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MARCADORES SOROLÓGICOS, GENÉTICOS E MOLECULARES NA HANSENÍASE: SUPORTE AO DIAGNÓSTICO CLÍNICO DE PACIENTES E VIGILÂNCIA DOS CONTATOS

ANDRÉ LUIZ LETURIONDO

MANAUS
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Tese apresentada ao Programa de Pós-Graduação em Medicina Tropical da Universidade do Estado do Amazonas, em convênio com a Fundação de Medicina Tropical Dr. Heitor Vieira Dourado**,** para obtenção do grau de *Doutor em Doenças Tropicais e Infecciosas.*

Orientadora: Profª Dra Carolina Chrusciak Talhari Cortez

Coorientador: Profº Dr Milton Ozório Moraes

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ANDRÉ LUIZ LETURIONDO

"Esta Tese foi julgada adequada para obtenção do Título de Doutor em Doenças Tropicais e Infecciosas, aprovada em sua forma final pelo Programa de Pós-Graduação em Medicina Tropical da Universidade do Estado do Amazonas em convênio com a Fundação de Medicina Tropical Dr. Heitor Vieira Dourado".

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_____

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_____

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Membro

DEDICATÓRIA

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O CONSELHO DIRETOR da FUNDAÇÃO DE AMPARO À PESQUISA DO ESTADO DO AMAZONAS, em reunião realizada em Manaus, no dia 26 de novembro de 2013, decidiu **HOMOLOGAR** o resultado da análise e julgamento das propostas submetidas à Chamada Pública FAPEAM/SUSAM-SES-AM/MS/CNPq nº001/2013, referente ao **Programa Pesquisa para o SUS: Gestão Compartilhada em Saúde - PPSUS**, recomendadas pela Comissão de Especialistas e pelo Comitê Gestor do Programa supra (ANEXO D).

RESUMO

A detecção precoce da hanseníase e o tratamento imediato com a poliquimioterapia são cruciais para alcançar as metas de zero transmissão e zero de incapacidades de grau II, da Organização Mundial da Saúde. A hanseníase é difícil de diagnosticar devido à variedade de formas clínicas e à falta de um teste padrão-ouro capaz de auxiliar o diagnóstico clínico. O conhecimento do perfil de resposta imunológica do hospedeiro e a identificação de variantes genéticas de suscetibilidade à hanseníase, poderão subsidiar um melhor entendimento de sua patogênese e talvez facilitar o diagnóstico precoce, tratamento e profilaxia. Nesse trabalho, foi avaliado a performance de dois testes sorológicos rápidos, (PGL1 e NDO-LID), na discriminação de casos de hanseníase e de indivíduos saudáveis, na Fundação Alfredo da Matta. Posteriormente, foi conduzido estudo caso-controle, em que foram testados dez SNPs, recentemente associados à hanseníase, em genes que regulam a resposta imune: *TLR1* (rs4833095), *NOD2* (rs751271, rs8057341), *TNF* (rs1800629), *IL10* (rs1800871), *CCDC122/LACC1* (rs4942254), *PACRG/PRKN* (rs9356058, rs1040079), *IFNG* (rs2430561), e *IL6* (rs2069845). A amostra populacional foi 1.379 indivíduos. Para o estudo sorológico, dentre os pacientes paucibacilares, a sensibilidade foi 34,0% e 32,0% para o NDO-LID e PGL1, respectivamente. Nos pacientes multibacilares, a sensibilidade do NDO-LID foi 73,6% e 81,0% para PGL1. Os testes sorológicos demonstraram especificidade de 75,9% para o PGL1 e 81,7% para o NDO-LID. O valor preditivo positivo (VPP), o valor preditivo negativo (VPN) e a acurácia nos pacientes multibacilares foram 47,9%, 93,1% e 80,2% respectivamente para o NDO-LID, e 43,4%, 94,6% e 76,8% para PGL1. Os testes sorológicos rápidos mostraram limitada capacidade no diagnóstico da hanseníase, contudo, o alto valor preditivo negativo dos testes indica grande probabilidade de exclusão da doença. No estudo genético, resultados estatisticamente significativos foram encontrados somente para as variantes dos genes *IFNG* e *NOD2*. O polimorfismo rs8057341 do gene *NOD2* foi estatisticamente significativo à proteção para o genótipo AA (OR = 0,56; 95% IC, 0,37–0,84; P =0,005) e limítrofe para o alelo A (OR = 0,76; 95% IC, 0,58–1,00; P = 0,053) e carreador (OR = 0,76; 95% IC, 0,58–1,00; P = 0,051). O SNP rs2430561 do gene *IFNG* foi associado com a suscetibilidade à doença para o genótipo AT (OR = 1,40; 95% IC, 1,06–1,85; P = 0,018) e carreador (OR = 1,44; 95% IC, 1,10–1,88; P = 0,008). Dessa forma, foi possível confirmar, em população geneticamente miscigenada do estado do Amazonas, forte associação de polimorfismos nos genes *NOD2* e *IFNG*. Perspectivas de novas variantes, associadas a importante efeito no fenótipo, podem ser identificadas, com a evolução da tecnologia de sequenciamento de genoma e de estudos funcionais, tanto para hanseníase como outras doenças infecciosas, ou inflamatórias.

Palavras-Chaves: Hanseníase, testes rápidos, Polimorfismos, Amazonas

ABSTRACT

Early detection of leprosy and prompt treatment with multidrug therapy are crucial to achieving the World Health Organization's goals of zero transmission and zero grade II disabilities. Leprosy is difficult to diagnose due to the variety of clinical forms and the lack of a gold standard test. Knowledge of the host's immune response profile and the identification of genetic variants of susceptibility to leprosy may support a better understanding of its pathogenesis and perhaps facilitate early diagnosis, treatment and prophylaxis. In this study, the performance of two rapid serological tests (PGL1 and NDO-LID) was evaluated in the discrimination of leprosy cases and healthy individuals at the Alfredo da Matta Foundation. A case-control study was further conducted and ten SNPs previously associated with leprosy susceptibility were tested: *TLR1* (rs4833095), *NOD2* (rs751271, rs8057341), *TNF* (rs1800629), *IL10* (rs1800871), *CCDC122/LACC1* (rs4942254), *PACRG/PRKN* (rs9356058, rs1040079), *IFNG* (rs2430561), and *IL6* (rs2069845). Population sample was 1,379 individuals. For the serological study, among paucibacillary leprosy patients, sensitivity was 34.0% and 32.0% for NDO-LID and PGL1, respectively. In multibacillary leprosy patients, NDO-LID sensitivity was 73.6% and 81.0% for PGL1. Serological tests demonstrated a specificity of 75.9% for PGL-1 and 81.7% for NDO-LID. The positive predictive value (PPV), negative predictive value (NPV) and accuracy in multibacillary patients were 47.9%, 93.1%, and 80.2% respectively for NDO-LID, and 43.4%, 94.6% and 76.8% for PGL1. Rapid serological tests showed limited ability to diagnose the disease; however, the high negative predictive value of the tests indicates a high probability of excluding leprosy. In the genetic study, statistically significant results were found only for the variants of the *IFNG* and *NOD2* genes. The rs8057341 polymorphism of the *NOD2* gene was statistically significant for protection for the AA genotype (OR = 0.56; 95% CI, 0.37–0.84; P =0.005) and borderline for the A allele (OR = 0.76; 95% CI, 0.58–1.00; P = 0.053) and carrier (OR = 0.76; 95% CI, 0.58–1.00; P = 0.051). The SNP rs2430561 of the *IFNG* gene was associated with disease susceptibility to the AT genotype (OR = 1.40; 95% CI, 1.06–1.85; P = 0.018) and carrier (OR = 1.44; 95% CI, 1.10–1.88; P = 0.008). In this way, it was possible to confirm a strong association of polymorphisms in the NOD2 and IFNG genes in a genetically mixed population in the state of Amazonas. Perspectives for new gene variants, possibly associated with a major effect on the disease phenotype (leprosy, other infectious or inflammatory diseases) could be identified upon the development of new genome sequencing technologies and functional studies.

Keywords: Leprosy, rapid tests, polymorphisms, Amazonas

RESUMO LEIGO

A hanseníase é uma doença milenar que ainda está presente na nossa sociedade, podendo levar a deformidades físicas e consequente estigmatização. Apesar da queda brusca após a implantação da poliquimioterapia no início da década de 1980, o número de casos tem se mantido praticamente constante nos últimos 10 anos, tanto no nível mundial como nacional. Por tratar-se de uma doença de amplo espectro clínico, muitas vezes o diagnóstico é difícil até mesmo para profissionais experientes. Até o momento não se tem uma ferramenta laboratorial de auxílio ao diagnóstico clínico, ou um marcador capaz de diferenciar exposição, infecção e doença. Recentes estudos têm tentado caracterizar biomarcadores, em diferentes populações, capazes de indicar prováveis candidatos ao adoecimento (entre os contatos, por exemplo) ou no diagnóstico precoce da hanseníase. Nosso estudo de replicação também seguiu essa mesma linha de colaboração, através da caracterização de biomarcadores sorológicos e genéticos na nossa população. Infelizmente, a utilização de testes sorológicos na identificação de doentes, com anticorpo anti-PGL1 positivo, não foi satisfatório. Porém, um resultado anti-PGL1 negativo tem uma grande probabilidade de afastar um suspeito de ter a doença. Essa característica do teste é importante, especialmente em uma região endêmica para a doença e com outras dermatoses semelhantes e confundidoras. No estudo genético, avaliamos dez polimorfismos de oito genes envolvidos com a resposta imune do hospedeiro, para verificar se a sua presença está associada com a hanseníase. Encontramos associação de dois polimorfismos em dois genes (*NOD2* e *IFNG*) envolvidos com proteção e risco, respectivamente. Por tratar-se de uma doença complexa, mais marcadores genéticos necessitarão ser avaliados, com a finalidade de compor um painel de marcadores genéticos que possa inferir maior risco de adoecimento no grupo mais vulnerável.

