINA; BRADAMANTE; LOVRIĆ, 2009).

1.6.1. Os citocromos P450 3A4 e 2D6

O sistema citocromo P450 (CYP450) é o principal sistema enzimático envolvido no metabolismo de substâncias endógenas e exógenas, desempenhando papel fundamental na biotransformação. Os substratos fisiológicos/endógenos incluem os esteroides, ácidos graxos, prostaglandinas, leucotrienos, e aminos biogênicos, enquanto os substratos exógenos incluem drogas, toxinas de plantas medicinais e produtos químicos tóxicos (MARUF et al., 2012; SAYED; IMAM, 2012). Sabe-se que o CYP450 está distribuído em diversos tecidos e órgãos, tais como fígado, intestino, pulmão, rim, cérebro, duodeno e placenta (AUDI; PUSSI, 2000; SAYED; IMAM, 2012).

As isoenzimas do CYP450 são classificadas dentro de famílias e subfamílias, de acordo com as semelhanças nas sequências protéicas (AUDI; PUSSI, 2000). As famílias são constituídas por enzimas que compartilham pelo menos 36% da sequência de aminoácidos e as subfamílias para enzimas com mais de 70% de similaridade. De acordo com a nomenclatura usualmente utilizada, o prefixo CYP é empregado para designar o sistema citocromo P450; o número arábico depois do prefixo CYP indica a família, a letra representa a subfamília e o último dígito é um número arábico que designa a isoenzima específica (por exemplo, CYP2D6) (AUDI; PUSSI, 2000). Os CYPs podem ser encontrados em mais de 50 formas, classificados em 3 grandes grupos; o segundo grupo que inclui as famílias CYP1 a 3, é menos conservado e responsável pelo metabolismo de fase I de 70 % a 80 % das drogas utilizadas na prática clínica com participação no metabolismo de inúmeros xenobióticos (AUDI; PUSSI, 2000; BOŽINA; BRADAMANTE; LOVRIĆ, 2009). As isoenzimas dessas famílias estão envolvidas em diversas reações do metabolismo de fase I, incluindo hidroxilação, demetilação e dealquilação e essas reações não são específicas para uma isoenzima (AUDI; PUSSI, 2000). Dentre as

enzimas das famílias CYP1 a 3 destacam-se as CYP3A4 e CYP2D6, duas das mais importantes EMD de fase I (SASSI et al., 2010).

O gene da CYP3A4, que metaboliza 50% das drogas, está localizado no cromossomo 7 (7q22.1) e é a CYP mais abundante no fígado. Além do fígado, o gene também se expressa no intestino delgado e duodeno. Dentre os substratos metabolizados por esta enzima, estão os hormônios esteroides, analgésicos, anti-histamínicos, antitumorais e imunossupressores. Por outro lado, a CYP3A4 também tem a função de inibir drogas e substâncias químicas como antifúngicos e inibidores de protease do HIV (BOŽINA; BRADAMANTE; LOVRIĆ, 2009). O polimorfismo mais estudado é o *CYP3A4*1B* (MARUF et al., 2012), que aumenta a transcrição do gene bem como a atividade da enzima (JIN et al., 2005; BHATNAGAR et al., 2010; HE et al., 2014).

O gene *CYP2D6* que está localizado no cromossomo 22 (22q13.2) contém 9 éxons e é mais expresso no fígado, intestino delgado e duodeno e em pequena quantidade em órgãos e tecidos como testículos, pele, ovário, rim, próstata, cérebro, endométrio, medula óssea, etc. A enzima CYP2D6 metaboliza 25% de todas as drogas conhecidas, a exemplo de antidepressivos, antiarrítmicos, analgésicos e anticancerígenos e como característico de sua família, seu gene é altamente polimórfico, influenciando a atividade da enzima (BOŽINA; BRADAMANTE; LOVRIĆ, 2009; SAYED; IMAM, 2012). Um dos alelos variantes mais importantes é o *CYP2D6*4*, que contribui para a diminuição da atividade dessa enzima, aumentando os riscos de intoxicação e reações adversas (BOŽINA; BRADAMANTE; LOVRIĆ, 2009; SAYED; IMAM, 2012).

1.6.2. A proteína transportadora de soluto SLC14A1

Os SLCs são famílias de proteínas transportadoras de solutos (EBBINGHAUS et al., 2017). A SLC14A1 também conhecida como *Urea Transporter - B* (UT-B) é uma família de transportadores de ureia, importante para a regulação da concentração da urina, devido a sua capacidade de filtrar a ureia (GARCIA-CLOSAS et al., 2011; STEWART, 2011; MATSUDA et al., 2015; EBBINGHAUS et al., 2017). O gene *SLC14A1* que codifica essa proteína está localizado no cromossomo 18 (18q12.3), contém 13 éxons e é amplamente expresso na membrana plasmática dos eritrócitos e pouco expresso nas células endoteliais do vaso reto descendente dos rins, no cérebro, orelha, testículos, intestino e bexiga urinária (SANDS, 2002; GARCIA-CLOSAS et al., 2011; RAFNAR et al., 2011; EBBINGHAUS et al., 2017). As proteínas transportadoras de ureia facilitam o seu transporte passivo, conduzido por um

gradiente de concentração através da membrana celular de algumas células renais e extra renais (ESTEVA-FONT; ANDERSON; VERKMAN, 2015).

Sabe-se que pequenas moléculas análogas da ureia podem também passar pelo canal UT-B como: a metilureia, formamida, metilformamida, acetamida, carbonato de amônio e acrilamida (ESTEVA-FONT; ANDERSON; VERKMAN, 2015). Polimorfismos em genes codificadores de proteínas transportadoras de ureia da família SLC14A foram associados a variabilidade na farmacocinética da HU em indivíduos com AF (ANGONA et al., 2013). A perda da função da UT-B, decorrente de alterações no gene *SLC14A1*, foi associada à resistência dos eritrócitos à lise na presença de concentração elevada de ureia (GARCIA-CLOSAS et al., 2011; ESTEVA-FONT; ANDERSON; VERKMAN, 2015). Walker e colaboradores demonstraram a correlação positiva entre o aumento da expressão de *UT-B* e a indução do gene da globina γ (*HBG*) nas células eritróides (WALKER; OFORI-ACQUAH, 2017). Além disso, polimorfismos no gene *UT-B* estão associados a variações nos parâmetros farmacocinéticos da HU em indivíduos com DF (WARE et al., 2011).

1.6.3. A catalase

A catalase é uma enzima abundante no fígado, rim e eritrócitos. Nos hepatócitos, a catalase é encontrada nos peroxisomas, enquanto que nos eritrócitos maduros, ela está localizada no citoplasma (FORSBERG et al., 2001). A catalase é um importante antioxidante endógeno, envolvido nas vias de neutralização de EROs (BABUSIKOVA et al., 2013; LIU et al., 2015). A catalase permite a detoxificação do peróxido de hidrogênio (H_2O_2) decompondo-o em água (H_2O) e oxigênio (O_2) e reduzindo assim os seus efeitos adversos (GHALY; GHATTAS; LABIB, 2012; MARY et al., 2014). Além disso, ela inativa vários mutagenes ambientais (MARY et al., 2014). Juntamente com a superóxido dismutase e as glutationas peroxidases, a catalase constitui o primeiro mecanismo de defesa contra o estresse oxidante (FORSBERG et al., 2001). Estudos *in vitro* demonstraram a conversão da HU em nitrito/nitrato pela ação da catalase (HUANG; KIM-SHAPIRO; KING, 2004; KING, 2005) e que a toxicidade da HU *in vivo* depende da sua atividade (JUUL et al., 2010). O gene da catalase (*CAT*) humana está localizado no cromossomo 11 (11p13.31), sendo constituído por 13 éxons (FORSBERG et al., 2001; GHALY; GHATTAS; LABIB, 2012). Polimorfismos raros no gene *CAT* estão associados à acatalasemia ou acatalasia, caracterizada pela ausência da enzima ou produção de uma catalase instável (FORSBERG et al., 2001). Polimorfismos na região promotora do gene podem levar à redução na taxa de transcrição bem como na atividade da enzima (LIU et al.,

2015), a exemplo do SNP *CAT*-262C>T, caracterizado pela substituição da citosina pela timina na posição 262 da região promotora do gene (PERIANAYAGAM et al., 2007).

2. JUSTIFICATIVA

De acordo com a Organização Mundial de Saúde (OMS), 270 milhões de indivíduos em todo o mundo (7% da população mundial) são portadores de genes que determinam hemoglobinas variantes. Estudos epidemiológicos têm demonstrado que, mundialmente, 300 a 400 mil crianças nascidas vivas apresentam o genótipo HbSS i.e, a AF (BANDEIRA et al., 2007; FERNANDES et al., 2010). O alelo β^S está amplamente distribuído na África, parte da Ásia, península Arábica, América e parte do sul da Europa. Em 2010, a estimativa da incidência da AF na África sub-sahariana foi de 230 mil, o que representa cerca de 75% dos casos de AF registrados mundialmente. Nesse mesmo ano, estimou-se que, só na África sub-sahariana, nasceram mais de 3,5 milhões de crianças com o traço falciforme (HbAS) (KATO et al., 2018). Além disso, a frequência do alelo β^S é mais elevada em países como a Nigéria, Gana, Gabão e Zaire, com 10 a 40 % de portadores, frequência maior que a descrita na Itália, Grécia, Oriente Médio e Índia (CHIKEZIE; CHIKEZIE; AMARAGBULEM, 2011; FOLASHADE; OMOREGIE, 2013). Nos Estados Unidos da América (EUA), em um total de 76 milhões de recém-nacidos nos últimos 20 anos, foram registrados cerca de 40 mil recém-nascidos com DF e mais de 1,1 milhão de recém-nascidos com o genótipo HbAS, sendo uma incidência de 1/1941 para a DF e 1/67 para o traço falciforme (KATO et al., 2018) . A incidência de indivíduos com DF na população afro-americana é de um a cada 360 recém-nascidos (KATO et al., 2018), enquanto na população hispano-americana a doença é encontrada na razão de um a cada 1.000 a 4.000 recém-nascidos (GONÇALVES et al., 2003). Na Europa, 20 a 25 mil indivíduos apresentam alguma forma de hemoglobinopatia (FRANCESCHI; CAPPELLINI; OLIVIERI, 2011).

No Brasil, a AF é a doença hereditária monogênica mais prevalente e constitui um problema de saúde pública (RAMALHO; GIRALDI; MAGNA, 2008; CALDAS et al., 2010). Segundo o Programa Nacional de Triagem Neonatal (PNTN), do Ministério da Saúde (MS), nascem anualmente no Brasil aproximadamente 3.500 crianças com AF (SILVA-PINTO et al., 2013) e 200 mil indivíduos portadores do genótipo HbAS (FELIX; SOUZA; RIBEIRO, 2010). Além disso, estima-se que 30 mil indivíduos são possuem a DF (KATO et al., 2018). As regiões sudeste e nordeste do país apresentam as maiores frequências de indivíduos HbAS, bem como prevalência elevada da DF (BANDEIRA et al., 2007; FERNANDES et al., 2010). A Bahia, por ser o estado brasileiro com número maior de afro-descendentes, possui a prevalência mais elevada da DF, com estimativa de um (1) indivíduo com a doença a cada 650 nascidos vivos (1/650) e a frequência de um (1) indivíduo HbAS a cada 17 nascidos vivos (1/17) (CANÇADO;

JESUS, 2007; JESUS, 2010). Em Salvador, estima-se que aproximadamente 9,8% dos recém-nascidos possuem o genótipo HbAS e 0,2% possuem a AF (CALDAS et al., 2010).

Diante do problema de saúde pública que representa a AF, existem várias abordagens que visam conhecer melhor o quadro clínico desses indivíduos, incluindo o tratamento com a HU. Diversos estudos mostraram que o uso da HU em indivíduos com AF pode melhorar o quadro clínico pela redução nas frequências de crises dolorosas, tempo de internação, necessidade de uso de hemoderivados e número de episódios de STA e AVC (SILVA-PINTO et al., 2013). Apesar do seu efeito benéfico, observa-se variabilidade interindividual na resposta ao tratamento, desde a ausência de resposta terapêutica até a toxicidade (WARE et al., 2002; WARE, 2010). Isso pode ser devido a vários fatores incluindo os genéticos. Polimorfismos em genes de EMD têm sido descritos na literatura por alterar a atividade enzimática, influenciar o metabolismo da droga e, consequentemente, levar a alterações no perfil farmacocinético, predispondo o indivíduo a reações adversas hepáticas (SHENG et al., 2014). Além disso, tem sido relatado que a melhora clínica ocorre antes do aumento da HbF, sugerindo a existência de outros mecanismos desencadeados, em paralelo, pela HU (BANDEIRA et al., 2004; SILVA-PINTO et al., 2013).

Dessa forma, tendo como base a importância do tratamento com a HU nos indivíduos com AF clinicamente graves, bem como a ausência de resposta terapêutica em alguns desses indivíduos durante o tratamento, faz-se necessário a realização do presente estudo, que irá permitir a identificação de biomarcadores genéticos que possam influenciar na resposta terapêutica, bem como no curso clínico a médio e longo prazo.

3. OBJETIVOS

3.1. GERAL

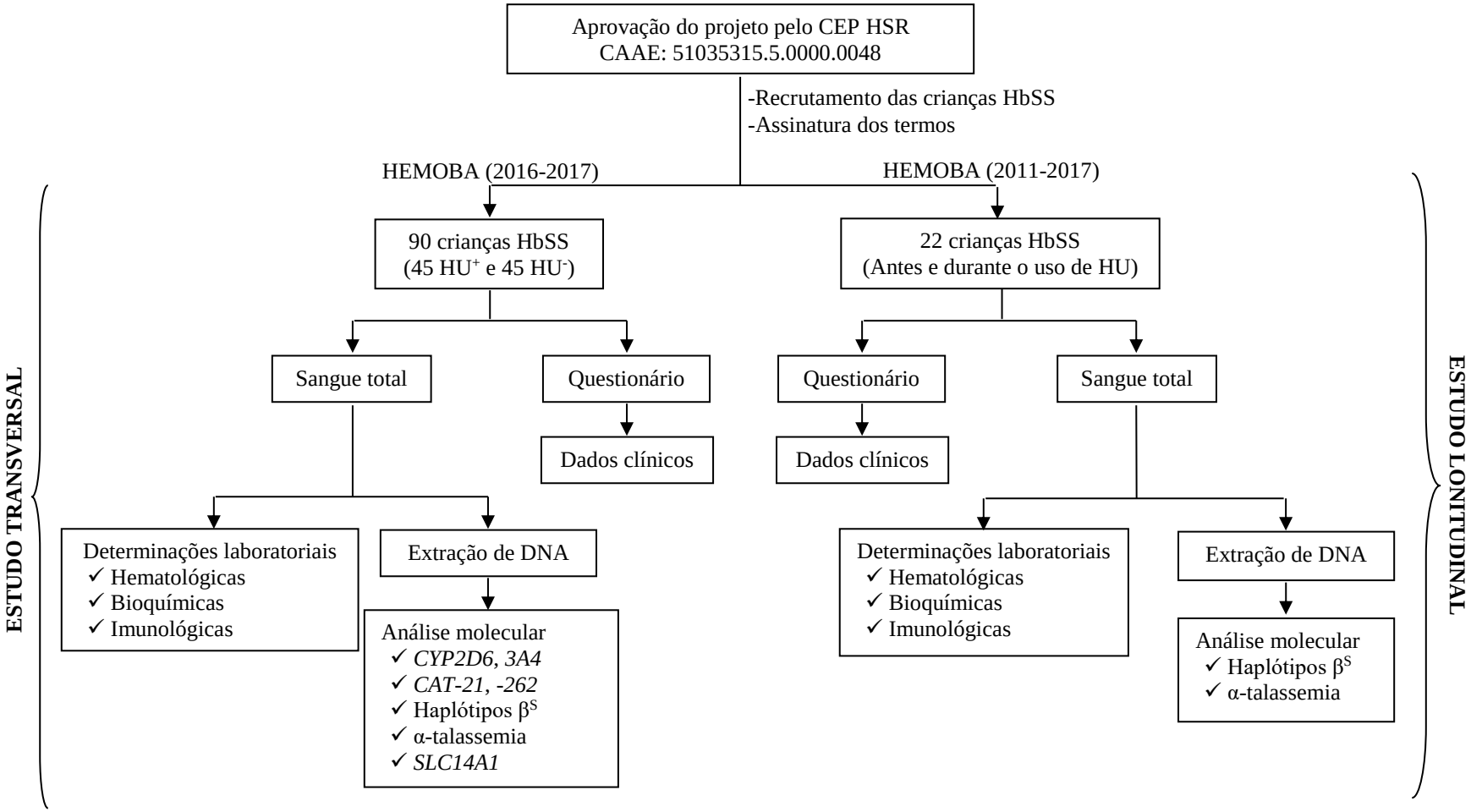
Investigar a farmacogenética da HU em crianças com AF.

3.2. ESPECÍFICOS

- Identificar genes candidatos envolvidos no metabolismo da HU;
- Investigar a influência dos haplótipos ligados ao grupo de genes da globina beta S e da α-talassemia sobre os efeitos da HU nos parâmetros laboratoriais em crianças com AF;
- Investigar a influência de polimorfismos em genes de EMD e proteína transportadora de soluto na resposta ao tratamento pela HU em crianças com AF.

4. DESENHO EXPERIMENTAL

Conferir o fluxograma a seguir.


Aprovação do projeto pelo CEP HSR
CAAE: 51035315.5.0000.0048
-Recrutamento das crianças HbSS
-Assinatura dos termos
HEMOBA (2016-2017)
HEMOBA (2011-2017)
90 crianças HbSS
(45 HU+ e 45 HU-)
22 crianças HbSS
(Antes e durante o uso de HU)
Sangue total
Questionário
Questionário
Sangue total
Dados clínicos
Dados clínicos
Determinações laboratoriais
✓ Hematológicas
✓ Bioquímicas
✓ Imunológicas
Extração de DNA
Determinações laboratoriais
✓ Hematológicas
✓ Bioquímicas
✓ Imunológicas
Extração de DNA
Análise molecular
✓ CYP2D6, 3A4
✓ CAT-21, -262
✓ Haplótipos βS
✓ α-talassemia
✓ SLC14A1
Análise molecular
✓ Haplótipos βS
✓ α-talassemia
ESTUDO TRANSVERSAL
ESTUDO LONITUDINAL

5. RESULTADOS

O presente estudo foi dividido em três capítulos conforme apresentado a seguir:

Capítulo I: *Hydroxyurea in the management of sickle cell disease: pharmacogenomics and enzymatic metabolism*;

Capítulo II: *Hydroxyurea alters hematological, biochemical and inflammatory biomarkers in Brazilian children with SCA: investigating associations with* β^S *haplotype and α-thalassemia*;

Capítulo III: *Hydroxyurea and polymorphisms in genes CYP2D6, CAT and SLC14A1: Enhanced therapeutic effects in patients with sickle cell anemia*.

CAPÍTULO I

Manuscrito intitulado: "*Hydroxyurea in the management of sickle cell disease: pharmacogenomics and enzymatic metabolism*"

Publicado no periódico *The Pharmacogenomics Journal*, em 2018.

Trata-se de um artigo de revisão que investigou estudos associados ao uso da HU em indivíduos com DF, realizando o levantamento dos fatores genéticos que podem influenciar a resposta ao tratamento, além das enzimas que participam do metabolismo da droga. Concluiu-se que o modelo proposto neste estudo (modelo de metabolismo diferencial da HU), em conjunto com os modelos de níveis basais diferencial de HbF e de susceptibilidade diferencial podem explicar a variabilidade na resposta à HU observada nos indivíduos. Também percebeu-se a necessidade de se identificar as enzimas envolvidas na via metabólica da HU, uma vez que isso facilitará os estudos de farmacogenômica e a identificação de genes candidatos para predizer a resposta à HU.

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REVIEW ARTICLE

Hydroxyurea in the management of sickle cell disease: pharmacogenomics and enzymatic metabolism

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Abstract

Hydroxyurea (HU) was approved to be used in the treatment of sickle cell disease (SCD) because of its anti-sickling potential. However, there is variability in HU response among SCD patients and this can be due to physiological, socioeconomic, environmental, metabolic and/or genetic factors. The present review focuses on the latter two. Three quantitative trait loci, *HBG2*, *BCL11A* and *HMIP*, have been suggested as important markers for HU response. Other genes (*ASS1, KLF10, HAO2, MAP3K5, PDE7B, TOX, NOS1, NOS2A*, *FLT1, ARG1, ARG2, UGT1A1, OR51B5/6*, *SIN3A*, *SALL2*, *SAR1A*, *UTB*, *OCTN1*, *CYP2C9*, *AQP9*, *MPO*, *CYP2E1*, and *GSTT1*) have also been considered. Studies implicate catalase, urease, horseradish peroxidase and enzymes of CYP450 family in HU metabolism. However, little is known about these enzymes. Therefore, further studies are needed to elucidate the metabolic pathway of HU, which will facilitate pharmacogenomic studies and help in identification of candidate genes for predicting HU response.

Introduction

Hydroxyurea (HU), or hydroxycarbamide, is a hydroxylated analogue of urea (Fig. 1; CAS Registry Number, 127-07-1) [1, 2], first synthesized in 1869 by Dresler and Stein and later tested in an experimental model in 1928 by Rosenthal, who suggested its myelosuppressive potential [3, 4]. HU has been used to treat myeloproliferative syndromes, particularly chronic myeloid leukemia, polycythemia vera and psoriasis [5, 6], as well as AIDS, since it inhibits DNA synthesis in human immunodeficiency virus type I (HIV-I) by reducing intracellular dNTP levels in activated lymphocytes [7, 8].

HU, due to its anti-sickling potential, was approved in 1999 by the U.S. Food and Drug Administration for the treatment of sickle cell disease (SCD) in patients with severe clinical profiles [9–11]. The benefits of HU in SCD patients have been attributed to increasing fetal hemoglobin (HbF) levels, which inhibits the polymerization of the variant hemoglobin S, leading to a reduction in the incidence of painful crises, as well as decreased rates of hospitalization, acute chest syndrome, blood transfusion and mortality among SCD patients [6, 9]. HU is also associated with increasing hemoglobin and mean cell volume of red cells; reducing white cell, platelet and reticulocyte counts; in addition to reducing expression of adhesion molecules and release of nitric oxide (NO) [12, 13]. However, increase in HbF levels and the clinical response induced by HU have been variable among different patients, necessitating elevated dosages and increasing toxicity [9, 14]. Differences in responses can be attributed to various factors, including physiological, socioeconomic and environmental factors. However, genetic factors have been considered as some of the most important determinants of variations in drug therapy response and tolerance [15]. Recently, studies in SCD patients showed that in addition to genomic variations within the β-globin gene (*HBB*), variants in modifier genes outside *HBB* are also significantly associated with increase in HbF levels, and, consequently, HU treatment response [15].

