UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

ESTUDO FARMACOGENÉTICO DA RESPOSTA AO TRATAMENTO DO ERITEMA NODOSO HANSÊNICO

Perpétua do Socorro Silva Costa

Porto Alegre, Agosto de 2018

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Tese submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da UFRGS como requisito parcial para obtenção do grau de Doutor em Ciências (Genética e Biologia Molecular).

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SUMÁRIO

LISTA DE ABREVIATURAS

3'-UTR	3' Untranslated Region - Região não traduzida 3'
ABCB1	*ATP-binding cassette sub-family B member 1 gene*
AP-1	Proteína ativadora 1
ASA	*Acetylsalicylic acid* – Acido acetisalicílico
BAAR	Bacilo Álcool-Ácido Resistente
BB	Hanseníase borderline-borderline
bFGF	Basic fibroblast growth factor – fator de crescimento de fibroblastos básico
BI	*Bacteriological index*
BL	*Borderline-lepromatous leprosy*
BT	Hanseníase borderline-tuberculóide
BV	Hanseníase borderline-virchowiana
CE	Ceará
Crbn	Proteína Cereblon
CRBN	Gene *CRBN*
CYP2C19	*Cytochrome P450 family 2 subfamily C member 19*
DNA	*Desoxiribonucleic acid* - Ácido desoxirribonucleico
ENH	Eritema Nodoso Hansênico
ENL	*Erythema Nodosum Leprosum*
ENLIST	*Study Group on Erythema Nodosum Leprosum*
GC	Glicocorticoides
GEE	*Generalized Estimating Equations*
GpP	Glicoproteína P
GR	Receptor de glicocorticoides
GRE	Elementos de resposta a glicocorticoides
hGRα	Human Glucocorticoid Receptor alpha– Receptor de glicocorticoide humano alfa
hGRβ	Human Glucocorticoid Receptor beta – Receptor de glicocorticoide humano beta
HIV	Human Immunodeficiency Virus – Virus da Imunodeficiência Humana

IFN-γ	Interferon gama
IL-1β	*Interleucin 1 beta* - Interleucina 1 beta
IL2R	*Interleucin 2 Receptor* - Receptor de Interleucina 2
IL-6	*Interleucin 6* - Interleucina 6
IL6R	*Interleucin 6 Receptor* -Receptor de Interleucina 6
Il-8	*Interleucin 8* - Interleucina 8
IL-10	*Interleucin 10* - Interleucina 10
IL-12	*Interleucin 12* - Interleucina 12
Il-17	*Interleucin 17* - Interleucina 17
IMiDs	*Immunomodulatory drugs*
INS/DEL	Inserção/Deleção
kg	kilogramas
LD	*Linkage disequilibrium*
LL	*Lepromatous leprosy*
LPS	Lipopolissacarídeo
mg	Miligramas
MA	Maranhão
MB	Hanseníase Multibacilar – *Multibacillary leprosy*
MM	Mieloma Múltiplo
MDT	Multidrug therapy
NF-κB	*Nuclear factor kappa B* – fator nuclear kappa B
NO	*Nitric oxid* - Oxido nítrico
NOD2	*Nucleotide-binding oligomerization domain containing 2*
NRAMP	*Natural resistance-associated macrophage protein*
NR3C1	*Nuclear receptor subfamily 3 group C member 1*
OMS	Organização Mundial de Saúde
PB	Hanseníase Paucibacilar – *Paucibacillary leprosy*
PCH	Programa Controle de Hanseníase
PCR	Reação em cadeia da polimerase
PQT	Poliquimioterapia
RFLP	*Restriction Fragment Length Polymorphism*
RNA	R*ibonucleic acid* - Ácido ribonucleico

RO	Rondônia
RR	Reação reversa
RS	Rio Grande do Sul
SNP	*Single nucleotide polymorphism* – polimorfismo de base única
TGF-β	*Transforming growth factor beta* / Fator de trasnaformação do crescimento beta
Th1	Linfócitos T *helper* 1/ Linfócitos T auxiliar tipo 1
Th2	Linfócitos T *helper* 2/ Linfócitos T auxiliar tipo 2
Th17	Linfócitos T *helper* 17/ Linfócitos T auxiliar tipo 17
TLR	*Toll like receptors*
TLR-1	*Toll like receptor 1*
TLR-2	*Toll like receptor 2*
TLR-3	*Toll like receptor 3*
TLR-4	*Toll like receptor 4*
TLR-5	*Toll like receptor 5*
TLR-6	*Toll like receptor 6*
TLR-7	*Toll like receptor 7*
TLR-8	*Toll like receptor 8*
TLR-9	*Toll like receptor 9*
TNF-α	Fator de necrose tumoral alfa
Treg	Linfócitos T regulatórios
TT	Hanseníase Tuberculóide-tuberculóide
VEGF	*Vascular endothelium growth factor* – fator de crescimento do endotélio vascular
VV	Hanseníase virchowiana-virchowiana
WHO	World Health Organization

RESUMO

O Eritema Nodoso Hansênico (ENH) é uma reação inflamatória que afeta pacientes de hanseníase virchowiana e borderline-virchowiana. Trata-se de uma complicação grave da hanseníase decorrente de seu potencial de causar deformidades e incapacidades. Os tratamentos mais utilizados para o ENH no Brasil são talidomida e prednisona, no entanto, a reação é de difícil controle se apresentando de forma recorrente ou crônica. Os pacientes necessitam de regimes prolongados desses medicamentos sendo sujeitos a complicações e manifestação de efeitos adversos. O objetivo desse trabalho foi realizar um estudo farmacogenético buscando avaliar variantes genéticas que possam influenciar na resposta ao tratamento do ENH com talidomida e prednisona. Através de uma revisão de literatura caracterizamos os medicamentos mais utilizados no tratamento para o ENH, descrevendo seus mecanismos de ação, dose e principais efeitos adversos associados. Todos os medicamentos utilizados no tratamento do ENH apresentam restrições de uso. No caso de prednisona e talidomida, ocorre o risco de efeitos adversos como a dependência para a prednisona, e neuropatia periférica e teratogenicidade para a talidomida. Além disso, nenhum medicamento é completamente eficaz na resolução do ENH. Somado a isso se verificou a falta de uma padronização do tratamento com essas drogas e, especialmente no Brasil, a necessidade de um controle mais efetivo desse tratamento principalmente com a talidomida devido à sua teratogenicidade. Foi realizado um estudo farmacogenético buscando identificar perfis genéticos mais susceptíveis a diferenças nas doses de talidomida e prednisona e à manifestação de efeitos adversos. Foram avaliadas amostras de DNA de pacientes de ENH de diferentes regiões do Brasil que usavam talidomida e/ou prednisona em algum momento do tratamento. Os polimorfismos de base única (SNPs) analisados foram de (1) genes envolvidos com metabolismo ou a ação dos medicamentos: genes *NR3C1* e *ABCB1*, no tratamento com prednisona; e os genes *TNF* e *CYP2C19,* no tratamento com talidomida; (2) gene *CRBN* que codifica a proteína Cereblon, um alvo da teratogenicidade da talidomida e associada ao efeito terapêutico da droga no Mieloma Múltiplo (MM); (3) gene *TLR-9* que codifica o receptor toll like receptor 9 (TLR-9), associado ao processo

inflamatório do ENH. Na análise de genes associados ao metabolismo e ao mecanismo de ação dos medicamentos, foi encontrada uma associação entre o polimorfismo do gene *ABCB1* (rs1045642) com a dose de prednisona e entre haplótipos do gene *TNF* (rs361525/ rs1800629/rs1799724/rs1800630/rs1799964) e o polimorfismo *CYP2C19*2* (rs4244285) com a variação da dose de talidomida durante o tratamento. Sugerimos assim que esses genes podem influenciar a resposta ao tratamento do ENH embora esses genes devam ser mais bem avaliados. Na avaliação do gene *CRBN*, se analisou polimorfismos rs1620675, rs1672770 e rs4183 das regiões flanqueadoras da porção do gene que codifica a região de ligação da proteína com a talidomida. Houve associação entre os polimorfismos rs1620675 e rs4183 e uma menor dose de talidomida no tratamento do ENH. Também houve associação de alelos e haplótipos desse gene com a manifestação de efeitos adversos. A partir desses resultados, sugerimos que Cereblon pode estar associado à eficácia terapêutica da talidomida no ENH, assim como tem sido descrito no MM. As análises do gene *TLR-9* sobre as doses de talidomida e prednisona no tratamento do ENH, revelaram uma associação dos haplótipos (rs5743836/rs352140) TG e CG com a dose de prednisona utilizada e com sua variação ao longo do tratamento do ENH. Esses haplótipos possuem o alelo G do polimorfismo rs352140, indicando a influência desse polimorfismo na ação da prednisona no tratamento do ENH. Os resultados obtidos nesse estudo sugerem uma influência farmacogenética no tratamento do ENH uma vez que nós identificamos variantes genéticas de genes relacionados com o metabolismo de prednisona e talidomida ou com o processo inflamatório do ENH. Esse tipo de estudo pode auxiliar na identificação de perfis de melhor resposta ao tratamento do ENH.

ABSTRACT

Erythema Nodosum Leprosum (ENL) is an inflammatory reaction that affects patients of Lepromatous (LL) and Borderline-lepromatous leprosy (BL). It is a serious complication of leprosy due to its potential to cause deformities and disability. The most used treatments for ENL in Brazil are thalidomide and prednisone, however, the reaction is difficult to control and presents as recurrent or chronic. Patients need prolonged regimens of these drugs being subjected to complications and manifestation of adverse effects. The aim of this study was to conduct a pharmacogenetic study to evaluate genetic variants that may influence the response to treatment of ENH with thalidomide and prednisone. Through a review of the literature we characterize the drugs most used in the treatment for ENL, describing its mechanisms of action, dose and main associated adverse effects. All medicines used to treat ENL have restrictions on use. In the case for prednisone and thalidomide, there is a risk of adverse effects such as dependence on prednisone and peripheral neuropathy and teratogenicity for thalidomide. In addition, no medication is completely effective in the resolution of ENL. Moreover, there is a lack of standardization of treatment with these drugs and, especially in Brazil, the need for a more effective control of thalidomide treatment mainly due to its teratogenicity. A pharmacogenetic study was conducted to identify genetic profiles more susceptible to differences in thalidomide and prednisone doses and to the manifestation of adverse effects. DNA samples from ENL patients from different regions of Brazil that used thalidomide and/or prednisone were evaluated at some point in the treatment. The single-base polymorphisms (SNPs) analyzed were from genes involved in different ENL processes: (1) genes associated with the metabolism or action of drugs: *NR3C1* and *ABCB1* genes, on treatment with prednisone; and the *TNF* and *CYP2C19* genes, on treatment with thalidomide; (2) *CRBN* gene encoding Cereblon, the target protein of thalidomide teratogenicity and associated with its therapeutic effect in Multiple Myeloma (MM); (3) *TLR-9* gene encoding toll receptor-like receptor 9 (TLR-9), associated with the inflammatory process of ENL. In the analysis of genes associated with metabolism and mechanism of action of the drugs, an association between the polymorphism of the *ABCB1* gene (rs1045642) with the dose of prednisone and between

haplotypes of the *TNF* gene was found (rs361525, rs1800629, rs1799724, rs1800630, rs1799964) and the CYP2C19*2 polymorphism (rs4244285) with the dose variation of thalidomide during treatment. We thus suggest that these genes may influence the response to ENL treatment although these genes should be better evaluated. In the evaluation of the *CRBN* gene, polymorphisms rs1620675, rs1672770 and rs4183 from the flanking regions of the gene portion coding for the protein binding region with thalidomide were analyzed. There was an association between polymorphisms rs1620675 and rs4183 and a lower dose of thalidomide in the treatment of ENL. There was also an association of alleles and haplotypes with the manifestation of adverse effects. From these results, we suggest that Cereblon may be associated with the therapeutic efficacy of thalidomide in ENL, as has been described in the MM. Analyzes of the *TLR-9* gene on the doses of thalidomide and prednisone in the treatment of ENL revealed an association of the haplotypes (rs5743836 / rs352140) TG and CG with the dose of prednisone used and its variation throughout the ENL treatment. These haplotypes have the G allele of rs352140 polymorphism, indicating the influence of this polymorphism on the action of prednisone in the treatment of ENL. The results obtained in this study show that genetic variants of genes related to the metabolism of prednisone and thalidomide or with the inflammatory process of ENL may influence the treatment of this condition. This type of study can help in the identification of profiles of better response to ENL treatment.

Capítulo I - Introdução

1. INTRODUÇÃO

1.1 HANSENÍASE

A Hanseníase é uma doença crônica, granulomatosa, causada pelo microorganismo intracelular obrigatório *Mycobacterium leprae.* Ela afeta a pele, o sistema nervoso periférico e ocasionalmente, outros órgãos e sistemas como sistema reticulo-endotelial, ossos e articulações, membranas mucosas, olhos, testículos, músculos e glândulas supra-renais. (Souza 1997; Walker and Lockwood 2007; Talhari et al. 2015). A doença é caracterizada por progressão lenta, alta infectividade e baixa patogenicidade do microorganismo. Além disso, apresenta baixa morbidade porque grande parte da população é naturalmente resistente ao patógeno (Corrêa et al. 2012; Lastória and Abreu 2014).

O *M. leprae* é um bacilo álcool-ácido resistente (BAAR) reto ou ligeiramente encurvado, de 1,5 a 8 µm de comprimento por 0,2 a 0,5 µm de largura (Goulart et al. 2002). Ele infecta principalmente macrófagos e células de Schwann. A presença de bacilos na pele produz as manifestações dermatológicas da doença e a infecção do nervo produz disfunção axonal e desmielinização, levando à perda sensorial e suas consequências de deficiência e deformidade (White and Franco-Paredes 2015).

As características clínicas da doença são determinadas pela resposta do hospedeiro ao *M. leprae*. Os sintomas geralmente se iniciam com neurite sensorial, mas pacientes não tratados que buscam cuidados médicos tardiamente apresentam distúrbios motores mais graves. Úlceras plantares, lesões ósseas líticas (nariz, falanges, etc.) e paralisias (nervo ulnar, lagoftalmo) podem ser complicações frequentes e que definem o quadro clínico da hanseníase descrito há séculos (Reibel et al. 2015).

Admite-se que as vias aéreas superiores constituem a principal porta de entrada e via de eliminação do bacilo. A pele erodida, eventualmente, pode ser porta de entrada da infecção. As secreções orgânicas como leite, esperma, suor e secreção vaginal podem eliminar bacilos, mas não possuem importância na disseminação da infecção (Araújo 2003). Além do contato humano, a única outra via de transmissão conhecida é o contato humano com tatus que tenham sido naturalmente infectados com *M. leprae* (da Silva et al. 2018).

A hanseníase tem alto impacto sobre a vida do paciente, pois tem risco potencial de levar a incapacidades e deformidades afetando principalmente a população economicamente ativa, interferindo no trabalho e vida social do paciente e causando perdas econômicas além de trauma psicológico (Lustosa et al. 2011).

1.1.1 Epidemiologia

A hanseníase está incluída entre as doenças infecciosas de notificação obrigatória, devido à sua incidência / prevalência, cronicidade, relevância social e econômica, morbidade (relacionada com as incapacidades e deformidades), e também porque é uma doença transmissível passível de tratamento e controle (Alves et al. 2014).

A doença pode atingir pessoas de todas as idades, de ambos os sexos, no entanto, raramente ocorre em crianças, a não ser em áreas de alta endemicidade. Além das características individuais, existem muitos determinantes sociais que estão associados à continuação dessa doença nessas áreas. Esses determinantes incluem fatores sociais e culturais, mas também as condições da vida cotidiana e as desigualdades estruturais que afetam a saúde geral e imunidade (White and Franco-Paredes 2015).

As variações geográficas são uma característica marcante da hanseníase em todos os níveis (World Health Organization 2012). A doença já esteve amplamente distribuída pela Europa e Ásia mas, atualmente, ocorre principalmente em países pobres nas regiões tropicais e temperadas quentes (Britton and Lockwood 2004). Em 1991, a Organização Mundial de Saúde (OMS) definiu a eliminação como a obtenção de um nível de prevalência inferior a um caso por 10.000 habitantes, a nível global. Com essa taxa de prevalência, acreditava-se que a transmissão da hanseníase seria reduzida e a doença desapareceria naturalmente. No entanto, a doença permaneceu como endemia em 15 países ao final do ano 2000, principalmente na Ásia, África e América do Sul (World Health Organization 1991; Araújo 2003; Britton and Lockwood 2004; Cruz et al. 2017).

A detecção de novos casos pela OMS caiu de 514.718 em 2003 para 244.796 em 2009. Entretanto essa taxa de diminuição está se tornando menor a cada ano (Suzuki et al. 2012). De acordo com o último relatório publicado, em 2016 a detecção de novos casos no mundo foi de 214.783. Desses, 27.356 casos ocorreram na região das Américas e 25.218 no Brasil. A maioria dos novos casos (82,6%) ocorreu em 3 países – India, Brasil e Indonésia – que são os países onde a hanseníase é mais endêmica (World Health Organization 2017a). Entretanto, existem muitas lacunas no relato de novos casos em países de todas as regiões. Excluindo países europeus com uma população total de 860 milhões, que só relataram 32 novos casos, 530 milhões de pessoas em 64 países não informaram novos casos de hanseníase em 2016. Assim, países de alta renda e boa infra-estrutura de sistemas de saúde, como a maioria dos países europeus, Estados Unidos da América, Austrália e Japão têm casos relatados de hanseníase, enquanto muitos países de baixa renda, onde geralmente existem as condições favoráveis para a hanseníase, não relataram novos casos (Salgado et al. 2018).

O Brasil apresenta o maior número de casos das Américas e é o segundo país em número absoluto de casos da doença (Corrêa et al. 2012; World Health Organization 2017a). Por isso, a doença é de vigilância e de notificação compulsória em todo o país. No entanto, estima-se que apenas 1 de cada 3 pacientes com hanseníase seja relatado. Isso, juntamente com fatores como, longo tratamento e abandono do tratamento, contribui para o aumento do problema da hanseníase na saúde pública (Corrêa et al. 2012).

A prevalência está diminuindo em muitos países e os dados atuais globais indicam que o objetivo de eliminação da hanseníase foi alcançado. No entanto, as taxas de detecção permanecem as mesmas em algumas áreas. Além disso, a proporção atual de novos casos de hanseníase em indivíduos com menos de 15 anos de idade indica que a transmissão da doença ainda é significativa na maioria dos países onde a hanseníase é endêmica, inclusive no Brasil (Cruz et al. 2017). Observa-se que crianças, menores de quinze anos, adoecem mais quando há uma maior endemicidade da doença. Assim, hanseníase em menores de 15 anos é um importante indicador epidemiológico, pois indica doença recente e focos ativos de transmissão da doença (Barreto et al. 2017).

As variações geográficas também ocorrem dentro dos países (World Health Organization 2012). No Brasil, a ocorrência da hanseníase está concentrada em regiões mais pobres, como norte, nordeste e centro-oeste (Bandeira et al. 2017). Já os estados do sul, Santa Catarina e Rio Grande do Sul, são os únicos estados que atingiram, em 2005, a meta de eliminação da doença como problema de saúde pública (Corrêa et al. 2012).

1.1.2 Classificação

A classificação da hanseníase é fundamental para determinar o prognóstico da doença, indicar quais os indivíduos podem transmitir a doença e o tipo de tratamento a ser adotado. Além disso, é importante para determinar com precisão a epidemiologia da doença (Walker and Lockwood 2007). Essa condição apresenta uma ampla gama de manifestações clínicas e histopatológicas que apresentam uma distribuição espectral associada a alterações imunológicas do hospedeiro (Scollard et al. 2006; Mendonça et al. 2008). Assim, várias classificações têm sido propostas para a hanseníase ao longo dos anos baseadas em novos conhecimentos adquiridos sobre a doença (Lastória and Abreu 2014). As classificações mais utilizadas são a de Ridley & Jopling e a da OMS.

A classificação de Ridley & Jopling (1966) adota subgrupos dentro do espectro e obedece a critérios clínicos e bacteriológicos, enfatizando os aspectos imunológicos e histopatológicos (Souza 1997). Esta classificação apresenta um espectro de cinco grupos em que formas polares são imunologicamente estáveis e são denominadas tuberculóide-tuberculóide (TT) e virchowiana-virchowiana (VV). A forma tuberculóide constitui o polo de resistência e é caracterizada por imunidade celular intensa, com poucos bacilos e um número limitado de lesões. Na outra extremidade, a forma virchowiana constitui o polo de suscetibilidade com resposta celular comprometida e predomínio da resposta humoral apresentando lesões cutâneas mais difusas com intenso crescimento do bacilo em macrófagos. As formas intermediárias, borderline-tuberculóide (BT), borderline-borderline (BB) e borderline-virchowiana (BV) são imunologicamente dinâmicas, apresentando características oscilantes entre os dois polos da doença (Ridley and Jopling 1966; de Sousa et al. 2017)

Em 1982 a OMS criou uma classificação operacional, a fim de determinar a quimioterapia, baseada na carga bacilar que, por sua vez, se relaciona às formas clínicas. Assim, com base na pesquisa de bacilos na linfa em diversos pontos do organismo, os pacientes foram classificados em paucibacilares (PB) e multibacilares (MB). O grupo paucibacilar incluía os casos de hanseníase indeterminada (I), borderline-tuberculóide (BT) e tuberculóide-tuberculóide (TT) na classificação de Ridley & Jopling com índice bacilar <2. Já o grupo multibacilar incluía os casos de hanseníase borderline-virchowiana (BV) e virchowiana-virchowiana (VV), segundo Ridley & Jopling com carga bacilar >2 (World Health Organization 2012). A partir de 1988 todos os casos com baciloscopia positiva passaram a ser considerados MB e todos os casos negativos PB. Em 1998, considerando que nem sempre a baciloscopia está disponível, a OMS modificou novamente essa classificação que passou a se basear na contagem de lesões na pele e quantidade de troncos nervosos comprometidos: os pacientes PB têm até cinco lesões na pele e/ou um tronco nervoso comprometido; enquanto os MB apresentam mais de cinco lesões e/ou um ou mais troncos nervosos acometidos (Massone and Brunasso 2012; World Health Organization 2012). O quadro 1 resume a classificação e as características clínicas da hanseníase.

Quadro 1: Classificação e características clínicas da Hanseníase

Clínica	Baciloscopia	Formas clínicas	Classificação operacional
Áreas de hiposensibilidade ou anestesia, parestesias, manchas hipocrômicas e/ou eritemohipocrômicas, com ou sem diminuição da sudorese e rarefação de pelos	Negativa	Indeterminada	Paucibacilar (PB) até cinco lesões de pele
Placas eritematosas, eritemato-hipocrômicas, bem delimitadas, hipossensíveis ou anestésicas, podendo ocorrer comprometimento de nervos	Negativa	Tuberculóide	
Lesões pré-foveolares (eritematosas planas com centro claro). Lesões foveolares (eritematopigmentares de tonalidade ferrunginosa ou pardacenta), apresentando alterações de sensibilidade	Positiva (bacilos e globias ou com raros bacilos) ou Negativa	Dimorfa	Multibacilares (MB) mais de cinco lesões de pele
Eritema e infiltração difusos, placas eritematosas infiltradas e de bordas mal definidas, tubérculos e nódulos, madarose, lesões das mucosas, com alteração de sensibilidade	Positiva (bacilos abundantes globais)	Virchowiana	

Adaptado de Ministério da Saúde 2009.

1.1.3 Diagnóstico

O diagnóstico da hanseníase é feito quando pelo menos um desses sinais cardinais se manifesta:

a) lesões ou áreas da pele com alteração da sensibilidade térmica e/ou dolorosa e/ou tátil;

b) espessamento de nervo periférico, associado a alterações sensitivas e/ou motoras e/ou autonômicas;

c) presença de bacilos *M. leprae,* confirmada na baciloscopia de esfregaço intradérmico ou na biopsia de pele (Cruz et al. 2017).

O diagnóstico clínico é realizado através do exame físico no qual se procede uma avaliação dermatoneurológica, buscando identificar sinais clínicos da doença, as lesões de pele próprias da hanseníase, pesquisando a sensibilidade nas mesmas. As áreas onde as lesões ocorrem com maior frequência são: face, orelhas, nádegas, braços, pernas e costas. Deve-se pesquisar a sensibilidade térmica, dolorosa, e tátil, que se complementam (Brasil 2002).