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LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES DE MEDIDA

ApoB	Apoproteína B
ASC	Análise de segregação complexa
ATG	Autophagy-related Genes
BAAR	Bacilo álcool-ácido resistente
BCG	Bacilo de Calmette-Guerin
CEP	Comitê de ética em pesquisa
DAMPs	damage-associated molecular patterns
DD	Dimorfa-Dimorfa
DV	Dimorfa-Virchowiana
Cts	Cycle threshold
ENH	Eritema Nodoso Hansênico
FUAM	Fundação Alfredo da Matta
GM-CSF	granulocyte-macrophage colony-stimulating factor
GWAS	Genome-wide association study
GWLS	Genome wide linkage studies
HBHA	Heparin-binding hemagglutinin
I	Indeterminada
IB	Índice baciloscópico
IDRI	Infectious Disease Research Institute
IFNG	Gene Interferon-gama
IL-6	Interleucina 6
IL-10	Interleucina 10
LAM	Lipoarabinomanana
LD	linkage disequilibrium
LID-1	Proteína de fusão recombinante para diagnóstico da hanseníase (proteínas de fusão ML0304 e ML2331)
LILRA2	leukocyte immunoglobulin-like receptor subfamily A member 2
LRRs	leucine-rich repeats
LTA	Linfotoxina alfa
MARCO	macrophage receptor with collagenous structure
Mb	Megabase
MB	Multibacilar
MDP	muramyl dipeptide
MHC	major histocompatibility complex
mL	Mililitro
ML Flow Test	Teste de fluxo lateral para M. leprae
NDO-LID	Conjugado de ND-O (dissacarídeo sintético mimético do PGL-1) e proteína LID-1 (proteínas de fusão ML0304 e ML2331)
NF-κB	factor nuclear-kappa B
NOD2	nucleotide-binding and oligomerization domain 2
OR	Odds ratio
PACRG	parkin coregulated
PAMPs	pathogen-associated molecular patterns
pb	pares de base
PCR	Polymerase chain reaction
PRKN	parkin RBR E3 ubiquitin protein ligase
PB	Paucibacilar
PEP	Post-Exposure Prophylaxis

PGL-1	phenolic glycolipid-1
PQT	Poliquimioterapia
PRRs	pattern recognition receptors
qPCR	quantitative real time PCR
RIG-1	retinoic-acid inducible gene I
RLEP	M. leprae-specific repetitive element
ROS	reactive oxygen species
RR	Reação Reversa
SINAN	Sistema de Informação sobre Agravos Notificação
SNPs	Single nucleotide polymorphism
SR-A	Scavenger receptors class A
T1R	Reação tipo 1
T2R	Reação tipo 2
TCLE	Termo de consentimento livre e esclarecido
TGF-β	Transforming growth factor beta
Th1	T helper 1
Th2	T helper 2
TLRs	Toll-like receptors
TNF	Tumor Necrosis Factor
Treg	Células T regulatórias
TT	Tuberculóide-tuberculóide
µL	microlitro
VDR	receptor de vitamina D
WHO	World Health Organization

SUMÁRIO

1 INTRODUÇÃO

A hanseníase é uma das enfermidades mais antigas da humanidade, com relatos registrados no antigo Egito, China e Índia, há aproximadamente três a quatro mil anos a.C., e com evidências ósseas sugestivas da doença, datadas de 2.000 a.C, na Índia(1). No Brasil, a doença chegou com o processo de colonização portuguesa, tendo os primeiros relatos descritos por volta do ano 1600. No estado do Amazonas, os registros iniciais são de 1854(2).

É uma doença granulomatosa crônica causada pelo *Mycobacterium leprae* (*M. leprae*), na maioria dos casos, e pelo *Mycobacterium lepromatosis* (*M. lepromatosis*), afetando principalmente pele e nervos periféricos. O diagnóstico e o tratamento precoce são importantes para interromper o ciclo de transmissão e evitar as incapacidades físicas e as deformidades(3), que levam à estigmatização e à discriminação.

O *M. leprae* apresenta alta infectividade e baixa patogenicidade, ou seja, muitos indivíduos são infectados pelo bacilo, mas poucos adoecem(4). Isso ocorre, em parte, devido a fatores socioeconômicos(5) e baixa variabilidade genética do microorganismo(6). Estudos recentes indicam que a condição genética do hospedeiro é um dos fatores mais relevantes na suscetibilidade à hanseníase e nas diferentes manifestações clínicas da doença(7).

Com a implantação da poliquimioterapia (PQT), há quatro décadas, houve, no mundo, redução significativa na prevalência e incidência da hanseníase. Mas, apesar dos esforços dos países onde a hanseníase é endêmica, e da Organização Mundial de Saúde (OMS) para melhorar a qualidade dos programas de controle da doença, a taxa de detecção de novos casos tem apresentado lenta redução nos últimos anos. Em 2011, 219.075 novos casos de hanseníase foram diagnosticados no mundo e em 2018, 208.641(8). A contínua transmissão ocorre, em grande parte, por meio de pacientes multibacilares ainda não-tratados(9).

Vários países endêmicos conseguiram atingir a meta estipulada pela OMS para a eliminação da doença como problema de saúde pública (menos de um caso para 10.000 habitantes). No entanto, em 2018, 127 países relataram casos novos(8). Houve aumento no número de casos com grau 2 de incapacidade física (GIF2) entre

2017 e 2018 em países como o Brasil, Angola, Moçambique e Nepal. Somente o Brasil detectou 2.109 casos, com GIF2, em 2018.

O diagnóstico clínico da hanseníase, em muitos casos, constitui grande desafio. As dificuldades encontradas ocorrem, não somente devido à falta de profissionais experientes no diagnóstico da hanseníase, mas também devido as características específicas da doença. As formas neurais puras, lesões de hanseníase indeterminada e lesões únicas em pacientes paucibacilares (PB) podem ser de difícil resolução clínica e facilmente confundidas com outras dermatoses comuns. Dermatoses como granuloma anular, sarcoidose, tuberculose cutânea, sífilis secundária, lúpus eritematoso sistêmico e pitiríase alba são alguns dos possíveis diagnósticos diferenciais(10). Aproximadamente 30% dos pacientes, muitos deles multibacilares (MB), não apresentam os sinais característicos da hanseníase, como por exemplo a perda de sensibilidade, o que dificulta o diagnóstico e favorece a transmissão da doença(11).

Campanhas estaduais ou municipais, com o objetivo de identificar novos casos de hanseníase, são importantes medidas de políticas públicas para reduzir a incidência da doença. Quando detectados em jovens, abaixo de 15 anos, pode-se fazer estimativas de início de transmissão e indicar a existência de um foco ativo na comunidade(12,13). A Fundação Alfredo da Matta, nesses 65 anos de história, tem enfrentado a hanseníase a partir de ações de assistência, vigilância e pesquisa. No recente estudo de Pedrosa e colaboradores(13), quando 34.547 crianças de escolas públicas, de Manaus, foram examinadas, 40 novos casos de hanseníase foram detectados. Esse achado revelou uma taxa de detecção, 17 vezes superior a taxa oficial. Na busca de uma possível fonte dessa infecção, mais sete novos casos de hanseníase, em contatos domiciliares, foram descobertos.

A OMS preconiza a vigilância dos contatos de pacientes com hanseníase pois essas pessoas apresentam risco de adoecimento maior do que a população geral. Quando essa estratégia é bem executada, ocorre aumento das taxas de detecção das formas clínicas menos graves(14). Uma atenção maior deve ser dada aos contatos domiciliares de pacientes multibacilares, que possuem um risco de 5 a 10 maior de adoecimento, do que a população geral (15,16) e incidência de, aproximadamente, 60% maior do que em contatos de PB (17) . Entre os contatos

domiciliares que adoecem, a maior parte deles, ocorre nos seis primeiros anos do caso primário, com o pico acontecendo no primeiro ano(17). Na coorte, realizado por Teixeira e colaboradores(17), 42.725 contatos domiciliares de pacientes com hanseníase, foram acompanhados no período de janeiro de 2007 a dezembro de 2014, utilizando informações coletadas da plataforma “Coorte de 100 milhões de brasileiros”(18) e correlacionadas com o Sistema de Informação de Agravos de Notificação (SINAN – Hanseníase)(12). Adoeceram, neste intervalo, 829 contatos (1,9%). E a taxa de incidência, com esse grupo de risco, foi superior em 50 vezes, quanto em comparação com a população geral do Brasil em 2017. Os principais fatores de risco associados à doença, destacados nesse estudo foram, a idade acima dos 50 anos e os contatos de pacientes que apresentam a forma multibacilar, grau II de incapacidade física ou reações do tipo 1 ou 2(17).

Identificar pacientes com infecção subclínica e/ou os prováveis contatos que tenham predisposição genética para adoecer, através de marcadores biológicos, pode constituir estratégia interessante no controle da hanseníase. No entanto, até o momento, não existe teste laboratorial padrão-ouro, capaz de detectar o *M. leprae* ou seus componentes celulares e que diferencie exposição, infecção e doença. Esse fato dificulta a intervenção precoce e consequente interrupção da cadeia de transmissão.

A dificuldade de identificar marcadores específicos de resposta imune celular e humoral consiste no principal impedimento para o desenvolvimento de testes que diferenciem infecção e doença. Além disso, face a baixa incidência da doença e ao longo tempo de incubação do bacilo, seriam necessários longos estudos longitudinais, os quais são, geralmente, de difícil execução.

Mas com os recentes avanços da imunogenética do hospedeiro, e o conhecimento do genoma do bacilo, novos testes diagnósticos estão sendo testados e replicados em diferentes populações.

Os testes imunológicos atualmente utilizados no auxílio ao diagnóstico da hanseníase são baseados na detecção de componentes específicos do *M. leprae* e do perfil de resposta do hospedeiro, como, na mensuração de anticorpos, de citocinas, quimiocinas ou o tipo resposta imune desenvolvido. Dentre esses testes, destacam-se: - a detecção do anticorpo anti-PGL-1, com o teste de fluxo lateral (*ML*

flow test)(19); - os níveis de citocinas e quimiocinas IP-10, IL-10 e CCL4 e anticorpos anti-PGL1, pelo teste de fluxo lateral (LFAs)(20); - a detecção de anticorpos mono ou policlonais de proteínas únicas do bacilo(21,22); - a mensuração dos níveis de produção de IFN-γ pelas células T, como o IGRA (*IFN-gamma releasing assays*)(23); - ou o uso de uma suspensão salina de antígenos solúveis do *M. leprae*, lepromina ou leprosina, com inoculação intradérmica(24). O uso desse teste intradérmico, quando positivo, indica que o indivíduo apresenta boa resposta imune celular. Apesar de não ser usado como diagnóstico para *M. leprae*, pelo fato de apresentar reação cruzada com antígenos de outras micobactérias, pode ser usado para classificação operacional da hanseníase(24).

Os testes sorológicos baseados da detecção do PGL1 ou nas suas formas sintéticas, tanto com a metodologia ELISA, quanto com o teste rápido, ainda são importantes ferramentas de auxílio ao diagnóstico clínico, principalmente para a classificação operacional(25). Esses testes também podem auxiliar no monitoramento da eficácia do tratamento da hanseníase(26). Além disso, os testes rápidos têm baixo custo quando comparados com os testes moleculares, são de fácil execução e não necessitam de equipamento especial ou refrigeração, podendo ser utilizado em campo(27,28).

A identificação da sequência genômica do *M. leprae* possibilitou a realização de testes como a reação em cadeia da polimerase (PCR) tanto na forma convencional(29,30), quanto a versão em tempo real (q-PCR)(30–32). Com esta técnica é possível detectar e quantificar concentrações baixas de DNA de *M. leprae*, aproximadamente de 30 fg (fentogramas), que é o equivalente ao DNA de 8,3 bacilos (33). Diferentes amostras clínicas como biópsias de lesões cutâneas, raspado dérmico, sangue, saliva, urina e da mucosa nasal podem ser utilizados como substrato para a realização da PCR. Esta técnica tem sido empregada nos grandes centros de referência, em estudos científicos e/ou como rotina laboratorial, como uma alternativa mais eficiente as metodologias tradicionais de auxílio ao diagnóstico clínico, como a baciloscopia e a histopatologia, por exemplo.

A PCR também tem sido útil na identificação de cepas resistentes as drogas administradas no tratamento, no acompanhamento de contatos com a identificação de bacilos viáveis, no diagnóstico diferencial da hanseníase com outras dermatoses,

na forma neural pura, e em pacientes com lesões atípicas e/ou histopatologia inconclusiva para hanseníase (34).