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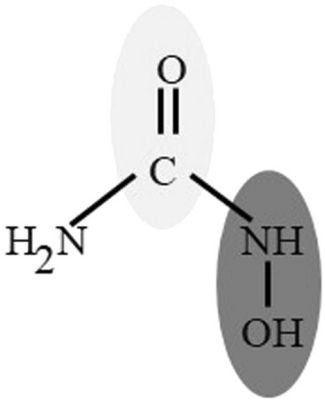

Fig. 1 Chemical structure of hydroxyurea. The areas of hydroxyurea involved in ribonucleotide reductase inhibition and cycling cell killing (grey-shaded area) as well as in nitric oxide production and thereby soluble guanylate cyclase stimulation (black-shaded area) are highlighted. Figure adapted from [17]

Some studies that investigated the enzymatic metabolism of HU, to better understand its mechanism of action, presented experimental evidence suggesting the participation of catalase, urease, horseradish peroxidase and enzymes of the CYP450 family [10, 16]. Pharmacogenomic studies performed later focused on quantitative trait loci (QTL) and modifier genes outside *HBB*, but paid little attention to the genes encoding enzymes that metabolize HU. The present review attempts to summarize findings concerning the genetic factors that influence HU response, such as QTL and the genes that influence HbF levels, in addition to placing a particular emphasis on genes encoding drug-metabolizing enzymes (DME) and transporters, as well as HU metabolism.

Three models attempting to clarify inter-individual variability in SCD patients on HU

In 2013, based on some evidence, Banan proposed two models in an attempt to explain the differential HU responses seen in SCD patients: (1) the differential susceptibility model, in which erythroid cells supposedly react differently to HU in responders and non-responders; (2) the differential baseline HbF model, in which HU increases HbF production in responders and in non-responders, yet only patients with high cellular levels of HbF respond to HU therapy [17]. In the present review, we propose a third model termed the differential HU metabolism model, in which SCD patients, regardless of their baseline HbF%, metabolize HU differently irrespective of whether they respond to HU treatment or not (Fig. 2). It is our belief that differences in HU metabolism may provide further insight into the variability observed in patient responses to HU, since it is known that single nucleotide polymorphisms (SNPs) in the genes encoding DME can lead to poor, intermediate, rapid or ultra-rapid HU metabolism, and, consequently, to differential pharmacokinetics and/or pharmacodynamics [18]. In addition, it was also reported that SNPs within genes encoding DME, transporters or targets could affect therapeutic response in SCD patients, thereby leading to efficacy or toxicity [19].

Biomarkers that affect baseline HbF levels and influence HU response

QTL

According to the differential baseline HbF model, genome-wide association studies (GWAS) have identified three important QTL associated with baseline HbF levels in patients with sickle cell anemia (SCA) and β-thalassemia, as well as in individuals with a normal hemoglobin pattern (HbAA): *HBG2*, *BCL11A* (developmental repressor of the γ-globin gene) and *HBS1L-MYB* [20–22]. The XmnIγ^G polymorphism (rs7482144) −158C > T of the γ^G-globin (*HBG2*) gene, located on chromosome 11p15, correlates with higher HbF levels in β-thalassemia and SCD patients, leading to decreased disease severity [17, 20, 23]. SNPs (e.g., rs11886868, rs46713993 and rs766432) in the intron 2 of the *BCL11A* gene located on chromosome 2p16 have been associated with high HbF levels in SCD patients and healthy individuals [17, 23]. SNPs (e.g., rs9399137 and rs4895441) in the *HMIP* region between genes *HBS1L* and *MYB*, located on chromosome 6q23, have also been associated with high HbF levels in SCD patients and healthy individuals [17, 23]. Wonkam and colleagues found associations between HbF levels and both *BCL11A* and *HMIP* SNPs in Cameroonian SCA patients [24].

Accordingly, it is likely that these QTL affect HbF levels in SCD patients on HU therapy [21, 23]. This hypothesis has been corroborated by several studies investigating associations between QTL and elevated levels of HbF in response to HU. In 2016, Friedrisch and colleagues analyzed Brazilian SCA patients and reported increased endogenous levels of HbF in association with both *BCL11A* and *HMIP*. Furthermore, *BCL11A* rs1427407, rs4671393 and rs11886868 were also associated with an increase of 2 to 4-fold in HbF level at maximum tolerated dose (MTD) [20]. Banan et al reported a strong correlation of TT genotype of *HBG2* and the C allele of *BCL11A* rs766432, with HU treatment response observed in Iranian β-thalassemia patients [25]. Moreover, the T allele of *HBG2* has also been associated with significant increase in hemoglobin (Hb) and HbF levels, in addition to HU therapy response in β-thalassemia intermedia patients, since the results showed that patients carrying the CT genotype presented, after HU therapy, an increase in Hb and HbF levels of 0.7 ± 1.26 mg/dl

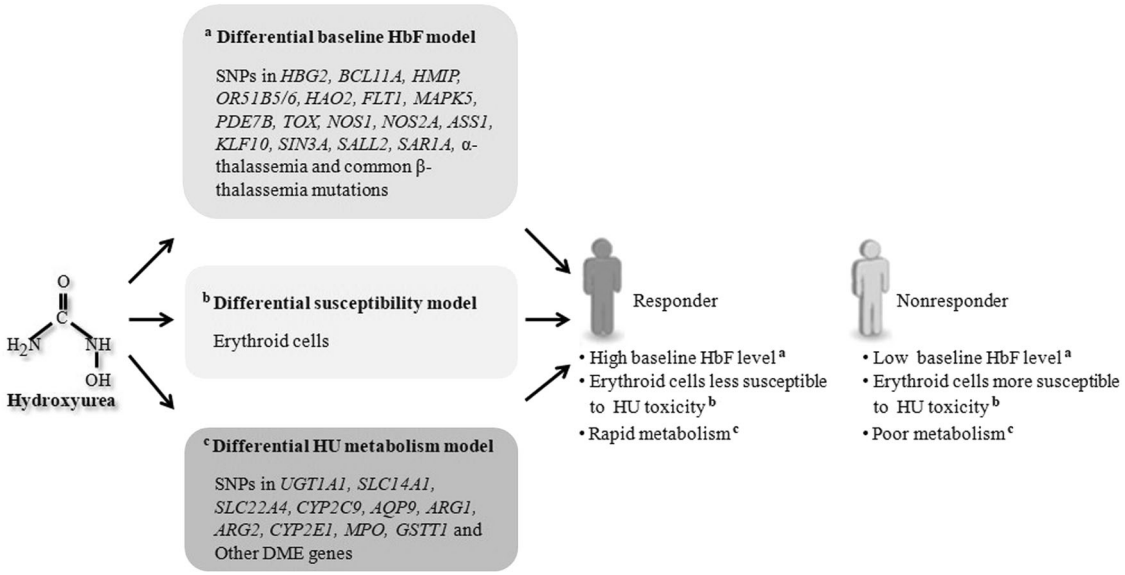

Fig. 2 Models attempting to elucidate differential hydroxyurea responses in SCD patients. Three models are presented, the differential susceptibility model, differential baseline HbF model and differential HU metabolism model. In the differential susceptibility model, it is supposed that erythroid cells can respond differently to HU in responders and non-responders. In the differential susceptibility model, the HbF production occurs only in patients with high cellular levels of HbF. In the differential HU metabolism model, we believe that, HU response will depend on capacity of each patient to metabolize HU. Hence, these three models can together determine the response of SCD patients on HU therapy. SCD patients can have a combination of models. *DME* drug metabolizing enzyme, *SNP* single nucleotide polymorphism

and 5.95 ± 14.8 mg/dl, respectively when compared with those carrying the CC genotype (0.26 ± 1.43 mg/dl and 0.8 ± 1.31 mg/dl, respectively) [26]. The TT variant genotype of *HBG2* has also been associated with higher HbF levels both pre and post-HU therapy in Kuwaiti Arab SCD and β-thalassemia patients on HU [21]. Recently, we found that SCA patients on HU therapy with the CC variant genotype of *BCL11A* rs766432 presented higher Hb concentration, red blood cell counts and hematocrit, in addition to lower direct bilirubin level and platelet count [23]. Furthermore, the multivariate linear regression analysis showed an independent and significant contribution of *HMIP* rs11759553 to modulate HbF levels.

However, other studies were unable to confirm this association, finding no significant correlations between the XmnIγ^G polymorphism and response to HU [27]. A study carried out in Iranian β-thalassemic patients also found no significant correlation between the XmnIγ^G polymorphism and HU response [28]. In a study conducted in SCD patients on HU, associations were found between the SNPs in the *BCL11A* gene (e.g., rs1427407, rs766432, rs4671393, rs7557939, and rs11886868) and baseline HbF% but none of them showed associations with MTD HbF levels [29]. A study involving Kuwaiti SCD and β-thalassemia patients on HU therapy reported that carriers of the C allele in *BCL11A* rs11886868, T allele in *HMIP* rs9376090, C allele in *HMIP* rs9399137 and A allele in *HMIP* rs9402686 had no significant differences in HbF levels either pre- or post-HU treatment when compared to carriers of respective wild-type alleles [21]. These findings seem to be consistent with the differential HU metabolism model, since no significant increases in HbF levels were observed in patients on HU therapy, regardless of whether patients were carriers of HbF QTL or not. Hence, it appears that in addition to HbF QTL, other genes are involved in the modulation of HbF production in SCD patients undergoing HU therapy.

Other genes associated with HbF production and HU response

It was reported that the olfactory receptors (*OR*) genes (*OR51B2*, *OR51B5* and *OR51B6*), located upstream of the *HBB* cluster locus control region may regulate the expression of the *HBG2* [30–32]. A GWAS performed by Green and colleagues in children with SCD on HU therapy found an association between *OR51B6* rs5024042 and baseline HbF [22]. On the contrary, in a study carried out in Cameroonian SCA patients, nonsignificant association was found between *OR51B5/6* rs5024042 and HbF level [24].

In a study carried out on SCA patients that received HU, it was discovered that SNPs within *HAO2*, *MAP3K5*, *PDE7B*, *TOX*, *NOS1*, *FLT1*, *ARG2* and *NOS2A* were significantly associated with the change of percent and/or absolute HbF. The most significant associations were found between SNP rs2182008 in *FLT1*, a vascular endothelial growth factor involved in cell proliferation and differentiation, and change of percent HbF as well as between SNP rs10483801 in *ARG2*, an enzyme involved in the

metabolism of drug, and the change of absolute HbF [14]. In another study involving SCD patients on HU therapy, SNPs *ARG1* rs17599586 and *ARG2* rs2295644 demonstrated significant associations with increased HbF levels [29]. A GWAS in hemoglobinopaties patients on HU treatment as well as erythroid progenitor cells from healthy and *KLF1*-haploinsufficient individuals treated with HU, found a significant association between intronic variants rs9483947 and rs9376230 of *MAP3K5*, a member of the p38 and the JNK MAPK pathway, and HU response [33].

The Krüppel-like family of transcription factors (KLF1, KLF2, KLF4, and KLF10) is a set of zinc finger DNA-binding proteins that can regulate the HbF production [34]. Research shows that KLF10 can repress the adult *HBB* gene interacting with SIN3A, leading to the repression of KLF1 activity, augmentation of γ-globin synthesis and hence HbF production [35]. Among the four SNPs identified within the *KLF10* gene in white population, it was discovered that *KLF10* rs3191333 can be considered as a pharmacogenomic biomarker [36, 37]. A pharmacogenomic study carried out earlier in Hellenic compound SCD/β-thalassemia patients as well as healthy and *KLF1*-haploinsufficient Maltese adults allowed the identification of *KLF10* rs3191333 (c.*141C > T) as an important pharmacogenomic biomarker, which facilitate the separation of responders and nonresponders to HU treatment [36]. Recently, in a cross-sectional study performed with Egyptian SCD and β-thalassemia (major and intermedia) patients on HU therapy, it was also found that *KLF10* gene plays a non-stand-alone role as HbF modifier and can be used as pharmacogenomic biomarker of HU treatment [38]. The participation of *SIN3A* gene in changes of *HBG2* expression was investigated in Hellenic β-hemoglobinopathies patients and it was found that the intronic *SIN3A* variants rs11072544 (C > T) and rs7166737 (T > C) were associated respectively with β-thalassemia disease severity and HU treatment response suggesting that they can be considered as pharmacogenomic biomarkers of HU response [35]. In a prospective study performed in SCA children treated with HU to MTD, Sheehan and colleagues using whole exome sequencing, investigated phenotype-genotype associations and they found out that a coding variant rs61743453 in *SALL2* (Spalt-like transcription factor) was associated with higher final HbF [39, 40].

The crucial role of the HU-induced small guanosine triphosphate-binding protein called secretion-associated and RAS-related (SAR) protein in *HBG2* induction has been demonstrated [41]. Report showed that some SNPs within the promoter region of *SAR1A*, a gene which acts in hemoglobin regulation, might contribute to the regulation of HbF expression and modulate the HU response in the patients [41, 42, 19]. A GWAS carried out in SCD patients identified SNPs in the promoter region of the *SAR* gene which were associated with the HU response [17].

In a recent study, it was suggested that SNPs (rs10901080 and rs10793902) within the *ASS1* gene, located on chromosomal region 9q34, can serve as pharmacogenomic biomarkers to predict HU treatment efficacy in compound SCD/β-thalassemia patients [43]. The G-T-T haplotype derived from the SNPs rs7860909, rs10901080 and rs10793902 was associated with higher HbF% and HU treatment efficacy. Furthermore, the in silico analysis performed by the authors revealed that these markers may induce NO biosynthesis, either altering splicing and/or miRNA binding, which consequently will activate the guanylate cyclase and increase γ-globin levels [43]. Multivariate analysis performed in SCA patients on HU therapy showed a strong influence of SNPs in *ASS* and *ARG1* on the change of HbF level, suggesting interactions between these genes and other genes for regulating the response to HU treatment [14].

Specific solute carrier membrane transporters (organic anion-transporting polypeptides—OATPs, cation/carnitine transporters—OCTNs, and urea transporters—UTs) regulate the transcellular traffic and accumulation of HU in the erythroid cells. The OATPs are more expressed in the intestine, liver and kidneys and associated with modulation of pharmacokinetics of HU, while UTB (encoded by *SLC14A1* gene) and OCTN1 (encoded by *SLC22A4* gene) are expressed principally in erythrocytes and bone marrow [44–46]. Walker and Ofori-Acquah carried out an experimental study which showed that the upregulation of UTB and OCTN1 in erythroid cells correlated with higher HU-mediated *HBG2* induction suggesting their important role in this process [47]. The result further showed that the OCTN1 expression mediated by HU was sustained and significantly correlates with *HBG2* induction, suggesting the pivotal role of OCTN1 in the efficacy of HU. Furthermore, the SNPs rs12605147 and rs2298720 in UTB demonstrated associations with pharmacokinetic parameters in SCD patients [29]. A GWAS performed in African-American SCA patients on HU therapy included various candidate genes (*ARG1*, *AQP9*, *ASS*, *PS1*, *SLC14A1*, *LOC57404*, *POR*, *CYP2C9*, and *CYP3A5*) with a potential role in HU metabolism and genes (*RRM1*, *RRM2*, *TK1*, *TYMS*, *MPO*, *SOD1*, *ABCB1*) mediating the effect of HU [48]. The results showed associations between SNPs in *CYP2C9* and *AQP9*, and HbF response to HU. In a recent study performed in Brazilian SCA patients on HU, we found independent associations between *MPO* rs2333227 and total cholesterol, *CYP2E1* rs3813867/rs2031920 and alpha 1-antitripsina, null *GSTT1* genotype and total bilirubin suggesting that these SNPs can be associated with alterations in lipid, inflammatory and hemolytic profiles, respectively, improving or compromising the treatment of the SCA patients under HU, depending on the SNP and parameter affected [49].

SNPs associated with alterations in clinical profile in response to HU therapy

There is evidence showing that beside factors such as reticulocytes count, frequency of vaso-oclusive events and α-thalassemia, genetic variants present in the promoter region of the uridine diphosphate (UDP)—glucuronosyl transferase 1A1 (UGT1A1), the main enzyme responsible for bilirubin conjugation, can be a risk factor for cholelithiasis [50–52]. In SCA patients on HU, an association was found between the $(TA)_7/(TA)_7$ repeats and higher bilirubin levels [52]. This result was confirmed by Adekile and colleagues that demonstrated that adults SCA patients under HU and carriers of $(TA)_7/(TA)_7$ repeats had higher levels of bilirubin [53]. Furthermore, a negative correlation was found between HbF% and bilirubin levels, suggesting contribution of this genotype in HU response [53]. Research from Italia and colleagues, showed that $(TA)_7/(TA)_7$ repeats were associated with elevated bilirubin levels among the different groups analyzed (SCA, β-thalassemia intermedia and HbE—β-thalassemia) and the use of HU therapy did not reduce the bilirubin levels under normal values [51]. Furthermore, the results showed that SCA and β-thalassemia intermedia patients on HU therapy with $(TA)_7/(TA)_7$ repeats and α-thalassemia had higher bilirubin levels in comparison with those without α-thalassemia [51]. However, further studies are needed to better understand this interaction between *UGT1A1* gene promoter polymorphism and HU response.

Report from Panigrahi and colleagues found that heterozygous $\alpha^{3.7}$ deletion carriers responded to HU therapy and were correlated with the XmnIγ^G polymorphism [54]. Hence, they suggested that besides the XmnIγ^G polymorphism, the $\alpha^{3.7}$ deletion can be associated with HU response in the patients. Genetic correlations between the XmnIγ^G polymorphism and common β-thalassemia mutations were investigated in different populations and the results were different according to the population studied. A research carried out in Iran showed that 61% of Iranian transfusion-dependent β-thalassemia patients on HU with molecular background favorable to HU (for example XmnIγ^G polymorphism), strongly associated with the IVS-II-I (G- > A) mutation in linkage with the haplotype I [+− − − − −] and internal β-globin framework 2, shifted from monthly blood transfusion dependency to a stable transfusion-free condition [55]. This result is supported by the works of Alebouyeh and colleagues that found a significant correlation between the XmnIγ^G polymorphism and IVSII-1 mutation [56]. They further demonstrated that the XmnIγ^G polymorphism and IVSII-1 mutation (homo- and/or heterozygosis) are important markers in most responding major β-thalassemic patients on HU.

One of the complications observed in patients treated with HU is cutaneous ulceration. In 2014, Crittenden and colleagues presented a case of a severe leg ulcer in an old woman with a history of myelodysplastic syndrome who has been on HU for 14 years, and homozygote for the C677T polymorphism of the methylene tetrahydrofolate reductase (*MTHFR*) gene, suggesting the implication of the *MTHFR* polymorphism or others thrombophilic genetic mutation in the development of cutaneous ulceration in patients on HU [57].

The above reports notwithstanding, little or no GWAS investigated association between genes encoding DME, especially those related to HU metabolism, and HU response (Table 1).

Enzymatic reactions of hydroxyurea

Metabolism of HU in liver and kidney

Currently, little is known about HU pharmacokinetics. It has been reported that approximately half of the drug is eliminated unchanged by the kidneys and the rest is metabolized in the liver [3]. Furthermore, it has been observed in mouse, that 30–50% of administered dose of HU-^{14}C was found in the urine as urea-^{14}C [58]. It has also demonstrated that mouse liver and kidney would convert HU to urea [58]. In 1970, Colvin and Bono suggested an enzymatic reduction of the hydroxylamine group and demonstrated the enzymatic reduction of HU to urea catalyzed by mouse liver tissue in the hepatic mitochondria [59]. Huang and colleagues demonstrated the efficient formation of NO from HU mediated by enzymes present in crude rat liver homogenate [10].

Potential enzymes involved in HU metabolism

In humans, the metabolic pathways of HU have not been established [3]. Fishbein and colleagues in 1965 reported that Davidson and Winter observed the breakdown of HU to hydroxylamine, ammonia, and carbon dioxide when treated with urease (Fig. 3) [60]. Another work suggests that urease greatly enhanced HbNO formation and that HU is partially metabolized to hydroxylamine, which quickly reacts with Hb to form methhemoglobin (metHb) and HbNO [61, 62]. It has been shown that 50% of HU dose administered intraperitoneally was metabolized in liver and kidney tissue, suggesting that NO formation from HU occurs in the liver rather than the blood [10]. In addition to acting as a substrate for urease, HU can act as an inhibitor of this enzyme [63].