A baciloscopia é o exame microscópico onde se observa o M*ycobacterium leprae* e é usada para detectar bacilos resistentes ao ácido e álcool (pesquisa de BAAR) em esfregaços cutâneos coletados em locais padronizados (lesões de pele, lóbulos nos ouvidos, cotovelos). É realizado pela técnica de coloração de Ziehl Neelsen, que consiste em colorir bacilos com corantes vermelhos e possibilitar a avaliação do índice morfológico (IM) e do índice bacteriano (IB) (Lastória and Abreu 2014).

O IM determina se o bacilo é viável ou não e é representado pela porcentagem de bacilos intactos em relação ao número total de bacilos analisados no estudo. O IB representa a carga quantitativa bacilar (número de bacilos) e é expresso de acordo com uma escala logarítmica variando de 0 a 6+. Esfregaços de pele identificam aqueles com doença multibacilar que são os mais infecciosos e também aqueles pacientes que apresentam recidivas clínicas (Moschella 2004; Lastória and Abreu 2014).

Nenhum teste laboratorial é considerado suficiente para diagnosticar a hanseníase. Assim, o estabelecimento de testes diagnósticos para detecção precoce da hanseníase, permitindo o tratamento adequado da hanseníase em

estágio inicial e da infecção pelo M. leprae, poderiam, fazer diferenças significativas na transmissão e nos resultados clínicos (van Hooij et al. 2017). A identificação de anticorpos séricos de imunoglobulina M (IgM) contra o glicolipídeo fenólico I (PGL-I), um antígeno específico de *M. leprae* pode ser útil para avaliar a exposição ao bacilo. Anticorpos anti PGL-1 correlacionam-se com o IB em pacientes com hanseníase e tem sido usado para apoiar sintomas da doença como um meio para categorizar pacientes com hanseníase. Além disso, podem ser marcadores para aqueles com maior risco de desenvolver a hanseníase, em uma triagem poderia ser usada para o diagnóstico precoce de contatos domésticos (Duthie et al. 2007; Fabri et al. 2016).

1.1.4 Tratamento

O tratamento da hanseníase compreende: quimioterapia específica, supressão dos surtos reacionais, prevenção de incapacidades físicas, reabilitação física e psicossocial (Araújo 2003).

A base para o tratamento atual é uma combinação de medicamentos, daí o nome poliquimioterapia (PQT). Os agentes de primeira linha são rifampicina, clofazimina e dapsona. A PQT foi introduzida pela OMS em 1982 após o aparecimento de resistência ao regime monoterápico da dapsona (Walker and Lockwood 2007). Isso foi um grande passo da OMS na história da hanseníase, pois resultou em um grande declínio da doença em regiões endêmicas (World Health Organization 2012). A PQT se tornou rapidamente o tratamento padrão da hanseníase e passou a ser fornecida gratuitamente por todos os países endêmicos desde 1995 (Kar and Gupta 2015).

Com a PQT, a OMS defende o uso de regimes de tratamento mais curtos para promover uma melhor adesão do paciente (Katoch 2002). A definição do esquema depende da classificação final do caso em paucibacilar (PB) ou multibacilar (MB) (Araújo 2003). O quadro 2 mostra o esquema de administração da PQT.

Existem tratamentos alternativos, disponíveis nos centros de referência, para pacientes com impossibilidade de usar os esquemas padronizados, incluindo o esquema ROM (rifampicina, ofloxacina eminociclina) para tratamento de lesão

única de pele em pacientes paucibacilares (Araújo 2003).

Quadro 2: Esquema poliquimioterápico (PQT) de tratamento da Hanseníase

Drogas	Paucibacilar - seis doses	Multibacilar - 12 doses
Rifampicina	600mg - dose mensal Supervisionada	600mg - dose mensal Supervisionada
Dapsona	100mg - dose diária Autoadministrada	100mg - dose diária Autoadministrada
Clofazimina		300mg - dose mensal supervisionada + 50mg - dose diária autoadministrada ou 100mg em dias alternados autoadministrada
Seguimento do caso	Comparecimentos mensais para a dose supervisionada e revisão dermatoneurológica na 6ª dose	Comparecimentos mensais para a dose supervisionada; revisão neurodermatológica na 6ª E 12ª doses
Critérios para alta	Alta por cura, após a 6ª. dose, que pode ser feita em até nove meses, independente do número de faltas consecutivas	Alta por cura, após a 12ª dose, que pode ser feita em até 18 meses, independente do número de faltas consecutivas

Adaptado de Ministério da Saúde, 2009 e Araújo,2003.

1.1.5 Episódios Reacionais

Os episódios reacionais ou reações hansênicas são episódios inflamatórios agudos que ocorrem no curso crônico da hanseníase (Ridley 1969). Eles podem resultar da reativação da resposta imune celular com a produção de mediadores inflamatórios, levando a graves danos nos tecidos e nos nervos (de Macedo et al. 2018). Esses episódios são sérias complicações da hanseníase porque essas reações são provavelmente a causa predominante de dano neurológico permanente levando a incapacidades e deformidades (Motta et al. 2012). Eles

contribuem significativamente para a carga da doença e precisam ser diagnosticados e tratados precocemente para prevenir dano neural e incapacidades permanentes (Kahawita et al. 2008)

Reações podem ocorrer a qualquer momento durante a progressão da doença, especialmente após o início da PQT, que leva à morte bacilar e, conseqüentemente, à liberação massiva de antígenos micobacterianos (de Macedo et al. 2018). Além disso, gestação, lactação e infecções concomitantes podem ser fatores potencialmente desencadeantes dos episódios reacionais (Motta et al. 2012; Scollard et al. 2015).

Há dois tipos bem reconhecidos de reações hansênicas: reação do tipo 1 que tem como manifestação característica a reação reversa (RR) e a reação do tipo 2, cuja principal manifestação é o Eritema Nodoso Hansênico (ENH).

1.2 ERITEMA NODOSO HANSÊNICO

O Eritema Nodoso Hansênico (ENH) é uma complicação imunológica séria, de difícil manejo que afeta pacientes de hanseníase BV e VV (Pocaterra et al. 2006; Kahawita and Lockwood 2008). Ocorre em aproximadamente 30% dos casos de hanseníase MB, pois a maior carga bacilar aumenta o risco de desenvolvimento da reação. A reação causa inflamação da pele, nervos e outros órgãos e mal-estar geral (Van Veen et al. 2009).

O ENH ocorre de maneira espontânea, mas pode estar associada a diversos fatores de risco como: infecções intercorrentes, anemia, estresse, puberdade, gestação, intervenção cirúrgica e uso de fármacos como antibióticos, progesterona e vitamina A (Valente and Vieira 2010). A reação se caracteriza por lesões eritematosas, dolorosas, de tamanho variável, incluindo pápulas e nódulos, localizadas em diferentes regiões do corpo. O ENH apresenta também comprometimento sistêmico e pode evoluir com neurite, orquite, epididimite, irite, iridociclite, artrite, linfadenite, dano hepático, edema dos membros inferiores, pré-tibialgia e febre (Valente and Vieira 2010). Geralmente, o ENH é diagnosticado clinicamente, mas uma biópsia da pele pode ser útil para o diagnóstico (Kahawita and Lockwood 2008).

O ENH pode ser agudo, recorrente ou crônico. O ENH agudo pode ser definido como um único episódio com duração inferior a 24 semanas. O ENH é recorrente se um paciente experimentou um segundo episódio de ENH ocorrendo 28 dias ou mais após a interrupção do tratamento para ENH. E o ENH é crônico se a reação ocorre por 24 semanas ou mais, período durante o qual um paciente necessitou de tratamento continuamente (Walker et al. 2014). Entretanto, poucos são os pacientes que apresentam um único episódio de ENH. A maioria dos indivíduos experimenta múltiplos episódios agudos de ENH ou a forma crônica por muitos anos (Van Veen et al. 2009). Isso tem um grande impacto físico, pois causa deformidades e incapacidades. Além disso, o tratamento prolongado pode apresentar efeitos colaterais que aumentam a morbidade e mortalidade (Voorend and Post 2013; Chandler et al. 2015). Também é uma condição que afeta indivíduos economicamente ativos tendo um grande impacto econômico e social nos pacientes e suas famílias (Chandler et al. 2015). Portanto, o ENH é um problema médico complexo que requer manejo cuidadoso por médicos experientes (Darlong et al. 2016).

Histologicamente, as lesões cutâneas do ENH frequentemente apresentam um intenso infiltrado perivascular de neutrófilos que se estende pela derme e tecido subcutâneo. Os neutrófilos são considerados a marca histológica do ENH. Também ocorre vasculite com edema do endotélio associada à infiltração de granulócitos na parede dos vasos. No entanto, nem todas as biópsias de pele do ENH mostram evidência de vasculite (Mabalay et al. 1965; Sehgal et al. 1986; Polycarpou et al. 2017)

Imunologicamente, o ENH é uma reação inflamatória sistêmica geralmente relacionada à deposição de imunocomplexos, porque apresenta características clínicas em comum com a reação de Arthus. Essa reação é uma hipersensibilidade do tipo III que envolve a deposição de imunocomplexos principalmente nas paredes dos vasos e também é caracterizada histologicamente por vasculite com um infiltrado de neutrófilos polimorfonucleares. Pacientes com ENH não tratados apresentam níveis séricos menores do componente C1q da cascata do complemento do que controles saudáveis e pacientes com hanseníase VV sem reação. Isso pode ser devido à sua utilização

para a formação do complexo antígeno-anticorpo na reação ENH. Da mesma forma níveis séricos diminuídos de C1q são encontrados em pacientes com outras doenças associados a imunocomplexos como glomerulonefrite aguda e lúpus eritematoso sistêmico (Negera et al. 2018b). No entanto não está claro se os imunocomplexos participam da imunopatogênese do ENH ou são apenas um epifenômeno (Wemambu et al. 1969; Polycarpou et al. 2017).

Diversas citocinas pró-inflamatórias também têm sido associadas à patogênese do ENH. O TNF-α parece ser um regulador da condição, enquanto há evidências substanciais que apoiam um papel para o IFN-γ também. Há também evidências de que outras citocinas, como IL-1β, IL-6, IL-8, IL-17 ou receptores de citocinas, como IL2R e IL6R também estejam envolvidas (Polycarpou et al. 2017).

Dados recentes sugerem que as respostas imunes mediadas por células (linfócitos T) também podem desempenhar um papel importante na patogênese da ENH (Negera et al. 2017b). Linfócitos T $CD4^+$ são os linfócitos T *helper* que quando ativados atuam como células efetoras produzindo citocinas. Já os linfócitos T $CD8^+$ correspondem as células T citotóxicas efetoras. Os linfócitos T $CD4^+$ também podem ser subdivididos em Th1, Th2, Th17 e Treg (células T reguladoras) de acordo com as citocinas produzidas (Polycarpou et al. 2017).

Pacientes de ENH apresentam maior ativação de linfócitos T em comparação com pacientes de hanseníase VV (Negera et al. 2017a). Além disso, eles possuem aumento do número de linfócitos T $CD4^+$ e diminuição do número de linfócitos T $CD8^+$, com aumentada relação $CD4^+/CD8^+$ (Polycarpou et al. 2017). O ENH também está associado com percentual reduzido de células T reguladoras e com aumento de células Th17 produtoras de IL-17. A IL-17 desempenha um papel fundamental na ativação e recrutamento de neutrófilos para o local da infecção em doenças inflamatórias. Ela é considerada uma citocina pró-inflamatória porque aumenta a produção de IL-6, IL-8, óxido nítrico (NO), TNF-α e IL-1β por vários tipos de células (Negera et al. 2017b).

Elementos da resposta imune inespecífica também têm sido associados ao ENH. Estudos genéticos demonstraram associações entre vários polimorfismos de um único nucleotídeo (SNPs) de genes de imunidade inata com ENH como o NOD2, que codifica um receptor citosólico que reconhece micobactérias; o gene

da proteína de macrófagos associada à resistência natural (NRAMP1) e os genes dos *toll like receptors (TLR)* (Almeida et al. 2010; Berrington et al. 2010; de Sales Marques et al. 2013; Polycarpou et al. 2017).

1.3 TRATAMENTO DO ERITEMA NODOSO HANSÊNICO

O principal objetivo do manejo do ENH é o controle da inflamação, o alívio da dor e a prevenção de outros episódios. Os casos leves podem ser tratados com anti-inflamatórios não-esteroidais (AINES). Aspirina é a droga mais usada nesses casos. Para os casos mais graves prednisona e clofazimina são as drogas mais utilizadas. A clofazimina é considerada um anti-inflamatório útil quando os corticosteroides são contraindicados ou precisam ser reduzidos. A colchicina e cloroquina também têm sido utilizadas com efeito limitado (Walker et al. 2007; Kahawita et al. 2008; Van Veen et al. 2009). No Brasil, as drogas mais utilizadas para o tratamento do ENH são a prednisona e a talidomida.

1.3.1 Prednisona

A prednisona é uma droga imunossupressora utilizada para o tratamento de doenças inflamatórias crônicas (Negera et al. 2018a). Na dose de 0,5-2,0mg/kg/dia, ela está indicada como tratamento de escolha para os casos moderados a graves de ENH. A prednisona geralmente age rapidamente, controlando a inflamação aguda e aliviando a dor. A dose inicial deve ser a mais baixa possível para controlar o ENH e ser gradualmente reduzida (Van Veen et al. 2009). No entanto, a maioria dos pacientes necessita de múltiplos ou prolongados regimes de prednisona devido à história natural da reação (Kahawita et al. 2008). Isso aumenta o risco de efeitos adversos tais como hipertensão, diabetes, síndrome de Cushing, gastrite, infecções fúngicas, osteoporose, catarata e dependência de esteroides (Sugumaran 1998; Van Veen et al. 2009).

Glicocorticoides suprimem a inflamação através de vários mecanismos celulares e moleculares. Seus efeitos sobre as células inflamatórias incluem: indução de apoptose, inibição de citocinas e inibição da migração de neutrófilos. Os mecanismos moleculares de ação dos glicocorticoides estão associados com a supressão de múltiplos genes inflamatórios que são ativados em doenças

inflamatórias crônicas, através da ligação de receptores de glicocorticoides a co-ativadores e recrutamento de histona desacetilase 2 para o complexo de transcrição ativado. Receptores de glicocorticoides ativados também interagem com sítios de reconhecimento no DNA para ativar a transcrição de genes anti-inflamatórios. Recentemente, se demonstrou que a prednisona atua modulando as citocinas pro-inflamatórias diretamente ou pela supressão de células imunológicas produtoras dessas citocinas (Torres et al. 2012; Negera et al. 2018a).

1.3.2 Talidomida

A talidomida foi originalmente desenvolvida na Alemanha em 1954 e posteriormente comercializada na Europa, Austrália e Canadá como sedativo e antiemético. No entanto seu uso por mulheres durante o primeiro trimestre da gestação levou a taxas alarmantes de defeitos nos ossos longos dos membros, ausência ou hipoplasia de olhos e orelhas, e anomalias cardíacas e gastrointestinais. Isso levou a droga a ser retirada do mercado no mundo todo no final de 1961 (Melchert and List 2007; Walker et al. 2007; Kowalski et al. 2015).

Os efeitos farmacológicos da talidomida vão além de seus efeitos neurosedativos e teratogênicos, e por essa razão ela foi posteriormente investigada em um grande número de doenças dermatológicas, reumatológicas e malignas (Melchert and List 2007). Em 1965, o médico israelense Jacob Sheskin descobriu acidentalmente que a talidomida é eficaz para o tratamento do ENH. No início da década de 1990, foi relatado que a talidomida inibe a produção de TNF-α e a replicação do HIV. Em 1994, foi demonstrada sua atividade anti-angiogênica, sugerindo que a droga tem um efeito anti-câncer. Em 1999, foi demonstrado que a talidomida é eficaz no tratamento de mieloma múltiplo, uma neoplasia de células B (Sheskin 1965; Sampaio et al. 1991; Makonkawkeyoon et al. 1993; D'Amato et al. 1994; Singhal et al. 1999; Ito et al. 2011).

Assim, sabe-se hoje que a talidomida apresenta propriedades terapêuticas anti-inflamatórias, imunomoduladoras e anti-angiogênicas (Ordi-Ros and Cosiglio 2014), sendo hoje classificada como uma droga imunomodulatória. O mecanismo de ação anti-inflamatória da talidomida envolve a inibição da expressão gênica

seletiva do TNF-α e, consequentemente, de suas funções. O TNF-α é uma potente citocina pró-inflamatória e imunoestimulatória de efeitos pleiotrópicos, podendo causar efeitos benéficos ou lesivos, dependendo da quantidade e do tempo de produção. O efeito inibitório parece envolver maior taxa de degradação do RNA mensageiro de TNF-α. Essa citocina tem sido uma das principais implicadas no mecanismo de patogênese do dano neural na hanseníase (Moreira et al. 1993; Penna et al. 2005).

A efetividade da talidomida no tratamento do ENH é primeiramente devida à sua ação sobre o TNF-α, mas outros mecanismos podem contribuir para seu efeito anti-inflamatório (Walker et al. 2007). Ela atua na redução da expressão de citocinas como IL-6, IL-1β, bFGF, VEGF, interferon-gama (INF-γ) e possivelmente fatores de transcrição como o NF-κB. Modula a expressão de moléculas de adesão e estimula a proliferação de células T citotóxicas e induz a produção de citocinas anti-inflamatórias (Mercurio et al. 2017)

Entre os efeitos colaterais da talidomida destaca-se a neuropatia periférica, sonolência, constipação intestinal e teratogenicidade que é a responsável pelo estigma no seu uso. No entanto, sabe-se que seus reconhecidos efeitos colaterais, exceto a teratogênese e a neuropatia, não representam uma complicação grave.

A talidomida no Brasil é a droga de escolha para o tratamento do ENH. A dose de talidomida recomendada para o controle das reações varia de 100 a 400 mg/dia (Putinatti et al. 2014). A dose é adequada à gravidade do quadro clínico e não há indicação de desmame nas normas. No entanto, devido aos graves efeitos teratogênicos, o medicamento à base de talidomida somente pode ser prescrito para mulheres em idade fértil após avaliação médica com exclusão de gravidez por meio de método sensível, e mediante a comprovação de utilização de, no mínimo, dois métodos efetivos de contracepção para mulheres em uso de talidomida, sendo pelo menos um método de barreira (Brasil. Ministério da Saúde 2011).

A hanseníase é a principal doença para a qual a talidomida é prescrita no Brasil, mas o medicamento não é comercializado, sendo distribuído apenas

através de programas específicos do Ministério da Saúde, e dispensado seguindo regras explícitas e rígidas (Brasil 2003; Brasil. Ministério da Saúde 2011).

Até o final da década passada, o Brasil figurava como único produtor de talidomida, com finalidade de suprir a demanda do Programa Controle de Hanseníase (PCH). Estima-se que, entre 1965 e 2001, cerca de 91.000 pacientes com ENH tenham recebido talidomida, fornecida pelo PCH. Trata-se da maior utilização da talidomida em serviço de saúde pública no mundo, tendo-se em vista ser o Brasil o único país endêmico de hanseníase que dispõe dessa droga (Vianna et al. 2015).

1.3.3 Outros medicamentos

Além da prednisona e da talidomida, clofazimina e pentoxifilina também são medicamentos úteis no tratamento do ENH. Entretanto eles não atuam tão rapidamente quanto prednisona e talidomida, sendo úteis no controle do ENH recorrente ou crônico e na redução do uso de esteroides (Lockwood 1996; Sampaio et al. 1998; Roy et al. 2015) Outras opções incluem metotrexato, azatioprina, etanercept e infliximab embora ainda sejam pouco utilizados na prática clínica (Nagar et al. 2015; Chowdhry et al. 2016; Jitendra et al. 2017).

Apesar de todas essas opções de medicamentos no tratamento do ENH, nenhum deles é completamente eficaz e existe uma variabilidade na reposta ao tratamento entre os pacientes de ENH. Portanto, estudos de farmacogenética que avaliem polimorfismos genéticos de genes associados à resposta aos medicamentos utilizados ou ao mecanismo do ENH podem ser úteis para a identificação de melhores opções de tratamento.

1.4 FARMACOGENÉTICA DO TRATAMENTO DO ENH

Estudos farmacogenéticos com talidomida e prednisona têm sido conduzidos em outras condições clínicas para identificar perfis genéticos que sejam mais suscetíveis a efeitos adversos e a diferenças na resposta ao tratamento. Os genes estudados são principalmente genes envolvidos no metabolismo desses medicamentos como os genes *NR3C1* e *ABCB1* para a prednisona e os genes *TNF* e *CYP2C19* para a talidomida. Além disso, o gene codificador da proteína

alvo da talidomida, *CRBN* e os genes de receptores da imunidade inespecífica, como os *Toll like receptors* (*TLR*) podem estar associados à resposta ao tratamento do ENH.

1.4.1 *NR3C1*

Os efeitos dos glicocorticoides ocorrem por meio de receptores de glicocorticoides (GRs) encontrados no citoplasma, núcleo e membrana celular. Esses receptores pertencem à subfamília do receptor nuclear 3, que não só transferem as informações encontradas na molécula do hormônio, mas também são importantes fatores de transcrição dependentes de ligantes. O GR, codificado pelo gene *NR3C1,* possui uma estrutura de três domínios: um domínio de transativação amino-terminal, que direciona a transativação dos genes-alvo; um domínio de ligação ao DNA, interagindo com elementos de resposta a glicocorticoides (GRE) no DNA; e um domínio carboxi-terminal, que contem sítios de ligação específicos para esteroides e proteínas do choque térmico, que permite a ligação com proteínas coativadoras (Manenschijn et al. 2009; Majer-Łobodzińska and Adamiec-Mroczek 2017).

Ao se ligar ao GR, os glicocorticoides podem atuar de duas formas: primeiro, o GR pode ativar ou reprimir a expressão gênica interagindo com sequências de DNA (os GREs) que estão presentes nas regiões promotoras de genes responsivos a esteroides. Esse mecanismo é chamado de transativação. Os efeitos colaterais do tratamento com GC são mediados principalmente pela transativação. Ao se ligar a GREs negativos, ocorre a transrepressão de genes alvo. O segundo mecanismo de ação do GR é a interação com outros fatores de transcrição, como a proteína ativadora 1(AP-1) ou o fator nuclear κB (NF-κB), reprimindo assim sua atividade transcricional, o que resulta na inibição de fatores de transcrição pró-inflamatórios. Esses efeitos transrepressivos da GR são o principal mecanismo que explica os efeitos antiinflamatórios dos GCs e elucida por que eles são amplamente utilizados no tratamento de doenças inflamatórias e autoimunes (Manenschijn et al. 2009).

O gene *NR3C1* humano está localizado no cromossomo 5 (região 5q31) e abrange aproximadamente 150Kb. O gene contém 9 éxons com a região

codificadora da proteína formada pelos éxons 2-9. O éxon 1 forma a região não traduzida 5'. O *splicing* alternativo de GR gera as isoformas hGRα e hGRβ, que são idênticas até o aminoácido 727, mas diferem em suas regiões C-terminal. A isoforma hGRα se liga a glicocorticoides, transloca-se para o núcleo e recruta co-reguladores para exercer efeitos transcricionais. No entanto, a isoforma hGRβ reside constitutivamente no núcleo e atua como um inibidor natural dominante negativo da isoforma hGRα. A isoforma hGRβ pode regular diretamente genes que não são regulados pela isoforma hGRα (Kadmiel and Cidlowski 2013).

O tratamento com glicocorticoides apresenta uma resposta variável observada entre os pacientes tanto quanto aos benefícios do tratamento quanto à manifestação de efeitos adversos. Variantes genéticas no receptor de glicocorticoides podem desempenhar um papel importante nessa variabilidade da resposta (Koper et al. 2014). Um grande número de polimorfismos no gene GR é conhecido. Alguns desses polimorfismos são funcionalmente relevantes, estando associados tanto com o aumento quanto com a diminuição da sensibilidade aos GC. Eles também podem estar associados a diferenças na composição corporal, parâmetros metabólicos, doenças autoimunes e doenças cardiovasculares. Neste contexto, destacam-se os polimorfismos *ER22/23EK* (rs6189 e rs6190), *N363S* (rs6195), *Bcl*I (rs41423247) e *GR-9β* (rs6198) (Manenschijn et al. 2009).