Alguns trabalhos, do tipo caso-controle, estudaram a distribuição da frequência de marcadores genéticos, como polimorfismos de base única ou SNPs (*Single Nucleotide Polymorphism*), em pacientes em comparação com indivíduos saudáveis. Assim, foi possível avaliar SNPs que conferem uma maior suscetibilidade de adoecimento(35,36). De fato, o fator genético do hospedeiro é apontado com uma das causas de desenvolvimento de doenças infecciosas, há bastante tempo, em estudos de segregação, de famílias e de gêmeos(37).

Estudos de caso-controle têm revelado importante associação de polimorfismos em genes candidatos, associando SNPs com a suscetibilidade à doença e/ou aos episódios reacionais. Dentre esses estudos, pode-se destacar SNPs nos genes: *HLA*, *VDR* (receptores da vitamina D), *TNF*, *TLR1*, *IL10*, *IFNG*, *NOD2*, *PACRG/PRKN* (anteriormente *PARK2*)(35,36,38–40).

1.1 Biologia do *Mycobacterium leprae*

A hanseníase é uma doença infecciosa crônica causada, principalmente, pelo *Mycobacterium leprae,* um bacilo intracelular obrigatório, que tem tropismo pelas células de Schwann, nos nervos periféricos, e pelos macrófagos, na pele(41). Também o bacilo pode causar manifestações oculares, otorrinolaringológicas, musculares, ósseas, nos testículos e vísceras abdominais(10,48,49) O termo hanseníase foi adotado no Brasil para diminuir o estigma associado com a doença e recebeu este nome em homenagem ao médico norueguês, Gerhard Armauer Hansen, que em 1873, identificou o bacilo como causador da doença.

Mais recentemente, em 2008, uma segunda espécie causadora da doença, o *Mycobacterium lepromatosis* (*M. lepromatosis*), com capacidade, também, de infectar nervos, foi identificado em duas amostras de pacientes do México, que morreram de hanseníase virchowiana difusa(42). Em seguida, em 2009, em um outro estudo, também liderado por Xiang Y. Han, realizaram o sequenciamento em 15 genes e 5 pseudo-genes, e revelaram que o *M. lepromatosis* é 90%

geneticamente idêntico ao *M. leprae*, e cuja diferença foi suficiente para caracterizá-lo como uma nova espécie(43). Desde então, essa nova espécie, já foi encontrada em vários pacientes do México(44), e em alguns casos individuais de Singapura(44) e Canadá(45). O sequenciamanento completo, do *M. lepromatosis*, ocorreu em 2015, com Singh e colaboradores(46). Recentemente, essa nova espécie foi identificada, pela primeira vez, em esquilos vermelhos, localizados na Escócia, apresentando lesões semelhantes à hanseníase(47).

O bacilo é levemente curvado, medindo de 1,5 a oito microns de comprimento e 0,2 a 0,5 micron de diâmetro. É um bacilo álcool ácido resistente devido ao fato de não descolorir a fucsina de Ziehl-Neelsen, a cor vermelha impregnada no bacilo, durante as etapas de lavagem com álcool ou ácido(50). Os bacilos podem apresentar-se em arranjos especiais, denominados de globias, isolados ou em agrupamentos variados, em esfregaços dérmicos ou em cortes histológicos.

O *M. leprae* não cresce em meios artificiais e a reprodução desse bacilo ocorre por fissão binária, com crescimento lento (entre 12 a 14 dias) em pata de camundongo(50). A temperatura ideal para a viabilidade e proliferação do *M. leprae* é entre 27 a 30^0C, explicando com isso, a predileção da bactéria por áreas mais frias do corpo humano, como lóbulos auriculares, pele, nervos periféricos e testículos(50). No meio ambiente, a viabilidade do bacilo é de até nove dias(51).

O exato mecanismo de transmissão do *M. leprae* ainda é desconhecido(52), mas é provável que o contágio inter-humano ocorra pelas vias aéreas superiores, com bacilos transportados pelo ar, em gotículas de aerossóis, por meio de um contato próximo e prolongado de pessoas doentes para indivíduos suscetíveis(10). Essa hipótese é validada por estudos que demostraram que adesinas, como a HBHA (*Heparin-binding hemagglutinin*), presente na superfície do bacilo, podem se fixar nas células epiteliais alveolares e nasais, mantendo o *M. leprae* viável(53,54).

A maioria dos indivíduos tem resistência natural ao bacilo(55) e poucas pessoas infectadas desenvolverão a doença (entre 1 a 5%). Os sinais clínicos geralmente aparecem entre dois a seis anos e, algumas vezes, em até 20 anos(56). Portadores sadios e indivíduos com infecção subclínica também podem transmitir o bacilo, principalmente para contatos próximos(57,58).

Alguns animais podem ser reservatórios naturais do *M. leprae* ou *M. lepromatosis*, como os tatus selvagens e os esquilos, podendo apresentar lesões características de hanseníase. Os primeiros casos relatados de *M. leprae* foram em tatus de vida selvagem (*Dasypus novemcinctus*), nos Estados Unidos, e, posteriormente, em diferentes espécies de tatus, em países da América Latina, como México, Brasil, Argentina e Colômbia(59).

Estudos recentes revelaram que os esquilos vermelhos (*Sciurus vulgaris*), no Reino Unido, estavam infectados com o *M. leprae* ou *M. lepromatosis.* Esses animais mostravam ainda características clínicas e laboratoriais compatíveis com a doença(47).

A presença de ácido ribonucleico (RNA) do *M. leprae*, que indica bacilo viável, foi demonstrada no solo e na água de áreas endêmicas de países como o Brasil e Índia. A identificação de *M. leprae,* viável por dias ou semanas, em amebas de vida livre sugere a possibilidade desses microorganimos serem potenciais vetores da transmissão do bacilo para os humamos(59). Essas potenciais vias de transmissão do *M. leprae* permanecem controversas.

O sequenciamento do genoma completo do *Mycobacterium leprae* foi realizado em 2001, por Cole e colaboradores(60). Não houve alteração significativa da arquitetura genômica do DNA do *M. leprae* quando comparado ao identificado em corpos preservados, dos séculos 12 e 13, em países europeus(6).

A comparação do genoma circular do *M. leprae,* com 3.268.203 pares de bases (pb), com o do *Mycobacterium tuberculosis* (4.411.532 pb), revelou extensa deleção de nucleotídeos durante a evolução. Houve consequente perda de importantes vias metabólicas e condições específicas de crescimento, tornando *M. leprae* um microorganismo intracelular obrigatório(60).

De acordo com o sequenciamento completo do genoma do *M. leprae*, 49,5% dos genes codificam proteínas; no *M. tuberculosis* são 90,8% dos genes. Dentre os 1604 genes que codificam proteínas, no *M. leprae*, 1439 são genes comuns para o *M. leprae* e *M. tuberculosis.* O conhecimento do genoma do bacilo foi importante para a identificação de proteínas específicas do *M. leprae*, as quais poderão,

eventualmente, servir para o desenvolvimento de testes imunológicos, vacinas e exames moleculares mais sensíveis e específicos.

1.2 Tratamento

O tratamento da hanseníase é gratuito e fornecido pelo Sistema Único de Saúde (SUS). É realizado em regime ambulatorial, independente da forma clínica, e preferencialmente nos serviços de atenção primária à saúde. No caso de intercorrências clínicas ou cirúrgicas, relacionadas ou não com a hanseníase, o atendimento é feito em um serviço especializado ambulatorial, se possível em centros de referência para a doença.

O histórico do tratamento da hanseníase começou no iníco da década de 1940, utilizando somente a dapsona como droga terapêutica. Mas a utilização da monoterapia, por longo período, desencadeou uma resistência crescente do patógeno. Essa constatação levou a Organização Mundial da Saúde a recomendar o uso do regime de poliquimioterapia (PQT), em 1981, com a utilização das drogas, dapsona, rifampicina e clofazimina. No Brasil, a poliquimioterapia foi estabelecida, oficialmente, somente em 1993(61).

O diagnóstico da hanseníase é clínico e epidemiológico, baseado na presença de pelo menos um dos três sinais cardinais da doença: 1- Alteração de sensibilidade de lesões infiltradas e avermelhadas e/ou de manchas hipocrômicas ou eritematosas; 2- Espessamento de nervos periféricos com associação de alterações motoras e/ou sensitivas e/ou autonômicas; 3- Presença do bacilo álcool-ácido resistente (BAAR) no raspado dérmico(62).

Nos locais onde a baciloscopia e a histopatologia são realizados como rotina, utiliza-se a classificação de Ridley & Jopling(63), a qual caracteriza o paciente cinco formas: tuberculoide-tuberculoide (TT), *bordeline*-tuberculoide (BT), *borderline-borderline* (BB), *borderline*-virchowiana (BV) e virchowiana-virchowiana (VV).

Em lugares onde a baciloscopia e/ou exame histopatológico não estão disponíveis, a OMS preconiza a utilização de parâmetros mais simples para classificar os pacientes, em: paucibacilares (PB) e multibacilares (MB). Essa

classificação é baseada no número de lesões, nervos comprometidos e a baciloscopia(64).

O Ministério da Saúde do Brasil, por meio da portaria 3.125, de 2010(65), estabeleceu a padronização terapêutica baseada na classificação operacional da doença (PB ou MB). O tratamento é realizado com a combinação de duas drogas, dapsona e rifampicina, durante 6 meses, para os pacientes paucibacilares. Nos multibacilares, o tratamento é realizado com a combinação de dapsona, rifampicina e clofazimina, durante 12 meses.

São considerados pacientes PB, os que apresentarem, no máximo, cinco lesões, baciloscopia negativa e nenhum envolvimento de nervos ou somente de um filete nervoso. Os pacientes MB são aqueles que apresentam mais de cinco lesões, baciloscopia positiva e têm mais de um nervo comprometido(65). Após um mês de tratamento, os pacientes tornam-se não infecciosos.

A estratégia para a prevenção da hanseníase, denominada de profilaxia pós-exposição, como a imunoprofilaxia com a vacina BCG (Bacilo de Calmette-Guerin) e/ou a quimioprofilaxia, no grupo de risco, tem sido estudada(66). O estudo COLEP (*Contact Leprosy Patient*)(67), realizado em região endêmica de Bangladesh, arrolou aproximadamente 19 mil contatos e os acompanhou por quatro anos. Esse estudo mostrou redução, estatisticamente significativa, somente nos primeiros dois anos, de 57% no número de casos de hanseníase no grupo que utilizou a rifampicina em dose única em relação ao grupo que recebeu apenas placebo.

Outra grande coorte, também com a população de Bangladesh, acompanhou contatos de pacientes com hanseníase que receberam e não receberam a profilaxia com rifampicina, por dois anos (68). Nesse estudo não houve diferença estatística entre os dois grupos de contatos. O uso da rifampicina, como quimioprofilaxia para a hanseníase, bem como re-vacinação com BCG, permanece controverso (66). Importante salientar, que, qualquer esquema de quimioprofilaxia deve ser executada somente por programas que tenham um amplo controle dos contatos, e desde que o seu caso índice permita a divulgação de seu nome. O esquema da quimioprofilaxia deve ser seguro para o indivíduo, eficaz, não ter grandes efeitos colaterais e não induzir resistência medicamentosa na hanseníase e tuberculose(69).

1.3 Imunopatologia da hanseníase

O amplo espectro das formas clínicas na hanseníase e suas reações hansênicas, oferecem oportunidade de investigar a complexa rede de comunicação entre os variados fenótipos celulares e citocinas envolvidos durante a resposta imune inata e adaptativa do hospedeiro.