Hepatic microsomes contain monooxygenases, which may be separated into two groups, the cytochrome (s) P-448 inducible by polycyclic aromatic hydrocarbons and the cytochrome (s) P-450, which predominate in the liver and accept as substrates hydrophilic and non-planar compounds [16]. Hence, HU is susceptible to being metabolized by a

Table 1 Summary of the association studies which have correlated SNPs located in genes within and outside the human globin gene cluster with HU therapy efficacy

Sample	HU therapy	Origin	Location	Gene and markers	Association with HU therapy response	References
SCD and β-thalassemia patients	No	NA	11p15	*HBG2*, XmnIγ^{G} rs7482144	High HbF level, decrease disease severity	[17, 20, 23]
SCD patients and healthy individuals	No	NA	2p16 6q23	*BCL11A* rs11886868, rs46713993, rs766432 *HMIP* rs9399137, rs4895441	High HbF level	[17]
SCA patients	No	Cameroon	2p16 6q23	*BCL11A* *HMIP*	Increased HbF level	[24]
SCD patients	Yes	NA	2p16	*BCL11A* rs1427407, rs766432, rs4671393, rs7557939, rs11886868	Baseline HbF% No association with MTD HbF levels	[29]
SCA patients	No	Brazil	2p16 6q23	*BCL11A* *HMIP*	Increased endogenous HbF level	[20]
	Yes		2p16	*BCL11A* rs1427407, rs4671393, rs11886868	Increase of HbF level	
β-thalassemia patients	Yes	Iran	11p15 2p16	*HBG2*, XmnIγ^{G} rs7482144 *BCL11A* rs766432	HU treatment response	[25]
β-thalassemia intermedia patients	Yes	Iran	11p15	*HBG2*, XmnIγ^{G} rs7482144	Significant increase in Hb concentration and HbF level; HU therapy response	[26]
SCA patients	Yes	Brazil	2p16	*BCL11A* rs766432	Significant increase in Hb concentration, RBC count and hematocrit, and significant decrease of DB levels and platelet count;	[23]
			6q23	*HMIP* rs11759553	High HbF level	
β-thalassemia patients	Yes	Iran	11p15	*HBG2*, XmnIγ^{G} rs7482144	No correlation with HU response	[28]
SCD and β-thalassemia patients	Yes	Kuwait	11p15	*HBG2*, XmnIγ^{G} rs7482144	Higher HbF levels pre- and post-HU therapy	
			2p16 6q23	*BCL11A* rs11886868 *HMIP* rs9376090, rs9399137, rs9402686	No significant difference in HbF levels pre- or post-HU treatment	[21]
Children SCD patients	Yes	NA	11p15.4	*OR51B6* rs5024042	Association with baseline HbF	[22]
SCA patients	No	Cameroon	11p15.4	*OR51B6* rs5024042	No association with HbF levels	[24]
SCD patients	Yes	NA	6q23.2 14q24.1	*ARG1* rs17599586 *ARG2* rs2295644	Increase HbF levels	[29]
Adults SCA patients	Yes	African-American	13q12.3 14q24.1 9q34.11 6q23.2	*FLT1* rs2182008 *ARG2* rs10483801 *ASS* rs7860909, rs10793902, rs10901080, rs543048 *ARG1* rs17599586, rs2781667, rs2246012	Change of percent HbF Change of absolute HbF Change of HbF level	[14]
Hemoglobinopathies patients; Erythroid progenitor cells from healthy and KLF1-haploinsufficient individuals	Yes	Western Greece	6q23	*MAPK5* rs9483947, rs9376230	HU response	[33]
SCD and β-thalassemia major and intermedia patients	Yes	Egypt	8q22.3	*KLF10*	HbF modifier; pharmacogenomic biomarker of HU therapy	[38]
SCD/β-thalassemia patients; Healthy and KLF1-haploinsufficient individual	No Yes	Greece Malta	8q22.3	*KLF10* rs3191333	Pharmacogenomic biomarker	[36]
β-hemoglobinopathies patients	No	Western–Greece	15q24.2	*SIN3A* rs11072544 and rs7166737	β-thalassemia disease severity and HU treatment response, respectively	[35]
SCA children treated with HU to MTD	Yes	NA	14q11.2	*SALL2* rs61743453	Higher final HbF	[39, 40]
Adults SCD patients	Yes	NA	10q22.1	*SAR1A* (SNPs in the promoter region)	Regulate HbF expression and modulate HU response	[17, 19]
SCD/ β-thalassemia patients	Yes	Western–Hellenic	9q34	*ASS1* rs10901080, rs10793902 *ASS1* rs7860909, rs10901080, rs10793902 –GTT haplotype	Predict HU treatment efficacy Higher HbF% and HU treatment efficacy	[43]
Human cell lines and oocytes overexpressing SLC transporters$_{\dagger}$	Yes	NA	12p12.1	*OATPs*	Modulation of HU pharmacokinetic	[44–46]

Table 1 (continued)

Sample	HU therapy	Origin	Location	Gene and markers	Association with HU therapy response	References
Erythroid cells[†]	Yes	NA	18q12.3 5q31.1	*SLC14A1* *SLC22A4*	Higher HU-mediated *HBG2* induction	[47]
SCD patients	Yes	NA	18q12.3	*SLC14A1* rs12605147 and rs2298720	Pharmacokinetic parameters	[29]
SCA patients	Yes	NA	10q24 15q22.1	*CYP2C9* *AQP9*	HbF response to HU	[48]
SCA patients	Yes	Brazil	17q22 10q26.3 22q11.23	*MPO* rs2333227 *CYP2E1* rs3813867/rs2031920 *GSTT1*	Alterations in lipid, inflammatory and hemolytic profiles, respectively	[49]
Pediatric SCA patients; Pediatric and adults SCA patients	Yes	NA	2q37.1	*UGT1A1* $(TA)_7/(TA)_7$ repeats	Higher bilirubin levels Influence HU response	[52] [53]
SCA, β-thalassemia intermedia and HbE/ β-thalassemia SCA, β-thalassemia intermedia	Yes	NA	2q37.1	*UGT1A1* $(TA)_7/(TA)_7$ repeats *UGT1A1* $(TA)_7/(TA)_7$ repeats and $\alpha^{3.7}$ deletion*	No reduction of bilirubin levels under normal values Higher bilirubin levels	[51]
Thalassemia intermedia patients	Yes	NA	16p13.3	$\alpha^{3.7}$ deletion	HU response	[54]
Transfusion-dependent β-thalassemia patients	Yes	Iran	11p15	*HBG2*, $XmnI\gamma^G$ rs7482144, IVS-II-I (G > A) in linkage with haplotype I […] and internal β-globin framework 2	Shift from monthly blood transfusion dependency to stable transfusion-free condition	[55]
Major β-thalassemia patients	Yes	NA	11p15	*HBG2*, $XmnI\gamma^G$ rs7482144 and IVS-II-I (homo- and/or heterozygosis)	HU response	[56]
Old woman**	Yes	NA	1p36.22	*MTHFR* C677T	Association with severe leg ulcer	[57]

SCA: sickle cell anemia; SCD: sickle cell disease; *HU* hydroxyurea, *MTD* maximum tolerated dose, *RBC* red blood cell, *DB* direct bilirubin, *Hb* hemoglobin, *HbF* fetal hemoglobin, *NA* not available *Co-heritance; **case report study; [†]experimental study

monooxygenase cytochrome (s) P-450 [9, 16]. In addition, Restituto and colleagues reported that 30 to 50% of HU dose administered is metabolized in the liver by the monooxygenated system [8].

A study with SCD patients on HU therapy showed evidence of in vivo NO formation from HU and identified peroxidases as potential oxidants for this conversion [63, 64]. Furthermore, Horseradish peroxidase and catalase catalyze the formation of NO and nitroxyl (HNO) from HU, which is then oxidized into NO [63]. Experiments carried out by Huang and co-workers using the 3 aminotriazole, a specific inhibitor of catalase, and heat-inactivated catalase demonstrated that, the catalase is necessary for the conversion of HU to nitrite/nitrate [63, 65]. The potential role of catalase in the in vivo metabolism of HU to NO was also revealed (Fig. 4) [10]. It was demonstrated in a study that the in vivo HU toxicity depends on catalase, which is a direct HU target since HU play a competitive inhibitor role of catalase-mediated hydrogen peroxide decomposition and HU decomposition *in vitro* can be accelerated by catalase [66].

Conclusion

In the present review, in addition to the previously proposed differential susceptibility model and differential baseline HbF model, we put forward for consideration a third model termed differential HU metabolism model to elucidate differential HU responses. These 3 models together can determine the response of SCD patients on HU therapy. In patients with hemoglobinopathies, various studies identified three important HbF QTL (*HBG2*, *BCL11A* and *HMIP*) and other genes involved in NO biosynthesis, genetic regulation

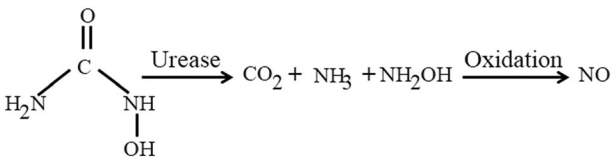

Fig. 3 Urease-mediated hydrolysis of hydroxyurea. Urease catalyzes the hydrolysis of hydroxyurea to hydroxylamine, carbon dioxide and ammonia following by nitric oxide production derived from reaction of oxidation. Figure adapted from [63]

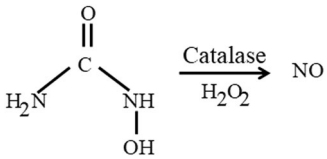

Fig. 4 Catalase-mediated hydrolysis of hydroxyurea. Hydroxyurea can be metabolized by catalase to produce NO in the presence of hydrogen peroxide

and drug metabolism (*ASS1, KLF10, HAO2, MAP3K5, PDE7B, TOX, NOS1, NOS2A*, *FLT1, ARG1, ARG2, UGT1A1*, *OR51B5/6*, *SIN3A*, *SALL2*, *SAR1A*, *UTB*, *OCTN1*, *CYP2C9*, *AQP9*, *MPO*, *CYP2E1*, and *GSTT1*). These genes considered as pharmacogenomic biomarkers can predict HU treatment efficacy. Despite the widespread use of HU and the diversity of its efficacy, the enzymes involved in its metabolic pathway are unknown. The few studies that investigated the enzymatic reactions of HU, had suggested the participation of enzymes such as catalase, urease, horseradish peroxidase and enzymes of CYP450 family. Therefore, further studies are necessary to elucidate the metabolic pathway of HU, which will allow identification and better selection of enzymes for pharmacogenomic studies in hemoglobinopathies patients under HU therapy.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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CAPÍTULO II

Manuscrito intitulado: "*Hydroxyurea alters hematological, biochemical and inflammatory biomarkers in Brazilian children with SCA: investigating associations with β^S haplotype and α-thalassemia*"

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Trata-se de um estudo que investigou o efeito da HU nos parâmetros hematológico, bioquímico e inflamatório bem como a influência dos haplótipos ligados aos genes da globina β^S e da α-talassemia no efeito da HU, em crianças com AF. Observou-se que, independente da presença ou ausência dos haplótipos ligados ao grupo de genes da globina β^S e da α-talassemia, a HU parece estar correlacionada com a melhoria nos perfis hemolítico e inflamatório dos indivíduos investigados. Além disso, os resultados demonstraram que a HU pode estar associada a alterações nos biomarcadores metabólicos.

PLOS ONE

RESEARCH ARTICLE

Hydroxyurea alters hematological, biochemical and inflammatory biomarkers in Brazilian children with SCA: Investigating associations with βS haplotype and α-thalassemia

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Abstract

This study investigated the effects of hydroxyurea (HU) on hematological, biochemical and inflammatory parameters in children with sickle cell anemia (SCA) in association with β^S haplotype and α-thalassemia. We included 22 children with SCA who were followed for an average of 14.5 months. Laboratory parameters were assessed by electronic methods, and molecular analysis was investigated by PCR-RFLP and allele-specific PCR. Results showed significant increases in hemoglobin, HbF, hematocrit, MCV, MCH, glucose, HDL-C and albumin levels, as well as significant decreases in MCHC and AST levels, WBC, neutrophils, eosinophils, lymphocytes and reticulocytes, in children during HU therapy. HbF levels were positively correlated with hemoglobin, hematocrit, MCV and total protein, yet negatively correlated with MCHC, RDW, AAT and AST during HU therapy ($p<0.05$). Children who carried the Central African Republic haplotype, in response to HU therapy, presented significant increases in hemoglobin, hematocrit, triglycerides and uric acid levels, as well as significant decreases in MCHC, AST and direct bilirubin levels, WBC, neutrophils, eosinophils, lymphocytes and reticulocytes. Those with the Benin haplotype presented increases in HbF and albumin levels, and a reduction in platelet counts ($p<0.05$). Children with α-thalassemia presented decreased ALT during HU use, while those without this deletion presented increases in hemoglobin, hematocrit, MCV, MCH, HDL-C and albumin, as well as decreases in MCHC, neutrophils, lymphocytes, reticulocytes and AST ($p<0.05$). Hence, regardless of its use in association with β^S haplotypes or α-thalassemia, HU seems to be linked to alterations in hemolytic, inflammatory, hepatic, lipid and glycemic profiles.

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Introduction

Sickle cell anemia (SCA) is one of the most common inherited monogenic diseases in the world, characterized by chronic hemolytic anemia, vaso-occlusive events (VOE) and chronic organ injury [1]. Clinical profile and life expectancy vary widely among individuals with SCA, which can be explained by several factors, including genetic modifiers, such as haplotypes linked with the beta S (β^S)-globin gene cluster and alpha 2 deletion of 3.7 kb thalassemia ($\alpha^{2\ del}{}_{3.7kb}$ thalassemia) [1]. The Benin (BEN), Bantu or Central African Republic (CAR), Senegal, Cameroon and Arab/Hindu haplotypes identified in the β^S-globin gene cluster have been associated with variability in fetal hemoglobin (HbF) levels, which is known to be a classic modulator of this disease [2–4]. Likewise, individuals with SCA who are carriers of α-thalassemia present increases in hemoglobin concentrations and red blood cell (RBC) count, as well as decreases in mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte counts and bilirubin levels [5–7]. However, α-thalassemia has also been associated with an elevated frequency of VOE, which may be due to increased hematocrit and, consequently, blood viscosity in individuals with SCA [7].

Among the three agents known to enhance HbF production (Sodium butyrate, 5-azacytidine and hydroxyurea—HU), HU, approved in 1998 by the U.S. Food and Drug Administration (FDA) for the treatment of individuals with clinically severe SCA, remains the most commonly used [3,8]. The main benefit associated with HU therapy is increased HbF levels, which have been directly associated with decreased HbS polymerization, reduced incidence of VOE, frequency and length of hospital stays, blood therapy and acute chest syndrome (ACS). HU therapy also reduces health care costs and increases survival rates [9–12]. Furthermore, studies have demonstrated the association of HU with a reduction in white blood cell (WBC) and reticulocyte counts, as well as bilirubin and lactate dehydrogenase (LDH) levels, in addition to increased MCV [3,4]. Although it is believed that increases in HbF levels mediates HU efficacy among individuals with SCA, it was reported that improvement in patients' clinical profiles appears prior to any significant increase in their HbF levels, suggesting that HU may modulate other laboratory parameters beside the classical increase seen in HbF [11]. Regarding HbF levels and the patient clinical profile, there is a great variability in response among individuals treated with HU, which may be due to genetic factors, including the β^S-globin gene cluster haplotype [13,14]. Moreover, despite evidence demonstrating its efficacy, HU is underused in younger individuals with SCA due to a range of issues, mainly side effects, which have not been completely elucidated [15,16].

Therefore, we sought to investigate the wider effect of HU on hematological, biochemical and inflammatory parameters in children with SCA who carry the β^S haplotype, with or without α-thalassemia, in a prospective study.

Materials and methods

Subjects and ethical aspects

The present prospective study included 22 children with SCA (HbSS). Laboratory parameters were assessed before and during HU treatment, and the median length of HU use was 14.5 months (6 to 72 months). The average age of the children was 8.5±3.4 years (median: 7.5 years) at the beginning of the study and 12 (54.55%) were female. All patients were seen at the outpatient service of the Fundação de Hematologia e Hemoterapia da Bahia (HEMOBA). They reported the prior regular use of folic acid, and, after study recruitment, HU was prescribed at doses lower than its maximum tolerated dose (MTD) and ranging between 10–25

mg/kg/day (median: 15 mg/kg/day). All legal guardians reported that HU treatment was very important to their children, and affirmed that the medication was taken regularly unless ordered to interrupt treatment by a physician, which strongly indicates HU compliance. For inclusion, children were required to be in steady state, characterized as the absence of acute crisis and without the use of blood therapy, for three months prior to blood collection procedures. Children on blood therapy and those with infections were excluded.

This study was approved by the Institutional Review Board of the São Rafael Hospital, and was conducted in accordance with the Declaration of Helsinki and its amendments. In addition, the legal guardians of all children signed a term of informed consent prior to enrollment in the study.

Laboratory methods

Blood samples were drawn at the time of each individual's enrollment by venipuncture, in the morning after 12 hours of fasting under standardized conditions. Hematological analyses were performed using a Cell Dyn-Ruby electronic cell counter (Abbott Diagnostics, Wiesbaden, Germany). Qualitative and quantitative hemoglobin profiles were determined by high-performance liquid chromatography (HPLC/Variant II; BIO-RAD, Hercules, CA, USA). Biochemical parameters were measured in serum by an immunochemistry assay using an A25 spectrophotometer analyzer (Biosystems SA, Barcelona, Spain). Inflammatory proteins, such as alpha-1 antitrypsin (AAT) and C-reactive protein (C-RP), were measured by immunochemistry using the Immage 800 System (Beckman Coulter, Fullerton, CA, USA). Serum ferritin was measured by immunoassay using an Access 2 Immunoassay system (Beckman Coulter, Fullerton, CA, USA).

Genomic DNA was extracted from peripheral blood using the Flexigen 250 kit (Qiagen, Hilden, Germany). β^S haplotypes were investigated by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) [17], and $\alpha^{2\ del\ 3.7kb}$ thalassemia was assessed by allele-specific PCR [18].

All analyses were performed at the Laboratório de Análises Clínicas and Laboratório de Pesquisa em Anemia, Faculdade de Farmácia, Universidade Federal da Bahia and the Laboratório de Investigação em Genética e Hematologia Translacional, Instituto Gonçalo Moniz–FIOCRUZ/BA.

Statistical analysis

Statistical analyses were performed using EpiInfo 7.0 and GraphPad Prism v5.0 software, with *p* values below 0.05 considered statistically significant. Distributions of quantitative variables were determined by the Shapiro-Wilk test. The mean values of variables (laboratory parameters), measured before and during HU therapy, were compared using the paired t-test for normal distribution, and Wilcoxon's test for non-normal distribution. Results were expressed as mean ± standard deviation. Pearson's correlation coefficient analysis was performed to assess the strength of linear relationships between two quantitative variables with normal distribution.

Results

Laboratory parameters of children with SCA before and during HU therapy

The laboratory parameters of the SCA children before and during HU therapy are shown in Table 1, and S1 Fig. After the onset of HU therapy, hematological analyses showed significant

Table 1. Laboratory parameters of SCA children, before and during HU therapy.

	Before HU, N = 22 M ± SD	During HU, N = 22 M ± SD	*p* value
Hemoglobin			
HbF, %	9.95±6.28	12.72±5.77	**0.04****
HbS, %	86.52±6.05	84.87±5.50	0.18*
Erythrogram			
RBC, $x10^9$/mL	2,74±0,63	2,74±0,57	0.89*
Hemoglobin, g/dL	8.15±1.30	8.77±1.24	**0.01***
Hematocrit, %	23.41±3.92	26.11±4.07	<**0.01***
MCV, fL	86.67±9.03	96.72±10.26	<**0.01***
MCH, pg	30.25±3.45	32.59±3.47	<**0.01***
MCHC, %	34.90±1.48	33.70±0.94	<**0.01***
Erythroblast (/10^2 leukocytes)	2.50±1.92	0.86±0.88	<**0.01****
Leukogram			
WBC, /mL	15283±3931	10772±3547	<**0.01***
Neutrophil, /mL	7275±2279	5174±2409	<**0.01***
Eosinophil, /mL	1097±816	508±439	<**0.01****
Lymphocyte, /mL	5717±2576	3958±1628	<**0.01***
Monocyte, mL	950±468	989±533	0.99**
Platelets			
Platelet, $x10^3$/mL	433±105	400±140	0.26*
Hemolysis			
Total bilirubin, mg/dL	2.54±1.77	2.69±1.65	0.30**
Direct bilirubin, mg/dL	0.54±0.29	0.43±0.24	0.13**
Indirect bilirubin, mg/dL	1.99±1.54	2.26±1.62	0.24**
Lactate dehydrogenase, U/L	1072±431.8	1026±375.3	0.51*
Reticulocyte, %	8.01±3.17	4.56±1.66	<**0.01***
Lipids and glucose			
Total cholesterol, mg/dL	120.0±26.0	124.7±29.6	0.28*
HDL-C, mg/dL	31.73±7.45	40.42±12.66	<**0.01****
LDL-C, mg/dL	69.96±19.69	64.35±23.62	0.20**
Triglycerides, mg/dL	92.23±51.44	106.5±55.65	0.05**
Glucose, mg/dL	71.00±9.35	83.86±9.58	<**0.01***
Renal panel			
Urea, mg/dL	18.86±7.48	21.04±8.85	0.43**
Creatinine, mg/dL	1.68±1.76	0.85±1.09	0.47**
Hepatic panel			
Aspartate aminotransferase, U/L	57.50±17.97	44.32±16.50	<**0.01***
Alanine aminotransferase, U/L	24.05±11.39	22.86±12.04	0.44**
Total protein, g/dL	7.84±0.70	8.17±0.77	0.23**
Albumin, g/dL	4.34±0.34	4.73±0.32	<**0.01***
Inflammatory markers			
Uric acid, mg/dL	2.48±1.72	3.41±1.77	0.07*
Ferritin, ng/dL	501.6±625.6	611.5±791.0	0.51**
C-reactive protein, mg/L	7.30±7.17	7.35±12.62	0.40**

(*Continued*)

Table 1. (Continued)

	Before HU, N = 22 M ± SD	During HU, N = 22 M ± SD	*p* value
Alpha 1 antitrypsin, mg/dL	168.3±39.98	104.2±47.41	<**0.01****

RBC: red blood cell, MCH: mean corpuscular hemoglobin, MCV: mean corpuscular volume, MCHC: mean corpuscular hemoglobin concentration, HbS: S hemoglobin, HbF: Fetal hemoglobin, WBC: white blood cell, HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol, VLDL-C: very low-density lipoprotein cholesterol, M ± SD: mean ± standard deviation, N: number of individuals

*Paired T-test

** Wilcoxon test

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increases in mean HbF, hemoglobin, hematocrit, MCV and MCH, in addition to significant reductions in reticulocytes and MCHC ($p<0.05$). In all, 14 out of 22 children (63.64%) presented increases in HbF levels during HU therapy. Furthermore, all children had significant decreases in WBC, neutrophils, eosinophils and lymphocytes during HU therapy ($p<0.05$). Biochemical analysis revealed elevated glucose, HDL-C and albumin, as well as decreased aspartate aminotransferase (AST), in the children following the introduction of HU therapy ($p<0.05$).

Correlations between HbF and laboratory parameters during HU therapy

Fig 1 shows the results of correlation analysis in SCA children undergoing HU therapy. HbF levels were found to be positively correlated with hemoglobin, hematocrit, MCV and total protein levels, yet negatively correlated with MCHC, RDW, AST and AAT ($p<0.05$). When performing correlation analysis in the children prior to the onset of HU therapy, no positive or negative associations were observed.

Laboratory parameters of children with SCA before and during HU therapy according to β^S haplotypes and α-thalassemia

The distribution analyses of β^S haplotypes and $\alpha^{2\ del\ 3.7kb}$ thalassemia showed that 45% were carriers of the CAR/CAR genotype, while 35% and 20% were CAR/BEN and BEN/BEN, respectively. Fourteen children (77.78%) presented the αα/αα genotype (without α-thalassemia) and 22.22% the -α/αα or -α/-α genotypes (with α-thalassemia).

As it is known that the response to HU can be affected by β^S haplotypes and α-thalassemia, we performed an association analysis using a dominant genetic model comparing all laboratory parameters of the children prior to and during HU therapy. This analysis showed that children with the CAR haplotype presented significant increases in hemoglobin, hematocrit, MCV, MCH, glucose, triglycerides, uric acid and albumin levels, as well as significant reductions in MCHC, AST, direct bilirubin and AAT levels, as well as WBC, neutrophils, eosinophils, lymphocytes and reticulocytes during HU therapy. Children who were carriers of the BEN haplotype presented elevated HbF, MCV, MCH, glucose and albumin levels, in addition to reduced platelets during HU therapy ($p<0.05$) (Table 2).

Regarding α-thalassemia, during HU therapy the children without α-thalassemia had elevated hemoglobin, hematocrit, MCV, MCH, glucose, HDL-C and albumin levels, in addition to decreased MCHC and AST, as well as WBC, neutrophils, eosinophils, lymphocytes and reticulocytes ($p<0.05$). Those with α-thalassemia presented significantly increased glucose levels and decreased WBC and eosinophils, as well as lower alanine aminotransferase (ALT) levels, during HU therapy ($p<0.05$) (Table 3).