O polimorfismo *ER22/23EK* é causado por duas mutações de ponto. A primeira é a transição GAG —> GAA no códon 22 que não causa alterações na cadeia polipeptídica. A outra variante, a transição AGG —> AAG, ocorre no códon 23 e leva à substituição de arginina por lisina no receptor. Esse polimorfismo está associado a uma diminuição da sensibilidade aos GC (Manenschijn et al. 2009; Majer-Łobodzińska and Adamiec-Mroczek 2017).

O polimorfismo *N363S* está localizado no códon 363 do éxon 2 e resulta de uma mudança de nucleotídeo AAT → AGT. Esta alteração produz uma alteração de aminoácidos de asparagina (N) para serina (S). Este polimorfismo mostra um aumento da capacidade de transativação *in vitro* e está associado ao aumento da sensibilidade aos GCs (Manenschijn et al. 2009; Koper et al. 2014).

O polimorfismo *Bcl*I é uma alteração de C>G localizada cerca 646 pb *downstream* do fim do éxon 2. O mecanismo molecular através do qual o

polimorfismo *Bcl*I exerce o seu efeito é desconhecido, mas ele foi associado a um fenótipo clínico consistente com o aumento da sensibilidade aos glicocorticoides (Manenschijn et al. 2009; Koper et al. 2014).

GR-9β é uma substituição de A>G localizada na região 3'-UTR do éxon 9β, o éxon terminal do RNAm da isoforma β. A substituição de nucleotídeos A para G está localizada em um motivo "ATTTA" (mudando para GTTTA). Este motivo "ATTTA" é conhecido por desestabilizar o RNAm e diminuir a expressão da proteína receptora *in vitro*. Assim, esse polimorfismo é associado com a redução da sensibilidade aos glicocorticoides (Koper et al. 2014).

1.4.2 *ABCB1*

O gene *ABCB1* codifica uma molécula transportadora de membrana, a glicoproteína P (PgP) que é expressa de maneira polarizada na membrana plasmática das células dos órgãos de barreira e eliminação, onde exerce funções protetora e excretora. A PgP atua no efluxo de moléculas das células, removendo substâncias tóxicas naturais do sangue para o lúmen do trato gastrointestinal, urina e bile, para proteger órgãos vitais de substancias tóxicas (Wang et al. 2003). Devido a sua localização nos órgãos de barreira e excreção, também desempenha um papel importante na eliminação de primeira passagem de fármacos administrados oralmente para limitar a sua biodisponibilidade por efluxo de fármacos. A Pgp está portanto, associada com resistência a medicamentos, participando do transporte ativo de antibióticos, drogas anticâncer, drogas antivirais, agentes imunossupressores, bem como os GCs do citoplasma para o exterior da célula. (Hodges et al. 2011; Gabryel et al. 2016).

A PgP faz parte do metabolismo dos glicocorticoides podendo influenciar sua farmacocinética, limitar sua absorção através da camada intestinal, distribuição no sistema nervoso central e excreção através do fígado ou do rim (Marino et al. 2009).

Existe uma grande quantidade de polimorfismos do gene *ABCB1* já identificados, relacionados tanto com a redução quanto com aumento na expressão de PgP. A superexpressão estaria associada a um aumento de resistência aos GCs. O polimorfismo C3435T (rs s1045642) no éxon 26 está

associado com uma redução na expressão e atividade da PgP aumentando a sensibilidade aos GCs (Marino et al. 2009).

1.4.3 *TNF*

O gene *TNF* está localizado no braço curto do cromossomo 6, na posição 6p21.3, codificando uma citocina pró-inflamatória multifuncional que é secretada principalmente por macrófagos. TNFα desencadeia uma série de processos imunológicos e inflamatórios e é associada às manifestações do ENH. Os estudos de farmacogenética da talidomida com polimorfismos do gene *TNF* se justificam pela ação da talidomida de bloquear a produção dessa citocina (Neben et al. 2002; Majumder et al. 2018).

A expressão do *TNF* é regulada ao nível transcricional, e os polimorfismos da região promotora do gene estão envolvidos na variabilidade genética da produção da citocina. Entre esses polimorfismos se destacam -1031T/C, -863C/A, -857C/T, - 308G/A e -238G/A (Neben et al. 2002; Majumder et al. 2018). Alguns estudos relatam que o alelo G do polimorfismo -308G/A e o alelo A do polimorfismo -238G/A estariam associados a uma maior expressão do TNF-α (Wu and McClain1997; Hajeer and Hutchinson 2000).

1.4.4 *CYP2C19*

Os CYP2Cs humanos são uma subfamília importante das enzimas P450 que metabolizam aproximadamente 20% das drogas usadas clinicamente. A subfamília CYP2C consiste em quatro membros em humanos (CYP2C8, CYP2C9, CYP2C18, CYP2C19). A enzima CYP2C19 é a principal responsável pela formação dos metabólitos 5-hidroxitalidomida (5-OH) e cis-5'-hidroxitalidomida (cis-5'-OH) em humanos. (Goldstein 2001; Ando et al. 2002a; Li et al. 2007).

Os polimorfismos do gene *CYP2C19* alteram a capacidade metabólica da enzima gerando os metabolizadores extensos e os metabolizadores lentos. Esses polimorfismos podem, portanto, ter consequências clínicas na resposta aos medicamentos metabolizados pela enzima CYP2C19, resultando em toxicidade de alguns medicamentos, ou alteração da eficácia de outros medicamentos

(Goldstein 2001). No caso da talidomida, pacientes com o genótipo de metabolizadores lentos podem apresentar concentração muito baixa ou ausência dos metabólitos ativos em podem receber pouco benefício terapêutico do tratamento com talidomida, apresentando pior desfecho clínico (Li et al. 2007).

1.4.5 *CRBN*

Recentemente, a proteína Cereblon (Crbn) foi identificada como alvo primário da talidomida. A talidomida se ligaria a Crbn e inibiria a sua função de E3 ubiquitina ligase. Entre as diversas questões que surgiram a partir dessa descoberta está a possível relação entre essa proteína e os efeitos terapêuticos da talidomida (Ito et al. 2010).

A proteína Crbn tem 442 aminoácidos e é expressa em vários tecidos e órgãos. Ela tem sido descrita como um componente do complexo de E3 ubiquitina que contêm proteínas, como DDB1, Cul4 e Roc1, que atuam na ubiquitinação e subsequente degradação de substratos. Crbn é codificada por um gene do mesmo nome (*CRBN*), que está localizado no braço curto do cromossomo 3. O gene é muito conservado e contém 11 éxons que se estendem em quase 30 kb. Até recentemente, as mutações neste gene apenas tinham sido associadas com uma forma recessiva de deficiência intelectual (Higgins et al. 2004; Ito et al. 2010).

A descoberta do Cereblon como alvo da talidomida gerou interesse considerável na definição se a expressão da proteína ou a presença de mutações no gene *CRBN* teria impacto nas respostas clínicas para essa droga e outras drogas imunomodulatórias, como lenalidomida e pomalidomida (Thakurta et al. 2014). Muitos estudos clínicos correlacionaram a maior expressão do gene *CRBN* em células de mieloma múltiplo com a resposta superior ao tratamento com um regime baseado em lenalidomida e um regime à base de pomalidomida, bem como maior sobrevida livre de progressão durante a terapia de manutenção com talidomida (Heintel et al. 2013; Huang et al. 2014; Schuster et al. 2014; Broyl et al. 2015). Assim, é possível que *CRBN* seja também um marcador útil sobre a resposta ao tratamento do ENH com talidomida.

1.4.6 *Toll Like Receptors*

Receptores Toll-like (TLRs) são proteínas transmembranares tipo 1, expressas em células imunes inatas que desempenham um papel crítico na resposta inflamatória a patógenos microbianos (Bochud et al. 2008). Eles reconhecem e respondem a diversas moléculas microbianas e permitem ao sistema imune inato discriminar entre grupos de patógenos e induzir uma cascata apropriada de respostas efetivas. O domínio extracelular do receptor reconhece uma vasta gama de produtos microbianos. Na ativação, o domínio intracelular provoca uma transdução de sinal complexa e ativa a transcrição de citocinas pró-inflamatórias. Essas citocinas desempenham um papel essencial na resposta imune inata do hospedeiro e determinam a ativação dos mecanismos imunes adaptativos (Bochud et al. 2008; Misch and Hawn 2008). Os TLRs individualmente reconhecem um repertório distinto, mas limitado, de produtos microbianos conservados; por exemplo, os pares receptores-ligandos bem caracterizados incluem TLR-4 e LPS (lipopolissacarídeo), TLR-5 e flagelina, TLR-1/TLR-2/TLR-6 e lipoproteínas, e TLR-3/TLR-7/TLR-8/TLR-9 e diferentes segmentos de ácido nucleico. Coletivamente, a família TLR completa permite que o hospedeiro detecte a infecção pela maioria (se não todos os tipos) de patógenos microbianos (Misch and Hawn 2008).

Diversos estudos têm demonstrado associações entre variações nos genes *TLR* e o risco de desenvolvimento da hanseníase e das reações hansênicas (Bochud et al. 2008; Bochud et al. 2009; Marques et al. 2013; Santana et al. 2017). Um receptor de reconhecimento de padrão específico com um papel potencial no ENH é o TLR-9, uma importante via de ativação da imunidade inata do hospedeiro durante infecções por micobactérias. Já se demonstrou que pacientes com ENH apresentam níveis mais altos de complexos de histonas e DNA humano e micobacteriano que poderiam atuar como ligantes do TLR-9 e que a ativação de TLR-9 desempenha um papel crucial na geração de citocinas pró-inflamatórias durante o ENH. Além disso, também foi demonstrado que o uso de inibidor E6446 dos receptores TLR7/TLR9 bloqueia de forma expressiva a produção de TNF-α, IL-6 e IL-1b em pacientes com ENH (Dias et al. 2016). Assim,

esses receptores podem ter um papel importante na patogênese do ENH e na resposta ao tratamento.

Capítulo II - Justificativa

2. JUSTIFICATIVA

A Hanseníase é uma doença endêmica no Brasil que ocupa o segundo lugar em número de casos da doença no mundo e apresenta diversas regiões com altos índices da doença. O Eritema Nodoso Hansênico (ENH) é uma reação inflamatória dolorosa que afeta pacientes de hanseníase multibacilar. Trata-se de uma das complicações mais graves da doença e uma importante causa de deficiências e incapacidades associadas à hanseníase. A reação está associada a uma carga física, psicológica, econômica e social para os pacientes afetados. Assim, em áreas endêmicas de hanseníase no país, o ENH constitui um importante problema de saúde pública.

O tratamento do ENH é um desafio, pois a reação é de difícil controle e nenhum medicamento atualmente disponível é completamente eficaz. Os pacientes apresentam uma variabilidade na resposta ao tratamento, as recorrências são frequentes e geralmente é necessário o uso prolongado dos medicamentos. Além disso, os medicamentos mais utilizados no Brasil, que são prednisona e talidomida estão associados a muitos efeitos adversos, como a dependência no caso da prednisona e neuropatia periférica no caso da talidomida. Tais efeitos podem complicar ainda mais o tratamento e a qualidade de vida do paciente. Deve-se também considerar a teratogenicidade da talidomida que é um fator adicionalmente preocupante, uma vez que o Brasil é o país que tem maior distribuição da droga no mundo em decorrência do tratamento do ENH e o único a registrar novos casos de embriopatia por talidomida.

Polimorfismos de genes que estão associados ao metabolismo desses medicamentos ou à patofisiologia da doença podem influenciar a variabilidade da resposta ao tratamento entre os pacientes. Nesse contexto, torna-se importante avaliar a existência de fatores genéticos que possam explicar as diferenças nas respostas clínicas dos pacientes com ENH em uso de prednisona e talidomida seja do ponto de vista de eficácia do tratamento ou da manifestação de efeitos adversos. A identificação desses fatores pode ser uma ferramenta importante na escolha do tratamento mais adequado e eficaz buscando diminuir o tempo do uso dos medicamentos e a diminuição da manifestação de efeitos adversos.

Capítulo III - Objetivos

3. OBJETIVOS

3.1 Objetivo Geral

Avaliar a existência de variantes genéticas que possam estar associadas à resposta ao tratamento do Eritema Nodoso Hansênico (ENH).

3.2 Objetivos Específicos

- Realizar uma revisão de literatura sobre os principais tratamentos do ENH.
- Identificar a associação entre diminuição de dose no tratamento do ENH com talidomida e/ou prednisona e os polimorfismos em *NR3C1, ABCB1*, *TNF, CYP2C19*.
- Associar o perfil de efeitos adversos relacionados ao uso de talidomida e prednisona com a frequência dos polimorfismos nos genes *TNF, CYP2C19*, *CRBN, NR3C1, ABCB1*.
- Avaliar a influência de polimorfismos do gene *CRBN* e o papel de *CRBN* como fator preditor da resposta ao tratamento do ENH com talidomida.
- Identificar a relação entre polimorfismos do gene *TLR-9* e o tratamento do ENH com talidomida e prednisona.

Capítulo IV – Artigo 1

Erythema Nodosum Leprosum: Update and Challenges on the Treatment of a Neglected Condition

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Erythema Nodosum Leprosum: Update and Challenges on the Treatment of a Neglected Condition

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ABSTRACT

Erythema Nodosum Leprosum (ENL) occurs due to the immunological complication of multibacillary leprosy and is characterized by painful nodules and systemic compromising. It is usually recurrent and/or chronic and has both physical and economic impact on the patient, being a very important cause of disability. In addition, ENL is a major health problem in countries where leprosy is endemic. Therefore, adequate control of this condition is important. The management of ENL aims to control acute inflammation and neuritis and prevent the onset of new episodes. However, all currently available treatment modalities have one or two drawbacks and are not effective for all patients. Corticosteroid is the anti-inflammatory of choice in ENL but may cause dependence, especially for chronic patients. Thalidomide has a rapid action but its use is limited due the teratogenicity and neurotoxicity. Clofazimine and pentoxifylline have slow action and have important adverse effects. Finally, there is no pattern or guidelines for treating these patients, becoming more difficult to evaluate and to control this condition. This review aims to show the main drugs used in the treatment of ENL and the challenges in the management of the reaction.

Key words: Leprosy, Erythema Nodosum, Treatment, Thalidomide, Challenges

1. INTRODUCTION

Leprosy is a chronic, granulomatous disease caused by *Mycobacterium leprae*, which affects the skin, peripheral nervous system and, occasionally, other organs (Britton and Lockwood, 2004; Souza, 1997). The disease is considered a public health problem marked by serious clinical manifestations and psychological repercussion generated by the deformities and physical incapacities (Brasil, 2008). Leprosy occurs in a variety of clinical forms which are distributed in a continuous spectrum related to the patient's immune response (Rodrigues Júnior et al., 2016; World Health Organization, 2012) and varies from a resistance pole (tuberculoid), with a cellular immune response, to a susceptibility pole (lepromatous), with a humoral immune response (Mendonça et al., 2008). Several different leprosy classifications considers these two ends of the spectrum and the intermediate manifestations (borderline or indeterminate forms) between them (Rodrigues Júnior et al., 2016). The Ridley-Jopling clinical classification system is the most widely used and classifies leprosy as indeterminate (I), tuberculoid (TT), borderline-tuberculoid (BT), borderline-boderline (BB), borderline-lepromatous (BL) and lepromatous (LL) based on patient's immune response (Ridley and Jopling, 1966). Besides this, there is an operational classification created by World Health Organization (WHO) in 1982, in order to facilitate treatment regimens, and modified in 1988. It is based on the counting of skin lesions and the number of compromised nerve trunks and classifies patients as paucibacillary (PB) or multibacillary (MB). PB patients have up to five skin lesions and/or a compromised nerve trunk and include indeterminate (I), polar tuberculoid (TT) and boderline-tuberculoid (BT) patients in the Ridley-Jopling classification. MB patients have more than five lesions and/or one or more affected nerve trunks and include boderline-boderline (BB), boderline-lepromatous (BL) and polar lepromatous (LL) (Table 1). (World Health Organization, 2012). However, there may be a discrepancy between the patient's bacillary load and the number of lesions and many multibacillary cases are misclassified as paucibacillary (Eichelmann et al., 2013). Thus, patients with BT leprosy are generally classified as paucibacillary but may have a high bacillary load in granuloma or nerve biopsies and should be treated as multibacillary patients (Barreto et al., 2007; Kumaran et al., 2015).

When classification is in doubt, the patient should be treated as having MB leprosy (World Health Organization, 2012).

Table 1: Leprosy Classification

RIDLEY & JOPLING	Celular Immunity			Humoral Immunity		
	I	**TT**	**BT**	**BB**	**BL**	**LL**
WHO	**PAUCIBACILLARY**			**MULTIBACILLARY**		
	<5 skin lesions BI: Negative			>5 skin lesions BI: Positive		

WHO: World Health Organization; I: Indeterminate leprosy; TT: Tuberculoid leprosy; Boderline-Tuberculoid leprosy; BB: Boderline-Boderline leprosy; BL: Bodeline-Lepromatous leprosy; LL: Lepromatous leprosy; BI: Bacteriological index.

In some cases, patient´s immune system develops varied signs and symptoms of inflammation arising from acute or chronic hypersensitivity in response to antigens of *Mycobacterium leprae.* These episodes are called leprosy reactions and may occur in the regular course of the disease even without the intervention of treatment (Balagon et al., 2010).

Leprosy reactions have serious complications because they are probably the predominant cause of permanent neurological damage, leading to disabilities and deformities (Motta et al., 2012). There are two well-recognized types of leprosy reactions: type 1 reaction, or reverse reaction (RR), and type 2 reaction, which the most frequent manifestation is Erythema Nodosum Leprosum (ENL) (Ministério da Saúde. Brazil, 2005).

ENL occurs due to the immunological complication of multibacillary leprosy, and manifests as painful subcutaneous erythematous nodules that can ulcerate. Furthermore, there may be signs of systemic involvement such as fever, inflammation of lymph nodes, neuropathy, joint involvement, testicular, ocular, hepatic, renal, extremity, among others (Naafs and Noto, 2012; Nicholls and Smith, 2002). In the skin, ENL manifests as erythematous, inflamed nodules and papules that may be superficial or deep. Bullous, pustular, ulcerated, hemorrhagic and necrotic forms may also occur. Some nodules can persist as chronic painful panniculitis leading to fibrosis and scarring (Kahawita et al., 2008; Wemambu et

al., 1969). The nodules affect mainly the face and the extremities even though they do not affect the scalp, axilla, inguinocrural, perineum, mucous and semi-mucous regions. In addition, the nodules have bilateral and symmetrical occurrences (Nery et al., 2006).

Systemic manifestations include iritis, iridocyclitis, neuritis, myositis, lymphadenitis, arthritis, dactylitis and orchitis. Hepatic and splenic damage (hepatosplenomegaly), generalized ganglionic infarction, acrofacial, lower limb or generalized edema, pre-tibialgia and fever can also occur. Moreover, rhinitis, epistaxis, insomnia and depression have also been observed (Hansenologia and Dermatologia, 2003; Ridley, 1969; Wemambu et al., 1969). ENL uveitis can result in cataracts, glaucoma, and ultimately blindness. Orchitis causes aspermia, impotence and infertility (Jolliffe, 1977; Teo et al., 2002). There may be cortical adrenal insufficiency as a consequence of continuous drug treatment (Goldgraber and Sulman, 1969). Patients may present low levels of the adrenal androgen dehydroepiandrosterone sulphate, which inversely correlated with some inflammatory cytokine levels (Leal et al., 2003). Individuals with ENL present urinary abnormalities more frequently than non-reactional patients. Renal lesions more frequently observed are glomerulonephritis, tubulo-interstitial-nephritis and amyloidosis. Amyloidosis is initially manifested by proteinuria, which progresses to nephrotic syndrome and chronic renal failure (Nakayama et al., 1995). ENL patients are in general chronically ill and fatigued, in chronic pain and suffer from insomnia (Teo et al., 2002). Table 2 shows the main ENL manifestations.

Table 2. Signs and Symptoms of Erythema Nodosum Leprosum

Organ or System	**Sign and Symptom**
Skin	Inflamed subcutaneous erythematous nodules or deep or superficial papules. Less of ten vesicles, blisters and pustules.
Joints	Polyarthritis or polyarthralgia
Lymph nodes	Lymphadenopathy
Eyes	Uveitis (iritis and iridocyclitis) that can progress to cataracts, glaucoma and blindness.
Liver	Hepatosplenomegaly
Testicles	Orchitis and epididymitis
Kidneys	Glomerunephritis, tubulo-interstitial-nephritis and amyloidosis which can progress to renal failure.
Respiratory system	Rhinitis, epistaxis, laryngitis
Bone	Dactylitis, pre-tibialgia
Muscles	Myositis
Nerves	Neuritis
Others	Acrofacial or generalized edema, fever, insomnia and depression.

ENL occurs only in borderline lepromatous (BL) and lepromatous (LL) patients which correspond to multibacillary (MB) patients. During the dapsone monotherapy era, ENL was reported in 50% of LL patients and 25% of BL patients. These numbers were reduced with the introduction of multidrug therapy

(MDT), probably due to the inclusion of clofazimine in the scheme (Becx-Bleumink and Berhe, 1992; Lockwood, 1996).

There is a wide variation on the occurrence of ENL in different regions of the world. In Brazil, ENL occurs in 37% of BL and LL leprosy patients, in Asian countries such as India, Nepal and Thailand, this frequency varies between 19-26%, and in African countries as Ethiopia is around 5% (Becx-Bleumink and Berhe, 1992; Fava et al., 2012; Kahawita et al., 2008; Nery et al., 1998; Pocaterra et al., 2006; Saunderson et al., 2000). Moreover, these frequencies differ not only between countries but also within countries (Voorend and Post, 2013). This variation occurs because, although there are adequate systems to estimate the overall prevalence of leprosy, they are not available to estimate the frequency or incidence of ENL. Hence, it is difficult to obtain reliable data (Voorend and Post, 2013). Differences in proportions among multibacillary (MB) cases may also be associated with this variability.

The natural course of the ENL reaction is between one and two weeks, but many patients experience multiple recurrences for months (Scollard et al., 2006). Because of this, ENL can be classified in three types: acute ENL, which comprises a single episode lasting less than 24 weeks; recurrent ENL, with a second or subsequent episode occurring 28 days or more following the end of treatment; and chronic ENL, with continuous episodes lasting longer than 24 weeks (Walker et al., 2014). These types may have different risk factors and require different therapeutic interventions. Defining these different types could be important in designing therapeutic interventions and health care planning (Pocaterra et al., 2006). In regards to severity, ENL can be classified as mild, moderate or severe (Table 3) and this classification is based on the clinical manifestations (Guerra et al., 2002).

Table 3. Classification of ENL according to the severity of the reaction

SEVERITY	CHARACTERISTICS

MILD	Less than 10 nodules per affected body segment, predominantly in the lower limbs and slightly painful to palpation with absence or mild intensity of systemic signs and symptoms.
MODERATE	10 to 20 nodules per affected body segment, necessarily more than one, accompanied by moderate fever (<38.4° C), with mild systemic symptomatology and local and/or regional lymph node involvement.
SEVERE	More than 20 nodules per affected body segment, painful even without palpitation, usually involving a large area of the integument, sometimes having ulcerated lesions, accompanied by significant systemic symptoms such as high fever (> 38.5° C), arthralgia, chills, headache, anorexia and generalized involvement of a lymph node.

Adapted from Guerra et al, 2002

In summary, ENL is a painful, multi-systemic and recurrent condition with high impact on the patient health and an increased risk to neurological damage and deformities. Therefore, adequate control of the reaction is necessary to prevent permanent disabilities in the patient. This review aims to show the main drugs used in the treatment of ENL and the challenges in the management of the reaction.

2. IMMUNOLOGICAL ASPECTS

ENL is a systemic inflammatory reaction characterized as acute episodes, which may be recurrent, in the chronic course of the disease (Campos and de Souza, 1954). At a immunological point of view, ENL is often described as a neutrophilic immune-complex-mediated condition because it has some clinical features in common with the Arthus reaction and its multisystem involvement resembling autoimmune diseases as systemic lupus erythematosus (Polycarpou et al., 2017; Wemambu et al., 1969). Many studies provide evidence of an association between immune complexes and ENL but they do not necessarily support that they are the trigger leading to ENL. Hence, it is unclear whether they

are involved in the pathogenesis of ENL or are simply an epiphenomenon (Polycarpou et al., 2017). The reaction also presents high concentrations of tumor necrosis factor alpha (TNF-α), neutrophil infiltration and complement activation (Mendonça et al., 2008).