O início da resposta imune começa quando um indivíduo passa de exposto, a um determinado patógeno, para infectado. Inicia-se assim, uma série de eventos imunológicos que visam eliminar esse agente infeccioso, começando por uma resposta imune inata, como primeira linha de defesa, e finaliza com uma específica resposta imune adaptativa. Tanto uma como a outra resposta imune são interdependentes e complementares, com a primeira direcionando para uma eficiente resposta adaptativa e essa por sua vez, potencializando as ações das células do sistema imune inato.

A mucosa do trato respiratório superior consiste na primeira barreira contra patógenos invasores, após exposição ao *M. leprae.* Mas somente a presença do DNA na mucosa nasal ou a soropositividade ao antígeno específico do *M. leprae* (PGL-1), em indivíduos saudáveis de uma área endêmica, não são indicativos de doença. Essas pessoas estão carreando o bacilo e colaborando com a transmissão do patógeno, ou estiveram em contato com pessoas bacilíferas, respectivamente(70–72).

Quando o *M. leprae* invade as vias aéreas superiores, a epiderme e nervos periféricos serão seus principais destinos. Na epiderme, um conjunto de células do sistema imune inato, como os macrófagos, células dendríticas, *natural killers*, entre outros, expressam receptores de reconhecimento padrão (*pattern recognition receptors*/PRRs), que identificam padrões moleculares conservados, comuns em determinados grupos de patógenos (*pathogen-associated molecular patterns*/PAMPs)(73), ou padrões moleculares associadas ao dano (*damaged-associated molecular patterns*/DAMPs). Diferentes PRRs são expressos em uma mesma célula, como os receptores de lecitina tipo-C, como o CD209, o *Nod-like receptor* (NLRs), o *retinoic-acid inducible gene I (*RIG-1) e o *Toll-like receptor* (TLRs)(74,75). Alguns membros da família dos TLRs, como o heterodímero TLR2/6

e TLR1/2, reconhecem PAMPs do *M. leprae*, como as lipoproteínas di ou tri-aciladas, respectivamente(75). Tanto o TLR1 como o TLR2 são mais expressos em células de lesões de pacientes com a forma clínica tuberculóide, quando comparados com os virchowianos(76,77). Em monócitos e células dendríticas houve uma redução da expressão do TLR2 pela IL4, e aumento da expressão do TLR1 pelo IFN-γ (Interferon-gama) e pelo Fator estimulador de Colônia granulócito-macrófago (GM-CSF/*granulocyte-macrophage colony-stimulating factor*)(76). Os diferentes TLRs são expressos em várias células, como os linfócitos, células de Schwann e células endoteliais, e, apresentam níveis elevados em monócitos, e principalmente macrófagos e células dendríticas, que são classicamente células apresentadoras de antígenos (APCs).

As APCs desempenham um importante papel na conexão da resposta imune inata com a adaptativa(78) e na formação do granuloma(79). Dependendo da sua distribuição tecidual, são denominadas de células de langerhans na epiderme ou células dendríticas dermais na derme(78,80). Outros fenótipos celulares participam da resposta inflamatória cutânea, como linfócitos Th CD4+ (auxiliares), linfócitos T CD8+ (citotóxicos), *Natural Killers* (NK), monócitos, T helper 17 (Th17), T regulatórias (Treg), células dendríticas, querotinócitos, células de Schwann e macrófagos(81).

Os macrófagos têm sido identificados como as principais células envolvidas na patogênese da hanseníase, responsáveis por atividades fagocíticas e bactericidas (81). Dependendo do perfil das citocinas pelo qual é estimulado, pode apresentar um comportamento M1 ou M2.

Quando ativados por citocinas pró-inflamatórias, como a IL-15, IFN-γ, TNF (*Tumor Necrosis Factor*), ou por agonistas do TLR, como as lipoproteínas di ou triaciladas do *M. leprae*, seu comportamento muda de MØ para um macrófago inflamatório M1, mais frequente na forma tuberculoide da doença. Desde modo é capaz de desempenhar inúmeras atividades como: - a de apresentador de antígenos; - produção de citocinas inflamatórias e quimiocinas; - liberação de moléculas coestimulatórias, regulados pelo fator de transcrição NF-ĸB (*factor nuclear-kappa B*); - ativação da via dos peptídeos antimicrobianos (catelicidinas e defensinas-beta 2 e 3 humanas) dependentes da vitamina D; - ativação da iNOS

(*induced nitric oxide synthase*), que induz a produção de NO (*nitric oxide*) e consequentemente radicais livres que destroem o patógeno intracelular (Figura 1)](73,82–84).

Por outro lado, o fenótipo M2, mais presentes na forma virchowiana, é ativado por citocinas como, a IL-4, IL-10, IL-13, IL-27 e TGF-β (*Transforming growth factor beta*), contribuindo com a atividade imunossupressora, de reparo tecidual e fibrose(82,83,85–88). Macrófagos estimulados pela IL-10 aumentam a fagocitose de lipoproteínas oxidadas de baixa densidade e da micobactéria, mas sem desencadear a via metabólica antimicrobiana, dependente de vitamina D(89,90). Esses fatores promovem um ambiente favorável intracelular para o bacilo, favorecendo sua sobrevivência(Figura1)(90).

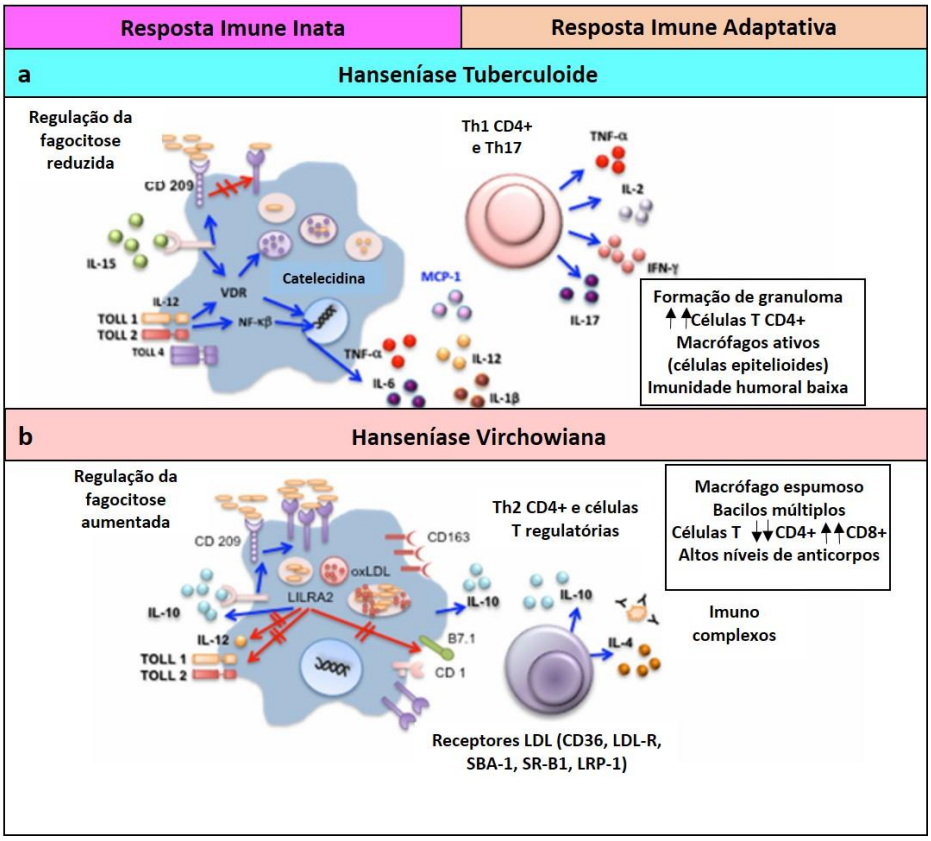


Figura 1 - Resposta imune inata e adaptativa do hospedeiro, em relação ao *Mycobacterium leprae,* nas formas clínicas polares da hanseníase: tuberculoide e virchowiana. Adaptado de Fonseca e colaboradores (90).

Esse aumento da carga bacilar é devido a uma maior expressão do receptor CD209 (*cluster of differentiation* 209), que é o responsável pela mediação da

captação do *M. leprae*(91). Concomitante, também estão mais expressos receptores de varredura (*scavenger*), como o CD163 (*cluster of differentiation* 163), SR-A (*Scavenger receptors class A*), CD36 (*cluster of differentiation* 36) e MARCO (*macrophage receptor with collagenous structure*). Todos esses receptores desempenham um papel na homeostase tecidual pelos macrófagos, com a ingestão de células apoptóticas, apoproteína B (ApoB), lipídios e lipoproteínas oxidadas de baixa densidade(92–94). No entanto, durante a manifestação da hanseníase, esses receptores ficam desregulados, com a ingestão exacerbada de lipídios, favorecendo o *M. leprae* com uma fonte de carbono(92). Os lipídios no interior do macrófago foi observado em cortes histológicos de lesões de pacientes multibacilares, dando um aspecto denominado de célula espumosa(95). Já o receptor de varredura CD163 é responsável pela ingestão do complexo haptoglobina-hemoglobina, fonte importante de ferro e essencial para a sobrevivência da micobactéria(96). Um outro receptor, mais expresso em lesões de virchowianos, é a LILRA2 (*leukocyte immunoglobulin-like receptor subfamily A member 2*), que quando ativado, promove a inibição do TLR1/2 e da IL-12 (Figura 1)(97).

Os linfócitos T CD4+ desempenham um papel fundamental no tipo de resposta imune adaptativa, se celular ou humoral, que será desencadeado pelo hospedeiro, resultando como desfecho clínico, as diversas formas de manifestação da hanseníase(41). As duas populações de linfócitos T CD4+ mais envolvidas na resposta imune ao *M. leprae*, são a T helper 1(Th1) e a T helper 2(Th2). Porém, continua uma lacuna de conhecimento o exato mecanismo de direcionamento da resposta imune adaptativa do hospedeiro. Essas células se originam da diferenciação das células T *naive* (Th0), quando estimuladas principalmente por citocinas IL-2 e IL-12 (Th1) e IL-4 (Th2)(98). Sabe-se que em pacientes tuberculoides há uma reduzida resposta humoral(99) e vigorosa resposta imune celular aos antígenos do *M. leprae.* Isso se deve a ativação de células Th1 CD4+ e da liberação de citocinas pró-inflamatórias, como a IL-2, IL-15, IFN-γ, TNF, que favorecem a eliminação da micobacteria e a contenção do bacilo, com a formação do granuloma, que é composto principalmente de macrófagos e linfócitos(100,101). Outro conjunto de células T específicas que atua em sinergia com as Th1, são as Th17. São células que liberam citocinas, como a IL-17A, IL17F, IL-21 e IL-22, que

estão envolvidas no recrutamento de neutrófilos, potencialização das células Th1 e na modulação da atividade do macrófago(102,103).

Por outro lado, em pacientes virchowianos, ocorre uma intensa resposta humoral, com formação de imunocomplexos, porém ineficiente na destruição do bacilo. A resposta imune adaptativa, nesses pacientes, é realizada principalmente pelas células Th2 CD4+ e T regulatórias FoxP3+ (T Reg), com produção de IL-4, IL-10 e TGF-β (*transforming growth fator-β*), que contribuem para a inibição da produção de citocinas proinflamatórias e inativação da resposta microbicida dos macrófagos, favorecendo com isso a sobrevivência do bacilo no interior da célula(90).