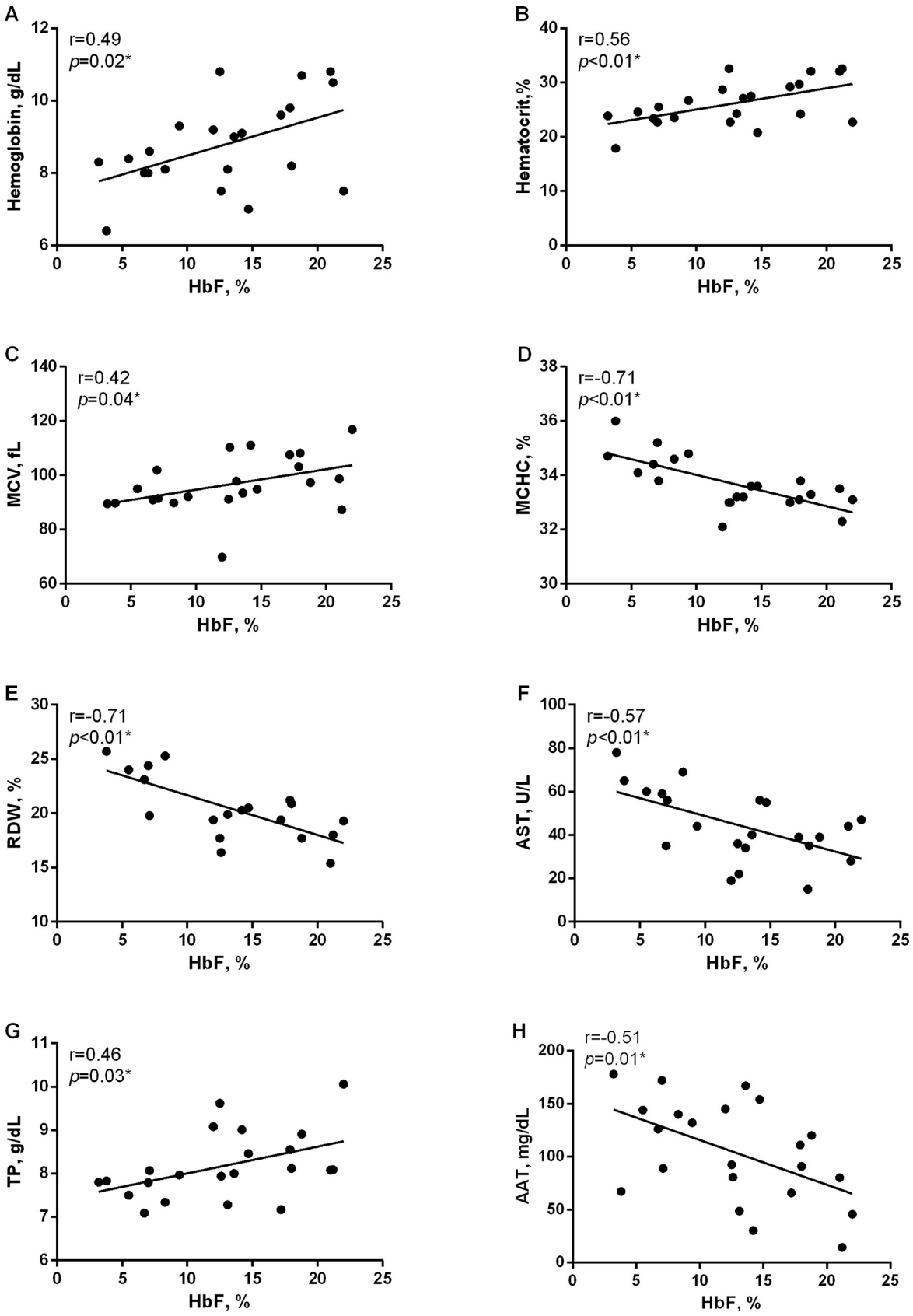
A
r=0.49
p=0.02*
Hemoglobin, g/dL
HbF, %
B
r=0.56
p<0.01*
Hematocrit,%
HbF, %
C
r=0.42
p=0.04*
MCV, fL
HbF, %
D
r=-0.71
p<0.01*
MCHC, %
HbF, %
E
r=-0.71
p<0.01*
RDW, %
HbF, %
F
r=-0.57
p<0.01*
AST, U/L
HbF, %
G
r=0.46
p=0.03*
TP, g/dL
HbF, %
H
r=-0.51
p=0.01*
AAT, mg/dL
HbF, %

Fig 1. Correlations between HbF levels and hematological and biochemical parameters in children with SCA during HU therapy. HbF levels were positively correlated with hemoglobin (**A**), hematocrit (**B**), MCV (**C**) and TP (**G**), while negatively correlated with MHCH (**D**), RDW (**E**), AST (**F**) and AAT (**H**). MCV: mean corpuscular volume, MCHC: mean corpuscular hemoglobin concentration, RDW: red cell distribution width, AST: aspartate aminotransferase, TP: total protein, AAT: alpha 1 antitrypsin, *Pearson's correlation.

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Discussion

The present study investigated the wider effect of HU on laboratory parameters in children with SCA, according to the presence of the β^S haplotype and α-thalassemia. It has been reported that individuals with SCA taking HU may present an improved clinical profile before significant increases in HbF levels are seen, and it is known that HU response can vary according to β^S haplotype and α-thalassemia [11,13,14].

As expected, HU intake resulted in a significant increase in HbF levels, which was observed in 14 (63.64%) children. This finding corroborates data from previous studies demonstrating that HU use was associated with higher HbF levels [19–22]. Moreover, increases in HbF were observed in some individuals [23–25], reinforcing the notion that not all individuals with SCA or SCD respond to HU therapy by initially presenting increased HbF levels. Additionally, HU was associated with higher hemoglobin, hematocrit, MCV and MCH, as well as lower MCHC and reticulocyte and erythroblast counts. These findings, also reported by previous studies [16,22,25–27], serve to confirm the association between HU therapy and an improvement in the hemolytic profile of individuals with SCA.

Table 2. Laboratory parameters of children with SCA according to β^S haplotype before and during HU therapy.

	CAR/CAR + CAR/BEN, n = 16			BEN/BEN, n = 4		
Parameter	**Before HU**	**During HU**	***p* value**	**Before HU**	**During HU**	***p* value**
HbF, %	10.57±6.68	12.14±5.46	0.27*	9.65±5.84	17.42±4.77	<**0.01***
Hemoglobin, g/dL	8.12±1.26	8.68±1.16	**0.03***	8.32±1.62	9.55±1.58	0.15*
Hematocrit, %	23.19±3.79	25.81±3.98	<**0.01***	24.13±5.38	28.73±4.63	0.07*
MCV, Fl	85.93±9.29	94.39±9.11	<**0.01***	87.30±10.32	104.45±11.62	<**0.01***
MCH, pg	30.13±3.39	31.90±3.29	<**0.01****	30.43±5.00	34.78±3.95	<**0.01***
MCHC, %	35.08±1.39	33.78±1.04	<**0.01***	34.68±1.88	33.30±0.29	0.50*
WBC, /mL	16004±3064	11149±2717	<**0.01***	10668±4978	6550±2646	0.14*
Neutrophils, /mL	7859±2064	5108±2035	<**0.01***	5161±2510	3288±1623	0.11*
Eosinophils, /mL	1186±836	600±459	<**0.01***	959±957	114±62	0.12**
Lymphocytes, /mL	5647±2181	4284±1653	**0.02***	3832±2049	2444±992	0.20*
Platelets, $x10^3$/mL	427±87	430±131	0.92*	363±83	250±107	**0.03***
Reticulocytes, %	8.07±3.39	4.79±1.71	<**0.01***	7.02±3.10	3.75±0.46	0.25**
Glucose, mg/dL	71.06±9.05	84.50±7.92	<**0.01***	76.25±9.21	88.00±13.52	**0.01***
Triglycerides, mg/dL	92.94±57.96	116.2±62.59	**0.01***	101.8±33.08	84.25±11.44	0.31*
Uric acid, mg/dL	2.45±1.78	3.63±1.88	**0.03****	2.97±1.54	3.41±0.84	0.70*
AST, U/L	61.00±17.60	43.25±16.03	<**0.01***	49.00±17.61	45.75±8.26	0.58*
Direct bilirubin, mg/dL	0.58±0.31	0.40±0.19	**0.01****	0.37±0.22	0.44±0.18	0.28*
Albumin, g/dL	4.40±0.35	4.68±0.30	<**0.01***	4.32±0.30	5.0±0.36	**0.02***
Alpha 1 antitrypsin, mg/dL	165.9±43.19	111.6±45.23	<**0.01****	167±33.20	62.08±29.05	**0.01***

HbF: Fetal hemoglobin, MCH: mean corpuscular hemoglobin, MCV: mean corpuscular volume, MCHC: mean corpuscular hemoglobin concentration, WBC: white blood cell, AST: aspartate aminotransferase, n: number of individuals

*Paired T test

** Wilcoxon test, Data presented as means ± standard deviation

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Table 3. Laboratory parameters of children with SCA according to $\alpha^{2del\ 3.7\ kb}$ thalassemia before and during HU therapy.

	αα/αα, n = 14			-α/αα and -α/-α, n = 4		
Parameter	**Before HU**	**During HU**	***p* value**	**Before HU**	**During HU**	***p* value**
HbF, %	10.54±6.82	13.26±5.73	0.12*	8.61±5.91	9.57±3.10	0.71*
Hemoglobin, g/dL	7.81±1.13	8.52±1.17	**<0.01***	8.35±0.94	9.15±1.20	0.39*
Hematocrit, %	22.21±3.64	25.21±3.77	**<0.01***	24.53±2.66	27.55±4.01	0.30*
MCV, Fl	89.68±7.44	100.00±8.41	**<0.01***	77.13±10.24	85.84±10.69	0.07*
MCH, pg	31.65±3.03	33.87±2.47	**<0.01***	26.20±2.82	28.67±4.21	0.18*
MCHC, %	35.28±1.52	33.91±0.91	**<0.01****	34.05±1.35	33.32±0.99	0.54*
WBC, /Ml	15952±3534	10963±3091	**<0.01***	10300±3339	7675±2384	**0.04***
Neutrophils, /mL	7716±2103	4766±1956	**<0.01***	5061±1882	3792±1499	0.08*
Eosinophils, /mL	1250±961	607±484	**0.01****	1074±381	328±316	**<0.01***
Lymphocytes, /mL	5648±2196	4401±1770	**0.04***	3493±1134	2815±947	0.27*
Reticulocytes, %	7.75±2.78	4.51±1.27	**<0.01***	7.32±5.64	3.55±1.47	0.17*
Glucose, mg/dL	72±7.74	83.07±7.29	**<0.01****	75.50±8.58	91.50±14.20	**0.04***
HDL-C, mg/dL	31.14±7.94	37.50±9.39	**0.02****	30.50±6.56	52.30±21.95	0.18*
AST, U/L	61.00±15.97	45.57±14.43	**0.01***	51.50±25.25	42.50±18.70	0.24*
ALT, U/L	25.36±12.18	26.50±12.84	0.82**	26.75±12.50	16.25±6.24	**0.04***
Albumin, g/dL	4.30±0.33	4.69±0.30	**<0.01***	4.47±0.26	4.82±0.52	0.24*

HbF: Fetal hemoglobin, MCH: mean corpuscular hemoglobin, MCV: mean corpuscular volume, MCHC: mean corpuscular hemoglobin concentration, WBC: white blood cell, AST: aspartate aminotransferase, ALT: alanine aminotransferase, n: number of individuals

*Paired T test

** Wilcoxon test, Data presented as means ± standard deviation

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The cytoreductive effect of HU, demonstrated by significant reductions in WBC, neutrophils, eosinophils and lymphocytes, is supported by other studies that also reported similar results [16,22,27,28]. In addition, we observed a significant decrease in levels of the anti-inflammatory protein AAT during HU therapy. These findings suggest an association between HU use and reduced WBC counts mediated and/or followed by decreases in AAT levels and, consequently, improvement in the inflammatory state generally presented by individuals with SCA [26].

In individuals with SCA, the anti-inflammatory role of HDL-C changes to a pro-inflammatory one when bound to free hemoglobin released during hemolysis; this has been associated with endothelial injury and worsening of the inflammatory profile [29,30]. In the present study, children also presented significant increases in HDL-C levels during HU therapy, with eight (36.36%) presenting HDL-C levels above reference values. This finding demonstrates the beneficial effect of HU on cholesterol, and corroborates data from our recent study showing an association between HU use and variation in cholesterol concentrations in individuals with SCA [3]. A previous study also showed that HU modulates the abnormalities of RBC membrane fatty acid composition, which in addition to the vasodilators, nitric oxide and Prostaglandin E2 generated by HU, may lead to clinical improvements prior to increases in HbF induced by HU therapy [31]. Surprisingly, the children also presented increases in glucose levels during HU therapy, although these remained within normal clinical ranges. This increase may be due to the changes observed in cholesterol concentrations in children during HU therapy, since it is known that glucose and lipid metabolism are linked to each other and that hypertriglyceridemia and low HDL-C may be the cause as well as the consequence of hyperglycemia [32]. In addition, significant decreases in AST levels and increased albumin were seen during the use of HU. Contrary to this finding, Colombatti and colleagues observed a

significant reduction in AST post-HU therapy [22], which may be due to differences in the clinical profiles of individuals in the respective study groups. Based on their findings, Ragg and colleagues suggested that, in addition to increased HbF observed in individuals with SCD treated by HU, some clinical improvements may be the result of a reduced imbalance in serum protein levels [33].

HbF levels were positively correlated with hemoglobin, hematocrit, MCV and total protein levels, in addition to negatively correlated with MCHC, RDW, as well as AST and AAT levels. A previous study demonstrated a negative correlation between HbF levels and hemolytic biomarkers, reticulocytes and LDH levels, in individuals with SCA [34]. Although we did not find this correlation, our results also suggest that the increases in HbF levels induced by HU are followed by improvements in the hemolytic and inflammatory profiles of the children with SCA studied herein. Furthermore, these findings suggest that, in addition to inducing HbF synthesis, HU can also alter metabolic and hepatic biomarkers by way of yet unknown direct or indirect mechanisms. A recent study performed in individuals with β-thalassemia demonstrated the effects of HU on the metabolic profile [35]. Previous studies also reported the effectiveness of HU for the treatment and prevention of proteinuria in individuals with SCD [36–38]. Nonetheless, further follow-up studies including larger numbers of individuals with SCA that evaluate HU dosage and length of use are needed to confirm the associations seen between HU and metabolic and hepatic biomarkers, as well as to investigate the clinical impact of these effects.

Regarding the distribution of haplotypes, 45% of the children were carriers of CAR/CAR genotype, while 35% and 20% had CAR/BEN and BEN/BEN genotypes, respectively, which corroborates previously reported frequencies in Brazilian individuals with SCA [13]. Our analysis of laboratory parameters, using the dominant genetic model, in children before and during HU therapy found significantly increased HbF levels only in carriers of the BEN haplotype on HU, which corroborates the results of a previous study [39]. These authors suggested that this may be explained by the high frequency of the favorable polymorphism *BCL11A* rs1427407 in these individuals. In the present study, HU was also associated with significant decreases in platelet counts in children with the BEN haplotype. In addition, children with the CAR haplotype presented significantly increased hemoglobin, hematocrit, triglycerides and uric acid, as well as significantly decreased MCHC, WBC, neutrophils, eosinophils, lymphocytes, reticulocytes, AST and direct bilirubin during HU therapy, which is suggestive of improvements in hemolytic, inflammatory and hepatic biomarkers. Regardless of haplotype, and in addition to being associated with significant increases in MCV and MCH, as well as significant decreases in AAT, HU therapy was also found to be associated with significantly increased levels of glucose and albumin.

Distribution analysis of $\alpha^{2\ del\ 3.7kb}$ thalassemia showed similar frequencies to those reported by Adorno and colleagues [40]. Using the dominant genetic model, laboratory parameter analysis in children before and during HU therapy revealed significantly decreased ALT during HU therapy in carriers of α-thalassemia, while those without α-thalassemia presented significant increases in hemoglobin, hematocrit, MCV, MCH, HDL-c and albumin, as well as significant decreases in MCHC, neutrophils, reticulocytes and AST. These findings corroborate previous studies suggesting that α-thalassemia attenuates the effect of HU [41–43], in addition to promoting a beneficial response to HU in SCA individuals without α-thalassemia. Contrary to our findings, other studies found significantly increased hemoglobin, hematocrit, MCV, MCH and HbF, as well as significantly decreased HbS, bilirubin and reticulocytes in SCA individuals with α-thalassemia on HU [25,44]. This discrepancy may be due to other intrinsic genetic factors in the individuals enrolled in the respective study groups. Our data further corroborate an association between HU and decreased WBC and eosinophils that was also

reported by a previous study [7]. However, we discovered increased glucose levels, regardless of the presence or absence of α-thalassemia.

Conclusion

The results of the present study confirm the association between HU therapy and higher HbF levels and suggest that exclusively focusing on HbF levels may not be the most suitable method of assessing HU response in children with SCA. Due to increases in HbF levels and/or via parallel pathways, HU therapy, whether in association or not with β^S haplotypes and α-thalassemia, is positively correlated with improvements in hemolytic and inflammatory profiles. Unexpectedly, our results also suggest that, at a dose under MTD, HU may also affect metabolic biomarkers, since it was found to be associated with changes in glucose, total protein, albumin, AST and HDL-C. The principal limitation of the present study is its relatively small sample size. Accordingly, the effects on metabolic biomarkers linked to HU therapy reported herein deserve further scrutiny in a larger study population.

Supporting information

S1 Fig. Laboratory parameters of SCA children, before and during HU therapy. All data presented statistical significant difference. MCH: mean corpuscular hemoglobin, MCV: mean corpuscular volume, MCHC: mean corpuscular hemoglobin concentration, HbF: Fetal hemoglobin, WBC: white blood cell, HDL-C: high-density lipoprotein cholesterol, AST: Aspartate aminotransferase, AAT: Alpha 1 antitrypsin.
(DOCX)

S1 File. Database.
(XLSX)

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CAPÍTULO III

Manuscrito intitulado: "*Hydroxyurea and polymorphisms in genes CYP2D6, CAT and SLC14A1: Enhanced therapeutic effects in patients with sickle cell anemia*"

Em revisão no periódico *Frontiers in Pharmacogenetics and Pharmacogenomics*, desde maio de 2020.

Trata-se de um estudo que investigou o efeito de polimorfismos nos genes *CYP2D6*, *CYP3A4*, *CAT*, *SLC14A1* na resposta à HU em crianças com AF. Observou-se que, os alelos variantes dos polimorfismos *CYP2D6* 1934G>A, *CAT* -262C>T e -21A>T e *SLC14A1* G>A rs2298720 estão associados ao aumento dos efeitos da HU sobre os parâmetros laboratoriais nos indivíduos com AF. Além disso, os resultados sugerem que a aceleração no metabolismo e/ou eliminação da HU podem reduzir seus efeitos.

Hydroxyurea and polymorphisms in genes *CYP2D6*, *CAT* and *SLC14A1*: Enhanced therapeutic effects in patients with sickle cell anemia

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Keywords: sickle cell anemia, hydroxyurea, *CYP2D6*, *CYP3A4*, *CAT*, *SLC14A1*, laboratory parameters

Abstract

Variability in HU response among patients with sickle cell anemia (SCA) may occur due to drug adherence and socioeconomic, environmental, physiological and genetic factors. This study aimed to investigate the effects of polymorphisms in genes *CYP2D6*, *CYP3A4*, *CAT* and *SLC14A1* on HU response in patients with SCA. In 45 SCA patients receiving HU, and 45 who did not, we evaluated hematologic and biochemical parameters by electronic methods and investigated polymorphisms by PCR-RFLP, allele-specific PCR and the TaqMan method. Patients undergoing HU treatment presented increases in MCV, MCH, hematocrit, HbF, hemoglobin and HDL-C levels, as well as decreases in MCHC, RDW, HbS, AST, total bilirubin, lactate dehydrogenase, reticulocytes, WBC, neutrophils, eosinophils, lymphocytes, monocytes, platelets and plateletcrit (p<0.05). In patients receiving HU who carried the *CYP2D6* 1934G>A polymorphism, we found significantly higher MCV, MCH, ALT and iron serum, as well as lower total cholesterol, LDL-C, uric acid, total protein, globulin and alkaline phosphatase. Patients on HU who carried the *CAT* -21A>T and -262C>T polymorphisms presented lower alpha 1-antitrypsin (AAT) concentrations and lymphocyte counts, respectively (p<0.05). Furthermore, carriers of heterozygote and variant genotypes of both *CAT* -21A>T and -262C>T presented reductions in lymphocyte and platelet counts compared to carriers of the wild type genotypes of both *CAT* -21A>T and -262C>T (p<0.05). Carriers of the *SLC14A1* G>A (rs2298720) polymorphism exhibited higher creatinine and lower AAT levels (p<0.05). Our results

suggest the association between these specific polymorphisms and the enhancement of HU effects in patients with SCA.

1 Introduction

Hydroxyurea (HU) is an antineoplastic drug primarily used to treat patients with myeloproliferative syndromes (Bandeira et al., 2004; Kovacic, 2011). In addition to its antimetabolite effects, studies have also demonstrated an anti-sickling action. Accordingly, HU was approved by the U.S. Food and Drug Administration for the treatment of patients with sickle cell anemia (SCA) in the context of a severe clinical profile (King, 2003; Huang et al., 2006; Kolliopoulou et al., 2017). Reports have demonstrated that HU treatment leads to increases in fetal hemoglobin (HbF), mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV), as well as decreases in white blood cell (WBC), platelet and reticulocyte counts. Furthermore, HU is also associated with reduced adhesion molecule expression and greater nitric oxide (NO) bioavailability (King, 2003; Rees et al., 2010; Sassi et al., 2010; Silva-Pinto et al., 2013; Yahouédéhou et al., 2019). Clinically, patients with SCA undergoing HU treatment present a lower incidence of painful crises, rates of hospitalization, acute chest syndrome episodes, blood transfusion and mortality (King, 2003; Rees et al., 2010; Sassi et al., 2010; Silva-Pinto et al., 2013). Despite these beneficial effects, studies have also demonstrated inter-individual variations in the therapeutic response to HU (Ware et al., 2002; King, 2003; Ma et al., 2007; Ware, 2010). This may be attributed to therapy adherence, or socioeconomic, environmental, physiological and genetic factors. Nonetheless, genetic factors have been highlighted as one of the most important determinants of variation in HU therapeutic response (Gravia et al., 2014; Yahouédéhou et al., 2018a).

Polymorphisms in genes encoding drug metabolizing enzymes (DME) and solute carriers may alter the bioavailability of drugs and metabolism, thereby influencing efficiency and toxicity (Sheng et al., 2014). Accordingly, we recently conducted a review of the literature focused on investigations into the metabolism of HU by enzymes of the CYP450 family and catalase, as well as reports detailing associations between the upregulation of urea transporter-B (UTB) and HU response in erythroid cells (Yahouédéhou et al., 2018a).

The CYP450 family consists of various isoenzymes essential for endo- or xenobiotic metabolism. CYP3A4, which is more abundant in the liver, can metabolize 50% of commercially available drugs and has steroid hormones, analgesics and antihistamines, as well as antitumor and immunosuppressive agents as substrates (Božina et al., 2009). This isoenzyme is encoded by the polymorphic *CYP3A4* gene, which is located on chromosome 7 (7q22.1) (Božina et al., 2009). One of the most investigated polymorphisms in this gene is *CYP3A4*1B* (*CYP3A4* -392A>G) (Maruf et al., 2012), associated with increased gene transcription and CYP3A4 activity (Jin et al., 2005; Bhatnagar et al., 2010; He et al., 2014). CYP2D6 is more abundant in the liver, and is encoded by the *CYP2D6* gene located on chromosome 22 (22q13.2). This isoenzyme metabolizes approximately 25% of all known drugs, including antidepressants, antiarrhythmics, analgesics and anticancer agents. Polymorphisms in *CYP2D6* have been reported to affect enzyme activity (Božina et al., 2009; Sayed and Imam, 2012). In addition, a *CYP2D6*4* variant (*CYP2D6* 1934G>A) has been linked to reduced CYP2D6 activity, which increases intoxication risk, as well as side effects, following exposure to xenobiotics (Božina et al., 2009; Sayed and Imam, 2012).