TNF-α is a potent pro-inflammatory and immune-stimulatory cytokine of pleiotropic effects that can cause beneficial or harmful effects depending on its concentration and time of production. TNF-α has been implicated in the mechanism of pathogenesis of neural damage that occurs in leprosy (Penna et al., 2005). However, the mechanism that triggers the reaction remains unknown (Fava et al., 2012).

In association with high levels of TNF-α, increased levels of interleukin 1 (IL-1) have also been found in the serum of ENL patients, suggesting a synergistic action between the two cytokines (Parida et al., 1992). The increase of interferon-gamma (IFN-γ) and IL-12, might be a contributing factor in the deleterious effects classically attributed to TNF-α (Moraes et al., 1999). In addition, there is an increase in the expression of IL-6, IL-8 and IL-10 (Mendonça et al., 2008; Yamamura et al., 1992).

Histologic analyses of ENL lesions present neutrophil infiltration, which is considered to be the histological hallmark of ENL. However, it remains unclear whether the neutrophil initiates ENL or is recruited to the site of the affected skin lesion under the action of chemokines secreted by other cell types (Mabalay et al., 1965; Polycarpou et al., 2017). In addition, multiple studies report an increased CD4+/CD8+ ratio in ENL patients in both skin and peripheral blood (Bach et al., 1981; Polycarpou et al., 2017; Wallach et al., 1982).

3. TREATMENT

The main objective of ENL management is to control acute inflammation and neuritis (Table 4), relieve pain and discomfort, prevent the development and extension of cutaneous, neural, ocular and visceral alterations and prevent the onset of new episodes (Hansenologia and Dermatologia, 2003; Lockwood, 1996; Mahajan et al., 2003). In all cases, the patient should rest and receive anti-inflammatory medication (Lockwood, 1996). Unfortunately, all currently available

treatment modalities have some drawback and are not effective for all patients (Lockwood, 1996; Mahajan et al., 2003).

3.1 Anti-inflammatory drugs

Mild cases can be treated with anti-inflammatory drugs. Acetylsalicylic acid (ASA) is the most commonly used drug in these cases, although indomethacin, chloroquine and colchicine have already been tested (Karat et al., 1969; Van Veen et al., 2009). The effects of indomethacin, chloroquine and acetylsalicylic acid on ENL control were similar. Acetylsalicylic acid has fewer adverse effects and patients in use of indomethacin have a lower incidence of visual disturbances (Karat et al., 1969). In a comparative study, colchicine was more effective compared to acetylsalicylic acid in inducing relief of neural and joint pain in moderate cases of ENL. However, colchicine treatment had diarrhea as a serious and limiting side-effect (Kar and Roy, 1988). Paracetamol at a dose of 1000mg every eight hours may be associated with the other treatments as an adjuvant in the control of pain and fever (Pulido Pérez and Suárez Fernández, 2015).

3.2 Thalidomide

The effect of thalidomide for ENL has been reported in at least 1,750 patients (6 controlled and 26 open-label clinical trials) (Villahermosa et al., 2005). It has a rapid action, controlling the symptoms within 24-48 hours (Sheskin, 1965). Thalidomide's mechanism of action is not yet completely understood. However, several clinical trials have highlighted its ability to selectively inhibit TNF-α production in monocytes (Moreira et al., 1993; Sampaio et al., 2002, 1991; Tramontana et al., 1995; Ye et al., 2006).

Thalidomide shows anti-inflammatory, immunomodulatory and anti-angiogenic activities (Ye et al., 2006). The mechanism of anti-inflammatory action of thalidomide involves the inhibition of the *TNF-α* gene expression and, consequently, its functions. This inhibitory effect seems to involve a higher degradation rate of the messenger RNA (Moreira et al., 1993; Penna et al., 2005; Sampaio et al., 1991), which is one of the main factors responsible for the effectiveness of thalidomide. It inhibits neutrophil numbers in the lesions by

decreasing neutrophil recruitment (Polycarpou et al., 2017; Sarno et al., 1993). The drug also acts on circulating T cells, decreasing the number of CD4 cells and increasing the number of CD8 cells (Lockwood, 1996; Shannon et al., 1992). It also has action on interferon-γ, IL-10 and IL-12, Cyclooxygenase 2 and on the pro-inflammatory transcription factor NF-kB (Franks et al., 2004).

The initial dose of thalidomide ranges from 100-400 mg daily ingested with water to at least 1 hour after a meal. The dose must be determined according to the patient's weight and those weighing less than 50 kg should start at the smallest dose. Thalidomide should be continued until identification of clinical improvement and symptoms disappearance. The drug can then be reduced by 50 mg every two to four weeks. If the patient requires maintenance, the lowest dose should be administered, and dose reduction should be attempted again every 3-6 months (Rosenbach and Werth, 2007).

Adverse effects of thalidomide are mainly teratogenesis, sedation and peripheral neuropathy (Franks et al., 2004). The use of thalidomide in the first trimester of pregnancy is teratogenic and can cause a range of defects in embryos exposed to the drug. Limb defects, including phocomelia (defects in the proximal elements of the limbs), accounts for the majority of cases. Other defects include cardiac and gastrointestinal anomalies, ear deformities, ocular defects, facial paralysis, anomalies or absence of internal organs (Dimopoulos and Eleutherakis-Papaiakovou, 2004; Smithells and Newman, 1992). Thus, the WHO Expert Committee recommends thalidomide to be administered only to men and postmenopausal women dependent on corticosteroids (WHO, 1998). However, for women in childbearing age, the drug should not be denied as long as the risks and benefits are assessed by the doctor and the patient and her partner are oriented about the drug and its teratogenicity property (Walker et al., 2007). In Brazil, the legislation determines that the thalidomide-based medicinal product should only be prescribed for women of childbearing age after medical evaluation excluding pregnancy through a sensitive method and by proving the use of, at least, two effective methods of contraception with at least one barrier method (Ministério da Saúde, 2011).

After teratogenesis, peripheral neuropathy is the most serious complication of the treatment (Wines et al., 2002). Its emergence arising from the use of thalidomide in other conditions is well documented in the literature, but such effect on leprosy is still controversial (Hansenologia and Dermatologia, 2003). Peripheral neuropathy typically associated with thalidomide includes symmetrical painful paresthesia of the hands and feet, often accompanied by sensory loss in the lower limbs (Calabrese and Fleischer, 2000; Wines et al., 2002). It correlates with the dose and is permanent in 50% of cases, and can progress for some time after drug discontinuation and any improvement that can occur are slow (Walker et al., 2007). It is difficult to differentiate leprosy and thalidomide-induced neuropathy as clinically as neuro-physiologically (Kaur et al., 2009).

Another adverse event in patients treated with thalidomide is thromboembolic complications including deep venous thrombosis and pulmonary embolism (Wu et al., 2005). The risk of occurrence of these events is increased when thalidomide is combined with other medications such as dexamethasone or chemotherapy (Hebe Petiti-Martin et al., 2013; Zangari et al., 2001). Venous thromboembolism associated with thalidomide use is reported to be less than 5% of the cases, but the risk increases to 12-17% when combined with corticosteroid therapy (Cavenagh et al., 2003; Yamaguchi et al., 2012; Zonder, 2006). Therefore, in Brazil, the Ministry of Health recommends the use of Acetylsalicylic acid 100 mg/day as prophylaxis in the combination of thalidomide and corticoid (Ministério da Saúde., 2016).

Dermatologic effects include rash, atrophic lesions, dry skin and mouth, and, rarely, toxic epidermic necrolysis and the Stevens-Johnson syndrome (Dimopoulos and Eleutherakis-Papaiakovou, 2004). Thalidomide frequently causes drowsiness and constipation that may limit the usefulness of the drug. The degree of somnolence is severe in up to 11% of patients. Other adverse effects that may lead to discontinuation of its use include dizziness, nausea, peripheral edema, neutropenia, amenorrhea, hypothyroidism, generalized weakness, fatigue, myalgia, headache, hypotension, increased appetite, pruritus, and weight gain (Clark et al., 2001; Melchert and List, 2007; Walker et al., 2007; Wu et al., 2005). Although many, these effects are rare, hence thalidomide is generally well

tolerated and effective on ENL treatment. In addition, it can offer an economic advantage since its use can avoid the costs of treating comorbidities associated with corticosteroid use (Darlong et al., 2016).

3.3 Corticosteroids

Corticosteroids act on inflammation by reducing recruitment and activation of neutrophils and macrophages and decreasing the production of various cytokines, including TNF-α (Rang and Dale, 2012). Prednisone is the anti-inflammatory of choice in moderate, severe and recurrent ENL, being readily available and having a rapid and defined therapeutic action (Mahajan et al., 2003). Prednisone acts rapidly on the control of acute inflammation and pain relief (Van Veen et al., 2009). There is no fixed regime for the use of prednisone in ENL (Darlong et al., 2016). Most doctors recommend an initial dose of 60 mg, but doses above 200 mg have been used (Lockwood, 1996). Overall, it is recommended initially to administer 1 to 1.5 mg/kg/day. This initial dose should be maintained for 15 to 30 days, when gradual withdrawal of the medication should begin. The dose of prednisone should be slowly reduced according to the response, being suggested to reduce 10 mg every 15 days and, after reaching the dose of 20 mg/day, to reduce 5 mg every 15 days. From 5 mg/day, the dose should be maintained for 15 consecutive days and then on alternate days for another 15 days (Ministério da Saúde., 2010).

Most patients require multiple or prolonged prednisone regimens due to the natural course of the disease (Kahawita et al., 2008). In patients with recurrent ENL the dose can be increased or maintained for an indefinite period of time (Darlong et al., 2016). A maintenance dose from 5 to 10 mg can be used for several weeks to prevent the recurrence of ENL (Walker et al., 2007). However, corticosteroids are not indicated to prevent recurrence of ENL as they only treat the active reaction (Darlong et al., 2016). A maintenance dose may be an additional problem to the course of the disease due to serious side effects associated with prolonged use of corticosteroids. Thus, this option, although effective, may cause dependence, especially for chronic patients (Hansenologia and Dermatologia, 2003; Lockwood, 1996). In addition, the induction of Cushing, acne, diabetes, osteoporosis, gastritis, cataract and immunosuppression can lead

to the risk of opportunistic infections including fungal infections and tuberculosis (Sugumaran, 1998).

Patients on chronic corticosteroid use should be closely monitored. This is because of the risk of developing an opportunistic infection. In addition, the levels of glucose, potassium, and calcium should be monitored periodically, especially when the patient has cramps. It is still important to perform parasitological examination, but irrespective of outcome, treatment should be instituted for *Strongiloides stercoralis* infestation, repeating periodically in every one-two months (Ura, 2007).

In patients with prolonged corticosteroid therapy, difficult to control, without improvement or with very intense symptoms, it is indicated the pulse therapy, which is the use of corticosteroids in a high dose intravenously. The most commonly used corticosteroids for pulse therapy are prednisone, dexamethasone and methylprednisone. Patients need monitoring and hydro electrolytic control, glycemic, blood pressure and heart rate. Therefore, pulse therapy should be performed in specialized centers, where the patient can be hospitalized, or in a day-hospital system (Ministério da Saúde., 2010). The dose of 1000 mg/day of methylprednisolone can be used for three consecutive days with monthly reinforcements of 1000 mg/day on a single day, or the dose of 500 mg/day for three consecutive days (minipulses) monthly, followed by lower oral doses of prednisone (0.5 mg/kg/day) between the pulses (Ministério da Saúde., 2010). Pulse therapy can also be used to facilitate withdrawal of the total oral dose of corticosteroid needed to control reactional episodes, reducing the incidence of side effects and the morbidity period (Nery et al., 2006).

3.4 Clofazimine

Clofazimine is considered a useful anti-inflammatory when corticosteroids are contraindicated or need to be reduced (ILEP, 1996; Van Veen et al., 2009). Its anti-inflammatory action occurs through inhibition of T lymphocyte proliferation (Cholo et al., 2012). However, this treatment takes 4 to 6 weeks to become effective and the dose of clofazimine required to control ENL is higher than the dose used in MDT (ILEP, 1996; Van Veen et al., 2009). Therapeutic regimens

indicate the use of clofazimine for three months, with a dose of 100mg three times a day in the first month, reducing to 100 mg every month. Another scheme proposes prolonged treatment of up to 15 months, with the initial dose being 300 mg daily for three months, and reducing the dose to 200 mg daily and, later, 100 mg daily in the interval of one to six months (Hansenologia and Dermatologia, 2003).

There is evidence of a reduction in the incidence of ENL with the establishment of MDT/WHO regimen, particularly as a result of the inclusion of clofazimine (Hansenologia and Dermatologia, 2003; Lockwood, 1996). In a study conducted in the Philippines, it was observed that ENL was generally more severe in patients who used MDT for one year than those who used it for two years. It was concluded that an extended period of clofazimine can reduce the severity of ENL in high-risk patients (i.e. those with the most multibacillary form of leprosy, with a high bacillary index) (Balagon et al., 2011). However, its main effect is to prevent new occurrence and reduce the dependence of corticosteroids (Hansenologia and Dermatologia, 2003). Therefore, it does not have effect on acute episodes, but might be effective on chronic ENL.

The adverse effects of clofazimine are mainly gastrointestinal problems and pigmentation of the skin and eyes (Cholo et al., 2012). Gastrointestinal effects include nausea, diarrhea, anorexia, constipation and weight loss, intestinal obstruction, gastrointestinal bleeding (Cholo et al., 2012; Moore, 1983). The skin pigmentation and dryness arise within a few weeks after the start of treatment and may take two years or more to disappear (Lockwood, 1996).

3.5 Pentoxifylline

Another drug used to treat ENL is pentoxifylline, a derivative of methylxanthine with a variety of anti-inflammatory effects (Hassan et al., 2014). It has a potentially important effect on ENL control, blocking the synthesis of TNF-α messenger RNA by inhibition of gene transcription (Sales et al., 2007; Strieter et al., 1988). This drug can also inhibit IL-1β, IL-6 and IL-8 (Neuner et al., 1994). It has a slow action period, reaching its therapeutic efficacy in 30 to 60 days. Pentoxifylline should not be used in patients with risk for a marked reduction in

blood pressure (severe ischemic heart disease, cerebral vasculature stenosis) or in patients with coagulation problems (Pulido Pérez and Suárez Fernández, 2015; Zargari, 2008). The use of pentoxifylline at a dose of 1200 mg per day is divided into three doses and may benefit the vasculitis predominant episodes (Hansenologia and Dermatologia, 2003). A study comparing pentoxifylline with thalidomide showed that the overall response to thalidomide was significantly better than pentoxifylline. However, pentoxifylline can be an alternative treatment for patients with contraindications to thalidomide use (Sales et al., 2007).

3.6 Other drugs

There are reports about the use of azathioprine, methotrexate, zinc, chimeric monoclonal anti-TNF-alpha antibody and infliximab for the treatment of ENL(Kahawita et al., 2008; Kar and Babu, 2004; Mathur et al., 1983; Verma et al., 2006).

Azathioprine is derived from 6-mercaptopurine and interferes with purine base production, DNA and RNA synthesis and cell division. It inhibits some T-cell functions, B-cell proliferation and antibody production. It also inhibits TNF-α and has anti-inflammatory action (Salmaggi et al., 1997; Verma et al., 2006; Younger et al., 1991). Some studies have demonstrated that azathioprine may be a good adjuvant as imunossupressive drug in the treatment for recurrent or chronic ENL. It may help in controlling the reaction, acting as a corticosteroid sparing agent and preventing recurrence of ENL with minimum side effects (Athreya, 2007; Jitendra et al., 2017; Verma et al., 2006). The most common symptomatic side effects of azathioprine are gastrointestinal, ranging from nausea to diarrhea but bone marrow depression may also occur (Patel et al., 2006).

Biological agents TNF-α inhinitors such as infliximab and etanercept, have been successfully used in cases of recurrent ENL in which prednisone, thalidomide and pentoxifylline failed (Chowdhry et al., 2016; Faber et al., 2006; Ramien et al., 2011; Santos et al., 2017). Infliximab (5 mg/kg) was used in a single case. The patient had improvement of symptoms within hours after starting treatment. Infliximab administration was repeated on weeks 2 and 6. After a follow-up of one year, no further episodes of ENL were described (Faber et al., 2006).

Etanercept was used in three cases in a dose of 50 mg/week during many weeks. In all cases there was improvement of symptoms after a few weeks and the use of Thalidomide and prednisone was interrupted or reduced (Chowdhry et al., 2016; Ramien et al., 2011; Santos et al., 2017). These results show that biological agents can be useful in patients with ENL, when it is difficult to control the condition, and are capable of reducing the need for thalidomide and prednisone, thus sparing patients of the side effects from both drugs (Santos et al., 2017). However, they must be used with great care because the immunosuppression increases the risk of *M. tuberculosis* and fungal infections (Faber et al., 2006). Moreover, they are expensive and their long term complications have not been assessed (Hossain, 2013). The use of etanercept is less costly, more convenient to the patient (comes in self-administered injections), and causes less immunosuppression in relation to infliximab (Chowdhry et al., 2016).

Methotrexate has been successfully used in patients of resistant ENL. It has potential as a steroid sparing agent (Hossain, 2013) and could provide a boosting effect to the action of thalidomide (Nagar et al., 2015). Thus, its combination with prednisolone or thalidomide could be additive and useful (Hossain, 2013; Nagar et al., 2015).

In a small pilot study in Ethiopia, cyclosporine showed promising results in the management of acute ENL, but did not appear to have a significant effect on the decrease in the use of corticosteroids in patients with chronic ENL. More studies are needed to determine if such results can be reproduced on a larger scale (Lambert et al., 2016).

Table 4. Characteristics of the main treatments available for ENL

Drug	Mechanism of action	Regimen of treatment	Most Frequent Adverse Effects
Prednisone	Inhibition of leukocyte recruitment and activity, activation of neutrophils and macrophages, and reduction in the production of TNF-α and IL-2, IL-4, IL-6 and IL-8	1 to 1.5 mg/kg/day for 15 to 30 days Reduction of 10 mg every 15 days up to the dose of 20 mg. Then decrease of 5 mg every 15 days.	Dependence, induced Cushing, acne, diabetes, osteoporosis, gastritis, cataract and immunosuppression, which can lead to the risk of opportunistic infections including fungal infections and tuberculosis, among others.
Thalidomide	Inhibition of the production of TNF-α and IL-6, IL-8, IL-10 and C-reactive protein.	100-400 mg/day Reduction of 50 mg every 2 to 4 weeks	Teratogenicity, peripheral neuropathy, thrombosis, drowsiness, dizziness, constipation, rash, edema, neutropenia, bradycardia, dryness, pruritus, headache, hypotension, increased appetite, mood changes, male sexual dysfunction, nausea, tachycardia, weight gain.
Pentoxifylline	Inhibition of IL-1, IL-6, IL-8 and TNF-α synthesis.	1200 mg/day No reduction	Dry mouth, constipation, anorexia, cholecystitis, septic meningitis, seizures, confusion, depression, anxiety, hypotension, edema, dyspnea, nasal congestion, nosebleed, breathing difficulty, rash,

			angioedema, urticaria, pruritus, brittle nails earache, scotoma, conjunctivitis, blurred vision, excessive salivation, malaise, leukopenia, bad taste, weight gain or loss, sore throat, nausea, headache.
Clofazimine	Inhibition of proliferation of T lymphocytes.	3-month schedule: 100 mg 3X daily Reduction of 100 mg every month 15-month schedule: Starting dose 300 mg x day for 3 months Reduction of 100 mg over a period from one to six months.	Skin pigmentation and gastrointestinal effects: abdominal pain, nausea, diarrhea, vomiting, gastrointestinal intolerance, bowel obstruction.

4. CHALLENGES

ENL reactions are complex medical emergencies that require careful management by experienced physicians (World Health Organization, 2006). They are usually recurrent or chronic and itis not possible to predict the clinical pattern of the disease that the individual will manifest, generating a problem for deciding which therapy should be instituted at the onset of the disease (Walker et al., 2007).

ENL has both physical and economic impact on the patient. Physical impact is associated with repeated episodes that impair the functions of various organs, causing deformities and disabilities. In addition, prolonged use of medications can cause many adverse effects, including dependence on corticosteroids and peripheral neuropathy due to thalidomide (Chandler et al., 2015). The economic impact refers to the ENL affecting young, economically productive young people who have restricted their ability to work and provide for their families, causing financial difficulties (Pocaterra et al., 2006; Walker et al., 2014). It is also recognized that ENL is a potentially morbid condition, having few works related to leprosy reactions and the risk of death though (Walker et al., 2014).

ENL is a major health problem in countries where leprosy is endemic. Nevertheless, there are few quality controlled studies on ENL treatment. In 2009, in a Cochrane review, only 13 randomized trial-type studies were found on treatment for ENL and most of the studies had been performed more than 20 years ago (Van Veen et al., 2009). However, there have been no further studies on ENL treatment since then, except for a recent pilot study in Ethiopia comparing the use of cyclosporine with prednisone (Lambert et al., 2016) and another in India, evaluating the use of thalidomide in patients who were already in use of corticosteroids (Darlong et al., 2016). Therefore, there is still a lack of data available for the baseline of the treatment and recommendation of new well-designed intervention studies (Walker et al., 2015).

Another challenge is a possible modification in multidrug therapy (MDT). It has already been shown that there is an increase in the incidence and severity of ENL in patients with shorter MDT regimens - when one year is compared to two year regimen (Balagon et al., 2011, 2010; Voorend and Post, 2013). In 2002, the WHO proposed a pilot study of a uniform 6-month MDT regimen (Shen et al.,

2012; World Health Organization, 2002). However, this possibility of time reduction of MDT may cause more MB patients to develop ENL, probably due to a higher bacillary load at discharge and a shorter time to treatment with clofazimine. This may mean more patients presenting reactions and manifesting more severe symptoms, more prolonged and with more recurrence.

An alarming fact is that although the symptoms of ENL are severe enough to force the patient to seek for treatment, the condition may not be recognized by physicians, becoming increasingly "rare". Patients may not report a history of leprosy, since the disease can occur after the end of MDT, or they may be unaware of it, since the diagnosis of leprosy can occur with the onset of leprosy reactions. Thus, diagnosis and appropriate treatment may be delayed (Saunderson et al., 2000).

In 2012 the International Study Group on Erythema Nodosum Leprosum (ENLIST) was created with the participation of reference centers for leprosy treatment in several countries (Walker et al., 2012). Their first results showed that ENL treatment varies considerably from one center to another (Walker et al., 2015). In Brazil, thalidomide is the first line of treatment, but this does not occur in any other country, where there is the use of prolonged oral corticosteroid regimens (Walker and Lockwood, 2015). However, even in Brazil, although there are specific recommendations from the Ministry of Health, it is difficult to follow a standardization of the use of thalidomide. The Health Care Operational Standards determine the decentralization of leprosy control actions with basic care by participating in the active case search, registration of patients, supervised treatment and preventive measures. On the other hand, the actions of higher complexity, such as cases of difficult diagnosis and treatment of leprosy reactions, should be performed in the reference center (Ministério da Saúde, 2002, 2001). Nevertheless, this process of decentralization has not been occurring adequately in all Brazilian states, with an increase of patients in referral centers (Pires et al., 2011). Thus, in endemic, mainly rural, northern and northeastern areas of the country, access to treatment of leprosy reactions is not easy. The distance from the centers brings difficulty in monitoring the patients after receiving the medication.

The use of thalidomide in Brazil is an extremely important issue. Brazil is the only endemic country for leprosy where the drug is available with the highest distribution provided by a public health service in the world and is a country that continues to report cases of thalidomide embryopathy in newborns, with many cases reported in recent decades (Penna et al., 2005; Schuler-Faccini et al., 2007; Vianna et al., 2013). Therefore, it is essential to guarantee strict measures to control the distribution and dispensation of the drug together with educational campaigns aiming health professionals and the population.