1.4 Epidemiologia

A hanseníase configura sério problema de saúde pública, em vários países, apesar da importante diminuição do número de novos casos após a implantação da PQT, no início da década de 1980. Nos últimos dez anos, o número de casos novos da doença tem se mantido praticamente constante. Houve leve decréscimo (17%), no número de casos registrados em 2009 (244.796) quando comparado aos reportados em 2019 (202.185)(8).

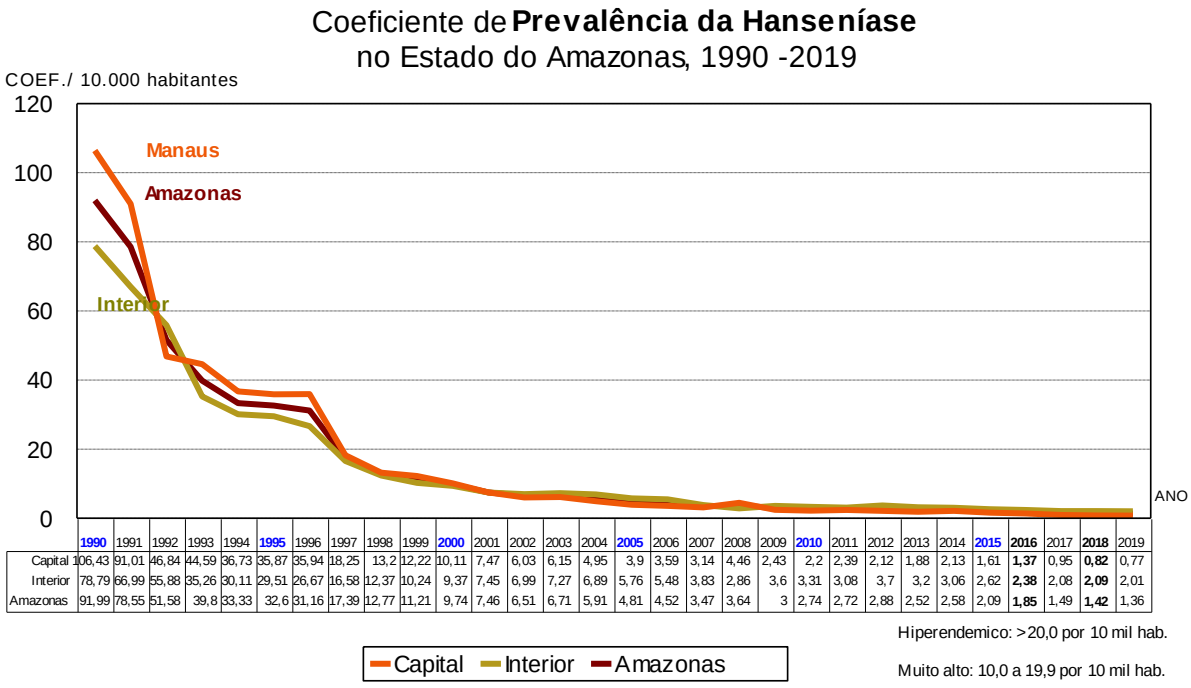
Três países relataram mais de 10.000 casos novos em 2019: Índia, Brasil e Indonésia. Esses países são responsáveis por 79% de todos os novos casos de hanseníase no mundo. O Brasil ocupa a segunda posição na detecção de novos casos: 27.863 em 2019. Nas Américas o Brasil lidera o número de casos (93% de todos os casos)(8).

Apesar do decréscimo de 30% da incidência, entre os anos de 2009-2018, o Brasil ainda apresenta alta endemicidade, com uma taxa de 13,70 por 100 mil habitantes, em 2018. Há variação da taxa de detecção de novos casos nas diferentes regiões do país, com a ocorrência das menores taxas nas regiões Sul e Sudeste, que apresentam parâmetro de média endemicidade (104).

Entre as regiões brasileiras, a região Norte ocupou a segunda posição na taxa de incidência e prevalência, em 2018, com 31,95 casos/100.000 e 3,34

casos/10.000 habitantes, respectivamente(104). O estado do Amazonas se destaca com importante declínio da prevalência (98,5%) e incidência (86,3%), nas últimas três décadas (Gráficos 1 e 2)(105). Atualmente, esse estado ocupa a 19ª posição, dentre os estados brasileiros, em relação à taxa de detecção de casos novos(104).

Gráfico 1 - Coeficiente de prevalência da Hanseníase da capital, interior e estado do Amazonas, no período de 1990 a 2019.



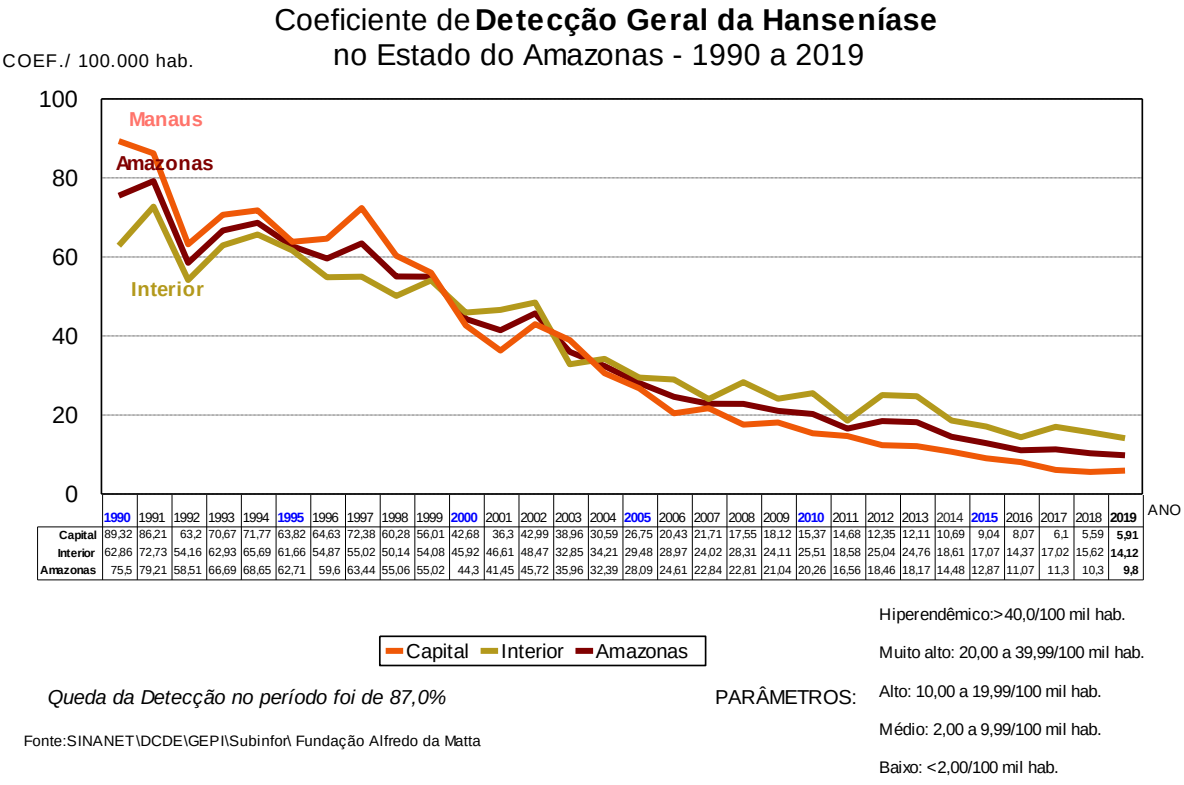
	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
Capital	106,43	91,01	46,84	44,59	36,73	35,87	35,94	18,25	13,2	12,22	10,11	7,47	6,03	6,15	4,95	3,9	3,59	3,14	4,46	2,43	2,2	2,39	2,12	1,88	2,13	1,61	**1,37**	0,95	**0,82**	0,77
Interior	78,79	66,99	55,88	35,26	30,11	29,51	26,67	16,58	12,37	10,24	9,37	7,45	6,99	7,27	6,89	5,76	5,48	3,83	2,86	3,6	3,31	3,08	3,7	3,2	3,06	2,62	**2,38**	2,08	**2,09**	2,01
Amazonas	91,99	78,55	51,58	39,8	33,33	32,6	31,16	17,39	12,77	11,21	9,74	7,46	6,51	6,71	5,91	4,81	4,52	3,47	3,64	3	2,74	2,72	2,88	2,52	2,58	2,09	**1,85**	1,49	**1,42**	1,36

Queda da Detecção no período foi de 98,5%

PARÂMETROS:
Hiperendemico: >20,0 por 10 mil hab.
Muito alto: 10,0 a 19,9 por 10 mil hab.
Alto: 5,0 a 9,9 por 10 mil hab.
Medio: 1,0 a 4,9 por 10 mil hab.
Baixo: <1,0 por 10 mil hab.

Fonte:SINANET\DCDE\GEPI\Subinfor\ Fundação Alfredo da Matta

Gráfico 2 - Coeficiente de detecção geral da Hanseníase da capital, interior e estado do Amazonas, no período de 1990 a 2019.



	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
Capital	89,32	86,21	63,2	70,67	71,77	63,82	64,63	72,38	60,28	56,01	42,68	36,3	42,99	38,96	30,59	26,75	20,43	21,71	17,55	18,12	15,37	14,68	12,35	12,11	10,69	9,04	8,07	6,1	5,59	**5,91**
Interior	62,86	72,73	54,16	62,93	65,69	61,66	54,87	55,02	50,14	54,08	45,92	46,61	48,47	32,85	34,21	29,48	28,97	24,02	28,31	24,11	25,51	18,58	25,04	24,76	18,61	17,07	14,37	17,02	15,62	**14,12**
Amazonas	75,5	79,21	58,51	66,69	68,65	62,71	59,6	63,44	55,06	55,02	44,3	41,45	45,72	35,96	32,39	28,09	24,61	22,84	22,81	21,04	20,26	16,56	18,46	18,17	14,48	12,87	11,07	11,3	10,3	**9,8**

Apesar do esforço dos profissionais de saúde no combate à doença, a hanseníase ainda é problema de saúde pública em vários estados e cidades do Brasil. O Amazonas apresentou média endemicidade (1,42/10.0000 hab.) na prevalência e alta endemicidade na incidência em 2018 (10.3/100.000 hab.).

1.5 Marcador Sorológico: testes rápidos nas doenças infecciosas

Os testes rápidos (TRs) começaram a ser desenvolvidos na década de 80, com a disseminação do vírus HIV e casos graves de AIDS. Mas foi a partir da década de 90, com a evolução tecnológica e melhoria da acurácia, que esses testes se tornaram mais populares. Os TRs utilizam, na maioria das vezes, a técnica da imunocromatografia, para identificação de variados analitos de diferentes áreas, como na biomedicina, alimentos, agricultura e meio ambiente(106). São dispositivos

portáteis, de baixo custo, não necessitam de refrigeração, simples de usar, rápida liberação de resultados (5-30 minutos) e podem ser realizados no ponto de atendimento, junto ao paciente (*Point-of-care*); entretanto, os resultados são qualitativos (sim/não) ou semi-quantitativos(106). Na ausência de um TR, o diagnóstico clínico de infecções, por exemplo, necessita ser confirmado por um teste laboratorial tradicional, com o resultado, na maioria das vezes, não saindo no mesmo dia. Neste intervalo, poderá ocorrer uma contínua transmissão do patógeno e um aumento das comorbidades no indivíduo.