Catalase, an important endogen antioxidant enzyme, is known to be involved in neutralization pathways of reactive oxygen species. Catalase is most abundant in the liver, kidney and erythrocytes (Forsberg et al., 2001; Babusikova et al., 2013; Liu et al., 2015). Studies have shown that this

antioxidant enzyme can convert HU into nitrite/nitrate (Huang et al., 2004; King, 2005) and that HU toxicity *in vivo* is dependent on catalase activity (Juul et al., 2010). Catalase is encoded by the *CAT* gene, located on chromosome 11 (11p13.31). Rare polymorphisms in this gene have been associated with a lack of enzyme production, as well as protein instability (Forsberg et al., 2001). Polymorphisms -21A>T and -262C>T located in the promoter region of the *CAT* gene can lead to reduced transcription and enzyme activity (Perianayagam et al., 2007; Liu et al., 2015).

UTB or Solute Carrier Family 14 Member 1 (SLC14A1) is a family of urea transporters important to the regulation of urine concentration (Garcia-Closas et al., 2011; Stewart, 2011; Matsuda et al., 2015; Ebbinghaus et al., 2017). UTB is encoded by the *SLC14A1* gene located on chromosome 18 (18q12.3). This protein is widely expressed on the plasma membranes of red blood cells and poorly expressed in endothelial cells of the descending rectum vessel in the kidney, brain, ear, testis, intestine and urinary bladder (Sands, 2002; Garcia-Closas et al., 2011; Rafnar et al., 2011; Ebbinghaus et al., 2017). It is known that molecules, such as methylurea, formamide, methylformamide, acetamide and acrylamide, may transit, rapidly and passively, via channel proteins such as UTB (Esteva-Font et al., 2015; Hou et al., 2017). A study performed in patients with SCA demonstrated an association between polymorphisms in the *SLC14A1* gene and variations in HU pharmacokinetics (Ware et al., 2011).

The present study aimed to investigate, in SCA patients undergoing HU treatment, possible associations between laboratory parameters and the following polymorphisms linked to alterations in gene expression and/or enzyme activity: *CYP3A4* -392A>G (rs2740574), *CYP2D6* 1934G>A (rs3892097), *CAT* -21A>T (rs7943316) and -262C>T (rs1001179), as well as *SLC14A1* G>A (rs2298720).

2 Materials and methods

2.1 Subjects and ethical aspects

The present cross-sectional study included patients with SCA (HbSS), all seen at the outpatient clinic of the Hematology and Hemotherapy Foundation of Bahia (HEMOBA), located in Salvador, Brazil. Forty-five (45) patients on HU treatment (HU^+), as well as 45 who were not using this treatment (HU^-), were enrolled. Twenty-two (48.89%) HU^+ patients were female compared to 20 (44.44%) in the HU^- group. The median age of the HU^+ group was 15 years (range: 9-22 years), versus 15.5 years (range: 7-21 years) in the HU^- group. All patients reported regular use of folic acid and were in steady-state, defined as the absence of acute crisis and no use of blood therapy in the three months prior to blood collection. HU dosage ranged between 10.5 and 27.6 mg/kg/day (median: 16.46). The average length of HU treatment was 31.7 months. Patients undergoing chronic transfusion therapy and those with active infection or inflammatory diseases were excluded from the study.

This research protocol received approval from the Institutional Review Board of São Rafael Hospital (protocol number: 1.400.527) and was conducted in compliance with the Declaration of Helsinki (1964) and its revisions. All individuals or their legal guardians provided a signed term of informed consent prior to enrollment in the study.

2.2 Laboratory parameters

Blood samples were collected by venipuncture in the morning, after 12h of fasting, under standardized conditions. Analyses were performed at the Clinical and Toxicological Analysis Laboratory (LACTFAR) and the Anemia Research Laboratory (LPA), both affiliated with the

Pharmaceutical School of the Federal University of Bahia (FACFAR-UFBA), as well as at the Laboratory of Investigation in Genetics and Translational Hematology, Gonçalo Moniz Institute (LIGHT-IGM).

Hematological parameters were evaluated using a Beckman Coulter LH 780 Hematology Analyzer (Beckman Coulter, Brea, California, USA). Qualitative and quantitative profiles of hemoglobin were assessed by high-performance liquid chromatography using an HPLC/Variant II hemoglobin testing system (BIO-RAD, Hercules, CA, USA). Biochemical parameters, as lipid profile, total proteins and fractions, total bilirubin and fractions, lactate dehydrogenase (LDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl-transferase (GGT), as well as renal profile and serum iron levels, were assessed using an A25 spectrophotometer autoanalyzer (Biosystems SA, Barcelona, Spain). Alpha-1 antitrypsin (AAT) and C-reactive protein (C-RP) levels were quantified using an Immage 800 system (Beckman Coulter, Fullerton, CA, USA). Serum ferritin was assessed on an Access 2 Immunoassay System (Beckman Coulter, Fullerton, CA, USA).

2.3 Molecular analysis

Molecular analyses were carried out on genomic DNA obtained from whole blood samples. *CYP3A4* -392A>G (rs2740574), *CYP2D6* 1934G>A (rs3892097), *CAT* -21A>T (rs7943316) and -262C>T (rs1001179) polymorphisms were investigated using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique (Maruf et al., 2012; Sayed and Imam, 2012; Liu et al., 2015). Primers and restriction enzymes used in the PCR-RFLP reactions are presented in table S1. The *SLC14A1* G>A (rs2298720) polymorphism was investigated using the TaqMan method in accordance with the manufacturer's instructions. Considering that beta S (β^S) haplotypes and alpha (α)-thalassemia-2 with the 3.7kb deletion ($\alpha^{2\ del\ 3.7kb}$ thalassemia) are associated with alterations in the laboratory parameters of patients with SCA (Camilo-Araújo et al., 2014; Darbari et al., 2014), these markers were also investigated. Beta S haplotypes and $\alpha^{2\ del\ 3.7kb}$ thalassemia were investigated by PCR-RFLP (Sutton et al., 1989) and allele-specific PCR (Chong et al., 2000), respectively.

2.4 Statistical analysis

All statistical analyses were performed using GraphPad Prism 6.0 and SPSS 17.0, with $p<0.05$ considered statistically significant. The Shapiro-Wilk test was used to determine quantitative variable distributions. Mean values between the two groups were compared using the unpaired t-test for variables with a normal distribution, while the Mann-Whitney *U* test was used for variables with non-normal distributions. ANOVA or Kruskal Wallis were used to compare mean values between more than two groups according to distribution. Frequencies of qualitative variables were also calculated. The Chi-square test ($\chi 2$-test) with Yates correction and Fischer's Exact test were used to investigate differences in genotypic and allelic frequencies between the two groups. Associations between parameters and polymorphisms were evaluated using codominant, dominant and recessive genetic models, and multivariate linear regression analysis was employed to investigate the influence of the investigated polymorphisms on laboratory parameters. Results were expressed as mean ± standard deviation (M±SD), median (minimum-maximum), or number or frequency where appropriate.

3 Results

3.1 Laboratory parameters of patients with or without HU treatment

The hematological and biochemical parameters of patients receiving, or not, HU treatment are presented in table S2. HU use was associated with an increase in HbF levels ($p<0.0001$) and,

consequently, a reduction in HbS levels ($p = 0.0001$). The analysis of biomarkers related to hemolysis and hepatic injury demonstrated an association between HU treatment and increases in hemoglobin ($p<0.0001$), hematocrit ($p<0.0001$), MCV ($p<0.0001$) and MCH ($p = 0.0004$), as well as reductions in reticulocytes ($p = 0.0419$), mean corpuscular hemoglobin concentration (MCHC) ($p<0.0001$), red blood cell distribution width (RDW) ($p<0.0001$), total bilirubin ($p = 0.0454$), AST ($p = 0.0023$) and LDH ($p = 0.0004$). Regarding leukocyte and platelet profiles, decreased levels of WBC ($p<0.0001$), neutrophils ($p = 0.0033$), eosinophils ($p = 0.0044$), lymphocytes ($p = 0.0243$), monocytes ($p<0.0001$), platelets ($p = 0.0101$) and plateletcrit ($p = 0.0005$) were seen in patients undergoing HU treatment compared to those who were not. HU was also associated with increased high-density lipoprotein cholesterol (HDL-C) levels ($p = 0.0137$).

3.2 Frequencies of investigated polymorphisms in patients with or without HU treatment

The analysis of genotypic and allelic distributions of the investigated polymorphisms revealed a lower frequency of the *CYP2D6* 1934GA+AA genotype in patients undergoing HU treatment compared to those who were not ($p = 0.0149$). Moreover, lower and higher frequencies of the *CYP3A4* -392G ($p = 0.0248$) and *CAT* -21T ($p = 0.0485$) variant alleles were respectively observed in patients on HU compared to those who did not receive this treatment (Table S3). *CAT* -21A>T, *CAT* -262C>T and *SLC14A1* G>A (rs2298720) were found to be in Hardy-Weinberg Equilibrium (HWE), while *CYP2D6* 1934G>A and *CYP3A4* -392A>G were not. The genotypic distribution of β^S haplotypes and α-thalassemia according to HU treatment are presented in table S4; no significant differences were observed between the two groups ($p>0.05$).

3.3 Associations between polymorphisms and laboratory parameters

Using a codominant genetic model, HU^+ patients who had the *CYP2D6* heterozygote (1934GA) or variant (1934AA) genotypes presented significantly increased MCV, MCH and iron serum, as well as reduced total cholesterol, low-density lipoprotein cholesterol (LDL-C), uric acid and alkaline phosphatase (ALP) compared with carriers of the wild type (1934GG) genotype. Furthermore, SCA HU^+ patients who were carriers of the heterozygote genotype, *CYP2D6* 1934GA, presented intermediary values in laboratory investigations compared with carriers of the *CYP2D6* wild type (1934GG) and variant (1934AA) genotypes (Figure 1). Using a dominant genetic model for analysis in HU^+ patients, associations between *CYP2D6* 1934G>A and significantly increased MCV, MCH, iron serum and ALT were observed, in addition to significant decreases in total cholesterol, uric acid, ALP, total protein and globulin (Figure 2). *CAT* -21A>T demonstrated a significant association with reduced AAT concentrations under both co-dominant and recessive genetic models, while *CAT* -262C>T was found to be significantly associated with reduced lymphocyte counts in HU^+ patients (Figure 3). HU^+ patients who were carriers of heterozygote and variant genotypes of both *CAT* -21A>T and -262C>T presented reductions in lymphocyte and platelet counts compared to carriers of the wild type genotypes of both *CAT* -21A>T and -262C>T (Figure 4). Finally, the *SLC14A1 G>A* (rs2298720) polymorphism was found to be significantly associated with elevated creatinine and reduced AAT in HU^+ patients (Figure 5). None of these associations was detected in patients who did not receive HU. Moreover, *CYP3A4* -392A>G was not found to be clinically significant with respect to the parameters investigated.

3.4 Multivariate linear regression analyses

Based on the findings of our association analyses among HU^+ patients, multivariate linear regression analyses were conducted, including β^S haplotypes and α-thalassemia, as well as some laboratory parameters as confounding variables. Our analysis using the dominant genetic model revealed

independent associations between the *CYP2D6* 1934G>A polymorphism and increases in MCV, MCH and iron serum levels, as well as decreases in total cholesterol, uric acid, total protein and globulin levels. We also observed an independent association between the *CAT* -21A>T polymorphism and AAT using the recessive genetic model. All statistically significant results from our regression analyses are presented in table 1.

4 Discussion

The present study investigated the possible influence of polymorphisms in genes encoding DME and solute carrier on SCA patients' response to HU treatment. As expected, in contrast to SCA patients who did not receive HU, those who received this treatment presented increased HbF levels and improvements in hemolytic and inflammatory profiles, as evidenced by significantly elevated hemoglobin, hematocrit, MCV and MCH, as well as decreased reticulocytes, MCHC, RDW, AST, total bilirubin, LDH, WBC, neutrophils, eosinophils, lymphocytes, monocytes, platelets and plateletcrit. These findings corroborate previous studies, which also demonstrated improvements in hemolytic, hepatic and inflammatory profiles in patients with SCA undergoing HU therapy (Santos and Maia, 2010; Voskaridou et al., 2010; Torres et al., 2012; Pallis et al., 2014; Belini Junior et al., 2015; Shome et al., 2016; Quarmyne et al., 2017; Colombatti et al., 2018). We further observed an increase in HDL-C concentration, demonstrating the effect of HU on lipid metabolism, which is consistent with our previous results (Yahouédéhou et al., 2018b, 2019).

The present analysis of genotype and/or allele distribution of *CYP3A4* -392A>G, *CYP2D6* 1934G>A and *CAT* -21A>T polymorphisms revealed frequencies divergent to those observed in other studies conducted in different patient populations, as well as healthy controls (Maruf et al., 2012; Sayed and Imam, 2012; Liu et al., 2015). Contrarily, the frequencies of the *CAT* -262C>T and *SLC14A1* G>A (rs2298720) polymorphisms observed herein corroborate previously published results (Angona et al., 2013; Liu et al., 2015). Furthermore, we found a significantly reduced frequency of the *CYP2D6* 1934GA+AA genotype in patients receiving HU compared to those who did not, as well as significantly lower and higher frequencies of the *CYP3A4* -392G and *CAT* -21T allelic variants, respectively, in patients on HU versus those who did not receive this treatment.

Association analyses of polymorphisms with laboratory parameters revealed interesting results. SCA patients who were carriers of the variant AA genotype presented more pronounced alterations in response to HU treatment compared to those with the wild type (GG) genotype, indicating an association between the *CYP2D6* 1934G>A polymorphism and an enhancement in HU effects. Furthermore, this enhancement was also found to be correlated with the number of variant A allele carried, as patients with the heterozygote (GA) genotype presented intermediary values on laboratory parameters compared to those with the wild type (GG) or homozygote variant (AA) genotypes. Reports have shown that the variant A allele leads to the incorrect splicing of RNAm and the formation of trunked protein, which reduces enzymatic activity (Sayed and Imam, 2012). Hence, our finding, together with the notion that patients with the wild type GG genotype are considered to be rapid metabolizers (Sayed and Imam, 2012), suggests that this isoenzyme may inactivate HU or accelerate its elimination, which could explain the lessened effects in response to HU seen in carriers of this genotype. Furthermore, in comparison to carriers of the wild type (GG) genotype, we observed that 95.65% of patients who received HU and were carriers of the heterozygote (GA) or homozygote variant (AA) genotypes presented MCV>98fL, which generally correlate positively with HbF levels (Borba et al., 2003) and, consequently, improved laboratory and clinical profiles. This further reinforces the association between the variant A allele and enhancements in HU effects.

Studies have demonstrated that HU is metabolized by catalase (Huang et al., 2004; Juul et al., 2010) and that the *CAT* -21A>T and -262C>T polymorphisms are associated with a reduction in or the absence of enzyme production (Liu et al., 2015). The present study observed associations between the *CAT*-21TT and *CAT*-262CT genotypes in patients receiving HU with respect to reductions in inflammatory biomarkers, such as AAT levels and lymphocyte counts. In addition, carriers of heterozygote and variant genotypes of both *CAT* -21A>T and -262C>T polymorphisms also exhibited significant reductions in lymphocyte and platelet counts compared to carriers of the wild type genotypes of both *CAT* -21A>T and -262C>T. We recently reported significant reductions in WBC, neutrophils, eosinophils and lymphocytes, as well as reduced AAT levels, in individuals undergoing HU therapy (Yahouédéhou et al., 2019). The concentrations of AAT, an acute-phase protein with anti-inflammatory properties, may be elevated in the context of an inflammatory response, as well as in response to tissue damage (Carvalho et al., 2017). Studies have demonstrated that AAT levels can decrease during HU therapy, either as a consequence or a cause reductions in WBC counts (Pallis et al., 2014; Yahouédéhou et al., 2019). Hence, our findings suggest the likelihood that lower catalase expression and activity may lead to higher HU bioavailability, which could explain the associations seen herein between the *CAT* -21A>T and -262C>T polymorphisms and improvement in inflammatory biomarkers.

A recent study demonstrated a correlation between the upregulation of *SCL14A1* and higher *HBG2* expression in erythroid cells treated with HU (Walker and Ofori-Acquah, 2017). Another study involving patients with sickle cell disease (SCD) reported an association between the *SLC14A1* G>A (rs2298720) polymorphism and alterations in HU pharmacokinetic parameters (Ware et al., 2011). In the present study, we found an association between the variant A allele of the *SLC14A1* G>A (rs2298720) polymorphism and elevated creatinine and reduced AAT concentrations in patients undergoing HU treatment. Our research group previously found a negative correlation between AAT and creatinine in patients with SCD, i.e. those with elevated AAT presented reduced creatinine serum concentrations (Carvalho et al., 2017). Moreover, it was reported that the accumulation of urea, an HU analog, might occur in human urothelial cells due to low *SLC14A1* expression (Hou et al., 2017). Considering these findings together, it is possible to speculate that the *SLC14A1* G>A (rs2298720) polymorphism may also be associated with diminished HU elimination, resulting in increased bioavailability. Moreover, as a result of reduced AAT concentrations, HU use may lead to an increase in creatinine serum levels, suggesting the beneficial effect of the variant A allele on inflammatory and renal dysfunction biomarkers.

In consonance with our hypothesis that some of the polymorphisms investigated herein could result in reduced HU metabolism or elimination, which results in increased bioavailability, a report suggested that high glutathione S transferase (GST) activity leading to ultra-rapid drug metabolism could reduce the bioavailability of intermediate metabolites, and, consequently, attenuate the cytotoxic effects of antineoplastic drugs on tumor cells (Safarinejad et al., 2011). Moreover, studies have also demonstrated the beneficial effects of *GST* genotypes on cancer survival, in association with low or absent enzymatic activity (Safarinejad et al., 2011). Indeed, the GSTs are a superfamily of isoenzymes crucial to the detoxification of xenobiotics and endobiotics, as these transferases catalyze reactions between glutathione and intermediate/toxic metabolites derived from phase I metabolism (Martínez-Guzmán et al., 2017).

Reports have shown that β^S haplotypes and α-thalassemia are associated with alterations in laboratory parameters of patients with SCA (Camilo-Araújo et al., 2014; Darbari et al., 2014). Taking this into account, we performed multivariate regression analyses using these genetic biomarkers as confounding variables, which confirmed the independent association of *CYP2D6* 1934G>A, *CAT* -

21A>T and -262C>T with several of these parameters. Accordingly, it will be interesting to investigate, in patients receiving HU who are carriers of these variant alleles, the clinical repercussions of the presently observed alterations in laboratory parameters.

5 Conclusion

The present study investigated HU response in patients with SCA who carry polymorphisms in genes encoding DME and solute carriers. Our results indicate that the *CYP2D6* 1934G>A, *CAT* -21A>T and -262C>T, and *SLC14A1* G>A (rs2298720) polymorphisms are associated with enhancements in HU effects on laboratory parameters. Accordingly, we suggest that these polymorphisms are linked to the reduced metabolism or elimination of HU, which may increase its therapeutic effects in patients with SCA. The future investigation of this hypothesis may help to fully elucidate the HU metabolic pathway and confirm these polymorphisms as genetic biomarkers of HU response.

6 Conflict of Interest

The authors declare that our research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 Author Contributions

SCMAY, EVA and MSG conceived and designed the experiments. SCMAY, JSSN, CCG, SPC, RPS, CVBF, LMF, USN and RMO collected the samples. SCMAY, JSSN, CCG, SPC, RPS, CVBF, LMF, CAF, CSAA and TSCR performed the experiments. SCMAY and JSSN analyzed the data. VMLN and LCR followed the patients. SCMAY drafted the manuscript. JSSN and CCG contributed to the writing of the manuscript. EVA and MSG supervised the study and critically revised the manuscript. All authors revised and approved the final version of the manuscript.

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Figure 1: Frequency of *CYP2D6* 1934G>A in patients with SCA undergoing HU therapy (**A**) and its association with laboratory parameters using the co-dominant genetic model (**B-H**). The co-dominant genetic model compared three genotype groups (wild type *vs* heterozygote *vs* variant). MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, LDL: low-density lipoprotein cholesterol, ALT: alanine aminotransferase, ANOVA or Kruskal-Wallis where appropriate.

Figure 2: Frequency of *CYP2D6* 1934G>A in patients with SCA undergoing HU therapy (**A**) and its association with laboratory parameters using the dominant genetic model (**B-J**). The dominant genetic model compared two genotype groups (wild type *vs* heterozygote/variant). MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, LDL: low-density lipoprotein cholesterol, ALT: alanine aminotransferase, unpaired t-test or Mann Whitney *U* test where appropriate.

Figure 3: Frequencies of *CAT* -21A>T (**A** and **C**) and -262C>T (**E**) and respective associations with laboratory parameters, using co-dominant (**B** and **F**), and recessive (**D**) models. The co-dominant genetic model compared three genotype groups (wild type *vs* heterozygote *vs* variant), while the recessive genetic model compared two genotype groups (wild type/heterozygote *vs* variant). AAT: alpha 1-antitrypsin, ANOVA, Kruskal-Wallis, unpaired t-test or Mann-Whitney *U* test where appropriate.

Figure 4: Number of SCA patients receiving HU who carry either two wild type or two variant alleles in both the *CAT* -21A>T and -262C>T polymorphisms (**A**) and association of the *CAT* haplotype with laboratory parameters (**B** and **C**). *CAT* 0: patients with both *CAT* -21AA and -262CC genotypes, *CAT* 1: patients with both *CAT* -21AA+TT and -262CT genotypes, unpaired t-test or Mann Whitney *U* test where appropriate.

Figure 5: Frequency of the *SLC14A1* G>A (rs2298720) polymorphism in patients with SCA undergoing HU therapy (**A**) and its association with laboratory parameters under dominant genetic model analysis (**B** and **C**). Two genotype groups (wild type *vs* heterozygote/variant) were compared. AAT: alpha 1-antitrypsin, unpaired t-test or Mann Whitney *U* test where appropriate.