5. CONCLUSION

ENL is an important public health problem in leprosy endemic countries with significant physical, economic and social impact on patients and the health system. All available treatments have caveats in their use: corticosteroids present serious adverse effects and risk of dependence; pentoxifylline and clofazimine take time to control the reaction; and thalidomide presents the risk of peripheral neuropathy and teratogenicity. In addition, somehow, they fail to effectively control the reaction, since most patients present ENL recurrently or chronically. Thus, further controlled studies on new forms of treatment for ENL are needed. In Brazil, where thalidomide is the treatment of choice, it is important to standardize care and monitoring treatment even in rural areas in order to guarantee patient improvement and avoid new episodes of teratogenicity. Therefore, the treatment should eliminate the symptoms of ENL and minimize the manifestation of adverse effects that can also be very dangerous to the patient.

Conflict of interest statement

The authors declare no conflict of interest.

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Capítulo V – Artigo 2

Evaluation of Polymorphisms and haplotypes in *TNF*, *CYP2C19*, *NR3C1* and *ABCB1* genes with the response to the treatment of Erythema Nodosum Leprosum.

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Evaluation of Polymorphisms and haplotypes in *TNF*, *CYP2C19*, *NR3C1* and *ABCB1* genes with the response to the treatment of Erythema Nodosum Leprosum.

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ABSTRACT

Erythema nodosum leprosum (ENL) is a systemic inflammatory reaction that affects about 30% of the patients with multibacillary leprosy. It is characterized by the involvement of several organs, being a very important cause of incapacities resulting from leprosy. It is a difficult-to-control reaction whose treatment in Brazil mainly involves prednisone and thalidomide. Both are effective drugs in ENL control, although the prolonged treatment might lead to significant health risks, such as corticosteroid tolerance and dependence, and teratogenicity and neurotoxicity related to thalidomide. In this study we conducted a pharmacogenetic evaluation to identify genetic profiles more susceptible to adverse effects and the differences in the doses of prednisone and thalidomide in ENL treatment. The genes evaluated in this study were: *NR3C1* and *ABCB1*, which are related to the treatment with prednisone, and the *TNF* and *CYP2C19* genes, to analyze the treatment with thalidomide. The sample consisted of 152 patients from different regions of Brazil who used thalidomide and/or prednisone at some time during treatment. The following single base polymorphisms (SNPs) were evaluated in this sample: *NR3C1* (rs6189, rs6190, rs6195, rs41423247, rs6198), *ABCB1* (rs1045642), *TNF* (rs361525, rs1800629, rs1799724, rs1800630, rs1799964), *CYP2C19* - *CYP2C19*2* (rs4244285), *CYP2C19*3* (rs4986893) and *CYP2C19*4* (rs28399504). The generalized estimating equation (GEE) method was used to evaluate the influence of polymorphisms and haplotypes on the drug dose variation throughout treatment. An association between the genotype TT of polymorphism *ABCB1* 3435C>T (rs1045642) (p=0.02) and prednisone dose was found in the recessive model. An association between the haplotypes 1031T/-863C/-857C/-308A/-238G (p=0.006) and 1031T/-863C/-857T/-308A/-238G (p=0.040) of the *TNF* gene and the *CYP2C19*2* polymorphism was also identified, regarding thalidomide dosage variation over the course of treatment. Although the dosage variation determined was discreet, these genes should be better studied to assess their effects and to evaluate them as markers of ENL severity and response to treatment.

Key words: Pharmacogenomics, prednisone, thalidomide, leprosy

INTRODUCTION

Leprosy is a chronic infectious disease that affects the skin and peripheral nerves caused by the intracellular microorganism *Mycobacterium leprae*. Its clinical manifestations depend on the interaction between the pathogen and the host immune response; they are distributed in a spectrum that goes from the tuberculoid (TT) pole, with a more limited scenario of the disease and few bacilli, to the lepromatous pole (LL), with higher number of lesions and greater bacillary load. Between these poles are the unstable forms of disease: indeterminate (I), borderline-tuberculoid (BT), borderline-borderline (BB) and borderline-lepromatous (BL) (Ridley and Jopling 1966). The disease can be worsened by reactional episodes, characterized by immunologically mediated inflammatory manifestations, which are considered the main causes of disability and deformities associated with leprosy. These reactions may be type I or reverse reaction (RR) or type II, the most common manifestation of which is erythema nodosum leprosum (ENL) (Fava et al. 2012; Sousa et al. 2012). Leprosy is a controlled disease in most developed countries, but it is still a serious public health problem in endemic countries such as Brazil (Ministério da Saúde. 2016).

ENL is a serious immunological complication that affects BV and VV leprosy patients (Pocaterra et al. 2006; Kahawita et al. 2008). Clinically, it is characterized by the sudden onset of painful erythematous nodules in the skin with fever, general malaise and systemic inflammation, usually occurring in a recurrent or chronic manner (Pocaterra et al. 2006). Its immunopathological mechanism is not yet fully understood, but high levels of the proinflammatory cytokine tumor necrosis factor-alpha (TNF-α) are associated with the pathogenesis of the reaction (Sarno et al. 1991; Oliveira et al. 2016).

The treatment of ENL is difficult, since the reaction is characterized by recurrent episodes or chronicity, being a challenge even for the most experienced physicians. In Brazil, prednisone and thalidomide are the drugs most used to treat this condition (Balagon et al. 2011; Negera et al. 2018). Prednisone usually works quickly controlling inflammation and relieving pain. However, its prolonged use is associated with adverse effects such as diabetes, hypertension, dependence, Cushing syndrome, acne, cataracts, gastritis, osteoporosis and immunosuppression, which may lead to fungal infections and tuberculosis

(Sugumaran 1998; Van Veen et al. 2009). Thalidomide also produces a rapid improvement of the symptoms (Feuth et al. 2008) and its concomitant use with prednisone causes a significant reduction in corticosteroid dosage and period of use (Hansenologia and Dermatologia 2003). Its efficacy in ENL is associated with the inhibition of TNF-α production (Moreira et al. 1993; Penna et al. 2005). However, there are serious problems associated with its use, which include teratogenicity and neurotoxicity (Lockwood 1996; Wu et al. 2005; Sharma and Kwatra 2016).

Pharmacogenetic studies with prednisone and thalidomide have been performed under other clinical conditions to identify genetic profiles that are associated with adverse effects and differences in response to treatment. *NR3C1* and *ABCB1* genes are among the main targets for research with prednisone. *NR3C1* encodes the glucocorticoid receptor, which is responsible for the effect of glucocorticoids on body cells (Koper et al. 2014; Majer-Łobodzińska and Adamiec-Mroczek 2017). The *ABCB1* gene encodes P-glycoprotein, which has glucocorticoids as substrates and is associated with drug resistance (Ambudkar et al. 2003; Marino et al. 2009). Studies evaluating the response to thalidomide treatment target especially the *TNF* and *CYP2C19* genes. *TNF* encodes TNF-α, which is important in the inflammatory process of ENL and hhose expression is decreased by the action of thalidomide (Neben et al. 2002; Du et al. 2010; Basmaci et al. 2016). *CYP2C19* is responsible for the hydroxylation of thalidomide metabolites in humans (Ando et al. 2002; Li et al. 2012). In leprosy endemic countries, such as Brazil, ENL has a major physical, psychological and social impact, affecting both patients and public health services (Costa et al. 2018). Moreover, the treatment is difficult, as few drugs are effective. Therefore, different approaches should be undertaken to improve the treatment of this condition, one of which is the assessment of the individual's genetic profile and the response to treatment with such drugs. Thus, this study evaluated polymorphisms in genes of prednisone and thalidomide metabolism, approaching their effects on the doses used and the manifestation of adverse effects in the treatment of ENL.

MATERIAL AND METHODS

Sample

The sample consisted of 152 individuals and the selection of patients took place in Porto Alegre (RS) in South Brazil, in Fortaleza (CE), Imperatriz and São Luís (MA) in Northeast Brazil, and in Porto Velho (RO) in North Brazil. Patients with ENL using thalidomide with or without prednisone at any dose were included as long as at least six visits were possible. All participants were duly informed about the purpose of the research and signed an informed consent form. This research was approved by the Ethics Committee of the Hospital de Clínicas of Porto Alegre under No.10-04410 and CAAE 21184413.0.0000.5327

Clinical and Demographic Data

Patients were evaluated for up to six visits (equivalent to about one consultation per month). The clinical and demographic data of the patients were obtained through information contained in the medical records; data were collected regarding sex, age, geographic origin, history of leprosy (time of diagnosis and the treatment used) and history of ENL (diagnosis, treatment, adverse effects, history of relapse and drug dosage).

Genetic Analyses

Saliva was used as biological material to DNA assessment with an Oragene® kit; DNA extraction was performed according to the manufacturer's instructions. Single base polymorphisms (SNPs) in *NR3C1*, *ABCB1*, *TNF* and *CYP2C19* genes were evaluated. The SNPs were selected due to their involvement in the metabolism of the evaluated drugs, pharmacogenetic studies with other diseases, and their involvement in the ENL.

Genotypic determination was performed through allelic discrimination, using the Custom TaqMan Genotyping Assay (Applied Biosystems, USA). The following polymorphisms were analyzed: *NR3C1* (rs6189, rs6195, rs41423247, rs6198), *ABCB1* (rs1045642), *TNF-alfa* (rs361525, rs1800629, rs1799724, rs1800630, rs1799964), *CYP2C19*2* (rs4244285), *CYP2C19*3* (rs4986893) and*CYP2C19*4* (rs28399504). The assay number for each polymorphism is described in Supplementary Table 1.

Statistical Analyses

The Hardy-Weinberg equilibrium was assessed for all polymorphisms by the chi-square test. The analysis of the effect of the variants on the dose of the medications during the studied period was carried out using the generalized estimating equations method (GEE). GEE is an analysis of repeated measures focused on average variation in response over time and on the impact of covariates on these changes. This method models the mean response as a linear function of covariates of interest through a transformation or binding function. It can be used in studies in which data is asymmetric or data distribution is difficult to verify due to a small sample size (Liang and Zeger 1986; Sortica et al. 2016). GEE was performed to evaluate the genetic influence on dose variation over time: (1) evaluating the *NR3C1* haplotypes and *ABCB1* polymorphism with prednisone dose; (2) evaluating the *TNF* haplotypes and *CYP2C19* polymorphism and treatment with thalidomide. In both models used in this study the following criteria were inserted as covariables: the origin of the patient, the concomitant use of multidrug therapy (MDT) for leprosy, the use of other medications and other treatments for ENL.

A criterion of gene expression was also applied for the clustering of *TNF* haplotypes. The number of alleles associated with higher expression of this gene was used to create these categories. According to previously published data, alleles associated with high *TNF* expression were -1031C (rs1799964), -863A (rs1800630), -857T (rs1799724), -308A (rs1800629) and -238A (rs361525). Therefore, low expression alleles were -1031T (rs1799964), -863C (rs1800630), -857C (rs1799724), -308G (rs1800629) and -238GA (rs361525), respectively (Wilson et al. 1997; Kamizono et al. 1998; Lindenau et al. 2017). *TNF* haplotypes with alleles associated with low gene expression were grouped as the "low-expression allele group," while haplotypes with alleles associated with high *TNF*-α expression were grouped as "1 or 2 high-expression alleles" and "3 or 4 high-expression alleles" according to the number of high-expression alleles included in the haplotype. GEE was also performed to evaluate the genetic influence on dosage variation over time evaluating the *TNF* expression allele groups and thalidomide.

The contribution of the genetic variants in the occurrence of adverse effects

caused by prednisone and thalidomide treatment was evaluated through Fisher's exact test. All tests were performed using SPSS version 18 (SPSS, www.spss.com, IIBM, USA).

The linkage disequilibrium (LD) for SNPs contained in the same gene was calculated using the Haploview 4.2 program (Barrett et al. 2005), and the haplotypes were inferred through the Bayesian algorithm implemented in the Phase 2.1.1 program (Stephens et al. 2001; Stephens and Donnelly 2003).

RESULTS

The clinical and demographic characteristics of the 152 study participants are presented in Table 1. The sample was divided into groups according to the treatment regimen used: Thalidomide (T) (n = 15), Thalidomide + Prednisone (T+P) (n=134) and Prednisone (n = 3). Once three subjects used only prednisone in the treatment, they were withdrawn from subsequent analyses. Most of the individuals were male (n = 115; 75.6%) and had lepromatous leprosy (n = 99; 66.4%). Sixty-six (44.3%) patients were on MDT for leprosy, and 91 (61%) used other medications during ENL treatment. The mean follow-up time was 189.49 days. The most frequent adverse effects of the treatment were neurological (n = 45, 29.6%), including neuritis, tremors, headaches and dizziness, followed by gastrointestinal (n = 34, 23.5%), which included diarrhea, abdominal pain, nausea, constipation and vomiting.

The ER22/23EK (rs6189) polymorphism in the *NR3C1* gene and the *CYP2C19*3* (rs4986893) and *CYP2C19*4* (rs28399504) polymorphisms were monomorphic in our sample and therefore excluded from further analysis. All variants analyzed were in Hardy-Weinberg equilibrium.

LD was identified between the *NR3C1* polymorphisms and for some polymorphisms in *TNF* gene: between -1031 T>C (rsrs1799964) and -863 C>A (rs1800630) and between -1031 T>C (rsrs1799964) and -857 C>T (rs1799724) (Supplementary Table 2). Five haplotypes for *NR3C1* and eight for the *TNF* (Table 3) were inferred. The frequency of TNF expression groups is also shown in Table 3. Supplementary Table 3 gives the complete description of the *TNF* haplotypes with alleles of high gene expression.

The analysis of the influence of the genetic variants on prednisone dose was done with the haplotypes of the *NR3C1* gene and the *ABCB1* 3435C>T (rs1045642) polymorphism. No association was identified between any haplotype of *NR3C1* and prednisone dosage over the course of treatment (Table 4). The *ABCB1* 3435C>T (rs1045642) polymorphism was associated with prednisone dose and its reduction over time (p=0.037), in the recessive model (Table 5). There was no association of any *NR3C1* haplotype or of the *ABCB1* 3435C>T (rs1045642) polymorphism with the manifestation of adverse effects with the use of prednisone.

Analysis of the genetic influence on thalidomide dosage was done with the *TNF* haplotypes, the groups of *TNF* expression alleles and the *CYP2C19*2* (rs4244285) polymorphism. When evaluating the influence of *TNF* haplotypes on thalidomide dosage, we identified an association of haplotypes -1031T/-863C/-857C/-308A/-238G (p=0.006) and 1031T/-863C/-857T/-308G/-238G (p=0.040) (Table 6). However, there was no association of the *TNF* expression allele groups with thalidomide dose (Table 7). Analysis of the influence of *CYP2C19*2* (rs4244285) showed association with thalidomide dose (p=0.002) in the recessive model (Table 7). There was no association of any *TNF* haplotype and *CYP2C19*2* (rs4244285) polymorphism with the manifestation of adverse effects occurred by thalidomide use.

DISCUSSION

ENL is a disabling condition with profound impact on the patients' lives. Currently, the guidelines available from the Brazilian National Ministry of Health for the treatment of ENL recommend the use of prednisone and thalidomide (Brasil 2017), however, there is no systematized protocol for the best therapeutic dose, neither determinations about how long it should be used. Furthermore, there is no description on the management of drug withdrawal. The adverse effects caused by thalidomide, especially teratogenesis and peripheral neuropathy, and prednisone justify the need for pharmacogenetic approaches that improve the prediction of the best treatment, not only to seek an improvement in life quality and in the prognosis of the patients, but also to identify which individuals will benefit from the use of these drugs.

This research aimed to identify genetic variants that may influence in the doses of prednisone and thalidomide used for the treatment of ENL, and to determine what are the more susceptible genetic profiles to adverse events as well. Regarding the use of prednisone, no association was found between the haplotypes of the *NR3C1* gene. However, an association of the 3435C>T polymorphism in *ABCB1* gene was found. It is important to note that there was no analysis of patients using prednisone only. Regarding the use of thalidomide, there was an association between *TNF* haplotypes and *CYP2C19*2* polymorphism and thalidomide dose.

NR3C1 encodes the glucocorticoid (receptor GR) and variants in this gene are potentially involved in the efficacy of glucocorticoid treatment. The N363S (rs56149945) and BclI (rs41423247) polymorphisms have already been associated with greater sensitivity to glucocorticoids, while the GR9β (rs6198) and ER22/23EK (rs6189) polymorphisms are associated with increased glucocorticoid resistance (Kaymak Cihan et al. 2017; Majer-Łobodzińska and Adamiec-Mroczek 2017; Herrera et al. 2018). However, the analysis of the haplotypes of the *NR3C1* gene showed no association with prednisone dose. This result is similar to what has been shown in previous studies in which no significant results were obtained (Manenschijn et al. 2009; Mwinyi et al. 2010).

In the case of the 3435C>T (rs1045642) polymorphism in the *ABCB1* gene, an association was found with prednisone dose in the recessive model. Individuals with the TT genotype of this polymorphism had an initial dosage of 6,69mg higher when compared to C allele carriers. At the same time, the TT genotype interaction was verified over time, indicating a greater dose reduction compared to the other C allele group. Prednisone is a substrate of P-glycoprotein (PgP), a transmembane transport protein encoded by the *ABCB1* gene, which functions as an efflux pump that protects cells from exogenous toxic molecules, moving them from the intracellular to the extracellular environment, including glucocorticoids (Bouatou et al. 2018). Polymorphisms in this gene may interfere with the Pg-P expression and function levels, directly affecting plasma levels and intracellular drug concentrations, thus affecting therapeutic response. (Ueda et al. 1992; Kroetz et al. 2003; Marino et al. 2009; Krupoves et al. 2011; Teeninga et al. 2016). According to Kimchi-Sarfatyet al., SNP 3435C>T alters the folding of the Pg-P

protein, changing substrate specificity. Other studies have shown that the transition from C to T in this polymorphism is associated with decreased intestinal expression of the molecule (Hoffmeyer et al. 2000; Ambudkar et al. 2003). This would lead to increased sensitivity to drugs that are substrates of Pgp, and could explain the decrease in prednisone dose. A recent study in the Brazilian population in treatment with warfarin demonstrated the need for lower doses of this drug in patients with the T allele (Tavares et al. 2018). However, the association of this polymorphism with drug dose reduction is still controversial, as it was not found in some studies (Cascorbi et al. 2001; Ameyaw et al. 2001; Bouatou et al. 2018).

In the analysis of the genetic variants and association with thalidomide dose, an association was found regarding the haplotypes of the *TNF* gene. ENL shows high levels of TNF-α, and thalidomide therapeutic effect in the control of the reaction is attributed to its capacity to inhibit TNF-α production (Moreira et al. 1993). Polymorphisms in the promoter region of *TNF* may affect the interaction of transcription factors with their binding sites, modulating the secretion of the cytokine (Wilson et al. 1997; Qidwai and Khan 2011; Gutiérrez-Hurtado et al. 2016). Patients with 1031T/-863C/-857C/-308A/-238G and 1031T/-863C/-857T/-308G/-238G haplotypes showed a mean initial dose of 29,900 and 17,578 mg, respectively. Patients with these haplotypes are those with the A allele in the-308 G>A polymorphism and T-allele in the -857 C>T polymorphism, which are polymorphisms associated with higher levels of TNF-α. However, clustering analysis of alleles according to TNF expression did not show any difference between the groups regarding thalidomide dose.

In *in vitro* studies, the -308 G>A polymorphism was associated with greater activation potential of *TNF* transcription than the common allele. Higher TNF-α levels are found in the carriers of the rare A allele of this polymorphism and in the *TNF* haplotypes containing the A allele (Bouma et al. 1996; Kroeger et al. 1997; Wilson et al. 1997; Cardoso et al. 2011; Youssef et al. 2018). A protective effect against leprosy and higher levels of TNF-α have also been associated with the A allele, especially in a Brazilian population (Cardoso et al. 2011). However, there are contrasting results whereas some studies do not show any association of this polymorphism with higher TNF-α levels and protective effect against leprosy, as it

occurs with the Asian populations (Oliveira et al. 2016; Areeshi et al. 2017). The -857 C>T polymorphism has also been associated with increased *TNF* expression. It has already been shown that the common C allele has less transcriptional activity, with the presence of the T allele leading to a change in the transcriptional function of the gene and increasing its expression. However, some studies have failed to show this association (van Heel 2002; Heidari et al. 2016). These conflicting results in relation to *TNF* polymorphisms and TNF-α levels may be related to the ethnic differences existing between the populations studied and the haplotypes with other genes of the HLA complex that may influence cytokine levels (Bouma et al. 1996; Santos et al. 2000; Cardoso et al. 2011; Hossein Ghaderian et al. 2011; Mekinian et al. 2011; Oliveira et al. 2016; Areeshi et al. 2017). Higher cytokine levels in carriers of these haplotypes explain the higher doses of thalidomide at the beginning of treatment.

Regarding the evaluation of the *CYP2C19*2* polymorphism, the enzyme CYP2C19 catalyzes thalidomide biotransformation in humans, resulting in the formation of the 5-OH and 5'-OH metabolites. Polymorphismos in these gene can segregate patients into subgroups that differ in their metabolic activities so that poor metabolizers may require higher therapeutic doses of thalidomide (Ando et al. 2002). In this study, the evaluation of the influence of the *CYP2C19*2* variant on thalidomide dosage showed a significant association with higher initial doses. In the dominant model, carriers of the A allele had an initial thalidomide average dose 26,480 mg higher than for those with the GG genotype (p=0.01). In the recessive model, the dose difference was 38 mg and patients with the AA genotype had a greater dose reduction over the course of treatment (p=0.002).

Although the polymorphisms that showed association in this study were related to a bigger dose variation, no remission of the condition was observed. The patients did not show resolution of the reaction after the average follow-up period observed here (189 days), needing continued treatment. This confirms previous studies showing a large proportion of patients presenting the reaction recurrently or chronically, hence requiring prolonged periods of treatment. Studies in other countries have shown variations in episodes recurrence: India with 92% of patients having multiple episodes in a period of 14 months, Nepal with duration of ENL

ranging from 1 to 62 months, and England with a mean duration of ENL of 60 months (Pocaterra et al. 2006; Feuth et al. 2008; Nabarro et al. 2016).

Allied with this scenario of a long-term treatment with multiple drugs with serious adverse events is the fact that there is still no systematic recommendation for thalidomide use in ENL in Brazil, which is still a challenge to be achieved in the country (Costa et al. 2018). In our study, there was a predominance of adverse effects among patients taking concomitant prednisone, corroborating what has already been found in other studies and showing the risk of prolonged use of this drug (Kaur et al. 2009). However, there was no association between the polymorphisms and haplotypes studied and the manifestation of adverse effects, similar to what has been demonstrated in other studies (Marino et al. 2009; Vangsted et al. 2010).

Epidemiological data from this study corroborated findings from other studies (Kumar et al. 2004; Feuth et al. 2008; Walker et al. 2015). There was a greater number of affected men, confirming that males might be associated with a higher incidence of the lepromatous form (Guerra et al. 2004). However, some studies did not show the male gender as a risk factor (Guerra et al. 2002; Voorend and Post 2013). Most individuals with ENL had lepromatous leprosy, indicating the bacillary index as a risk factor for the development of the reaction as demonstrated in other studies (Guerra et al. 2004; Pocaterra et al. 2006; Sales et al. 2007; Chandler et al. 2015; Walker et al. 2015).

This study had some limitations that need to be taken into account. First, the Brazilian population is very admixed, and this study was carried out with individuals from different regions of the country, who have a diverse genetic background. This was taken into account in the analyses. In addition, a considerable number of patients were on MDT for leprosy. The use of clofazimine, which has anti-inflammatory action in MDT, may contribute to a reduction in the drug dosage. Another limitation was our small sample size which might have impeded the identification of small-effect alleles previously described for the polymorphisms studied. However, to our knowledge this is the first study that sought to evaluate genetic variants that may influence response to ENL treatment. Although the sample size was limited for the analysis of genetic variants, few

studies present this number of samples for patients with ENL, emphasizing the neglected character of this disease.

In conclusion, polymorphisms in the *ABCB1*, *TNF* and *CYP2C19* genes may influence doses of prednisone and thalidomide, respectively, in the treatment of ENL. Although these results are preliminary and still lack clinical significance for improvements in patient management, they demonstrate pharmacogenetic studies can identify genetic variants that influence the treatment response and the genetic profiles that best respond to the treatment with prednisone and thalidomide. This opens new perspectives for improving the management of ENL aiming to provide a better quality of life for the patient.