Na área biomédica, a utilização de TRs é cada vez mais frequente, melhorando a abordagem ao paciente na atenção primária e colaborando na identificação da doença de uma forma rápida e precisa, como na identificação dos principais agentes causadores das Infecções Sexualmente Transmissíveis (ISTs), como o HIV, o *Treponema pallidum*, a *Chlamydia trachomatis* e a *Neisseria gonorrhoeae*. Van Der Pol e colaboradores(107) avaliaram a performance de um TR, baseado na detecção molecular de *Chlamydia trachomatis* (CT) e *Neisseria gonorrhoeae* (NG), e compararam com outros três testes comerciais de amplificação de ácidos nucleicos (NAATs). A permorfance foi equivalente aos testes moleculares realizados em laboratório, com uma sensibilidade para CT e NG de 96,1% e 100%, e especificidade de 99,1% e 99,9% respectivamente, em 1.523 mulheres que frequentaram as clínicas de ISTs. Enquanto que nos homens, a sensibilidade para CT e NG foram de 92,5% e 97,3%, e a especificidade foi de 99,3% e 100%, respectivamente, em 922 homens que procuraram atendimento em clínicas de ISTs.

Os estudos de validação dos TRs são importantes, como fizeram Van Der Pol e colaboradores(107). Somente testes que apresentem uma acurácia satisfatória na identificação de um determinado marcador, é que permitirá elaborar estratégias mais assertivas para tratamento, aconselhamento e em alguns casos, isolamento social.

A validação dos TRs para a identificação de anticorpos IgM/IgG para o SARs-CoV-2, ainda está ocorrendo. A despeito da urgência de identificar infectados e doentes desta pandemia, houve uma liberação do uso desses kits comerciais, pelos órgãos fiscalizadores. Somente com a realização de um inquérito sorológico a nível populacional, com TRs validados e de acurácia desejada, é que conheceremos a real magnitude desta doença, identificando quem está imunizado ou aqueles que

são suscetíveis. A validação também deve passar pelos mais diversos testes moleculares (RT-PCR) colocados no mercado. Estes testes indicam infecção atual e sua imprecisão comprometem os esforços de contenção da pandemia. Resultados falso-positivos colocam o indivíduo em quarentena e triagem dos contatos desnecessariamente. Enquanto que resultados falso-negativos, expõem indivíduos assintomáticos a infectar outras pessoas(108).

Uma endemia que continua presente nas últimas décadas no país é a tuberculose (TB), causada pelo *Mycobacterium tuberculosis.* Como estratégia para reduzir a sua incidência e mortalidade, um teste laboratorial de diagnóstico, rápido e eficaz, tem sido o objetivo nesses últimos anos. Tentativas de usar um *Point of care* (POC), que reconhece um componente da parede celular da *M. tuberculosis*, o lipoarabinomanana (LAM), em amostras de urina, tem sido proposto. Na prática, esses testes apresentaram uma baixa sensibilidade, com uma média de 44%, variando de 21% em pacientes ambulatoriais a 54% de hospitalizados(109). Portanto, esses testes têm limitações que devem ser superadas para que possam ser aplicadas de maneira adequada como POC.

Na hanseníase, a idéia de criar um TR para o diagnóstico da doença foi baseada na descoberta, no início da década de 1980, de um componente de parede celular, específico do *M. leprae,* o glicolipídio fenólico-1 (PGL-1, phenolic glycolipid-1)(19), cuja composição química é o 3,6-di-*O*-metila-β-D-glicopiranosil-(1→4)-2,3-di-*O*-metila-α-L-ramnopiranosil-(1→2)-3-*O*-metila-α-L-ramnopiranose(110). O determinante antigênico do PGL-1, capaz de induzir altos títulos de anticorpos no hospedeiro, é a parte di e trissacarídica da molécula(111,112). Essa descoberta permitiu a síntese química do glicídio e a produção de variados neoglicolipídeos, conjugados com a Albumina Sérica Bovina (BSA) ou Albumina Sérica Humana (HSA), como o ND-O-BSA ou ND-O-HSA(113) e o NT-P-BSA(114), unidos por ligantes específicos, como os radicais octil (O) ou fenol (P). Esses variados formatos de neoglicolipideos podem ser produzidos em maior quantidade, com um custo menor do que o PGL1 nativo, tornando viáveis em aplicações que vão do diagnóstico clínico a grandes estudos epidemiológicos. As técnicas mais empregadas e desenvolvidas para detectar a presença de anticorpos contra o *M. leprae*, utilizando tanto antígeno nativo quanto o semi-sintético, foram o ELISA (*enzyme-linked immunosorbent assay*) e o teste de fluxo lateral (*ML flow*). O ELISA,

quando desenvolvida *in house*, é laboriosa e requer padronização, que pode variar, por limitações na diferença e concentração de reagentes. No entanto, o seu baixo custo e o resultado quantitativo, tornam um teste único para estudos epidemiológicos em grande escala, e para monitoramento da eficácia do tratamento de pacientes(115). Enquanto que o *ML flow* é um teste rápido, imunocromatográfico, simples de usar, com resultados saindo entre 10 a 15 minutos, e pode ser aplicado junto ao paciente.

Além do PGL-1 e seus antígenos análogos, novos componenentes com potenciais antigênicos têm sido avaliados, como o lipoarabinomanana e as proteínas ML0405 e ML2331(22,116). Isso permitiu conhecer o perfil de resposta humoral entre as diversas formas clínicas da doença(22). A proteína quimérica formada pela fusão das proteínas (ML0304 e ML 2331), denominada de LID-1 (*Leprosy IDRI Diagnostic 1*), teve melhor sensibilidade do que as proteínas isoladamente(117). E quando adaptadas para a técnica ELISA, pode ser utilizada como uma ferramenta de controle da efetividade do tratamento, através da mensuração do decréscimo da titulação de anticorpos, que ocorre desde o inicio da poliquimioterapia(117).

Devido à natureza glicolipídica do PGL1 ou das suas variações com o açúcar dissacarídeo ou trissacarídeo, conjugados com albumina sérica bovina ou humana, a característica de anticorpos detectada é predominantemente IgM. Enquanto que a detecção de anticorpos IgG, em resposta ao bacilo *M. leprae*, pode ser identificada através do teste sorológico LID-1.

Inúmeros estudos foram realizados posteriormente, procurando entender como seria a resposta imunológica do hospedeiro diante desses antígenos, principalmente nas formas polares da hanseníase. A acurácia dos testes em detectar anticorpos contra antígenos do *M. leprae*, como o PGL-1 (natural ou sintético) ou das proteinas do LID-1, tanto pela técnica do ELISA, como da imunocromatografia utilizada em TRs (*ML flow*), variam conforme a apresentação clínica ou classificação operacional (Tabela 1).

Tabela 1 - Acurácia dos testes sorológicos, utilizando as técnicas, ELISA ou ML flow, com carreadores BSA ou HSA

Antígenos	Resultados	Voluntários	População	Ref
NT-P-BSA (ML flow)	PB➔ S=40% MB ➔ S=97,4% E=88%	85 PB;114 MB 234 CE	Brasil,Indonésia e Filipinas	(118)
PGL1 (ELISA)[a] ND-O-HSA (ELISA)[b] NT-P-BSA (ML flow)[c]	PB ➔ S^a=22,7% / S^b=31,8% / S^c=0 MB ➔ S^a=86,4% / S^b=76,4% / S^c=90% E=98% para ambos ELISA	44 PB; 110 MB 52 CE	Brasil	(119)
NT-P-BSA (ML flow)	PB ➔ S=44,9% MB ➔ S=92,7% E=98%	69 PB; 69 MB 101 CE	Brasil e Nepal	(28)
ND-O-LID (ML flow)[a] NT-P-BSA (ELISA)[b]	PB ➔ S^a=21,2% MB ➔ S^a=87% E^a=97% PB➔ S^b=15,4% MB ➔ S^b=83% E^b=100%	104PB;108MB 101 CE	Brasil	(120)
ND-O-LID (ELISA)	PB ➔S=27% MB ➔ S=90,6% E=85,9%	50PB; 146 MB 117 CE	Colômbia e Filipinas	(121)
ND-O-LID (ML flow)[a] ND-O-BSA (ML flow)[b]	PB➔S^a=32,3% MB ➔ S^a=87% E^a=98,4% PB➔ S^b=6,5% MB ➔ S^b=82% E^b=90,5%	62 PB;208 MB 63 CE	Filipinas	(122)
NT-P-BSA (ELISA)[a] NT-P-BSA (ML flow)[b] NT-P-HSA (ELISA)[c] NT-P-HSA (ML flow)[d]	PB➔ S^a=25,7% MB ➔ S^a=77% E^a=100% PB➔ S^b=29,2% MB ➔ S^b=87% E^b=100% PB➔S^c=24,0% MB ➔ S^c=74% E^c=98,5% PB➔S^d=31,6% MB ➔ S^d=81% E^d=95,6%	342 PB; 486 MB 69 CE	Brasil	(123)
PGL1 (ELISA)[a] ND-O-HSA (ELISA)[b] LID1 (ELISA)[c] ND-O-LID (ELISA)[d]	PB➔ S^a=37,2% MB ➔ S^a=88% E^a=NR PB➔S^b=19,6% MB ➔ S^b=77% E^b=92,3% PB➔ S^c=7,8% MB ➔ S^c=79% E^c=97% PB➔S^d=29,4% MB ➔ S^d=88% E^d=60,1%	51 PB; 43 MB 2.494 CE	Brasil	(124)
LID1 (ELISA)[a] ND-O-LID (ELISA)[b]	PB ➔ S^a=16 % MB ➔ S^a=88% E^a=99% PB ➔ S^b=6% MB ➔ S^b=65% E^b=98%	32 PB; 66 MB 98 CE	Brasil	(125)
ND-O-LID (ML flow)[a] ND-O-LID (ELISA)[b]	MB ➔ S^a=81,4% E^a=96,4% MB ➔ S^b=86,2% E^b=93,8%	145 MB 224 CE	Filipinas	(126)
ND-O-BSA (ELISA) [a] LID1 (ELISA)[b] ND-O-LID (ML flow)[c]	MB ➔ S^a=57,9% E^a=65,7% MB ➔ S^b=47,4% E^b=72,7% MB ➔ S^c=65,8% E^c=39,6%	38 MB 245 CE	Brasil	(127)

Abreviaturas: ELISA, *enzyme-linked immunosorbent assay*; ML flow, teste rápido de fluxo lateral; LID1, Leprosy IDRI Diagnostic 1; BSA, Albumina sérica bovina; HSA, Albumina sérica humana; ND-O, dissacarídeo ligado ao radical octil; NT-P, trissacarídeo ligado ao radical fenol; MB, multibacilar; PB, paucibacilar; CE, controle endêmico; S, sensibilidade; E, especificidade

A sensibilidade dos TRs também pode variar, conforme a amostra clínica. Amostras de soro de pacientes com hanseníase têm uma positividade ligeiramente superior ao sangue, com amostras do mesmo indivíduo(28,118). A capacidade dos testes em detectar anticorpos é mais frequente nos multibacilares e fraca nos paucibacilares. Isso se deve a forte resposta humoral desenvolvida nas formas clínicas mais graves da doença, associadas com a elevada carga bacilar(120). No entanto, o teste não pode ser usado como diagnóstico na hanseníase e sim no

processo de diagnóstico. Uma determinada parcela de indivíduos da área endêmica ou de contatos domiciliares estarão positivos sem nunca desenvolver hanseníase, indicando apenas exposição ao bacilo.