559 **Table 1**: Multivariate linear regression models of *CYP2D6* 1934G>A and *CAT* -21A>T and -262C>T
560 polymorphisms in patients with SCA undergoing HU treatment

Dependent variables	Independent variables	β	R	*p*1	*p*2
***CYP2D6* 1934G>A**					
MCV, fL	*CYP2D6**	0.42	0.54	**0.006**	**0.008**
	HAP	0.09		0.544	
	Tal	-0.34		**0.031**	
MCH, pg	*CYP2D6**	0.41	0.57	**0.006**	**0.004**
	HAP	0.13		0.364	
	Tal	-0.40		**0.010**	
Cholesterol total, mg/dL	*CYP2D6**	-0.33	0.53	**0.028**	**0.008**
	HAP	-0.04		0.788	
	Tal	0.42		**0.008**	
Uric acid, mg/dL	*CYP2D6**	-0.41	0.43	**0.007**	**0.018**
	HAP	-0.10		0.480	
Iron serum, mcg/dL	*CYP2D6**	0.33	0.39	**0.030**	**0.035**
	HAP	0.20		0.170	
Total protein, g/dL	*CYP2D6**	-0.32	0.44	**0.032**	**0.013**
	HAP	-0.30		**0.044**	
Globulin, g/dL	*CYP2D6**	-0.31	0.54	**0.030**	**0.004**
	HAP	-0.38		**0.009**	
	Hemoglobin	-0.26		0.066	
***CAT* -21A>T**					
AAT, mg/dL	*CAT***	-0.39	0.40	**0.011**	**0.035**
	HAPCAT	0.51		0.731	
***CAT* -262C>T**					
Lymphocytes, /mL	*CAT**	-0.22	0,51	0.144	**0.014**
	HAP	0.34		**0.028**	
	Tal	-0.35		**0.026**	

561 MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, HAP: β^S haplotype, Tal: $\alpha^{2\ del\ 3.7kb}$
562 thalassemia, R: coefficient of determination, β: coefficient of regression, *p*1: *p* value of the independent
563 variable, *p*2: *p* value of the model, *: dominant genetic model, **: recessive genetic model

Supplementary Material

Table S1: Primers and restriction enzymes used in PCR-RFLP reactions to investigate selected polymorphisms

Polymorphism	Method	Primer	Restriction enzyme	Reference
CYP3A4 -392A>G	PCR-RFLP	F: 5'- GGA ATG AGG ACA GCC ATA GAG ACA AGG GGA -3' R: 5'- CCT TTC AGC TCT GTG TTG CTC TTT GCT G -3'	MboII	Maruf et al., 2012
CYP2D6 1934G>A	PCR-RFLP	F: 5'- GCC TTC GCC AAC CAC TCC G -3' R: 3'- AAA TCC TGC TCT TCC GAG GC -3'	MvaI	Sayed and Imam, 2012
CAT -21A>T	PCR-RFLP	F: 5'- AAT CAG AAG GCA GTC CTC CC -3' R: 5'- TCG GGG AGC ACA GAG TGT AC -3'	HinfI	Liu et al., 2015
CAT -262C>T	PCR-RFLP	F: 5'- AGA GCC TCG CCC CGC CGG ACC G -3' R: 5'- TAA GAG CTG AGA AAG CAT AGC T -3'	SmaI	Liu et al., 2015

Table S2: Laboratory parameters of patients with SCA receiving or not HU therapy

Parameter	HU^+ (N=45) Mean ± SD	HU^- (N=45) Mean ± SD	*p* value
Hemoglobin			
HbF, %	11.90±5.70	7.05±4.67	**<0.0001***
HbS, %	85.05±5.64	89.72±5.20	**0.0001***
Hemolysis			
RBC, $x10^9$/mL	2.75±0.52	2.71±0.52	0.7159*
Hemoglobin, g/dL	8.93±1.12	7.95±0.82	**<0.0001****
Hematocrit, %	26.74±3.57	23.32±2.87	**<0.0001***
Reticulocyte, %	124963±67866	153473±62318	**0.0419***
MCV, fL	98.60±12.65	86.49±9.61	**<0.0001***
MCH, pg	33.07±4.45	29.89±3.65	**0.0004***
MCHC, %	33.52±0.86	34.55±1.10	**<0.0001***
RDW, %	20.77±3.62	24.56±3.31	**<0.0001***
Erythroblast (/10^2 leukocytes)	1.59±5.04	1.33±2.66	0.4384**
Leukocytes			
WBC, /mL	9694±3069	12393±2162	**<0.0001****
Neutrophil, /mL	4511±2048	5811±2009	**0.0033***
Eosinophil, /mL	367±358	612±427	**0.0044***
Lymphocyte, /mL	3808±1365	4460±1319	**0.0243***
Monocyte, mL	843±470	1325±586	**<0.0001***
Platelets			
Platelet, $x10^3$/mL	388±152	443±86	**0.0110****
Plateletcrit, %	0.29±0.12	0.35±0.07	**0.0005****
MPV, fL	7.88±0.87	8.08±0.82	0.2519*
PDW, %	16.37±0.75	16.18±0.56	0.2181*
Hemolytic plus hepatic			
Total bilirubin, mg/dL	2.65±1.77	3.36±1.55	**0.0454***
Direct bilirubin, mg/dL	0.38±0.15	0.43±0.18	0.1532*
Indirect bilirubin, mg/dL	2.26±1.76	2.92±1.51	0.0597*
Lactate dehydrogenase, U/L	991.58±344.18	1289.35±420.10	**0.0004***
Iron serum, mcg/dL	113.29±50.81	100.62±50.51	0.2388*
Aspartate aminotransferase, U/L	41.31±16.53	52.78±18.09	**0.0023***

Table S2: Continued

Lipids and glucose			
Total cholesterol, mg/dL	118.44±25.26	123.35±26.20	0.3678*
HDL-C, mg/dL	38.00±8.45	33.60±8.14	**0.0137***
LDL-C, mg/dL	59.74±21.06	66.14±22.05	0.1630*
Triglycerides, mg/dL	99.48±43.45	117.87±54.12	0.0791*
Glucose, mg/dL	83.82±9.30	82.22±7.37	0.3681*
Renal			
Urea, mg/dL	18.69±7.99	16.26±4.42	0.3229**
Creatinine, mg/dL	0.47±0.12	0.43±0.15	0.1486*
Hepatic			
Alanine aminotransferase, U/L	19.07±11.83	21.24±10.71	0.3624*
γ-glutamine aminotransferase, U/L	23.91±15.88	26.13±19.02	0.5490*
Total protein, g/dL	8.44±0.79	8.29±0.84	0.3759*
Albumin, g/dL	4.76±0.33	4.79±0.34	0.6893*
Globulin, g/dL	3.68±0.71	3.51±0.65	0.2294*
Alkaline phosphatase, U/L	124.42±61.01	133.38±71.83	0.5255*
Inflammatory			
Uric acid, mg/dL	4.02±1.35	3.95±1.08	0.8053*
Ferritin, ng/dL	407.20±345.49	243.10±364.39	0.0960*
C-reactive protein, mg/L	6.34±9.45	4.54±3.41	0.5328**
Alpha 1 antitrypsin, mg/dL	77.09±44.11	75.65±43.89	0.8806*

RBC: red blood cell, MCH: mean corpuscular hemoglobin, MCV: mean corpuscular volume, MCHC: mean corpuscular hemoglobin concentration, HbS: variant S hemoglobin, HbF: Fetal hemoglobin, RDW: red cell distribution width, HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol, WBC: white blood cell, MPV: mean platelet volume, PDW: platelet distribution width, * Independent T test, ** Mann Whitney *U* test

Table S3: Genotypic and allelic frequencies of SNPs in patients with SCA receiving or not HU therapy

SNP		Genotype, N (%)					Allele, N1 (%)			
		Total	HU$^+$	HU$^-$	*p* value$^{\#}$		Total	HU$^+$	HU$^-$	*p* value
***CYP2D6* 1934G>A**	GG	09 (10.34)	08 (18.60)	01 (2.27)	**0.0149**	G	88 (50.57)	47 (54.65)	41 (46.59)	0.3619
	GA	70 (80.46)	31 (72.10)	39 (88.64)		A	86 (49.43)	39 (45.35)	47 (53.41)	
	AA	8 (9.20)	04 (9.30)	04 (9.09)						
	GA+AA	78 (89.66)	35 (81.39)	43 (97.73)						
***CYP3A4* -392A>G**	AA	32 (36.36)	20 (44.44)	12 (27.91)	0.1644	A	96 (54.54)	57 (63.33)	39 (45.35)	**0.0248**
	AG	32 (36.36)	17 (37.78)	15 (34.88)		G	80 (45.46)	33 (36.67)	47 (54.65)	
	GG	24 (27.27)	08 (17.78)	16 (37.21)						
	AG+GG	56 (63.64)	25 (55.55)	31 (72.09)						
CAT* -21A>T	AA	26 (29.55)	09 (20.45)	17 (38.64)	0.1019	A	98 (55.68)	42 (47.73)	56 (63.64)	**0.0485**
	AT	46 (52.27)	24 (54.55)	22 (50.00)		T	78 (44.32)	46 (52.27)	32 (36.36)	
	TT	16 (18.18)	11 (25.00)	05 (11.36)						
	AT+TT	62 (70.45)	35 (79.54)	27 (61.36)						
CAT* -262C>T	CC	78 (87.64)	38 (86.36)	40 (88.89)	0.9682	C	167 (93.82)	82 (93.18)	85 (94.44)	0.9693
	CT	11 (12.36)	06 (13.64)	05 (11.11)		T	11 (6.18)	6 (6.82)	5 (5.56)	
	TT	0 (0)	0 (0)	0 (0)						
	CT+TT	11 (12.36)	06 (13.64)	05 (11.11)						
SLC14A1* G>A	GG	64 (71.11)	29 (64.45)	35 (77.78)	0.2449	G	153 (85.0)	73 (81.11)	80 (88.89)	0.2104
	GA	25 (27.78)	15 (33.33)	10 (22.22)		A	27 (15.0)	17 (18.89)	10 (11.11)	
	AA	1 (1.11)	1(2.22)	0 (0)						
	GA+AA	26 (28.89)	16 (35.55)	10 (22.22)						

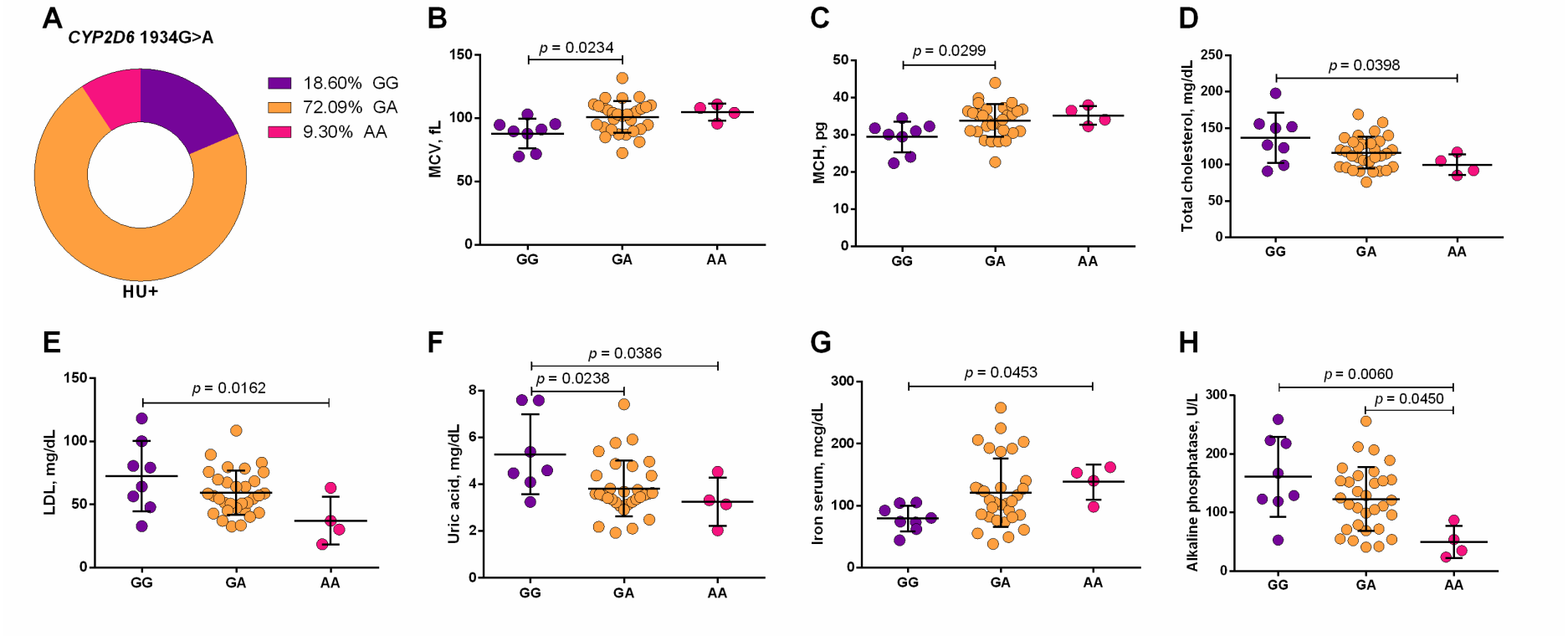
HU: hydroxyurea, SNP: single nucleotide polymorphism, N: number of individual, N1: number of chromosomes, * the polymorphism was in Hardy-Weinberg Equilibrium, $^{\#}$ *p* value refers to comparisons between wild type genotype *vs* heterozygote/variant genotypes, χ2-test with Yates correction and Fischer's Exact test performed where appropriate

Table S4: Genotypic frequencies of β^S haplotype and α-thalassemia in SCA patients receiving or not HU therapy

	Patients with SCA		
	HU^+, N (%)	**HU^-, N (%)**	***p* value**
β^S haplotype			
BEN/BEN	15 (33.33%)	21 (46.67%)	0.282
CAR/BEN and CAR/CAR	30 (66.67%)	24 (53.33%)	
$\alpha^{2del\ 3.7kb}$ thalassemia			
αα/αα	30 (75%)	30 (75%)	1.000
-α/αα and -α/-α	10 (25%)	10 (25%)	

SCA: sickle cell anemia, HU: hydroxyurea, BEN: Benin haplotype, CAR: Central African Republic haplotype, %: frequency, N: number, χ2-test with Yates correction was performed

Figure 1.TIF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CYP2D6 1934G>A
18.60% GG
72.09% GA
9.30% AA
HU+
B
p = 0.0234
MCV, fL
GG
GA
AA
C
p = 0.0299
MCH, pg
D
p = 0.0398
Total cholesterol, mg/dL
E
p = 0.0162
LDL, mg/dL
F
p = 0.0386
p = 0.0238
Uric acid, mg/dL
G
p = 0.0453
Iron serum, mcg/dL
H
p = 0.0060
p = 0.0450
Alkaline phosphatase, U/L

Figure 2.TIF

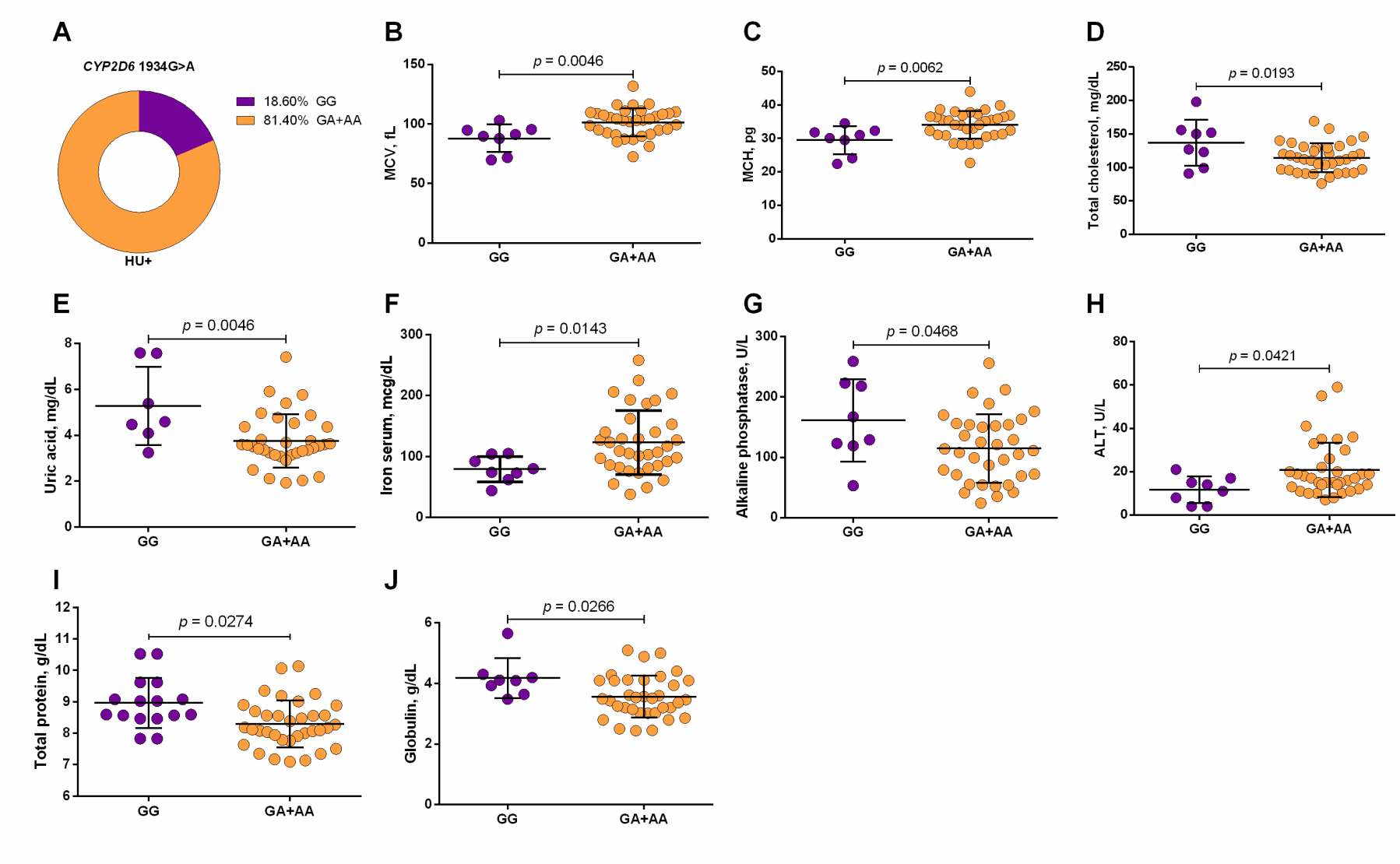

A
CYP2D6 1934G>A
18.60% GG
81.40% GA+AA
HU+
B
p = 0.0046
MCV, fL
GG
GA+AA
C
p = 0.0062
MCH, pg
D
p = 0.0193
Total cholesterol, mg/dL
E
p = 0.0046
Uric acid, mg/dL
F
p = 0.0143
Iron serum, mcg/dL
G
p = 0.0468
Alkaline phosphatase, U/L
H
p = 0.0421
ALT, U/L
I
p = 0.0274
Total protein, g/dL
J
p = 0.0266
Globulin, g/dL

Figure 3.TIF

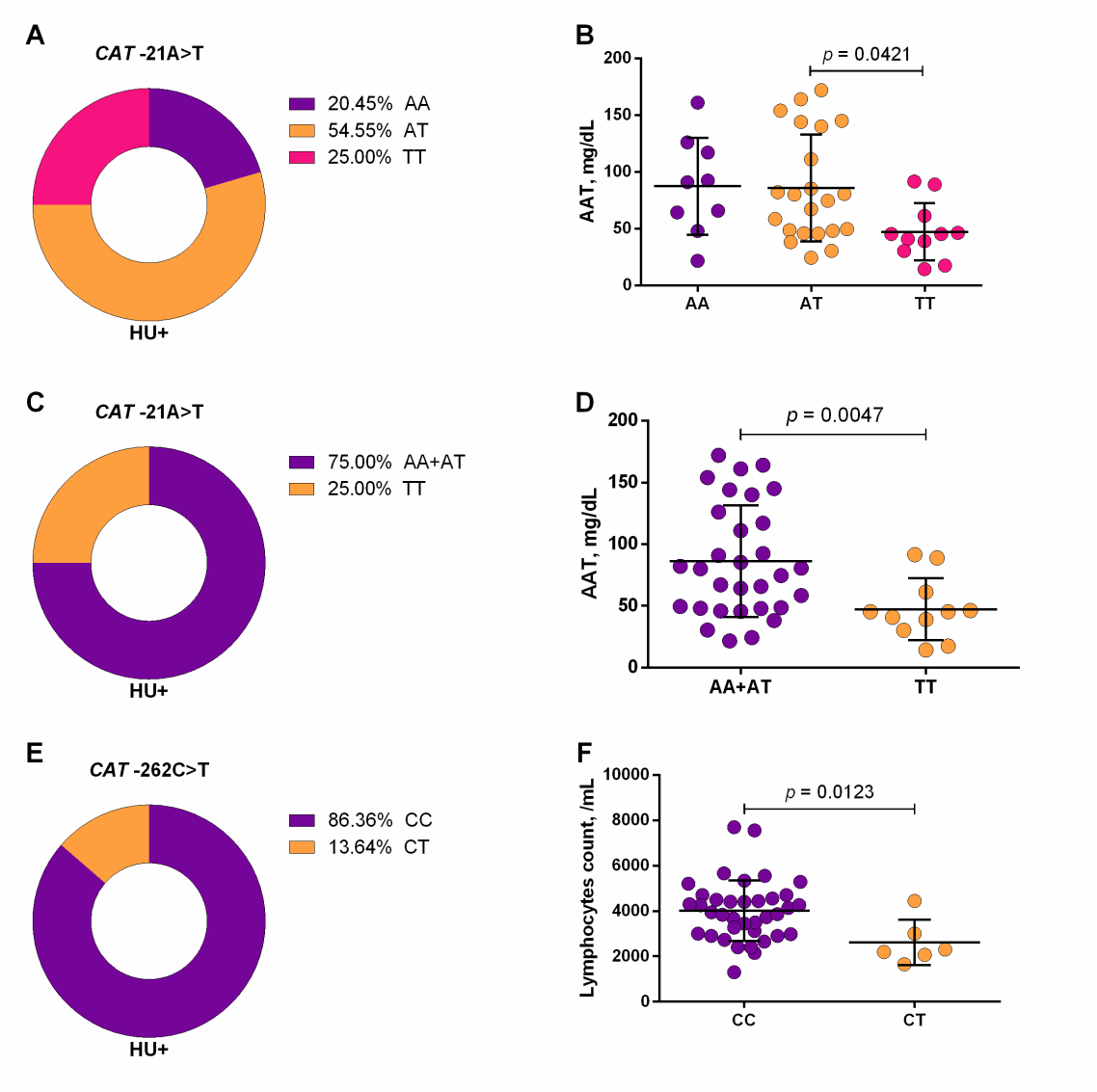

A
CAT -21A>T
20.45% AA
54.55% AT
25.00% TT
HU+
B
p = 0.0421
AAT, mg/dL
200
150
100
50
0
AA
AT
TT
C
CAT -21A>T
75.00% AA+AT
25.00% TT
HU+
D
p = 0.0047
AAT, mg/dL
200
150
100
50
0
AA+AT
TT
E
CAT -262C>T
86.36% CC
13.64% CT
HU+
F
p = 0.0123
Lymphocytes count, /mL
10000
8000
6000
4000
2000
0
CC
CT