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Table 1: Clinical and Demographic Characteristics of ENL Patients

Characteristic	All (n=152)	T[A] (n=15; 9.8%)	T +P[B] (n=134; 85.9%)	P[C] (n=3; 1.9%)
Male [n (%)]	115 (75.6)	14 (93.3)	101 (75.2)	1 (0,65)
Multidrug therapy for leprosy[n (%)]	66 (44.3)	9(60)	57 (42.5)	3 (100,0)
Other medications [n (%)]	91 (61)	2 (13,3)	88 (66.2)	2 (66,6)
Days of consultation [Mean (min/max)]	189.49(0/1463)			
Prednisone dose[Median (min/max)]	20 (0/80)	-	20 (0/80)	20 (28/80)
thalidomide dose[Median (min/max)]	100 (0/400)	100 (0/400)	118.5(0/400)	-
South[n (%)]	42(29.6)	8(53.3)	34(25.3)	-
Northeast[n (%)]	95(62.5)	5(33.3)	88(65.6)	2(66.6)
North[n (%)]	15(9.8)	2(13.3)	12(9)	1(33.3)
Leprosy:				
Lepromatous[n (%)]	99 (66,4)	8 (53.4)	88 (65.6)	3 (100,0)
Boderline lepromatous [n (%)]	32(21,4)	3(20)	29 (21.6)	0 (0)
Indeterminate [n (%)]	2 (1.3)	-	2(1.49)	0 (0)
Adverse effects:				
Neurological[n (%)]	45 (29.6)	3 (20.0)	42(31.3)	-
Gastrointestinal[n (%)]	34(22.3)	3 (20.0)	31 (23.1)	-
Musculoskeletal[n (%)]	27 (17.7)	1 (6.7)	27 (20.1)	-
Ocular[n (%)]	23 (15.1)	0(0)	23(17.1)	-
Edema[n (%)]	17 (11.1)	1(6.6)	16 (11.9)	-
Dermatological[n (%)]	16 (10.5)	5(33.3)	11 (8.2)	-
Immunological[n (%)]	7 (4.6)	1 (6.6)	6 (4.4)	-
Respiratory[n (%)]	2 (0.8)	0 (0)	2 (1.4)	-

TA: Treatment with thalidomide; T+PB: Patient on treatment with thalidomide and prednisone; PC: treatment with prednisone only.

Table 2: Genotypic and allelic frequencies of*NR3C1, ABCB1, TNF-α* and *CYP2C19* polymorphisms in ENL patients on treatment with thalidomide.

Gene/Polymorphisms	Alleles/Genotypes	Frequency
		N (%)
***NR3C*1 N363S (rs56149945)**	AA	146 (98)
	AG	3 (2)
	GG	0 (0)
	A	295 (98.9)
	G	3 (1.1)
***NR3C1*BclI (rs41423247)**	GG	78 (52.3)
	CG	65 (4..6)
	CC	6 (4)
	G	219 (74)
	C	77 (26)
***NR3C1*GR9β (rs6198)**	TT	106 (72.1)
	TC	37 (25.2)
	CC	4 (2.7)
	T	249 (83.6)
	C	43 (14.4)
***ABCB1*3435C>T (rs1045642)**	CC	53 (35.6)
	CT	69 (47.3)
	TT	27 (18.1)
	C	175 (58.7)
	T	123 (41.3)
***TNF* - 1031 T>C (rsrs1799964)**	TT	85 (57.4)
	CT	56 (37.8)
	CC	7 (4.7)
	T	226 (76.4)
	C	70 (23.6)
***TNF*– 863 C>A (rs180063)**	CC	90 (60.8)
	AC	50 (33.8)
	AA	8 (5.4)
	C	230 (77.7)
	A	66 (22.3)
***TNF*- 857C>T (rs1799724)**	CC	114 (76.5)
	TC	33 (22.1)
	TT	2 (1.3)
	C	261 (87.6)
	T	37 (12.4)
***TNF*-308 G>A (rs1800629)**	GG	126 (85.1)
	AG	21 (14.2)
	AA	1 (0.7)
	G	275 (92.3)
	A	23 (7.7)
***TNF* -238 G>A (rs361525)**	GG	140 (94)
	AG	9 (6)
	AA	0 (0)
	G	289 (97)
	A	9 (3)

CYP2C19*2 (rs4244285)	GG	106 (76.3)
	AG	30 (21.6)
	AA	3 (2.2)
	G	252 (87.5)
	A	36 (12.5)

Table 3: Frequencies of the haplotypes of the *NR3C1* and *TNF* genes and groups of *TNF* alleles[3] in ENL patients

Gene	Haplotype	N (%)
NR3C1[1]	ACT	76 (25.5)
	ACC	1 (0.3)
	AGT	175 (58.7)
	AGC	43 (14.4)
	GGT	3 (1)
TNF[2]	TCCAG	23 (7.7)
	TACGG	1 (0.3)
	CACGG	61 (20.5)
	TCCGG	166 (55.7)
	CCCGG	2 (0.7)
	TATGG	4 (1.3)
	TCTGG	33 (11.1)
	CCCGA	8 (2.7)
TNF Groups	No HE alleles	42 (28.2)
	1-2 HE alleles	88 (59.1)
	3-4 HE alleles	19 (12.8)

1.Haplotypes in the following order: rN363S/BclI/GR9β
2.Haplotypes in the following order: -1031/-863/-857/-308/-238.
3. HE = alleles associated with high expression of *TNF*

Table 4: Analysis of interaction between haplotypes of *NR3C1* and time related to estimated prednisone dose in the dominant model using the generalized estimating equation model (GEE)[1]

HAPLOTYPE[2]	**B**	**SE**	***P*-value**
GGT	-0.392	0.238	0.100
AGC	0.217	0.232	0.350
AGT	0.017	0.111	0.882
ACC	0.073	0.228	0.747
ACT	R[3]		
Time	-0.015	0.005	0.003
GGT*Time	0.003	0.002	0.253
AGC*Time	0.004	0.004	0.273
AGT*Time	0.003	0.002	0.264
ACC*Time	0.004	0.004	0.300
ACT*Time	R[3]		

Dependent variable: thalidomide dose.
1. Model: Origin, MDT, Other medications, Prednisone dose, Haplotype, Time, Haplotype*Time
2. Haplotypes in the following order: N363S/BCR/I/GR9B
3. Reference

Table 5 : Analysis of interaction between *ABCB1*3435C>T (rs1045642) genotype and time related to estimated prednisone dose in recessive and dominant genetic model using the generalized estimating equation model (GEE)* in erythema nodosum leprosum treatment

POLYMORPHISM	**MODEL**	**INTERACTION**	**B**	**SE**	**P-value**
3435 C>T (rs1045642)	Dominant	CC carrier	-3.485	2.92	0.233
		Time	-0.011	0.046	0.013
		CCcarrier *Time	0.001	0.009	0.935
	Recessive	TT carrier	**6.693**	3.22	**0.038**
		Time	0.032	0.01	0.001
		TTcarrier*Time	**-0.023**	0.11	**0.037**

Dependent variable: Prednisone dose.
*Model: Origin, MDT, Other medications, Thalidomide dose, Genotype, Time, Genotype*Time

Table 6: Analysis of interaction between haplotypes of *TNF* and time related to estimated thalidomide dose in the dominant model using the generalized estimating equation model (GEE)[1]

HAPLOTYPE[2]	**B**	**SE**	***P*-value**
CCCGA	16.298	13.988	0.244
TCTGG	17.578	9.996	0.079
TATGG	37.174	22.447	0.098
CCCGG	46.587	30.732	0.130
TCCAG	**29.900**	11.027	**0.009**
CACGG	17.124	9.686	0.077
TACGG	26.051	23.245	0.262
TCCGG	R[3]		
Time	0.020	0.322	0.526
CCCGA*Time	-0.121	0.690	0.079
TCTGG*Time	-0.068	0.033	**0.040**
TATGG*Time	-0.006	0.064	0.920
CCCGG*Time	-0.128	0.119	0.284
TCCAG*Time	-0.113	0.041	**0.006**
CACGG*Time	0.003	0.034	0.932
TACGG*Time	-0.011	0.065	0.867
TCCGG*Time	R[3]		

Dependent variable: Thalidomide dose.
1. Model: Origin, MDT, Other medications, Prednisone dose , Haplotype, Time, Haplotype*Time
2. Haplotypes in the following order: -1031/-863/-857/-308/-238
3. Reference

Table 7: Analysis of interaction between groups of alleles associated with *TNF* expression and time related to estimated thalidomide dose in the dominant model using the generalized estimating equation model (GEE)[1]

Group of alleles	**B**	**SE**	***P*-value**
3-4 HE[3] alleles	34.505	16.857	>0.05
1-2 HE alleles	10.390	10.005	
No HE alleles	R[2]		
Time	-0.006	0.020	0.772
3-4 HE alleles	-0.010	0.044	0.817
1-2 HE alleles	-0.038	0.030	0.214
No HE alleles	R[2]		

Dependent variable: Thalidomide dose.
1. Model: Origin, MDT, Other medications, Prednisone dose, Group, Time, Group*Time
2. Reference
3. Alleles associated with high expression of *TNF*.

Table 8: Analysis of interaction between CYP2C19*2 genotype and time related to estimated thalidomide dose to recessive and dominant genetic model using the generalized estimating equation model (GEE)* in erythema nodosum leprosum treatment

POLYMORPHISM	MODEL	INTERACTION	B	SE	*P*-value
CYP2C19*2	Dominant	Carrier AG+AA	26.480	10.233	0.010
		Time	-0.017	0.017	0.313
		Carrier AG+AA*Time	-0,031	0.037	0.405
	Recessive	Carrier AA	**37.988**	26.102	0.146
		Time	-0.024	0.015	0.108
		Carrier AA*Time	-0.266	0.084	**0.002**

Dependent variable: Thalidomide dose .
*Model: Origin, MDT, Other medications, Prednisone dose, Genotype, Time, Genotype*Time

Supplementary Table 1: Polymorphisms and assays analyzed

GENE	POLYMORPHISM	RS	Position[1]	Assay
NR3C1	N363S	rs56149945	Chr[2] 5:143399752	C__26841917_40
	BclI	rs41423247	Chr 5:143399010	Custom[3]
	GR9β	rs6198	Chr 5:143278056	C___8951023_10
	ER22/23EK	rs6189	Chr 5:143400774	C_175679493_10
ABCB1	3435 C>T	rs1045642	Chr 7:87509329	C___7586657_20
TNF	- 1031 T>C	rsrs1799964	Chr 6:31574531	C___7514871_10
	- 863 C>A	rs1800630	Chr 6:31574699	Custom
	- 857C>T	rs1799724	Chr 6:31574705	C__11918223_10
	-308 G>A	rs1800629	Chr 6:31575254	C___7514879_10
	-238 G>A	rs361525	Chr 6:31575324	C___2215707_10
CYP2C19	CYP2C19*2	rs4244285	Chr 10:94781859	C__25986767_70
	CYP2C19*3	rs4986893	Chr 10:94780653	C__27861809_10
	CYP2C19*4	rs28399504	Chr 10:94762706	C__30634136_10

1.Position of the polymorphism on the chromosome
2.Chr: Chromosome
3.Custom: Custom assay

Supplementary Table 2: Linkage disequilibrium values (D') of polymorphisms in genes *NR3C1* and *TNF*

Gene	Variant 1	Variant 2	D'
NR3C1	GR9β (rs6198)	*Bcl*I (rs41423247)	1.0
	GR9β (rs6198)	*N363S* (rs6195)	1.0
	*Bcl*I (rs41423247)	*N363S* (rs6195)	1.0
TNF	*(-1031 C>T)* rs1799964	*(-863A>C)* rs1800630	0.901*
	(-1031 C>T) rs1799964	*(- 857C>T)* rs1799724	1.0*
	(-1031 C>T) rs1799964	*(-308C>T)* rs1800629	0.735
	(-1031 C>T) rs1799964	*(-238 A>G)* rs361525	1.0*
	(-863A>C) rs1800630	*(- 857C>T)* rs1799724	0.5
	(-863A>C) rs1800630	*(-308C>T)* rs1800629	1.0
	(-863A>C) rs1800630	*(-238 A>G)* rs361525	0.75
	(- 857C>T) rs1799724	*(-308C>T)* rs1800629	1.0
	(- 857C>T) rs1799724	*(-238 A>G)* rs361525	1.0
	(-308 G>T) rs1800629	*(-238 A>G)* rs361525	0.081

***(LOD>2)**

Supplementary Table 3: Characterization of TNF haplotypes according to the number of alleles associated with high expression.

TFN haplotypes	N	Expression	HEA*
TCCAG/TCCAG	1	- - - + -/- - - + -	2
TCCAG/CACGG	6	- - - + -/+ + - - -	3
TCCAG/TCCGG	10	- - - + -/- - - - -	1
TCCAG/TCTGG	2	- - - + -/- - + - -	2
TCCAG/CCCGA	2	- - - + -/+ - - - +	3
TACGG/TCCGG	1	- + - - -/ - - - - -	1
CACGG/CACGG	4	+ + - - -/+ + - - -	4
CACGG/TCCGG	40	+ + - - -/- - - - -	2
CACGG/TATGG	4	+ + - - -/- + + - -	4
CACGG/CCCGA	3	+ + - - -/+ - - - +	4
TCCGG/TCCGG	42	- - - - -/- - - - -	0
TCCGG/CCCGG	2	- - - - -/+ - - - -	1
TCCGG/TCTGG	27	- - - - -/- - + - -	1
TCCGG/CCCGA	3	- - - - -/+ - - - +	2
TCTGG/TCTGG	2	- - + - -/- - + - -	2

Haplotypes in the following order: rs1799964/rs1800630/rs1799724/rs1800629/rs361525. The high-expression alleles in these variants were C, A, T, A, A, respectively.

*__HE:__ number of alleles associated with high expression.

Capítulo VI – Artigo 3

CRBN variants influence response in the treatment of Erythema Nodosum Leprosum with thalidomide.

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CRBN variants influence response in the treatment of Erythema Nodosum Leprosum with thalidomide

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ABSTRACT

Thalidomide is an immunomodulatory and anti-inflammatory drug currently used to treat conditions such as erythema nodosum leprosum (ENL) and multiple myeloma (MM). Cereblon protein has been described as the target of thalidomide with regard to its teratogenicity and is required for the efficacy of thalidomide and other immunomodulatory drugs (IMiDs) in multiple myeloma. However, there are no studies on the role of CRBN in the effect of thalidomide in ENL. The objective of this study was to evaluate the relation between *CRBN* gene polymorphisms and the dose of thalidomide used in the treatment of ENL and on the manifestation of adverse effects during treatment for ENL. The polymorphisms rs1620675, rs1672770 and rs4183 from regions flanking the part of the gene encoding the portion of CRBN that binds thalidomide were evaluated. The study was conducted with 148 ENL patients treated with thalidomide. The evaluation of the influence of polymorphisms and haplotypes on thalidomide dose variation over the course of treatment was performed using the generalized estimating equations method (GEE). An association between the polymorphisms rs1620675 (p=0.043) and rs4183 (p=0.030) and a lower dose of thalidomide in ENL treatment was identified. Also an association of the allele A rs1672770 with gastrointestinal adverse effects and of the haplotype A/A/DEL (rs1620675/rs1672770/rs4183) with dermatological problems was observed. We conclude that as in MM, *CRBN* gene may be a factor that influences the efficacy of thalidomide in ENL and that *CRBN* polymorphisms are potential markers of response to ENL treatment.

Key-words: Cereblon, thalidomide, erythema nodosum leprosum, pharmacogenomics

INTRODUCTION

Thalidomide is a glutamic acid derivative produced initially in the 1950s as an anticonvulsant and subsequently used as a sedative and antiemetic in early pregnancy (Melchert and List 2007; Mercurio et al. 2017). However, its teratogenic effect soon became known when reports of birth defects in children born to mothers who used thalidomide during pregnancy appeared in 1961 (Mcbride 1961; Lenz et al. 1962). Thus, it was withdrawn from the market, but soon its importance was rediscovered according to the report of drug efficacy in the treatment of erythema nodosum leprosum (ENL) (Sheskin 1965).

ENL is an inflammatory reaction characterized by painful nodules on the skin that can ulcerate, and by systemic involvement with fever and general malaise and effects on various organs (Pocaterra et al. 2006; Costa et al. 2018). It affects about 30-50% of patients with multibacillary leprosy and is a potentially disabling condition (Chandler et al. 2015). Thalidomide is effective in treating ENL, rapidly reducing symptoms such as fever and night sweats and improving skin lesions (Sheskin 1965; Melchert and List 2007). By 1999, Thalidomide was also proved to be effective in the treatment of multiple myeloma (MM) (Singhal et al. 1999) and later for other conditions. The effectiveness of thalidomide in ENL is initially due to its action on TNF-α, but other mechanisms may contribute to its anti-inflammatory effect (Moreira et al. 1993; Knobloch et al. 2017).

Recently, the Cereblon protein (CRBN) was described as the primary target of thalidomide teratogenicity (Ito et al. 2010). This molecule is part of an E3-ubiquitin ligase complex ($CRL4^{CRBN}$), acting as a substrate receptor that recognizes specific targets for ubiquitination, leading to further degradation by the ubiquitin-proteasome system (Yang et al. 2018). More recently, it was determined that CRBN is necessary for the efficacy of thalidomide and its analogues lenalidomide and pomalidomide (named immunomodulatory drugs - IMiDs) in the treatment of MM (Zhu et al. 2011; Lopez-Girona et al. 2012; Huang et al. 2014). Several studies have investigated the Thalidomide-CRBN interaction with regard to the teratogenic, immunomodulatory and therapeutic effects of the drug. However, there are no studies on the effect of the interaction Thalidomide-CRBN on ENL.

The *CRBN* gene encodes a protein 442 amino acids long, has 11 exons and is highly conserved, hence, polymorphisms in its coding regions are rare. Therefore, some studies have analyzed non-coding regions of the gene that may be associated with the control of gene expression (Butrym et al. 2016; Szudy-Szczyrek et al. 2018). Three polymorphisms that flank the exons encoding the thalidomide-binding region of CRBN have been identified by bioinformatics tools as possible modulators of splicing sites with the potential to affect either the expression or activity of the protein: two in intron 10 (rs1620675 and rs1672770) and one in the 3'UTR region (rs4183) (Vianna et al. 2016). Here, we evaluated the influence of these three *CRBN* variants on the dose variation of thalidomide in patients with ENL.

MATERIAL AND METHODS

Sample

The sample consisted of 148 ENL patients selected in several regions of Brazil: Porto Alegre (RS), South Brazil; Fortaleza (CE) and São Luís (MA), cities in Northeast Brazil; and Porto Velho (RO), in North Brazil. The patients included had used thalidomide at different doses and had a follow-up of up to six visits. All participants were informed about the research objectives and signed an informed consent form. This study was approved by the Ethics Committee of the Hospital de Clínicas of Porto Alegre under number 10-04410.

Analysis of clinical and demographic data

Up to six consultations annotated in the patient's medical record were analyzed with the collection of demographic data, including: sex, age and region of origin; history of leprosy (moment of diagnosis and treatment used); and history of ENL (diagnosis, treatment, adverse effects, history of relapse and dose of medications used).

Genetic Analyses

DNA was extracted from saliva samples using the Oragene DNA Extraction Kit (DNA Genotek), according to the manufacturer's instructions. A pair of primer was designed to amplify a fragment of 682 base pairs containing the region encompassing the three studied *CRBN* polymorphisms: forward 5'-TGTGGTCTTGGCAACCAGCAATTT-3' and reverse 5'-ACTGCCGTTCATGCTTGTTTCCT-3'. This region was amplified by polymerase chain reaction (PCR). The fragment obtained was visualized on a 2% agarose gel, purified and sequenced using the same primers.

Sequences were visualized and analyzed using CodonCodeAligner®, version 3.0.1 (CodonCode Corporation, Dedham, MA, USA). The hg19 sequence deposited in GenBank was used as the reference sequence. When there was doubt about the variant, sequencing was repeated for confirmation.

Statistical Analyses

The chi-square test was used to evaluate Hardy-Weinberg equilibrium for all polymorphisms. The generalized estimating equations method (GEE) was used to evaluate the influence of *CRBN* polymorphisms on thalidomide dose. This method is a repeated measures analysis focused on average changes in response over time and on the impact of covariates on these changes. GEE can model the average response of variables as a linear function of covariates of interest through a transformation or link function and can be used in studies where the data is asymmetric or the data distribution is difficult to verify due to the small-size sample. The covariates inserted in the model were: the place of origin of the patient, concomitant use of multidrug therapy (MDT) for leprosy, and the use of other medications and other treatments for ENL.

The evaluation of the effect of *CRBN* on the occurrence of adverse effects due to thalidomide treatment was conducted using Fisher's exact test. All statistical analyses were performed with SPSS version 20 (SPSS, www.spss.com, IIBM, USA).

MLocus software was used to calculate linkage disequilibrium (LD) for the polymorphisms (Long et al. 1995), and haplotypes were inferred using the Bayesian algorithm of the Phase 2.1.1 program (Stephens et al. 2001; Stephens and Donnelly 2003).

RESULTS

In the present study, 148 ENL patients were included, where 115 (75.6%) were male, and 99 (66.4%) presented lepromatous leprosy, the form associated with a higher bacillary load. The demographic and clinical characteristics of the sample are presented in Table 1.

The genotypic distributions were in Hardy-Weinberg equilibrium and the allelic and genotypic frequencies of the polymorphisms are shown in Table 2. A high linkage disequilibrium between the polymorphisms studied was identified (Supplementary Table 1), and four haplotypes were identified in the sample (Table 3).

GEE analyses using the dominant and recessive models to evaluate the influence of polymorphisms on thalidomide dose were performed (Table 4). We found an association of the rs1620675 polymorphism in the recessive model (p=0.043) and of the rs4183 polymorphism in the dominant model (p=0.030) with thalidomide dose and its variation over time (Table 4). Individuals with the CC genotype in rs1620675 polymorphism received an initial dose of about 43.27 mg less than that received by patients heterozygous and homozygous for the A allele. There was also an association of the rs4183 polymorphism and 3'UTR region with thalidomide dose. Patients homozygous for insertion required an initial dose of thalidomide averaging 45.41 mg less compared to patients who had the deletion in this region of the gene. No association was found between haplotypes and thalidomide dose.

Analysis of the association between genetic variants and the manifestation of adverse effects showed an association between the rs1627770 T allele and gastrointestinal adverse effects (p=0.03; Table 5). These effects were present in 51 (28.8%) patients who carried the T allele. The analysis of the association between haplotypes and adverse effects showed an association with dermatological adverse effects (p=0.019) with haplotype A/A/DEL (rs1620675/rs1672770/rs4183) (Table 6). These effects were present in 11 patients (24.4%) who carried this haplotype. There was no association between genotypes and manifestation of adverse effects.

DISCUSSION

This study aimed to identify genetic variants of the *CRBN* gene that might influence in response of the treatment of ENL with thalidomide. We found that rs1620675 polymorphism is associated with thalidomide dose in the recessive model and that rs4183 polymorphism is associated with thalidomide dose and its variation over time in the dominant model.

CRBN is composed of 11 exons extending over 30 Kb. Thalidomide binds to CRBN in a region of 104 amino acids (339-442) located in the C-terminal portion, encoded by exons 9, 10 and 11 (Ito et al. 2010). This gene is extremely conserved

and no polymorphism was found in the coding region (Butrym et al. 2015; Vianna et al. 2016). The polymorphisms studied are located in regions adjacent to the region encoding the portion of the protein that binds thalidomide, an A>C substitution (rs1620675) and a G>A substitution (rs1672770), both in intron 10, and an insertion/deletion (INS/DEL) of four nucleotides in (-/GTTA) in the 3'UTR region adjacent to exon 11 (rs4183).