A busca de um teste diagnóstico, ou prognóstico, que possa identificar casos de hanseníase, tão logo quanto possível após a sua infecção, é uma das principais recomendações da Organização Mundial de Saúde.

Somente com a detecção precoce e o tratamento imediato, de pacientes assintomáticos ou pré-sintomáticos, é que será possível reduzir a transmissão da infecção na comunidade e as incapacidades físicas. Vários estudos têm focado na identificação do anti-PGL1, como um biomarcador de prognóstico para a doença, em contatos domiciliares, porém, os resultados têm sido divergentes(128).

A revisão sistemática e meta-análise, realizada por Penna e colaboradores(128), buscou consenso entre os oito trabalhos selecionados. Este estudo mostrou que entre os contatos que adoeceram, aqueles que apresentavam anticorpos anti-PGL1, tiveram três vezes mais chance de desenvolverem a doença, do que os contatos com sorologia negativa. Porém, a sensibilidade ficou abaixo de 40%, para todos os estudos, e o valor preditivo positivo máximo, alcançado em apenas um estudo, foi de 17,5%. Assumindo que em uma eventual quimioprofilaxia, a eficácia fosse de 100%, significaria que, a escolha de contatos para receber esse medicamento, baseado apenas do resultado positivo da sorologia, seria efetiva em menos da metade dos contatos que adoeceram, e desnecessário em mais de 80% deles. Isso também se confirmou no estudo de Barreto e colaboradores(129), quando acompanharam durante dois anos, contatos domiciliares, de jovens estudantes, diagnosticados com hanseníase, de duas cidades do estado do Pará. Concluíram que a chance de risco de os contatos soropositivos desenvolverem a doença, foram 2,7 vezes maiores do que os soronegativos, indicando que se acompanhassem 10 soropositivos, durante dois anos, a probabilidade de adoecer (>90%), seria de no mínimo um contato.

Richardus e colaboradores(130), em um estudo longitudinal de seis anos, com 224 contatos de pacientes com hanseníase, de Bangladesh, também concluiram que o anti-PGL1 não foi um bom preditor de adoecimento. Verificaram

que não houve diferença do nível de anticorpos, entre os grupos, com ou sem o desfecho da doença.

A maior dificuldade, nos ensaios sorológicos, é na identificação de pacientes paucibacilares, que apresentam baciloscopia negativa e exame histopatológico, geralmente, inconclusivo. Nesses pacientes, há maiores níveis de IFN-γ, em relação aos multibacilares. Porém, o diagnóstico precoce baseado somente nesta citocina, não é fidedigno, uma vez que seus contatos, exibem similaridade de produção de IFN-γ(131).

Devido ao amplo espectro de apresentação clínica da hanseníase, e do tipo da resposta imune que o indivíduo pode desencadear, a escolha de biomarcadores de prognóstico, necessariamente, tem que refletir a resposta imunológica, inata ou adaptativa (humoral ou celular). Testes baseados na combinação desses biomarcadores, podem aumentar a eficiência em diferenciar: paciente, de contato; virchowianos, de tuberculoides; ou indivíduos infectados pelo *M. leprae*, de não infectados(20,132). Um novo teste de fluxo lateral, o UCP-LFAs (*Up-converting phoshor lateral flow assays*), combinando quatro marcadores imunológicos (anticorpos anti-PGL1, IL-10, IP-10 e CCL4), foi avaliado, recentemente, por Hooij e colaboradores(20), em uma coorte, em Bangladesh. Os resultados foram promissores, tendo o teste a capacidade de identificar, significativamente, indivíduos infectados pelo *M. leprae*, hanseníase *per se* ou pacientes multibacilares. A combinação de marcadores, como, o IP-10, a CCL4 e a IL-10, foi indicativa de infecção pelo bacilo; os marcadores, CCL4 e IP-10, com a hanseníase *per se*; e a indicação de pacientes multibacilares, com a combinação do anti-PGL1 IgM, da IL-10 e do IP-10, capacitando na distinção entre pacientes multibacilares e paucibacilares. Os marcadores CCL4 e IP-10 são marcadores de resposta celular do hospedeiro, e uma inclusão em testes que detectam o anti-PGL1, mostra uma esperança em identificar os pacientes paucibacilares(20).

Os pacientes, com hanseníase, também podem ser acometidos por episódios inflamatórios agudos, antes, durante ou depois do tratamento. O diagnóstico rápido e o tratamento imediato, reduz o dano neural e o risco de incapacidades permanentes. Corstjens e colaboradores(133), avaliaram um multiplex, UCP-LFA, com dois marcadores, o IP-10 e o anti-PGL1 IgM, para o monitoramento

intraindividual do diagnóstico precoce das reações hansênicas. O teste mostrou-se útil no diagnóstico inicial, permitindo o tratamento eficaz, apesar do número reduzido de pacientes testados.

1.6 Estudo molecular: A importância da PCR na hanseníase

O desenvolvimento de testes diagnósticos em hanseníase sempre foi um desafio, principalmente devido à incapacidade de crescimento do *Mycobacterium leprae* em meios artificiais, ou seja, *in vitro*. Somente após o desenvolvimento de protocolos que possibilitaram o crescimento do bacilo da hanseníase em pata de camundongo(134) e em tatus(135), foi possível desenvolver novos agentes quimioterápicos, confirmar a resistência às drogas administradas e conhecer as estruturas moleculares e antigênicas da bactéria(34).

Os primeiros ensaios de PCR na identificação do *M. leprae* ocorreram há três décadas(136), e foram aprimoradas após o sequenciamento completo do bacilo em 2001(60), permitindo, com isso, a comparação entre genomas de outras micobactérias, como o *Mycobacterium tuberculosis*(137). A melhoria de sensibilidade e especificidade no diagnóstico por PCR, tanto convencional e principalmente, em tempo real (q-PCR)(34), se deve ao aprimoramento dos protocolos de extração de DNA, amplificação e identificação de diferentes genes e amostras clínicas testadas. Portanto, a purificação do DNA com kits comerciais, a amostra biológica adequada e representativa da lesão e uma melhor eficiência na amplificação de tamanhos menores do genoma por PCR tempo real, foram fundamentais para a utilização desta técnica como ferramenta de auxílio ao diagnóstico clínico(34). A biópsia de pele continua sendo a melhor escolha de amostra biológica, não só pela maior quantidade de bacilos encontrados, mas também, pela capacidade de manter a reprodutibilidade do teste após o armazenamento em álcool a 70^0GL. Mas outras amostras clínicas são importantes, isto é, quando o paciente não tem lesões ou se apresenta de uma forma atípica. Nesses casos, têm que ter a clareza no variado grau de sensibilidade nas amostras, como, nervos, urina, mucosa oral e nasal, sangue e tecido ocular(138–142) e sua correta correlação com a doença.

Diferentes sequências, de genes distintos, foram avaliadas para verificar a sensibilidade do teste molecular, como, o gene que codifica o antígeno 36-kDa(143), o antígeno 18-kDa(144), o *16S rRNA*, o *RLEP* (uma sequência repetida do genoma), o *Ag85B* e o *sodA*(30–32), entre outros genes do *M. leprae*. Martinez e colaboradores(32) foram os primeiros a realizarem um estudo comparativo, entre quatro ensaios por q-PCR, para a identificação do bacilo, nas biópsias de pele, oriundas de pacientes, com diferentes formas clínicas da hanseníase, de voluntários saudáveis e de indivíduos com outras dermatoses, sem a hanseníase. A sensibilidade de detecção do *RLEP* (87,1%), foi superior aos genes *Ag85B* e *16S*, porém a especificidade não teve o mesmo desempenho, ficando inferior na comparação com o *Ag85B* e *16S*, os quais foram de 100%. Portanto, o uso desse marcador pode levar os pacientes a terem resultados falso-positivos, levando a um tratamento desnecessário e triagem em todos os seus contatos. Uma hipótese para essa baixa especificidade pode estar na sequência de identificação do gene. Trata-se de uma região bastante conservada, e por isso, sequências homólogas podem estar identificando outras espécies de micobactérias, gerando assim, resultados falso-positivos, como aconteceu com o gene *IS6110*, na identificação do *Mycobacterium tuberculosis*(145). Para evitar diagnósticos incorretos de indivíduos que não tem a doença, a utilização de marcadores como o *16S* e o *Ag85B* são escolhas mais sensatas como alvo molecular, no processo do diagnóstico clínico.

A utilização da técnica molecular de PCR, ou q-PCR, como ferramenta de auxílio ao diagnóstico clínico de casos difíceis é cada vez mais necessária para a elucidação e o tratamento imediato, ou, para evitar o tratamento inadequado(146). Os pacientes com as Formas Neurais Puras (FNP) enquadram-se nesse perfil, devido a não apresentarem lesões da pele, à baciloscopia negativa e à histopatologia geralmente inconclusiva(140,147). No estudo de Jardim e colaboradores(140), o resultado do PCR foi positivo em 47% (n=23) das 49 biópsias das FNP, sendo superior aos achados histopatológicos do nervo, que mostraram granuloma epitelioide em 14% dos pacientes, BAAR em 16% e infiltrado inflamatório não específico e/ou fibrose em 39%. Cunha e colaboradores(147), também encontraram uma frequência semelhante, do DNA do bacilo nas biópsias neurais (50%), e método superior a histopatologia e baciloscopia. Portanto, a detecção do bacilo pela técnica de PCR, em biópsias de nervos, pode ser uma alternativa de

diagnosticar, em parte, a forma neural da hanseníase. Outro teste laboratorial adicional para ajudar no diagnóstico da FNP é a detecção de anticorpos contra o PGL1, podendo complementar com os resultados do teste molecular(148). A detecção do bacilo nos estágios iniciais da infecção neural possibilita o tratamento imediato, evitando a fibrose e consequente dano neural permanente e irreversível(147).

Os pacientes paucibacilares, com lesão única, têm sido também um constante desafio de diagnóstico clínico. Infiltrado inflamatório em nervos da derme nem sempre são visíveis nestas lesões, tornando o exame histopatológico limitado. Interessante foi o estudo de Barbieri e colaboradores(146), que realizaram o q-PCR, para identificação do *M. leprae*, e revisaram histologicamente as 66 amostras de lesão única. Quando os resultados da revisão da histopatologia foram cruzados com os resultados do teste molecular, verificaram que seis pacientes que receberam a poliquimioterapia, foram negativos na q-PCR, com uma amostra com baixa probabilidade de ser hanseníase na histopatologia e cinco consideradas como outras dermatoses. E em dois pacientes que não receberam o tratamento, foram positivos na q-PCR, sendo um deles considerado de baixo risco de ser hanseníase e outro classificado como outra dermatose na histopatologia. Se consideramos o q-PCR como padrão-ouro, esses seis pacientes provavelmente receberam a poliquimioterapia desnecessariamente; e os dois pacientes não tratados, com o teste q-PCR positivo, foram subdiagnosticados. A sensibilidade do q-PCR, em identificar pacientes paucibacilares, de fato, é superior a histopatologia. Mas, a utilização desses dois testes laboratoriais, aumenta a acurácia do diagnóstico clínico, sem uma perda de especificidade(149). Outra maneira de melhorar a acurácia de um teste, seria utilizar mais de um alvo molecular (multiplex), na identificação do *M. leprae*, como sugeriram Pathak e colaboradores(150), na identificação precoce da hanseníase, principalmente de pacientes paucibacilares.