Figure 4.TIF

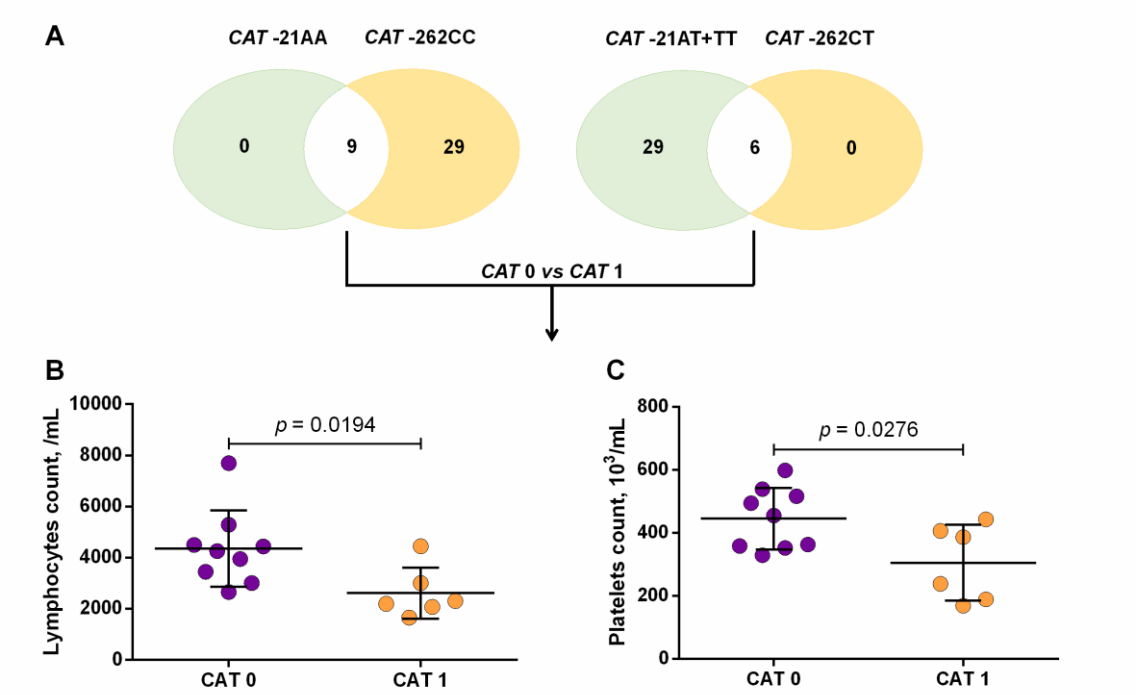
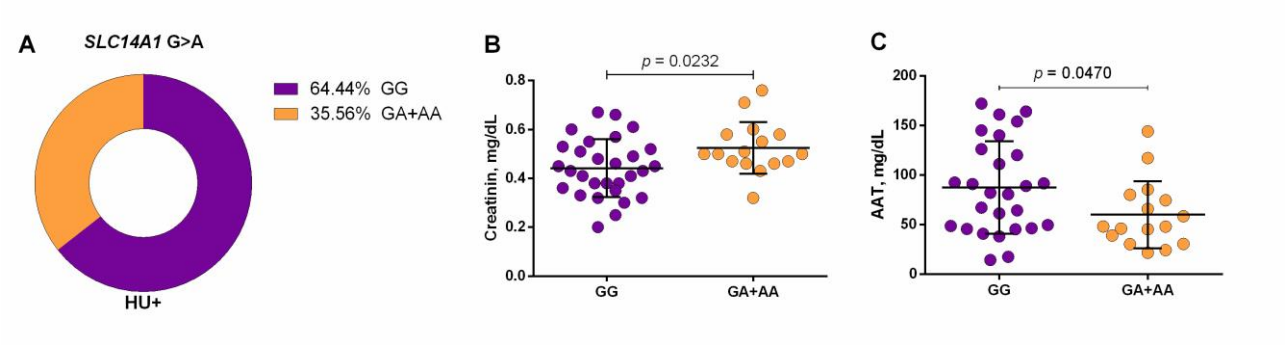

A
CAT -21AA
CAT -262CC
CAT -21AT+TT
CAT -262CT
0
9
29
29
6
0
CAT 0 vs CAT 1
B
Lymphocytes count, /mL
10000
8000
6000
4000
2000
0
p = 0.0194
CAT 0
CAT 1
C
Platelets count, 10³/mL
800
600
400
200
0
p = 0.0276
CAT 0
CAT 1

Figure 5.TIF


A
SLC14A1 G>A
64.44% GG
35.56% GA+AA
HU+
B
p = 0.0232
Creatinin, mg/dL
0.8
0.6
0.4
0.2
0.0
GG
GA+AA
C
p = 0.0470
AAT, mg/dL
200
150
100
50
0
GG
GA+AA

6. DISCUSSÃO

Os indivíduos com AF que fazem tratamento com a HU apresentam variabilidade na resposta à droga (MA et al., 2007) e isso pode ser devido a diversos fatores, incluindo aqueles fisiológicos, socioeconômicos, ambientais e/ou genéticos. O presente estudo propôs investigar a farmacogenética da HU em crianças com AF, com ênfase nos genes que codificam as EMD e protéinas transportadoras de solutos.

Inicialmente, exploramos os resultados das pesquisas sobre a variabilidade na resposta à HU nos indivíduos com DF, de um modo geral, realizando o levantamento dos fatores genéticos que podem influenciar a clínica e, principalmente, a resposta desses indivíduos ao tratamento, além das enzimas que participam do metabolismo da HU. Em um estudo publicado em 2013, o autor tentou explicar a variabilidade na resposta ao tratamento com a HU em pacientes com a DF propondo dois modelos: o modelo de susceptibilidade diferencial e o modelo de HbF basal diferencial (BANAN, 2013). No primeiro modelo, o autor supõe que os precursores eritróides respondem de maneira diferente à HU nos pacientes respondedores e nos pacientes não respondedores, enquanto no segundo modelo supõe-se que os respondedores possuem níveis basais de HbF elevados em comparação aos não respondedores. Isso significa que o paciente para ser respondedor, precisaria ter nível basal de HbF elevado e precursores eritróides menos susceptível à citoxicidade da HU.

Nessa mesma linha de raciocínio de BANAN, estudos anteriores, realizados em pacientes com DF, identificaram genes associados ao aumento do nível de HbF, nomeados loci de traço quantitativo (QTL: *quantitative trait loci*), incluindo o gene da globina gama (*HBG2*), o gene *BCL11A* e a região inter-gênica *HBS1L-MYB*, como biomarcadores de resposta à HU (GREEN et al., 2013; ADEKILE et al., 2015; FRIEDRISCH et al., 2016). Além desses, outros genes envolvidos na biossíntese do NO, na regulação gênica e outros mecanismos celulares (*ASS1*, *KLF10*, *HAO2*, *MAP3K5*, *PDE7B*, *TOX*, *NOS1*, *NOS2A*, *FLT1*, *ARG1*, *ARG2*, *UGT1A1*, *OR51B5/6*, *SIN3A*, *SALL2* e *SAR1A*) também foram identificados (YAHOUÉDÉHOU et al., 2018b). Entretanto, estudos mostraram que alguns pacientes, mesmo sendo portadores desses marcadores clássicos de resposta, continuam apresentando uma má resposta diante do tratamento pela HU (WARE et al., 2011; KARIMI et al., 2012; ADEKILE et al., 2015). Dessa forma, surge a hipótese de que, existiria outros marcadores genéticos que explicariam a variabilidade inter-individual na resposta à HU.

Levando em consideração os dados da literatura que demonstram a influência de polimorfismos em genes de EMD no desfecho de alguns tratamentos, propomos então um

terceiro modelo chamado, modelo de metabolismo diferencial da HU. Nesse modelo, supõe-se que variação no metabolismo da HU, irá influenciar a resposta ao tratamento, independentemente do nível basal da HbF ou da susceptibilidade dos precursores eritróides à HU. Essa hipótese baseia-se no fato que polimorfismos em genes de EMD e proteínas transportadoras de solutos estão associados a diferentes tipos de metabolismo: lento, intermediário, rápido e ultrarrápido. Além disso, sabe-se que variabilidade no metabolismo e transporte de uma droga bem como de seus metabólitos podem influenciar tanto a farmacocinética quanto a farmacodinâmica da droga e, consequentemente, determina o desfecho do tratamento, que pode variar da eficácia terapêutica até a ocorrência de toxicidade (KUMKHAEK et al., 2008; BOŽINA; BRADAMANTE; LOVRIĆ, 2009). Na nossa busca, identificamos poucos estudos de associação que investigaram a participação de genes de EMD e proteínas transportadoras de solutos na resposta à HU de indivíduos com AF (YAHOUÉDÉHOU et al., 2018b). Esses estudos identificaram genes como *CYP2C9*, *AQP9*, *MPO*, *CYP2E1*, *GSTT1*, *UTB* e *OCTN1* que podem influenciar a resposta do paciente à HU. O número pequeno de estudo de associação envolvendo genes de EMD e proteínas transportadoras de solutos nos indivíduos com AF em uso de HU demonstra a falta de informação acerca do metabolismo da HU e a necessidade de identificar as enzimas envolvidas no seu metabolismo (YAHOUÉDÉHOU et al., 2018b). Estudos anteriores demonstraram a participação das enzimas como o CYP450, catalase, urease e peroxidase, porém mais estudos são necessários para elucidar por completo a sua via metabólica (ANDRAE, 1984; KING, 2005; WARE et al., 2011). Isso irá facilitar a seleção dos genes candidatos para os estudos de farmacogenética.

No intuito de validar nosso modelo, foram realizados dois tipos de estudos, com objetivos específicos diferentes. No primeiro estudo, que foi um estudo longitudinal, a questão era investigar os efeitos da HU nos parâmetros hematológico, bioquímico e inflamatório em associação com dois marcadores clássicos, os haplótipos β^S e a α-talassemia. No segundo estudo, que foi um estudo de corte transversal, foram investigados os efeitos de polimorfismos em genes de EMD e proteínas transportadoras de solutos na resposta à HU em crianças com AF. Esses genes foram selecionados com base nos resultados da revisão de literatura.

Nos dois tipos de estudos, antes de investigar a influência dos genes na resposta ao tratamento, foram realizadas análises de caracterização dos parâmetros laboratoriais das crianças. Os resultados dessas análises confirmaram alguns dados da literatura além de trazer novos achados. Como esperado, o uso da HU pelas crianças resultou no aumento significativo dos níveis de HbF. Entretanto, esse efeito não acontece em todas as crianças como foi possível

observar no estudo longitudinal. Das crianças analisadas no estudo longitudinal, apenas algumas chegaram a apresentar um aumento no seu nível de HbF durante o tratamento com a HU. Esses achados corroboram os dados da literatura que demonstraram a associação entre o uso da HU e o aumento da HbF (CHAND et al., 2014; RODGERS et al., 1990; SHOME et al., 2016) e reforçam a noção de que nem todos os pacientes respondem à HU apresentando inicialmente níveis elevados de HbF. Além disso, a HU foi associada ao aumento dos níveis de hemoglobina, hematócrito, VCM e HCM, bem como redução de CHCM, RDW, bilirubina total e indireta, LDH e das contagens de reticulócitos e eritroblastos. Esses achados, também foram relatados por estudos anteriores (SHOME et al., 2016; QUARMYNE et al., 2017; COLOMBATTI et al., 2018), e confirmaram a associação entre o uso da HU e a melhora no perfil hemolítico dos indivíduos com AF.

A HU esteve associada à redução significativa das contagens de leucócitos, neutrófilos, eosinófilos, linfócitos, monócitos, plaquetas e plaquetócrito. Esses resultados corroboram os dados da literatura que demonstraram o efeito cito/leucorredutor da HU nos pacientes (SANTOS; MAIA, 2011; QUARMYNE et al., 2017; COLOMBATTI et al., 2018). Além disso, no estudo longitudinal, as crianças apresentaram uma redução significativa nas concentrações da proteína anti-inflamatória AAT, durante o tratamento com a HU. Os achados sugerem então a associação entre o uso de HU e redução na contagem de leucócitos mediada e/ou acompanhada da redução nos níveis de AAT e, consequentemente, reduz o perfil inflamatório, geralmente apresentado pelos indivíduos com AF.

No presente estudo, as crianças também apresentaram aumento significativo nos níveis de HDL-C durante o tratamento com a HU. Das crianças incluídas no estudo longitudinal, algumas apresentaram níveis de HDL-C acima do valor de referência. Esse achado demonstra o efeito da HU no metabolismo lipídico e corrobora os dados de estudo nosso, recentemente publicado, no qual foi demonstrada a associação entre o uso de HU e variação nas concentrações de colesterol total e frações em indivíduos com AF (YAHOUÉDÉHOU et al., 2018a). DAAK e colaboradores, em estudo prévio, demonstraram que a HU pode modular a composição de ácidos graxos da membrana das hemácias, e isso, combinado à ação dos vasodilatadores, óxido nítrico e prostaglandina E2 gerados pela HU, podem levar a melhorias clínicas antes de se perceber qualquer aumento no nível de HbF (DAAK et al., 2011).

Contrário a nossa expectativa, os resultados do estudo longitudinal demonstraram a associação do uso da HU com o aumento dos níveis de glicose. Entretanto, esses níveis estavam dentre do valor de referência. Esse aumento pode ser o resultado das alterações nas concentrações de colesterol, apresentadas pelas crianças durante o tratamento com a HU. Sabe-

se que os processos metabólicos da glicose e dos lipídios estão ligados entre si e que a hipertrigliceridemia e o HDL-C baixo podem ser a causa bem como o resultado da hiperglicemia (PARHOFER, 2015). Além disso, observamos redução significativa nas concentrações de AST e aumento significativo nas concentrações de albumina, nas crianças, durante o uso da HU. COLOMBATTI e colaboradores demonstraram a associação entre a HU e a redução significativa na concentração de AST (COLOMBATTI et al., 2018). RAGG e colaboradores sugeriram que, além do aumento da HbF, a HU pode levar a melhora clínica através da redução das alterações nas concentrações de proteína sérica (RAGG et al., 2016).

Os resultados da caracterização demonstram também a correlação positiva entre a HbF e os níveis de hemoglobina, hematócrito, VCM e proteína total, bem como sua correlação negativa com os níveis de CHCM, RDW, AST e AAT. Isso confirma a associação da HU com a melhora no perfil hemolítico e inflamatório dos pacientes e sugere que a HU pode também levar a alterações nos biomarcadores metabólicos e hepáticos por meio de mecanismos diretos ou indiretos ainda desconhecidos. Alguns dados da literatura corroboram a nossa hipótese, uma vez que relataram o efeito benéfico do uso da HU no tratamento e prevenção da proteinúria em indivíduos com DF (FITZHUGH; WIGFALL; WARE, 2005; LAURIN et al., 2014; WARE et al., 2017).

Após essa primeira análise de caracterização dos grupos, foram então realizadas análises de associação entre os polimorfismos escolhidos (haplótipo β^S, $\alpha^{2\ del\ 3.7kb}$ talassemia, *CYP3A4* -392A>G, *CYP2D6* 1934G>A, *CAT* -21A>T, *CAT* -262C>T e *SLC14A1* G>A rs2298720) e os parâmetros laboratoriais, os quais mostraram resultados interessantes.

Dados da literatura demonstram que a resposta dos indivíduos com AF à HU pode variar de acordo com o tipo de haplótipo β^S e a α-talassemia (BRUN et al., 2003; VICARI; BARRETTO DE MELLO; FIGUEIREDO, 2005). No presente estudo, foi possível observar o aumento significativo nos níveis de HbF em crianças portadoras do haplótipo BEN, durante o tratamento com a HU, corroborando os resultados de estudo anterior (BERNAUDIN et al., 2018). Como sugerido pelos autores desse trabalho, isso pode ser devido à presença do polimorfismo *BCL11A* rs1427407, associado a nível elevado de HbF, nos indivíduos com o haplótipo BEN. Essa hipótese encaixa-se no modelo de HbF basal diferencial de BANAN, que supõe que os pacientes que respondem melhor à HU possuem níveis basais elevados de HbF em comparação aos não respondedores (BANAN, 2013). Além do aumento na HbF, essas crianças também apresentaram redução significativa na contagem de plaquetas. As portadoras do haplótipo CAR apresentaram, durante o tratamento com a HU, aumento significativo de hemoglobina, hematócrito, triglicérides e ácido úrico e, redução significativa de CHCM,

leucócitos, neutrófilos, eosinófilos, linfócitos, reticulócitos, AST e bilirrubina direta. Além disso, independentemente do haplótipo, a HU estava associada ao aumento significativo no VCM, HCM, glicose e albumina, bem como redução significativa da AAT. Esses achados demonstram de um modo geral que a HU teria mais efeitos nos portadores do haplótipo CAR em comparação aso portadores do haplótipo BEN.

As crianças portadoras da α-talassemia apresentaram, durante o tratamento com a HU, redução significativa na concentração de ALT. Contrário a esse grupo, aquelas sem α-talassemia apresentaram níveis significativamente elevados de hemoglobina, hematócrito, VCM, HCM, HDL-C e albumina, bem como redução significativa de CHCM, neutrófilos, reticulócitos e AST. Esses resultados sugerem que a deleção no gene da globina α pode comprometer a ação da HU nos pacientes. Alguns estudos haviam sugerido que a α-talassemia pode atenuar o efeito da HU (STEINBERG et al., 1995; VASAVDA et al., 2008, 2012). Entretanto, estudos realizados em indivíduos com AF e portadores de α-talassemia demonstraram a associação da HU com o aumento significativo da hemoglobina, hematócrito, VCM, HCM e HbF, bem como redução significativa da HbS, bilirrubina e dos reticulócitos (SHEEHAN et al., 2013; SHOME et al., 2016). Isso pode ser devido a outros fatores genéticos intrínsecos às diferentes populações de estudo. No presente estudo, a HU esteve associada a redução significativa de leucócitos e eosinófilos, nas crianças portadoras de α-talassemia, corroborando os resultados de DARBARI e colaboradores (DARBARI et al., 2014). Além disso, independentemente da presença ou ausência de α-talassemia, as crianças apresentaram aumento dos níveis de glicose.

O alelo variante A do polimorfismo *CYP2D6* 1934G>A está associado ao processamento incorreto do RNAm e à formação de uma proteína troncada, com redução da atividade da enzima (SAYED; IMAM, 2012). A análise de associação entre o polimorfismo *CYP2D6* 1934G>A e os parâmetros laboratoriais demonstrou que o genótipo variante AA está associado ao aumento dos efeitos da HU. Além disso, esse efeito está correlacionado com o número de alelo variante A, uma vez que as crianças portadoras do genótipo heterozigoto GA tinham valores intermediários, quando comparado aos genótipos selvagem GG e variante AA. Os resultados também mostram que quase todas as crianças portadoras do alelo variante A e em uso de HU apresentaram nível de VCM elevado, em comparação às portadoras do alelo selvagem G. BORBA et colaboradores demonstraram a correlação positiva entre o VCM e a HbF e, consequentemente, a sua associação com melhora nos perfis laboratoriais e clínicos dos pacientes. Dessa forma, o menor efeito da HU nas portadoras do genótipo selvagem GG nos leva a sugerir que a *CYP2D6* pode inativar a HU e/ou acelerar sua eliminação.

Sabe-se que os polimorfismos *CAT* -262C>T e -21A>T estão associados à ausência e/ou redução na produção da catalase (LIU et al., 2015). No presente estudo, os genótipos variantes -21TT e -262TT estiveram associados, respectivamente, à redução na contagem de linfócitos e concentração de AAT, nas crianças em uso de HU. A presença de ambos os genótipos variantes também foi associada a reduções na contagem de linfócitos e plaquetas. Na análise de caracterização dos parâmetros laboratoriais das crianças, observou-se que a HU pode levar à redução da concentração da proteína anti-inflamatória AAT, a qual pode ser a causa ou a consequência da redução das contagens de leucócitos, neutrófilos, eosinófilos e linfócitos nesses pacientes (PALLIS et al., 2014). Esses achados sugerem que a redução na expressão e/ou atividade da catalase pode levar ao aumento da biodisponibilidade da HU, resultando na melhora dos efeitos da HU nas portadoras dos genótipos variantes.

Estudos experimentais, realizados em células eritróides tratadas com a HU, demonstraram a correlação positiva entre a expressão do gene *SLC14A1* e a expressão do gene *HBG2* (WALKER; OFORI-ACQUAH, 2017). WARE e colaboradores descobriram a associação entre o polimorfismo *SLC14A1* G>A rs2298720 e alterações nos parâmetros farmacocinéticos (WALKER et al., 2011). HOU e colaboradores relataram que a redução na expressão do gene *SLC14A1* nas células uroteliais humanas pode levar ao acúmulo da uréia nessas células (HOU et al., 2017). No presente estudo, os resultados demonstram a associação do alelo variante A do polimorfismo *SLC14A1* G>A rs2298720 com creatinina elevada e AAT reduzida nas crianças em uso de HU. Estudo anterior demonstrou a correlação negativa entre AAT e creatinina em indivíduos com DF (CARVALHO et al., 2017). Com base nesses achados, podemos também sugerir a associação do polimorfismo *SLC14A1* G>A rs2298720 com o aumento da biodisponibilidade da HU.

Corroborando nossa hipótese, que sugere a associação da redução da expressão/atividade da CYP2D6, CAT e SLC14A1 com a redução na eliminação de HU e aumento de sua biodisponibilidade, SAFARINEJAD e colaboradores também sugeriram a associação entre o aumento na atividade da GST e a redução do efeito citotóxico das drogas anticancerígenas (SAFARINEJAD; SHAFIEI; SAFARINEJAD, 2011). As GSTs compreendem uma superfamília de isoenzimas importantes na desintoxicação de xenobióticos e endobióticos, que catalisam a reação entre a glutationa e os metabólitos intermediários/tóxicos derivados do metabolismo de fase I (MARTÍNEZ-GUZMÁN et al., 2017).

Nenhuma associação foi encontrada entre os polimorfismos nos genes de EMD (*CYP2D6* 1934G>A, *CAT* -21A>T, *CAT* -262C>T e *SLC14A1* G>A rs2298720) e os parâmetros laboratoriais, nas crianças que não faziam tratamento com a HU. Além disso, o polimorfismo

CYP3A4 -392A>G não demonstrou nenhuma associação significativa com os parâmetros laboratoriais em ambos os grupos (crianças em uso e sem uso de HU). Esses achados confirmam então nosso modelo (modelo de metabolismo diferencial da HU), uma vez que os alelos variantes 1934A, -21T, -262T e A dos polimorfismos *CYP2D6* 1934G>A, *CAT* -21A>T, *CAT* -262C>T e *SLC14A1* G>A rs2298720, respectivamente, estiveram associados ao aumento dos efeitos da HU.