CRBN acts as a substrate receptor as part of the E3 ubiquitin ligase complex ($CRL4^{CRBN}$), which controls the expression of target proteins by their ubiquitination and degradation. According to Ito et al. (2010), CRBN is necessary for the teratogenic effect of thalidomide. In addition, this protein is also important for the anti-proliferative effect of thalidomide and other IMiDs in MM (Gandhi et al. 2014). It is postulated that when thalidomide binds to CRBN, it inhibits its function causing teratogenic effects by preventing the degradation of proteins that play a crucial role in embryonic development (Ito et al. 2011; Schuster et al. 2014). In the case of MM, thalidomide binding to CRBN promotes recruitment of the transcription factors Ikaros and Aiolos to the ubiquitin-ligase complex, resulting in increased ubiquitination and degradation of these transcription factors in T cells and MM cells (Chamberlain et al. 2014; Dimopoulos et al. 2018). In addition, some studies have associated low *CRBN* mRNA expression with poorer clinical response to IMiDs, suggesting a potential role of CRBN as a predictive biomarker for treatment response (Heintel et al. 2013; Huang et al. 2014; Schuster et al. 2014; Dimopoulos et al. 2018). Since the 1990s, it is known that one of the main effects of thalidomide is to decrease the *TNF* mRNA half-life, which explains some of its therapeutic effects. Using *CRBN* knockdown, it has been shown that the inhibitory effect of IMiDs on TNF-α production was also impaired in the silencing of *CRBN* (Lopez-Girona et al. 2012; Zhu et al. 2013)

The polymorphisms evaluated in our study were identified in a previous study as possible splicing sites (Vianna et al. 2016). The association found in the present study indicated that polymorphisms in these regions may interfere with *CRBN* gene expression or CRBN activity, which may modulate the response to thalidomide treatment. High levels of CRBN may indicate a better response and need for lower doses of thalidomide over the course of treatment, as found in our

study. Studies performed with MM showed that polymorphisms in non-coding regions of *CRBN* were also associated with response to thalidomide therapy (Butrym et al. 2016; Szudy-Szczyrek et al. 2018).

In this study, we also found an association between the A allele of rs1672770 with manifestation of gastrointestinal adverse effects (p=0.03). These gastrointestinal effects consisted of diarrhea, vomiting, nausea, heartburn, constipation and inappetence. The most common of these symptoms was constipation, a commonly reported side effect of thalidomide (Wines et al. 2002; Walker et al. 2007). There was also an association of the haplotype A/A/DEL (rs1620675/rs1672770/rs4183) with dermatological adverse effects (p=0.019). Such effects consisted of pruritus, dry skin, dermatitis and hair loss. Among these, dry skin was the most common, present in 10 individuals (21.3%) who had the A/A/DEL haplotype (rs1620675/rs1672770/rs4183). Dry skin is a side effect also described in the use of thalidomide (Dimopoulos and Eleutherakis-Papaiakovou 2004). The association of adverse effects with the polymorphisms studied may also be related to differences in *CRBN* expression. However, it should be taken into consideration that these effects may also be associated with MDT for leprosy due to the use of clofazimine (Goulart et al. 2002; Cholo et al. 2012). Both MDT and use of other medications during ENL treatment may influence the appearance of adverse effects. In the analysis of the influence of polymorphisms on thalidomide dose, MDT and other medications were used as model covariates, but this was not possible in the case of influence analysis regarding adverse effects.

The epidemiological data of this study were in agreement with the data found in other studies (Pocaterra et al. 2006; Feuth et al. 2008). Most of the individuals with ENL had lepromatous leprosy and were male. This indicates that men may be more affected by lepromatous leprosy and that the bacillary index is a risk factor for the development of the reaction as demonstrated in other studies (Guerra et al. 2004; Pocaterra et al. 2006; Walker et al. 2015). In addition, many patients were on MDT during treatment for ENL, confirming that the reaction manifests mainly in the first year of illness, during MDT (Voorend and Post 2013; Dias et al. 2016).

Some limitations should be considered in our study. The use of clofazimine in MDT for leprosy may interfere with both dose reduction of drugs because of its

anti-inflammatory effect and in the manifestation of adverse effects. In addition, a retrospective study was carried out in which the clinical data of patients, thalidomide dose and manifestation of adverse effects were obtained through the analysis of medical records. The lack of standardization in the description of this information or even the absence of a description of the adverse effects could not be ruled out. Peripheral neuropathy, one of the main adverse effects of thalidomide, was not evaluated in this study due to its retrospective nature, making it difficult to differentiate neuropathy due to the disease itself or due to the use of thalidomide. Accordingly, the evaluation of the effect of polymorphisms on the onset of peripheral neuropathy should be performed by a prospective study to reduce potential biases. Another limitation was that this study was carried out with individuals from different regions of the country and the heterogeneous genetic background of the Brazilian population might have been underestimated in the analysis and interpretation of the results.

On basis of the results of this study, we identified some genetic variants of *CRBN* that can alter the expression or activity of the protein and affect response to Thalidomide treatment. This also shows that CRBN may be necessary for the action of thalidomide in ENL as already described in MM. ENL is a chronic and difficult-to-control condition. Thalidomide is an effective drug but has restrictions on its use due to peripheral neuropathy and its teratogenicity. Therefore, it is important to identify useful markers in predicting treatment response to limit its use to patients who will benefit most from treatment. To our knowledge, this is the first study to evaluate the association of genetic variants of *CRBN* with thalidomide treatment in ENL patients. There are still many gaps to be filled in our knowledge of the mechanism of action of thalidomide and on how CRBN participates in this process. However, this study shows that evaluation of *CRBN* and its expression may help to understand the action of thalidomide in ENL.

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Table 1: Clinical and demographic characteristics of ENL patients

Characteristic	**N=148**
Male [n (%)]	115 (75.6)
Multidrug therapy for leprosy [n (%)]	66 (44.3)
Other medications [n (%)]	91 (61)
Days of consultation [Mean (min/max)]	189.49 (0/1463)
Thalidomide dose [Median (min/max)]	100 (0/400)
South [n (%)]	41 (29.6)
Northeast [n (%)]	93 (62.5)
North [n (%)]	14 (9.8)
Leprosy:	
Lepromatous [n (%)]	99 (66.4)
Boderline-lepromatous [n (%)]	32 (21.4)
Indeterminate [n (%)]	2 (1.3)
Adverse Effects:	
Neurological[A]	45 (30.4)
Grastrointestinal[B]	34 (23)
Musculoskeletal[C]	27 (18.2)
Ocular[D.]	23 (15.5)
Edema	17 (11.5)
Dermatological[E.]	16 (10.8)
Fever	7 (4.7)
Respiratory[F]	2 (1.3)

A. Drowsiness, paresthesias, dizziness, tremor, neuritis, tingling, headache; B. diarrhea, vomiting, nausea, constipation and inappetence; C. myalgia, arthralgia and weakness; D. decreased visual acuity and eye irritation; E. pruritus, dry skin, dermatitis and hair loss; F. dyspnea and dry cough.

Table 2: Genotypic and allelic frequencies of *CRBN* polymorphisms in ENL patients on treatment with thalidomide.

Polymorphism	Alleles/Genotypes	Frequency N (%)
rs1620675	AA	41 (27.9)
	CA	71 (48.3)
	CC	35 (23.8)
	A	153 (52)
	C	141 (48)
rs1672770	AA	61 (41.8)
	GA	66 (45.2)
	GG	19 (13)
	A	188 (64.4)
	G	104 (35.6)
rs4183	INS/INS	36 (24.5)
	INS/DEL	75 (51)
	DEL/DEL	36 (24.5)
	INS	147 (50)
	DEL	147 (50)

Table 3: Haplotype frequencies of *CRBN*

Haplotype	N	(%)
C/A/INS	142	(48)
A/G/INS	4	(1.4)
A/A/DEL	47	(15.9)
A/G/DEL	103	(34.8)

Haplotypes in the following order: rs1620675/rs1672770/rs4183

Table 4: Analysis of interaction between *CRBN* genotype and time related to estimated thalidomide dose for the recessive and dominant models using the generalized estimating equation model (GEE)* in erythema nodosum leprosum treatment

POLYMORPHISM	MODEL	INTERACTION	B	SE[1]	P-value
rs 1620675	Dominant	Carrier AA	11.771	11.891	0.322
		Time	-0.015	0.0196	0.436
		Carrier AA*Time	-0.027	0.0345	0.428
	Recessive	Carrier CC	**-43.273**	18.456	**0.019**
		Time	-0.026	0.017	0.118
		Carrier CC*Time	0.107	0.053	**0.043**
rs1672770	Dominant	Carrier TT	-22.280	14.273	0.119
		Time	0.024	0.0335	0.471
		Carrier TT*Time	0.050	0.0375	0.183
	Recessive	Carrier CC	12.299	15.00	0.412
		Time	0.011	0.0265	0.687
		Carrier CC*Time	-0.051	0.044	0.244
rs 4183	Dominant	Carrier INS/INS	**-45.41**	17.7319	**0.010**
		Time	0.086	0.0504	0.089
		Carrier INS/INS*Time	-0.113	0.0518	**0.030**
	Recessive	Carrier DEL/DEL	13.966	11.957	0.243
		Time	0.009	0.0284	0.751
		Carrier DEL/DEL*Time	-0.017	0.0346	0.622

Dependent variable: Thalidomide dose
*Model: Origin, MDT, Other medications, Thalidomide dose, Genotype, Time, Genotype*Time
SE: Standard error

Table 5: Frequency of gastrointestinal adverse effects[A] according to the rs1672770 alleles

Allele	Absence	Presence	P-value[B]
	N (%)	**N (%)**	
A	126 (71.2)	51 (28.8)	0.030
G	86 (83.5)	17 (16.5)	

A. Gastrointestinal adverse effects: diarrhea, vomiting, nausea, constipation and inappetence; B.Fisher's exact test

Table 6: Frequency of dermatological adverse effects[A] according to *CRBN* haplotypes

Haplotype[B]	Absence	Presence	P-value[C]
	N (%)	**N (%)**	
C/A/INS	123 (90.4)	13 (9.6)	0.019
A/G/INS	4 (100)	0 (0)	
A/A/DEL	34 (75.6)	11 (24.4)	
A/G/DEL	93 (92.1)	8 (7.9)	

A. Dermatological adverse effects: pruritus, dry skin, dermatitis and hair loss ; B. haplotypes in the following order: rs1620675/rs1672770/rs4183 ; C. Fisher's exact test

Supplementary Table 1: Linkage disequilibrium values (D') of polymorphisms in *CRBN*

Variant 1	Variant 2	D'
rs1672770	**rs1620675**	1.0
rs1620675	**rs4183**	0.926
rs1672770	**rs4183**	1.0

Capítulo VII – Artigo 4

Association of Toll Like Receptor 9 haplotypes with treatment of Erythema Nodosum Leprosum

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Association of Toll Like Receptor 9 haplotypes with treatment of Erythema Nodosum Leprosum

Manuscrito a ser submetido à revista Immunology Letters

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ABSTRACT:

Toll-like receptors (TLRs) are elements of innate immune system that can activate various inflammatory and adaptive immune pathways. Erythema Nodosum Leprosum (ENL) is a severe inflammatory reaction associated with high cytokine levels that occurs in some patients with multibacillary leprosy (MB). Toll-like Receptor 9 (TLR-9) may be associated with ENL pathogenesis by stimulating the release of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α). *TLR-9* gene polymorphisms have been associated with autoimmune, infectious and inflammatory diseases. Among *TLR-9* gene polymorphisms, T-1237C (rs5743836) and G2848A (rs352140) are sufficient to determine the major haplotypes of TLR-9. The aim of this study was to evaluate the influence of these TLR-9 polymorphisms on the treatment of ENL with thalidomide and prednisone. A total of 148 ENL patients treated with thalidomide and / or prednisone were recruited, and clinical information data on leprosy, ENL, treatment and possible adverse effects were collected. The evaluation of the influence of haplotypes on the variation of thalidomide and prednisone dosage throughout the treatment was carried out using the Generalized Estimating Equations (GEE) method. An association between haplotypes (rs5743836 / rs352140) TG ($p = 0.02$) and CG ($p = 0.024$) was identified with the dosage of prednisone used throughout ENL treatment. There was no association between *TLR-9* haplotypes and treatment with thalidomide or with manifestation of adverse effects. Our results suggest that *TLR-9* polymorphisms may influence the treatment of ENH with prednisone. However, the effect of this association and its clinical application should be better characterized.

Key words: Toll like receptor, haplotypes, prednisone, erythema nodosum leprosum

INTRODUCTION:

Erythema Nodosum Leprosum (ENL) is a leprosy reaction presenting a severe, painful and multisystemic inflammatory complication that especially affects patients with multibacillary leprosy (MB) (Nery et al., 1998; Pocaterra et al., 2006). ENL is characterized by systemic symptoms such as fever, neuritis, bone pain and general malaise, and is associated with high levels of cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) (Dias et al. 2016; Polycarpou et al. 2017; Negera et al. 2018). The treatment of ENL is based on the decrease of these cytokines and the most commonly used drugs are prednisone and thalidomide. Prednisone acts by inhibiting the activation of transcription factors, such as nuclear factor kappa (NF-κB), which regulates many genes, including those encoding TNF-α, IL-1, IL-2, and the enzyme inducible nitric oxide synthase (iNOS), involved in inflammatory responses (Andersson et al. 2005). Thalidomide acts on ENL mainly through the inhibition of *TNF-α* expression, decreasing the levels of this cytokine (Moreira et al. 1993).

Toll-like receptors (TLRs) are a family of ten transmembrane receptors that play a key role in innate immunity through the recognition of various types of ligands and molecular patterns specific to pathogens during infections and endogenous of the human organism during inflammatory processes and tissue damage (Rao et al., 2015, Wu et al., 2000). In interacting with their ligands these receptors activate mechanisms of adaptive immunity through intracellular signaling pathways that culminate in the induction of the expression of inflammatory cytokines, chemokines, interferons (IFNs), and the upregulation of costimulatory molecules (Kawai and Akira 2007; Lyn- Cook et al., 2014). TLR-9 receptor recognizes unmethylated CpG motifs found in viral and bacterial DNA and acts by promoting the activation of NF-κB, which induces proinflammatory cytokines TNF-α, IL-6 and IL-1β (Etem et al., Hamdy et al., 2011). Recently, higher levels of TLR-9 and its ligands have been identified in ENL patients. Also, the ability of E6446, a synthetic TLR-9 antagonist in inhibit the secretion of proinflammatory cytokines such as TNF-α was observed in patients of ENL, similar to that occurring in treatment with thalidomide. This phenomenum suggests the involvement of the innate immunity in the pathophysiology of ENL (Dias et al., 2016). The *TLR9* gene

has several single nucleotide polymorphisms, but two have been described as sufficient to distinguish the four commonest *TLR9* haplotypes: rs5743836 and rs352140 (Lazarus et al., 2003). In this context, the objective of this study is to evaluate the influence of these *TLR9* polymorphisms on thalidomide and prednisone dose variation in ENL treatment.

MATERIAL AND METHODS

Sample

This study was approved by the Ethics Committee of the Hospital de Clínicas of Porto Alegre under number 10-04410. The sample consisted of 148 ENL patients selected in different regions of Brazil: Porto Alegre - RS, southern Brazil, in Fortaleza - CE, Imperatriz and São Luís - MA, cities of Northeast Brazil and in Porto Velho - RO, northern Brazil . The patients included were under treatment for ENH with thalidomide and/or prednisone at any dose. The characteristics collected included demographic data (sex, age and origin), history of leprosy (moment of diagnosis and treatment used); history of ENL (diagnosis, treatment, adverse effects, history of relapse and medication regimens used), and occurrence of adverse effects of treatments. These information were collected for up to six visits (equivalent to about one consultation per month).

Genetic Analysis

DNA was extracted from saliva samples using the Oragene DNA Extraction Kit (DNA Genotek®), according to the manufacturer's instructions. The rs352140 polymorphism of *TLR9* was analyzed by PCR-RFLP using the restriction endonuclease BstUI, and the polymorphism rs5743836 was genotyped by bi-directional PCR allele-specific amplification (BI-PASA), both assays were based on protocols previously described by Santos et al. (Dos Santos et al., 2012).

Statistical Analyzes

The chi-square test was used to evaluate the Hardy-Weinberg equilibrium expectation for polymorphisms. Generalized Estimating Equation (GEE) method was used to evaluate the influence of *TLR*9 haplotypes on the dose variation of

thalidomide and prednisone over time. Through GEE, the average response can be modeled as a linear function of covariates of interest through a transformation or link function. The covariates inserted in the model were: the origin of the patient, the concomitant use of multidrug therapy (MDT) for leprosy, use of other medications and other treatments for ENL.

The linkage disequilibrium (LD) for both SNPs in the samples was calculated through the Haploview 4.2 program (Barrett et al., 2005) and haplotypes were inferred through the Bayesian algorithm implemented in the Phase 2.1.1 program (Stephens et al. ; Stephens and Donnelly 2003). The evaluation of the effect of haplotypes on the occurrence of adverse effects to treatment with thalidomide and / or prednisone was performed using Fisher's Exact Test. All tests were performed through SPSS® version 18 (SPSS, www.spss.com, IIBM, USA).

RESULTS

The sample consisted of 148 participants and was characterized by a predominance of males (75.6%). About 66% (n = 99) of the patients presented lepromatous leprosy (VV), and 44.3% (n = 66) were using multidrug therapy for leprosy during treatment for ENL. Patients were followed up for an average of 190 days. The most common adverse effects were neurological (30.4%) and gastrointestinal (23%).

The genotypic distributions of the polymorphisms were in Hardy-Weinberg equilibrium. A high linkage disequilibrium between polymorphisms was identified and haplotype analysis identified four haplotypes (Table 2).

No influence of genetic variations of *TLR9* or its haplotypes on the dose of thalidomide was observed. Also no influence of such variants or haplotypes on the occurrence of adverse effects related to thalidomide or prednisone use was observed.. However, GEE analysis showed that *TLR-9* haplotypes (rs5743836 / rs352140) CG and TG influence on prednisone doses ($p = 0.024$ and $p = 0.02$, respectively; Table 3). Patients with the CG haplotype presented an initial dose of prednisone averaging in 5.23mg lower than those with TA haplotype. On the other hand, patients with TG haplotype had an initial dose of prednisone averaging in 2.18mg lower than patients with TA haplotype. These same haplotypes, CG and

TG, also showed a difference in the dose variation of prednisone over time (p = 0.002 and p = 0.025, respectively). The CG haplotype had increased dose throughout the treatment and the TG haplotype had a lower dose reduction (Figure 1).

DISCUSSION

Excessive activation of TLRs has already been implicated in the pathogenesis of chronic inflammatory and autoimmune diseases, such as systemic lupus erythematosus and psoriasis, that typically intercalate periods of inflammatory activity with periods of remission, as occurs in ENL (Barrat and Coffman 2008; Dias et al. 2016). A recent study observed a high TLR-9 expression in ENL lesions. Also, this study shown that *in vitro* stimulation of inflammatory infiltrate cells with TLR-9 ligands increased levels of TNF-α, IL-6 and IL-1β cytokines. Thus, these results suggest that the high levels of these cytokines in ENL may, at least in part, be related to higher levels of TLR-9 (Dias et al., 2016). In the present work we sought to evaluate the role of polymorphisms that change the expression of *TLR9* gene on the treatment of ENL with thalidomide and prednisone. The T-1237C polymorphism (rs5743836) is a T> C substitution in the promoter region that creates a potential NF-κB binding site, modifying the transcriptional activity of the gene (Mollaki et al. 2009). However, some studies associate higher transcriptional activity to C allele while others associate to T allele (Novak et al. 2007; Ng et al. 2010). The G2848G polymorphism (rs352140) is a G>A change that is located at position 2848 in exon 2 of the gene. This polymorphism does not change the amino acid and does not alters the regulatory site, but carriers of the A allele have already been associated with a higher expression of *TLR9* generating more inflammatory response. This association is attributed to possible linkage disequilibrium with another functional polymorphisms (Kikuchi et al., 2005; Paradowska et al., 2016).

Analysis of the influence of haplotypes on prednisone treatment showed that CG haplotype (rs5743836 / rs352140) and TG haplotype patients had an initial prednisone dose lower than the TA haplotype. These haplotypes also showed a difference in dose variation over time. The TG haplotype presented a dose

variation of prednisone more discreet than the other haplotypes. On the other hand, the CG haplotype presented increased dose of prednisone throughout the treatment. Glucocorticoids have strong anti-inflammatory effects on both innate and acquired immunity (Guiducci et al., 2010). They inhibit B and T lymphocyte responses and monocyte and neutrophil effector functions. In addition, they may directly or indirectly interact with proinflammatory transcription factors NF-κB and activator protein 1 (AP-1) and reduce its activity (Guiducci et al., 2010; Schijvens et al., 2018). They can thus regulate cytokines, chemokines, enzymes, adhesion molecules among others (Czock et al., 2005). Prednisone can act on ENL by modulating the proinflammatory cytokines either directly or by suppressing the immune cells that produce these cytokines (Negera et al., 2018). The haplotypes that were identified as being associated to prednisone dose had the 2848 G allele (rs352140). The allele A of this polymorphism has already been associated with higher *TLR-9* expression and higher inflammatory status (Kikuchi et al., 2005). Here we found an association between haplotypes that include the 2848 G allele (rs352140) and a lower initial dose of prednisone probably associated with a lower inflammatory state associated with that allele. However, the association of these haplotypes with the dose over time showed discrete dose reduction or an increase in the dose of prednisone. Guiducci et al demonstrated the TLR-9 associated with resistance to glucorocorticoids (Guiducci et al., 2010). Otherwise, Broering et al. demonstrated that glucocorticoids modulate the activation of TLRs in a manner specific to each cell type resulting in the negative regulation of TLR expression (Broering et al., 2011). A study with renal transplant patients showed that 2848 G allele (rs352140) carriers have a poor response to glucocorticoids and a higher risk of transplant rejection (Kim et al. 2013). Thus, it can be hypothesized that although initially the individual needs a lower dose of prednisone due to a more favorable inflammatory state, the dose of prednisone needs to be increased due to the low response to treatment of the allele carriers. That is, this variant may either favor a lower inflammatory response or present a more discreet response to treatment with prednisone.

The GEE analysis did not reveal an association between TLR9 haplotypes and thalidomide dose. Thalidomide has anti-inflammatory and immunomodulatory

action, however its exact mechanism of action is not completely known. Thalidomide's inhibitory action on TNF-α has already been described through the degradation of its mRNA (Moreira et al., 1993). However, it may also inhibit other proinflammatory cytokines such as transforming growth factor beta (TGF-β), IL-1β, IL-6 while increasing levels of IL-10 (Shannon et al. al. 1992; Liu et al., 2017). Dias et al. reported an action of a TLR-9 antagonist on inhibition of TNF-α that resembles what was seen in regard to thalidomide in ENL lesion cells (Dias et al., 2016). Here we found no involvement of TLR-9 in the dose of thalidomide. The lack of association with thalidomide in this study could be attributed to several factors, such as lower effect size on medication doses, lack of standardization of thalidomide treatment, heterogeneity of the sample in both disease complexity and geography. The Brazilian population is mixed and has a different genetic background in the different analyzed regions. Another limiting factor is the sample size, which may reveal little differences, especially in clinical and geographic subgroups,

Although we have not find evidence of association of TLR9 with thalidomide, this does not mean that there are no inhibitory pathways for TNF-α and other cytokines involving the TLR9 receptor and that have an impact on ENL treatment, including thalidomide. For example, apremilast, an inhibitor of phosphodiesterase 4 (PDE4) in immune cells, has among its actions the inhibition of TLR-9 signaling and the transcriptional activity of NFκB (Schafer et al., 2014). Thus, it is possible that TLR-9 is a therapeutic target for inflammatory conditions, including leprosy and ENL. Therefore, other studies are needed to identify how this innate immunity pathway can influence the development of the reaction and response to treatment. Here we suggest that TLR-9 gene polymorphisms may influence the treatment of ENL with prednisone, however, this association should be better characterized in other studies, especially in relation to treatment response in 2848 G allele carriers (rs352140).

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Table 1: Clinical and demographic characteristics of ENL patients

Characteristic	**T[A] (n=15; 10.1%)**	**T +P[B] (n=133; 89.8%)**
Male [n (%)]	14 (93.3)	101 (75.9)
Multidrug therapy for leprosy [n (%)]	9(60)	57 (42.8)
Other medications [n (%)]	2 (13,3)	88 (66.1)
Days of consultation [Mean (min/max)]		
Prednisone dose [Median (min/max)]	-	20 (0/80)
Thalidomide dose [Median (min/max)]	100 (0/400)	118.5(0/400)
Patient Origin		
South [n (%)]	8(53.3)	34(25.5)
Northeast [n (%)]	5(33.3)	88(66.1)
North [n (%)]	2(13.3)	12(9)
Leprosy:		
Lepromatous [n (%)]	8 (53.4)	88 (66.1)
Boderline-lepromatous [n (%)]	3(20)	29 (21.8)
Indeterminate [n (%)]	-	2(1.5)
Adverse Effects:		
Neurological[A]	3 (20.0)	42(31.5)
Grastrointestinal[B]	3 (20.0)	31 (23.3)
Musculoskeletal[C]	1 (6.7)	27 (20.3)
Ocular[D.]	0(0)	23(17.3)
Edema	1(6.6)	16 (12)
Dermatological[E.]	5(33.3)	11 (8.2)
Fever	1 (6.6)	6 (4.5)
Respiratory[F]	0 (0)	2 (1.5)

A. Drowsiness, paresthesias, dizziness, tremor, neuritis, tingling, headache; B. diarrhea, vomiting, nausea, constipation and inappetence; C. myalgia, arthralgia and weakness; D. decreased visual acuity and eye irritation; E. pruritus, dry skin, dermatitis and hair loss; F. dyspnea and dry cough.

Table 2: Frequencies of the haplotypes of the *TLR-9*

Haplotype	**N**	**(%)**
CG	8	(2.7)
TG	156	(52.7)
CA	49	(16.6)
TA	83	(28.0)

Haplotypes are shown as follow : rs5743836/rs352140

Table 3: Analysis of interaction between haplotypes of *TLR-9* and time related to dose of thalidomide and prednisone estimated using the Generalized Estimates Equation model (GEE)

DRUG[1]	**HAPLOTYPE**[A]	**B**	***SE***	***P*-value**
	CG	-5.229	2.31	**0.024**
	TG	-2.178	0.938	**0.020**
	CA	0.028	0.8762	0.975
	TA	R		
Prednisone[1]	Time	-0.016	0.0043	**<0.01**
	CG*Time	0.024	0.0077	**0.002**
	TG*Time	0.007	0.0033	**0.025**
	CA*Time	0.002	0.0036	0.486
	TA*Time	R[3]		
	CG	8.736	11.32	0.440
	TG	-3.943	7.524	0.600
	CA	1.334	4.69	0.776
	TA	R		
Thalidomide[2]	Time	-0.002	0.187	0.015
	CG*Time	-0.024	0.0365	0.902
	TG*Time	0.011	0.0228	0.502
	CA*Time	0.023	0.0193	0.240
	TA*Time	R[B]		

1. Dependent variable: Dose of Prednisone.
   Model: Origin, MDT, Other medications, Dose of Thalidomide, Haplotype, Time, Haplotype*Time
   A. Haplotypes in the following order: rs5743836/rs352140
   B. Reference
2. Dependent variable: Dose of Thalidomide
   Model: Origin, MDT, Other medications, Dose of Prednisone, Haplotype, Time, Haplotype*Time
   A. Haplotypes in the following order: rs5743836/rs352140
   B. Reference

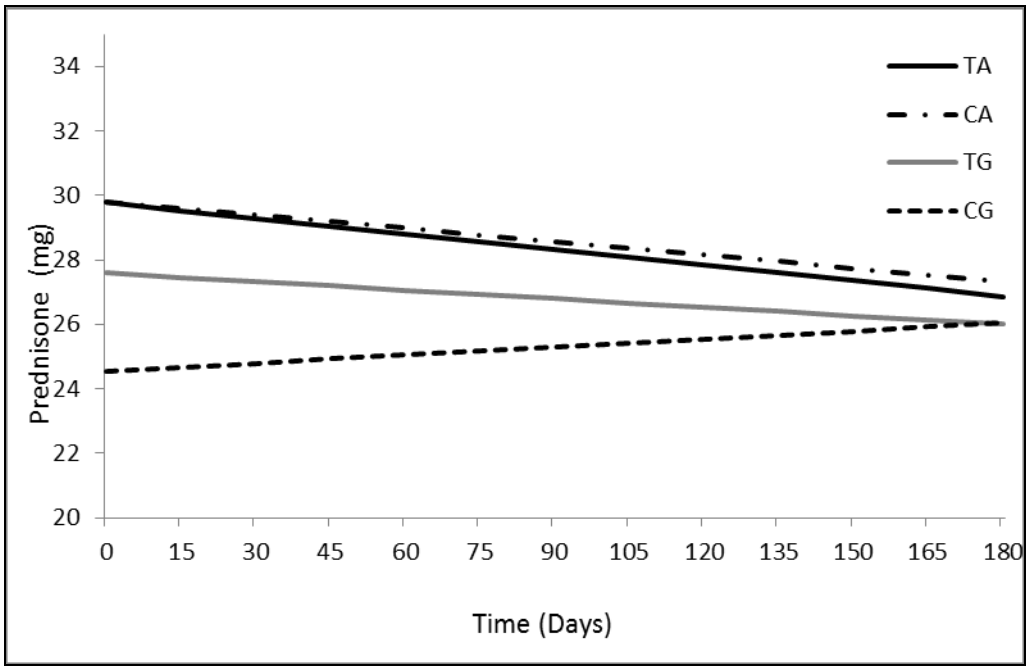

Figure 1: Line chart of the prednisone dose averages with TLR9 haplotypes discriminated by the times evaluated.

Capítulo VIII - Discussão

8. DISCUSSÃO

Em um país onde a hanseníase é endêmica como o Brasil, o Eritema Nodoso Hansênico é um problema que atinge milhares de pessoas. Trata-se de pacientes em idade economicamente ativa que, devido ao potencial incapacitante das reações, podem ter sua capacidade de trabalhar e prover suas famílias limitadas, sofrendo dificuldades financeiras (Chandler et al. 2015). Eles também são sujeitos a longos períodos de tratamento, com altas doses de medicamentos que podem gerar efeitos adversos. Além disso, esses pacientes sofrem com o estigma decorrente da doença e das incapacidades que traz uma carga psicológica e isolamento social. O ENH é, portanto, uma condição que tem um profundo impacto negativo sobre a vida dos pacientes. Nesse contexto, a fim de contribuir para um melhor direcionamento do tratamento do ENH e melhora da qualidade de vida dos pacientes esse trabalho buscou: (1) avaliar as opções de tratamento utilizados no Brasil e os desafios no manejo do ENH; (2) identificar variantes gênicas que possam influenciar no resultado do tratamento do ENH.

1. Opções de tratamento utilizados no Brasil e os desafios no manejo do ENH.

No Capítulo IV - artigo 1 foi realizada uma revisão da literatura para caracterizar os medicamentos mais utilizados no tratamento do ENH, principalmente as opções mais utilizadas no Brasil. Foram descritas as principais características, mecanismos de ação e efeitos adversos associados aos medicamentos disponíveis para o tratamento do ENH. Os medicamentos mais utilizados no Brasil são prednisona e talidomida.

Esse artigo chamou especial atenção para os desafios referentes ao tratamento, considerando que é difícil prever o padrão clínico da reação que o indivíduo irá manifestar. Entre esses desafios está a falta de dados disponíveis para o embasamento adequado do tratamento e a ausência de novos estudos de intervenção já que todos os medicamentos atualmente disponíveis falham de alguma forma no controle da reação.

O artigo também destacou a possível modificação da poliquimioterapia (PQT) para hanseníase que atualmente ocorre em esquemas separados para os

casos paucibacilares (PB) e multibacilares (MB), para um esquema único de PQT de 6 meses para todos os casos. Isso poderia levar a um aumento do número de casos de ENH, pois esse tratamento pode não ser suficiente para garantir a eliminação da carga bacilar em pacientes MB. Além disso, isso reduziria o tempo de tratamento com a clofazimina que é um medicamento da PQT com ação anti-inflamatória cuja ação pode reduzir o número de casos de ENH em pacientes MB. Recentemente o Ministério da Saúde no Brasil, sinalizou com a possibilidade de implantação do regime único de PQT de 6 meses no Brasil, entretanto, a última recomendação da Organização Mundial de Saúde não sugere a adesão a tal regime. Segundo a OMS, evidências de potenciais riscos e benefícios de um esquema mais curto foram limitadas e inconclusivas, com possibilidade de aumento no risco de recidivas (World Health Organization 2017b).

Finalmente o artigo destacou a dificuldade de padronização de tratamento no Brasil mostrando que em algumas regiões de áreas endêmicas do país há dificuldade no acesso e no controle do tratamento do ENH principalmente no que se refere à talidomida. É importante lembrar que, apesar da existência de uma rígida legislação no Brasil sobre a dispensação da talidomida, novos casos de embriopatia por talidomida foram registrados nos últimos anos em regiões endêmicas de hanseníase, decorrentes do seu uso no tratamento do ENH (Schuler-Faccini et al. 2007; Vianna et al. 2013). Isso torna fundamental o estabelecimento de um protocolo adequado e padronizado de tratamento e de monitoramento a fim de melhorar a reposta do paciente e evitar novos casos de embriopatia, já que a talidomida é amplamente utilizada no Brasil, sendo muitas vezes utilizada como primeira opção terapêutica para o tratamento do ENH.

2. Identificação de variantes genéticas que possam influenciar no tratamento do ENH.

Considerando a variabilidade na resposta ao tratamento do ENH e dificuldade de controle da reação, nós buscamos avaliar variantes genéticas que possam influenciar nas doses de prednisona e talidomida utilizadas no tratamento e na manifestação de efeitos adversos. As análises foram feitas a partir de

amostras de pacientes de ENH com tratamento utilizando talidomida e/ou prednisona.

No capitulo V, artigo 2, foram avaliados genes associados ao metabolismo dos medicamentos mais utilizados no tratamento do ENH no Brasil, prednisona e talidomida. Os genes *NR3C1* e *ABCB1* foram avaliados em relação ao tratamento com a prednisona e os genes *TNF* e *CYP2C19*, em relação ao tratamento com a talidomida. Foi encontrada uma associação entre a dose de prednisona e o polimorfismo do gene *ABCB1* 3435 G>A, mostrando uma redução da dose ao longo do tratamento. O gene *ABCB1* codifica a glicoproteína-P, uma bomba de efluxo de moléculas exógenas e metabólitos tóxicos nas células incluindo glicocorticoides. Essa proteína pode modular a eficácia do tratamento já que seu excesso nas células pode causar resistência a medicamentos. Esse polimorfismo estaria associado a menores níveis da proteína nas membranas celulares, aumentando a resposta ao medicamento o que explicaria a redução da dose de prednisona durante o tratamento (Ambudkar et al. 2003; Tavares et al. 2018). Já o gene *NR3C1* codifica o receptor de glicocorticoides (GR) e seus polimorfismos poderiam modular a resposta à prednisona já que estão associados à resistência ou sensibilidade aos glicocorticoides (Herrera et al. 2018). Entretanto, não foi identificada associação entre os seus haplótipos e a dose de prednisona utilizada no tratamento dos pacientes.

Na análise com talidomida foi encontrada uma associação entre dois haplótipos do gene *TNF* e do polimorfismo *CYP2C19*2* com a variação da dose de talidomida durante o tratamento. A citocina pró-inflamatória TNF-α é um importante mediador no ENH e o efeito da talidomida na reação está associado à sua inibição (Moreira et al. 1993). Os haplótipos que mostraram influência sobre a dose de talidomida possuem polimorfismos associados a maior expressão de TNF-α. Isso confirma o maior estado inflamatório dos pacientes associados a níveis mais altos da citocina e a necessidade de maiores doses de talidomida no tratamento já que ela atua inibindo o TNF-α. Também foi encontrada uma associação entre o *CYP2C19*2* com a dose de talidomida e sua variação durante o tratamento. O gene *CYP2C19* participa do metabolismo da talidomida e sua atividade enzimática polimórfica para formar os metabólitos ativos e/ou

intermediários pode segregar a população de pacientes em subgrupos que diferem em sua capacidade metabólica, os metabolizadores extensos e os metabolizadores lentos (Goldstein 2001; Ando et al. 2002a; Ando et al. 2002b). O metabolizador lento pode exigir doses relativamente altas de talidomida para fins terapêuticos como a associação encontrada com esse polimorfismo. Concluímos, portanto que os polimorfismos dos genes estudados podem influenciar as doses necessárias para o tratamento do ENH com prednisona e talidomida.

Apesar da eficácia terapêutica da talidomida em diversas doenças e de sua reconhecida teratogenicidade, seu mecanismo de ação nunca foi completamente esclarecido. A proteína Cereblon foi identificada como alvo da sua teratogenicidade e reconhecida como necessária para o seu efeito terapêutico no Mieloma Múltiplo (Ito et al. 2010; Lopez-Girona et al. 2012). Desde então diversos estudos têm sido realizados buscando compreender como a interação talidomida/Cereblon pode modular sua resposta terapêutica. Assim, no capítulo VI - artigo 3, nós avaliamos polimorfismos das regiões flanqueadoras da porção do gene *CRBN* associada à ligação com a talidomida. Foi encontrada associação entre polimorfismos desse gene e a dose de talidomida utilizada no tratamento bem como a manifestação de efeitos adversos. Embora esses polimorfismos estejam em regiões não codificantes do gene *CRBN*, não modificando a estrutura da proteína, eles podem afetar a estabilidade do mRNA e os sítios de ligação a microRNAs e fatores de transcrição (Vianna et al. 2016). Isso pode modular a expressão da molécula ou sua atividade e interferir na interação Cereblon/ talidomida e influenciar no efeito terapêutico da droga. Assim, a nossa hipótese é que variantes em *CRBN* também podem modular o efeito terapêutico da talidomida no ENH.

A participação do receptor toll like 9 (TLR-9), um mediador da imunidade inata, no processo inflamatório do ENH foi recentemente descrita. TLR-9 ao reconhecer seus ligantes ativa NF-κB promovendo a produção de citocinas pró-inflamatórias. No capítulo VII – artigo 4, foi analisada a influência de haplótipos do gene *TLR-9* no tratamento do ENH com talidomida e prednisona a partir de dois polimorfismos do gene T-1277C e G2848A. A análise revelou associação de dois haplótipos com as doses de prednisona utilizadas no tratamento do ENH. Esse

haplótipos, CG e TG (rs5743836/rs352140), apresentaram doses médias iniciais de prednisona menores que o haplótipo TA. Também apresentaram diferenças na variação da dose de prednisona. Assim, alelo G do polimorfismo G2848A foi associado com uma resposta reduzida aos glicocorticoides. Não foi encontrada nenhuma associação dos haplótipos com o uso da talidomida ou com a manifestação dos efeitos adversos. A identificação dessa associação *TLR9* e prednisona pode indicar que variantes nesses gene podem influenciar o tratamento do ENH com prednisona, mas sugerimos que essa associação deve ser mais bem caracterizada.

Algumas características desse trabalho não permitem generalizações diretas sobre os resultados aqui identificados e suas aplicações práticas. As informações clínicas dos pacientes, incluindo doses de medicações e manifestação de efeitos adversos, foram obtidas através da análise de prontuários. Portanto, não se pode descartar a obtenção informações inadequadas ou a falta de algumas informações devido a erros no preenchimento dos registros. Outra limitação é que esse estudo foi realizado com amostras de diversas regiões do Brasil, e já se sabe que o background genético é heterogêneo na população brasileira devido à miscigenação, que pode interferir nos resultados. Apesar de algumas análises terem considerado essa heterogeneidade, não se pode descartar esse viés. O mesmo se aplica ao uso da clofazimina e da dapsona na poliquimioterapia para a hanseníase que podem interferir nas doses utilizadas devido à sua ação anti-inflamatória. Outra limitação é o pequeno tamanho amostral que pode interferir na identificação de associação de alelos de pequeno efeito, especialmente em subgrupos da doença. Há também de se considerar que o significado clínico dos achados desse estudo sobre as doses de prednisona e talidomida das associações aqui descritas são ainda limitados e não aplicáveis do ponto de vista clínico, confirmando a complexidade da reação. No entanto, as diferenças encontradas mostram uma perspectiva de futuros estudos que possam caracterizar perfis genéticos de melhor resposta ao tratamento.

A complexidade do ENH como reação inflamatória de difícil controle torna necessária a busca de medidas que possam melhorar o resultado do tratamento seja por novas opções de tratamento ou por formas de predizer como o paciente

pode reagir ao tratamento. Nesse sentido, a farmacogenética pode ser uma ferramenta útil, pois busca tornar os tratamentos individualizados a fim de se obter uma melhor resposta com mais segurança, minimizando os riscos de efeitos adversos. Os resultados obtidos nesse trabalho mostram que o tratamento do ENH com talidomida e prednisona pode ser influenciado por diversos polimorfismos genéticos. Esse é o primeiro trabalho a buscar características genéticas que possam melhorar a determinação do tratamento dos pacientes a fim de melhorar o desfecho clínico, o que é incomum em doenças negligenciadas, mas claramente desejável do ponto de vista populacional. Embora esses resultados ainda sejam incipientes, as análises de variantes em genes envolvidos no metabolismo dos medicamentos utilizados e nos mecanismos patofisiológicos da reação mostram que o uso dessa ferramenta pode ser útil para auxiliar a predizer a evolução do tratamento.

Capítulo IX- Conclusões

9. CONCLUSÕES

Os resultados obtidos nesse trabalho permitiram cumprir os objetivos propostos.

- Realizar uma revisão de literatura sobre os principais tratamentos do ENH.

A revisão de literatura nos permitiu caracterizar os medicamentos disponíveis para o tratamento do ENH. Também identificamos a necessidade de padronização do tratamento no Brasil com um melhor monitoramento desse tratamento, principalmente no que diz respeito à distribuição da talidomida.

- Identificar a associação entre diminuição de dose no tratamento do ENH com talidomida e/ou prednisona e os polimorfismos em *NR3C1, ABCB1*, *TNF, CYP2C19*.
- Associar o perfil de efeitos adversos relacionados ao uso de talidomida e prednisona com a frequência dos polimorfismos nos genes *TNF, CYP2C19*, *CRBN, NR3C1, ABCB1*.

Através da análise dos polimorfismos conseguimos identificar a influência de polimorfismos dos genes *ABCB1*, *TNF* e *CYP2C19* sobre as doses de prednisona e talidomida utilizadas no tratamento do ENH. Não foi identificada influência desses polimorfismos e do polimorfismo *NR3C1* sobre a manifestação de efeitos adversos.

- Avaliar a influência de polimorfismos do gene *CRBN* e o papel de *CRBN* como fator preditor da resposta ao tratamento do ENH com talidomida.

Identificamos a influência dos polimorfismos do *CRBN* sobre o uso da talidomida no ENH. Esse resultado nos mostra que o Cereblon também pode ser necessário para o efeito terapêutico da talidomida no ENH como ocorre no Mieloma Múltiplo.

- Identificar a relação entre polimorfismos do gene *TLR-9* e o tratamento do ENH com talidomida e prednisona.

Identificamos que o polimorfismo G2848A do *TLR-9* influencia a reposta ao tratamento com a prednisona embora isso precise melhor caracterizado.

Capítulo VII - Perspectivas

10. PERSPECTIVAS

Esse trabalho abordou a complexidade do tratamento do ENH frente à realidade brasileira como país onde a hanseníase é endêmica e mostrou a importância da identificação de variantes genéticas que possam auxiliar a predizer a evolução do tratamento. Frente aos resultados encontrados, acreditamos que esse trabalho possa auxiliar a responder algumas perguntas sobre o mecanismo de ação da talidomida e da prednisona no ENH, mas também tenha aberto caminho para a realização de novos estudos sobre a influência genética no tratamento do ENH. As perspectivas de novos estudos envolvem:

1. A análise da expressão de *CRBN* em pacientes de ENH antes e depois do tratamento com talidomida para avaliar o potencial de Cereblon como um biomarcador de resposta a tratamento com talidomida. .

2. Avaliar variantes em outros genes que codificam TLRs que também estejam associados a respostas inflamatórias similares às que ocorrem no ENH ou que já tenham sido associados a respostas na Hanseníase, como por exemplo, *TLR-1*, *TLR-2*, *TLR-4* e *TLR-7*.

3. Analisar genes associados à angiogênese que também podem participar da resposta ao tratamento com talidomida.

4. Analisar genes associados ao mecanismo de apoptose que podem estar associados à resposta ao tratamento do ENH com prednisona.

5. Avaliar a expressão de microRNAs durante o tratamento do ENH para a identificação de biomarcadores da resposta ao tratamento com a talidomida e prednisona.

Capítulo XI - Referências Bibliográficas

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Capítulo XII - Anexos

12. ANEXOS

12.1 ANEXO 1

ESTUDO FARMACOGENÉTICO DA RESPOSTA AO TRATAMENTO DO ERITEMA NODOSO HANSÊNICO

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO PARA PESSOAS COM ERITEMA NODOSO DA HANSÊNICO

Justificativa e objetivo da pesquisa

O eritema nodoso hansênico (ENH), ou reação tipo 2, é uma reação de difícil controle que ocorre em alguns pacientes com hanseníase, e caracteriza-se por diversos sintomas, como mal estar geral, febre, dor, nódulos e espessamento dos nervos. O tratamento é baseado no uso de talidomida e prednisona, entretanto, ambos mostram uma grande quantidade de efeitos colaterais que tornam difícil a vida do pacientes e prejudicam a continuidade do tratamento, além de muitas vezes não solucionarem os sintomas.

Nosso objetivo nesse trabalho é analisar algumas partes do material genético (DNA) que estão envolvidas no metabolismo desses medicamentos e avaliar a existência de uma possível relação entre essas variantes genéticas e a resposta ao tratamento da reação com os medicamentos utilizados para tratar essa doença.

Procedimentos que serão realizados

Será coletada amostra de saliva. A amostra será estudada para a análise genética conforme mencionado acima. As amostras serão armazenadas e analisadas no Departamento de Genética da Universidade Federal do Rio Grande do Sul, campus do Vale, sob a responsabilidade da Prof. Dra. Fernanda Sales Luiz Vianna e a Profa. Dra. Lavínia Schüler-Faccini; elas serão utilizadas somente para o que foi disposto neste estudo.

Riscos, desconfortos potenciais e instruções de coleta

Não é esperado nenhum desconforto maior durante a coleta. Entretanto, não é permitido comer, beber, mastigar pastilhas ou fumar por 30 minutos antes da coleta. A maioria das pessoas demora de 2-5 minutos para produzir a quantidade necessária saliva. Alguns indivíduos podem ter dificuldades na produção de saliva, e nesse caso, devem colocar na boca ¼ de colher de sobremesa de açúcar na boca antes da coleta. A coleta de saliva não deve exceder o período de 30 minutos.

Benefícios Esperados

Este estudo poderá no futuro beneficiar o pacientes através da escolha do tratamento ajustada ao perfil genético do paciente. Sabemos, entretanto, que outros fatores além dos genéticos influenciam na resposta ao tratamento do ENH e efeitos colaterais.

Procedimentos Alternativos

Eu entendo que tive o direito de recusar a participar dessa pesquisa e que minha recusa não afetará de nenhuma maneira meus cuidados médicos em relação ao ENH.

Pelo presente Consentimento, declaro que fui esclarecido, de forma detalhada, livre de qualquer forma de constrangimento e coerção, dos objetivos, da justificativa, dos procedimentos que serei submetido, dos riscos, desconfortos e benefícios da presente pesquisa, assim como dos procedimentos alternativos aos quais poderia ser submetido, todos acima listados.

Fui igualmente informado:

- Da garantia de receber esclarecimento a qualquer dúvida acerca dos procedimentos, riscos, benefícios e outros assuntos relacionados a pesquisa;
- Da liberdade de retirar meu consentimento, a qualquer momento, e deixar de participar do estudo, sem que isso traga prejuízo à continuação do meu cuidado e tratamento;
- Da segurança de que não serei identificado e que se manterá o caráter confidencial das informações relacionadas com minha privacidade;

Os pesquisadores responsáveis por esse projeto de pesquisa são Pesquisadora Dra. Fernanda Sales Luiz Vianna e a Profa. Dra. Lavínia Schüler-Faccini (51 33089826), tendo sido este documento revisado e aprovado pelo Comitê de Ética da Secretaria Municipal de Saúde de Porto Alegre (5132895517)

Data ___/___/___

Nome do paciente:

Assinatura do Paciente

Pesquisadores responsáveis

Fernanda Sales Luiz Vianna / Lavínia Schüler-Faccini