7. CONCLUSÃO E PERSPECTIVA

Os resultados do presente estudo reforçam a necessidade de incluir além da HbF, outros parâmetros na avaliação da resposta da HU em crianças com AF e mostram a associação entre a HU e alterações no perfil metabólico através do aumento da glicose, HDL-C, proteína total e albumina e redução da AST. Além disso, os resultados mostram a relevância da identificação de genes de EMD e proteínas transportadoras de solutos que participam da via metabólica e do transporte da HU bem como de estudos voltados à investigação dos efeitos de polimorfismos nesses genes em pacientes com AF que são tratados pela HU, visto que não há uma compreensão completa acerca da farmacocinética, farmacodinâmica e toxicidade desse medicamento. Foi possível evidenciar a influência dos polimorfismos *CYP2D6* 1934G>A, *CAT* -21A>T e -262C>T e *SLC14A1* G>A rs2298720 na resposta ao tratamento pela HU nas crianças com AF, corroborando o modelo proposto neste trabalho, o modelo de metabolismo diferencial da HU. Entretanto, faz-se necessário a realização de estudos adicionais visando a investigação da implicação clínica das alterações metabólicas induzidas pela HU, além da associação dos polimorfismos investigados à farmacocinética e farmacodinâmica da droga.

Dessa forma, os próximos passos serão: a padronização do método de quantificação da HU nas amostras de plasma de crianças com AF em uso de HU; identificação do perfil farmacocinético de cada criança e ajuste da dose caso seja necessário; avaliação da metabolômica das crianças durante o tratamento e; avaliação periódica dos parâmetros laboratoriais e clínicos, entre outros marcadores. Além disso, os dados obtidos serão associados a polimorfismos em genes de EMD e protéinas transportadoras de solutos e a parâmetros laboratoriais a fim de identificar biomarcadores genéticos de resposta à HU.

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APÊNDICES

Outros trabalhos em colaboração desenvolvidos no período do doutorado.

Manuscrito 1

Genetic modulation of fetal hemoglobin in hydroxyurea treated sickle cell anemia

American Journal of Hematology 2017; 92: E70–E72. (Publicado).

Manuscrito 2

Heme-mediated cell activation: the inflammatory puzzle of sickle cell anemia

Expert Review of Hematology 2017; 10: 1-10. (Publicado).

Manuscrito 3

Genetic Polymorphisms Associated with Environmental Exposure to Polycyclic Derivatives in African Children

Disease Markers 2018; 2018:1-9. (Publicado).

Manuscrito 4

Differential gene expression analysis of sickle cell anemia in steady and crisis state

Annals of Human Genetics 2018, 2018: 1-8. (Publicado).

Manuscrito 5

Effect of N(Epsilon)-(carboxymethyl)lysine on Laboratory Parameters and Its Association with β^S Haplotype in Children with Sickle Cell Anemia

Disease Markers 2019, 2019: 1-8. (Publicado).

Manuscrito 6

Hydroxyurea alters circulating monocyte subsets and dampens its inflammatory potential in sickle cell anemia patients

Scientific Reports 2019, 9: 1-11. (Publicado).

Manuscrito 7

Sickle cell disease: A distinction of two most frequent genotypes (HbSS and HbSC)

PLOS ONE 2020, 15: 1-15. (Publicado).

ANEXO I

Alterações em genes de enzimas metabolizadoras de drogas em pacientes com anemia falciforme em uso de hidroxiuréia acompanhados em um estudo longitudinal prospectivo

QUESTIONÁRIO PARA PACIENTES

Nome: {NOME} _____ Sigla: {sig} _____ Telefone: ( ) _____

Endereço:_____

Registro: {REG} _____ Nº Pront. HEMOBA: {PRON} _____ Data de Nasc.: __/____/____

Idade: {I} _____ Gênero: {GENER} ( ) Masculino [0] ( ) Feminino [1]

Gordura corporal {BF}: _____ Hidratação {BW}: _____

Massa muscular {BM}: _____ Massa óssea {BB}:_____

01. Qual a sua cor? {cor} ( ) Branca[0] ( ) Negra[1] ( ) Parda[2] ( ) Amarela[3] ( ) Indígena[4]

02. Você estuda? {EST} ( ) NÃO [0] ( ) SIM [1]

03. Nível de escolaridade: {NESC} ( ) Alfabetiz.[0] ( ) Até 4 FM[1] ( ) Até 8 FM[2] ( ) Até 3 MD[3]

04. Número de irmãos:{NIRM} ( ) 0 [0] ( ) 1 [1] ( ) 2 [2] ( ) 3 [3] ( ) 4 ou + [4]

05. Familiares com DF? {FDFALC} ( ) Nenhum[0] ( ) Pai [1] ( ) Mãe [2] ( ) Irmão [3]

06. Idade primeira menstruação: {IPM} ( ) Não menst.[0] ( ) 09-11[1] ( ) 12-14 [2] ( ) 15-17 [3]

07. Já engravidou? {ENGRA} ( ) NÃO [0] ( ) SIM [1]

08. Está grávida? {GRA} ( ) NÃO [0] ( ) SIM [1]

09. Usa anticoncepcional? {ANTICO} ( ) NÃO [0] ( ) SIM [1]

10. Menstruação é regular? {MREG} ( ) NÃO [0] ( ) SIM [1]

11. Idade do 1° diagnóstico de Doença ( ) <6 m [0] ( ) 6m - 4anos [1] ( ) 5 - 9anos [2]
Falciforme: {ID} ( ) 10 - 14anos [3] ( ) 15 - 17anos [4]

12. Eletroforese de Hb {EHB} ( ) AA[0] ( ) SS[1] ( ) SC[2] ( ) SB+[3] ( ) SB[4] ( ) SD[5]

13. Já esteve internado? {INTER} ( ) NÃO [0] ( ) SIM [1]

Se SIM, quantas vezes? {QINTER} ( ) 1 [0] ( ) 2-5 [1] ( ) 6-10 [2] ( ) 11 ou + [3]

Qual especialidade? {ESPEC} ( ) Cardiologia [0] ( ) Oftalmologia [1] ( )Neurologia[2]
( ) Infectologia [3] ( ) Pneumologia [4] ( ) Cirurgia [5] ( ) Angiologia [6]
( ) Nefrologia [7] ( ) Clínica da Dor [8]( ) Outras [9]

14. Já teve pneumonia? {PNEU} ( ) NÃO [0] ( ) SIM [1]

Se SIM, quantas vezes? {QPNEU} ( ) 1[0] ( ) 2-3[1] ( ) 4-6[2] ( ) 7 ou + [3]

Se SIM, teve febre? {FEBRE} ( ) NÃO [0] ( ) SIM [1]

Anormalidade no RX? {ARX} ( ) NÃO [0] ( ) SIM [1]

Quando internado, usou medicação? {MPNEU} ( ) NÃO [0] ( ) SIM [1]

Quais?{DESCMPNEU}_____

15. Teve ou tem esplenomegalia? {ESPLE} ( ) NÃO [0] ( ) SIM [1]

Em que período? {PERIOESPLE} ( ) <6m[0] ( ) 6m-1ano [1] ( ) 2-3a [2] ( ) 4-5a [3] ( ) >6a [4]

Teve crise de sequestro esplênico? {SEQESPLE} ( ) NÃO [0] ( ) SIM [1]

Se SIM, quantas vezes? {QSEQESPLE} _____

16. Faz uso profilático de Penicilina? {PROP} ( ) NÃO[0] ( ) SIM [1]

Se SIM, qual? {QPEN} ( ) Penicilina V oral [0] ( ) Penicilina benzatina [1]

Se Sim, há quanto tempo? {QTPEN} ( ) até 1 ano [0] ( ) + de 1 ano a 3 anos [1] ( ) + 3 anos a 5 anos [2]
( ) + 5 anos a 7 anos [3] ( ) + de 7 anos [4]

17. Já teve AVC? {AVC} ( ) NÃO [0] ( ) SIM [1]

Se SIM, quantas vezes? {QAVC} ( ) 1 [0] ( ) 2 [1] ( ) 3 [2] ( ) 4 ou + [3]

Se SIM, sequelas do AVC? {SEQAVC} ( ) NÃO [0] ( ) SIM [1]

Já fez ressonância magnética? {RESSOMAG} ( ) NÃO [0] ( ) SIM [1]

Alguma alteração? {ALTRESSOMAG} ( ) NÃO [0] ( ) SIM [1]

18. Esplectomizado? {ESPLECTO} ( ) NÃO [0] ( ) SIM [1]

Esplenectomia: {TIPOESPLECTO} ( ) Total [0] ( ) Parcial [1]

19. Apresenta asma? {ASMA} ( ) NÃO [0] ( ) SIM [1]

Se SIM, quantas crises nos últimos 06 meses? {QASMA} ( ) 0[0] ( ) 1-3[1] ( ) 4-7[2] ( ) 8ou+[3]

Faz uso regular de nebulização?{NEBU} ( ) [0] NÃO ( ) SIM [1]

20. Tem crises de dor? {CRISDOR} ( ) [0] NÃO ( ) SIM [1]

Se SIM, quantas crises nos últimos 06 meses? {QCRISDOR} ( ) 0[0] ( ) 1-3[1] ( ) 4-7[2] ( ) 8ou+[3]

Quando foi a última crise? {ULTCRISDOR} ( ) <1mês [0] ( ) 1-3m [1] ( ) 4m ou+[2]

Usa medicação para a dor? {MDOR} ( ) NÃO [0] ( ) SIM [1]

Prescrita por um médico? {PRESMDOR} ( ) NÃO [0] ( ) SIM [1]

Assistido por especialista em dor? {ESPECDOR} ( ) NÃO [0] ( ) SIM [1]

Faz tratamento com hidroxiuréia? {HIDROXI} ( ) NÃO [0] ( ) SIM [1]

Se SIM, quanto tempo? {QTHIDROXI} _____

Se SIM, qual dose? {DHIDROXI} _____

21. Faz uso de alguma medicação? {MEDIC} ( ) NÃO [0] ( ) SIM [1]

Se SIM, qual? {DESCMEDIC}_____

Com que frequência? {FREQMEDIC} ( ) Diário [0] ( ) Dias alternados[1] ( ) Semanal[2]
( ) Quinzenal[3] ( ) Mensal [4] ( ) Bimestral[5] ( ) Semestral [6]

22. Vaso-Oclusão: {VO} ( ) NÃO [0] ( ) SIM [1] Quantas vezes? {QVO} _____

Fez uso de alguma medicação? {MVO} ( ) NÃO [0] ( ) SIM [1]

23. Retinopatia: {RETIN} ( ) NÃO [1] ( ) SIM [2]

Se SIM, fez uso de alguma medicação? {MRETIN} ( ) NÃO [0] ( ) SIM [1]

Faz consultas periódicas com oftalmo? {CONSOFTAL} ( ) NÃO [0] ( ) SIM [1]

24. Infecções: {INFEC} ( ) NÃO[0] ( ) SIM [1]

Quais? {DESCINFEC} ( ) Rinite [0] ( ) Sinusite [1] ( ) Otite [2]
( ) Faringite [3] ( ) Amigdalite [4] ( ) Outros [5]

Fez uso de alguma medicação? {MINFEC} ( ) SIM [0] ( ) NÃO [1]

25. Priapismo: {PRIAP} ( ) NÃO [0] ( ) SIM [1]

N° de vezes: {QPRIAP} ( ) Até 4 [0] ( ) 05-09 [1] ( ) 10 ou +[2]

Fez uso de alguma medicação? {MPRIAP} ( ) NÃO [0] ( ) SIM [1]

26. Úlcera maleolar: {ULCMALEO} ( ) NÃO [0] ( ) SIM [1] Quantas vezes? {QULCMALEO} _____

Idade da primeira úlcera: {IDULC} ( ) Até 4 anos [0] ( ) 5-9 [1] ( ) 10 ou + [2]

Tratou a úlcera? {TRATULC} ( ) NÃO [0] ( ) SIM [1]

Qual tratamento? {QUALTRAT} _____

27. Síndrome torácica aguda: {SDTOR} ( ) NÃO [0] ( ) SIM [1]

Quantas vezes? {QSDTOR} ( ) Até 2 [0] ( ) 03-05 [1] ( ) 06 ou + [2]

28. Alterações ósseas: {ALTOSSEA} ( ) NÃO [0] ( ) SIM [1]

Quais? {DESCALTOSSEA} _____

29. Insuficiência Renal Aguda: {INSRENAG} ( ) NÃO [0] ( ) SIM [1]

Quantas vezes? {QINSRENAG} ( ) Até 2 [0] ( ) 03-05 [1] ( ) 06 ou + [2]

30. Insuficiência Renal Crônica: {INSRENCRO} ( ) NÃO [0] ( ) SIM[1]

Idade diagnóstico: {IDINSRENCRO} ( ) Até 5 anos [0] ( ) 06-11[1] ( ) 12 ou + [2]

31. Alterações cardíacas: {INSCARD} ( ) NÃO [0] ( ) SIM[1]

Qual alteração? {QUALALTCA}

_____

Idade diagnóstico: {IDINSCARD} ( ) Até 5 anos [0] ( ) 06-11 [1] ( ) 12 ou + [2]

Fez eletrocardiograma? {ELETRO} ( ) NÃO [0] ( ) SIM [1]

Fez ecocardiograma? {ECOCARD} ( ) NÃO [0] ( ) SIM [1]

32. Sequestro hepático: {SEQHEP} ( ) NÃO [0] ( ) SIM [1] Quantas vezes? {QSEQHEP} _____

33. Insuficiência respiratória: {INSRESP} ( ) NÃO [0] ( ) SIM [1] Quantas vezes? {QINSRESP} _____

34. Distúrbio do sono? {DISTSONO} ( ) NÃO [0] ( ) SIM [1]

35. Litíase biliar: {LITIBILI} ( ) NÃO [0] ( ) SIM [1] Quantas vezes? {QLITIBILI} _____

36. Cirurgia: {CIRURG} ( ) NÃO [0] ( ) SIM [1]

Quais? {QUALCIRURG}

_____

Se SIM, fez uso de profilaxia antibiótica? {PROFANTIB} ( ) NÃO [0] ( ) SIM [1]

37. Completou o calendário vacinal? {CALVAC} ( ) NÃO [0] ( ) SIM [1]

Fez uso das seguintes vacinas? {USOVAC} ( ) 7 valente [0] ( ) 23 valente [1] ( ) Meningo [2]
( ) Haemophilus [3]

38. Faz uso de hemoderivados? {HEMODER} ( ) NÃO [0] ( ) SIM [1]

Se SIM, quantas vezes ao ano? {QHEMODER} _____

39. Possui outra patologia? {PATOLOG} ( ) NÃO [0] ( ) SIM [1]

Quais? {DESCPATOLOG} ( ) Hipertensão [0] ( ) Diabetes [1] ( ) Obesidade [2] ( ) Outras [3]

40. Você trabalha? {TRAB} ( ) NÃO [0] ( ) SIM [1]

Tipo de profissão: {QTRAB} _____

Se SIM, manipula alguma substância química? {SUBQUIM} ( ) NÃO [0] ( ) SIM [1]

Qual? {QSUBQUIM} _____ Frequência? {FREQSUBQUI} _____

Manipula diretamente esta subst? {MANIDIRE} ( ) NÃO [0] ( ) SIM [1]

41. Pratica esportes? {ESPOR} ( ) NÃO [0] ( ) SIM [1]

42. Faz uso de bebida alcoólica? {BEBE} ( ) NÃO [0] ( ) SIM [1]

Se SIM, que frequência? {FREQBEBE} _____

43. Você fuma? {FUMA} ( ) NÃO [0] ( ) SIM [1]

Se SIM, que frequência? {FREQFUMA} _____

44. Faz uso de alguma droga? {DROGA} ( ) NÃO [0] ( ) SIM [1]

Em caso de SIM, que frequência? {FREQDROGA} _____

45. Além dos seus pais quantos membros da família ou parentes são apegados a vc? {APEG}( ) 01[0]
( ) 02 – 03 [1] ( ) 04 – 06[2] ( ) 07 – 10[3] ( ) nenhum[4]

46. Quantos amigos vc têm aproximadamente? {AMIGO} ( ) 01[0] ( ) 02 – 03 [1] ( ) 04 – 06[2]
( ) 07 – 10[3] ( ) nenhum[4]

47. Com que frequência vc se reúne com seus parentes, amigos ou vizinhos? {REUNI}

( ) Diariamente ou quase todos os dias [0] ( ) Várias vezes na semana [1]

( ) Várias vezes no mês [2] ( ) Várias vezes por ano [3] ( ) Quase nunca [4]

Data da próxima consulta no HEMOBA: ____/____/____

ANEXO II. Condições das reações de PCR-RFLP

Reagentes PCR		***CYP2D6* 1934G>A**	***CYP3A4* –392A>G**	***CAT*-21A>T**	***CAT*-262C>T**
Tampão 10x		5µL	5µL	5µL	5µL
MgCl2 50mM		2,5µL	2,5µL	2µL	2,5µL
dNTPs 2mM		5µL	5µL	5µL	5µL
Primers 25pM	*CYP2D6* D/R	0,25µL/0,25µL			
	CYP3A4 D/R		0,2µL/0,2µL		
	CAT21 D/R			0,3µL/0,3µL	
	CAT262 D/R				0,3µL/0,3µL
Taq 5U/µL		0,3µL	0,3µL	0,3µL	0,3µL
DNA		1µL	1µL	1µL	1µL
H2O qsp 25µL		35,7µL	35,8µL	36,1µL	35,6µL
Termociclagem		94°C-4min 94°C-1min 60°C-1min 72°C-1min } 35x 72°C-10min 4°C-...	94°C-5min 94°C-45s 57°C-45s 72°C-1min } 35x 72°C-10min 15°C-...	95°C-5min 95°C-30s 62,5°C-1min 72°C-45s } 35x 72°C-7min 4°C- …	95°C-5min 95°C-30s 56°C-30s 72°C-45s } 35x 72°C-10min 4°C- …
Padrões de banda de PCR		355pb	385pb	250pb	185pb
PCR-RFLP					
Produto PCR		20µL	20µL	20µL	20µL
Tampão da enzima		2µL	3µL	3µL	3µL
*Mva*1 (*BstN*I)		0,2µL			
*Mbo*II			0,5µL		
*Hinf*I				0,5µL	
*Sma*I					0,6µL
H2O qsp 15µL		7,8µL	6,5µL	6,5µL	6,4µL
Padrões de bandas de corte		Normal (GG): 250pb+105pb Hetero (GA): 355pb+250pb+105pb Mutante (AA): 355pb	Normal (AA): 175pb+169pb+41pb Hetero (AG): 210pb+175pb+169pb+41pb Mutante (GG): 210pb+175pb	Normal (AA): 177pb+73pb Hetero (AT): 250pb+177pb+73pb Mutante (TT): 250pb	Normal (CC): 155pb+30pb Hetero (CT): 185pb+155pb+30pb Mutante (TT):185pb

qsp: quantidade suficiente para

ANEXO III**. Descrição dos polimorfismos investigados e métodos utilizados.**

Genes	**Polimorfismos**	**Método utilizado**	**Primer**	**Referência**
CYP3A4	-392A>G	PCR-RFLP	F: 5'- GGA ATG AGG ACA GCC ATA GAG ACA AGG GGA -3' R: 5'- CCT TTC AGC TCT GTG TTG CTC TTT GCT G -3'	MARUF et al, 2012
CYP2D6	1934G>A	PCR-RFLP	F: 5'- GCC TTC GCC AAC CAC TCC G -3' R: 3'- AAA TCC TGC TCT TCC GAG GC -3'	SAYED et IMAM, 2012
CAT	-21A>T	PCR-RFLP	F: 5'- AAT CAG AAG GCA GTC CTC CC -3' R: 5'- TCG GGG AGC ACA GAG TGT AC -3'	LIU et al, 2015
CAT	-262C>T	PCR-RFLP	D: 5'- AGA GCC TCG CCC CGC CGG ACC G -3' R: 5'- TAA GAG CTG AGA AAG CAT AGC T -3'	LIU et al, 2015
*SLC14A1**	G>A rs2298720	TaqMan	--	--

CYP2D6: citocromo p450 2D6; *CYP3A4*: citocromo p450 3A4; *CAT*: catalase ; *SLC14A1*: solute carrier 14A1; G: guanina; C: citosina; T: timina; A: adenina; PCR-RFLP: *polymorphism chain reaction - restriction fragment lenght polymorphism*; * O polimorfismo foi investigado pelo método TaqMan de acordo com as recomendações do fabricante (ThermoFischer).

ANEXO III - PCR-RFLP para Haplótipos

Reagentes

Reagente	Quantidade por amostra
Tampão	5,0 µL
$MgCl_2$ 50mM	2,5 µL
dNTP 2 mM	5,0 µL
Primers (3 ou 5 ou 6 ou 8 ou 10 ou 12) 25 pmol/µL	0,5 µL
Primers (4 ou 6 ou 7 ou 9 ou 11 ou 13) 25 pmol/µL	0,5 µL
Taq 5U/µl	0,25 µL
DNA	1,5 µL
H2O qsp 50µl	34,75 µL

Termociclagem

Desnaturação	94ºC,10 min.
35ciclos	94ºC, 45 seg. Temp. Variável de acordo com a Tab. 1 45 seg. 72ºC, 1min 30 seg.
Extensão	72ºC, 10 min 4ºC …

RFLP

Reagentes	Quantidade por amostra
Produto de PCR	20 µL
BSA*****	0,3 µL
Tampão	3 µL
Enzima de restrição de acordo com tab. 1	
Xmn I	0,2 µL
Hinc II	0,1 µL
Hind III ou *Hinf* I	0,2 µL
H_2O	Qsp 30 µL

***** Verificar as reações que necessitam BSA na tab. 1

Tabela. Padrão de banda de PCR e corte

Gene	Primer	Fragmento (pb)	Após digestão	Temp. de pareamento	Enzima
5'γ^G	3 e 4	650	450+200	57ºC	*Xmn* I*
γ^G/γ^A	5 e 6	780	440+340	60ºC	*Hind* III
γ^G/γ^A	6 e 7	760	360+400	62ºC	*Hind* III
Ψβ	8 e 9	700	360+340	60ºC	*Hinc* II
3' ψβ	10 e 11	590	470+120	57ºC	*Hinc* II
5'β	12 e 13	380	240+140	57ºC	*Hinf* I

*Acrescentar BSA

ANEXO IV - PCR para Talassemia $\alpha^{2\ del\ 3.7kb}$

Reagentes

Reagente	Quantidade por amostra
Tampão 10X	1,25 μL
$MgCl_2$ 50mM;	0,5 μL
dNTP 10mM	0,25 μL
Primer A (Comum)	0,125 μL
Primer B ou C	0,125 μL
Q solution 5X	2,5 μL
Taq DNA Polimerase (5U/μL);	0,1 μL
DNA	Aprox. 1 μL (100ng/μL)
Água para PCR	qsp. 12 μL

Termociclagem

Abertura inicial	98ºC, 3 min 85ºC, 3 min
5 ciclos	98ºC, 30 seg 66ºC, 1min30seg 72ºC, 3 min
30 ciclos	96ºC, 30 seg 66ºC, 30 seg 72ºC, 3 min
Extensão	72ºC, 15 min 15ºC, infinito

Interpretação dos resultados

Tubo A+B	Tubo A+C	Paciente
+	-	Homozigoto para a talassemia $\alpha_2^{3,7Kb}$
+	+	Heterozigoto para a talassemia $\alpha_2^{3,7Kb}$
-	+	Portador de genes α normais
-	-	Problemas na reação