O uso do q-PCR como ferramenta de auxílio ao diagnóstico clínico, de alguns pacientes com a forma clínica indeterminada ou tuberculoide, também pode ser muito útil. Foi o que revelou no estudo de Martinez e colaboradores(32), que encontraram uma sensibilidade nas amostras de paucibacilares (indeterminados e tuberculoides) de 50%. Neste mesmo estudo, os autores sugeriram a utilização do teste molecular na detecção precoce da hanseníase, ou seja, na infecção subclínica,

devido terem encontrado DNA do *M. leprae* em amostras definidas como “outras dermatoses”, mas que desenvolveram hanseníase, após 10 anos de acompanhamento.

O q-PCR como ferramenta para a mensuração da expressão do RNA micobacteriano vem evoluindo nos últimos anos, culminando com a determinação da taxa RNA/DNA por q-PCR, para a determinação da viabilidade bacteriana(31). Essa técnica tem-se mostrado bastante promissora para monitorar a efetividade da poliquimioterapia, ou em pacientes suspeitos de resistência medicamentosa(151,152). Esse ensaio também pode ser usado como preditor das formas clínicas da hanseníase(31), como nos contatos domiciliares, principalmente se a quantificação do RNA se elevar em amostras subsequentes, indicando claro risco de desenvolver a patologia(34). Neste caso, uma vigilância clínica mais próxima é a mais adequada, ou sugerir, talvez, quimioprofilaxia. Por enquanto, ainda não há nenhuma associação da presença de DNA do *M. leprae* com o desenvolvimento da doença, entre os contatos, por exemplo(153). Uma tentativa de aumentar o valor preditivo do diagnóstico da hanseníase poderia estar na associação de um teste molecular de identificação de DNA com um teste sorológico, como o anti-PGL1 (34,154).

Outra importante utilização da técnica molecular é no diagnóstico diferencial da hanseníase com outras dermatoses, que apresentem lesões hipocrômicas ou granulomatosas, de difícil resolução clínica, como, a tuberculose cutânea, a sarcoidose, o granuloma anular, a leishmaniose cutânea, a sífilis secundária, a pitiríase alba, entre outras doenças dermatológicas que a histopatologia não consegue elucidar(34). São dermatoses confundidoras com a hanseníase que o teste molecular poderá confirmar ou afastar a possibilidade de ter a doença, devido a sua alta sensibilidade e especificidade.

A testagem em pata de camundongo é ainda o padrão-ouro para avaliar a suscetibilidade das drogas à hanseníase, porém ela é laboriosa, demorada, requer amostras de biópsias com bacilos viáveis, e necessita ser enviada ao laboratório, num intervalo de no máximo uma semana, e na forma refrigerada. Uma suspensão bacilar é preparada a partir da biópsia, inoculada em pata de camundongo e entre 24 a 30 semanas é avaliada a resistência bacteriana(155). Porém, nas últimas duas

décadas, novas metodologias para avaliar a suscetibilidade das drogas administradas na hanseníase, foram introduzidas. A tecnologia de amplificação de DNA, por PCR convencional, seguida do sequenciamento da região genômica do *M. leprae*, vem sendo consolidada na identificação e monitoramento de cepas resistentes(155). Mutações, que levam a resistência as drogas administradas, têm sido reveladas, e os ensaios baseados no DNA para identificá-las, têm sido desenvolvidos. Já foram confirmadas, em pata de camundongos, que determinadas mutações presentes dentro de uma limitada região de DNA, dos genes *folp1*, *gyrA* e *rpoB*, conferem resistência a dapsona, a rifampicina e as quinolonas, respectivamente(155).

Até o momento, não existe um teste comercial para detectar *M. leprae* nas mais variadas amostras clínicas do paciente. Muitos laboratórios desenvolvem seus próprios protocolos de detecção, *in house*, impossibilitando a comparação da sensibilidade e especificidade entre os centros de referência. Com a identificação de vários genes específicos do bacilo, sua utilização em ensaios que envolvam máquinas automatizadas, poderia ser desenvolvida na hanseníase, aumentando a capacidade de decisão clínica no diagnóstico da doença. Como acontece com o GeneXpert MTB/RIF, utilizado na identificação do *Mycobacterium tuberculosis* e resistência a rifampicina, simuntaneamente(34).

1.7 Estudo Genético: a relação dos polimorfismos na hanseníase

1.7.1 Genética da suscetibilidade humana a doenças infecciosas

Nas últimas décadas ocorreram grandes avanços no desenvolvimento de vacinas, medicamentos e diagnóstico laboratorial para as doenças infecciosas, que, junto com melhorias no nível educacional e sócio-econômico da população, levaram a um diagnóstico precoce, tratamento mais adequado e melhorias na expectativa de vida. Apesar dessa evolução, a erradicação das doenças infecciosas ainda continua como um grande desafio(156). Para entender esse cenário, deve-se levar em conta a complexa inter-relação de fatores ambientais e humanos, que determinam a imunidade para a infecção ou o desfecho para a doença(156). É interessante notar

que somente uma parcela de indivíduos expostos desenvolve a doença(157,158).

Evidências científicas sugerem que o perfil genético dessas pessoas seja um dos principais fatores responsáveis pelo desfecho clínico da doença(156–158). Isso ficou bem caracterizado nas imunodeficiências primárias, que são desordens tipicamente monogênicas (Mendelianas), cuja mutação em determinados genes, resultam em falha na resposta imunológica e predisposição a inúmeras doenças infecciosas, como a infecção pelo vírus Epstein-Barr, *Neisseria sp,* tripanossomíase e na suscetibilidade mendeliana a infecções micobacterianas (MSMD – *Mendelian susceptibility to mycobacterial disease*) (156,159–161). A MSMD é uma imunodeficiência rara, cujos indivíduos acometidos são altamente suscetíveis a micobactérias fracamente virulentas, como as micobactérias ambientais e a BCG (Bacilo de Calmette-Guerin), que é uma vacina que tem por base, cepas atenuadas do *Mycobacterium bovis*(159). Mutações nos genes do circuito *IL12/IL23/ IFNG e STAT1*, estão associados à MSMD(159).

É possível verificar a contribuição de fatores ambientais e do hospedeiro, no desenvolvimento de uma determinada doença, em estudos que comparam a taxa de concordância entre gêmeos monozigóticos e dizigóticos, ou entre crianças adotadas e biológicas(160). Em estudos com gêmeos monozigóticos e dizigóticos, a influência do fator genético ficou evidente, com as taxas de concordância maiores em monozigóticos, em diferentes doenças infecciosas, tais como, a poliomielite, a tuberculose e a hanseníase (162–164). Por fim, vários genes têm sido associados com diferentes doenças, tais como, a Hepatite B (*HLA*, *UBE2L3*, *CD40*, *INTS10*)(165), tuberculose (*HLA*, *INFG*, *IL10*, *MRC1*)(166), Malária (*HBB*, *DARC*, *SLC4A1*)(167) e a hanseníase (*IL23R*, *LTA, LACC1-CCDC122*, *NOD2*, *PACRG/PRKN*(168–171). Porém, ainda permanece para ser descoberto um fator genético que implique em uma grande variação do fenótipo, principalmente nas doenças complexas(172).

É cada vez mais frequente a descoberta de novos genes envolvidos em diferentes doenças infecciosas. Isso se deve à utilização de ferramentas de genética molecular, envolvendo a utilização de marcadores genéticos, principalmente os do tipo SNPs. Polimorfismos de nucleotídeo único são marcadores genéticos que apresentam variações nas bases nitrogenadas do DNA, ou seja, polimorfismos ou variantes alélicas numa frequência superior a 1%.

Estudos moleculares de ligação e de associação têm revelado regiões genômicas, em distintos genes associados, a diversas doenças infecciosas. Os estudos de ligação ampla do genoma, GWLS (*Genome wide linkage studies*), verificam se há relação na cossegregação de segmentos cromossômicos e a doença, ao longo das gerações de uma família. Uma limitação desses estudos é distinguir qual gene do segmento cromossômico, contendo dezenas ou até centenas de genes, exercerá efeito na doença. Por outro lado, os estudos de associação genômica ampla, GWAS (*Genome wide association studies*), identificam diferenças nas frequências alélicas entre grupos independentes, ou seja, indivíduos com e sem a doença, de uma determinada população. Esses estudos permitem identificar variantes genéticas associadas com o risco aumentado de desenvolver a doença. A grande vantagem dessa metodologia é de permitir analisar, simultaneamente, mais de 500.000 SNPs, em milhares de indivíduos(167).

Os SNPs são considerados a forma mais abundante de variação no genoma humano, entre 15 a 20 milhões [http://www.genome.gov] e devido à importância de alguns deles estarem associados ao maior risco de desenvolver doenças, existe um projeto internacional, o *HapMap* [http://www.hapmap.org], para catalogar em grande escala os SNPs do genoma humano.

1.7.2 Genética da suscetibilidade humana à hanseníase

Na hanseníase, como nas doenças infecciosas humanas, o patógeno é necessário, mas não suficiente para o desenvolvimento da doença. Estudos recentes indicam que aproximadamente 5% dos indivíduos expostos ao *M. leprae* irão apresentar os fenótipos conhecidos(10).

Os fatores ambientais e principalmente o *background* genético do hospedeiro, contribuem para a manifestação clínica da doença(158,173). Os fatores ambientais, como o estado nutricional, a vacinação e o grau de exposição ao bacilo, influenciariam fortemente o risco de infecção, mas não o risco de adoecimento(10). Por outro lado, a baixa variabilidade genética do *M. leprae* em diferentes amostras de pacientes de diversas regiões do mundo(174), e a diversidade de fenótipos clínicos da hanseníase, incluindo a hanseníase *per se*, sugerem fortemente uma

associação do *background* genético do hospedeiro controlando a infecção, a doença e seus estados reacionais(10,175).

As primeiras evidências de que a genética do hospedeiro influencia a suscetibilidade à hanseníase, vem de estudos genéticos epidemiológicos observacionais, como o estudo realizado na India, que evidenciou a alta taxa de concordância da doença e suas formas clínicas, entre os gêmeos monozigóticos (80%) em relação aos dizigóticos com menos do que 20%(164).

Em adição, a técnica de Análise de Segregação Complexa (ASC) tem sido realizada em diferentes populações, utilizando modelos matemáticos para inferir indivíduos afetados pela doença em diferentes *pedigrees*. O estudo mais recente utilizando este modelo de análise foi com a população completa da Vila de Santo Antônio do Prata, uma ex-colônia de hansenianos localizada no interior do estado do Pará. O estudo demostrou que a transmissão da hanseníase segue padrão de herança codominante, com efeito do gene principal influenciando a suscetibilidade a hanseníase(176).

Outro estudo de ASC, realizado na ilha caribenha Desirade, demostrou padrão codominante ou recessivo(177). O efeito do gene principal não significa apenas um gene envolvido na hanseníase, mas provavelmente a combinação de conjunto de genes exercendo forte efeito genético bastante evidente e distinto de outros fatores que possam influenciar na manifestação da doença.

No entanto, esses estudos observacionais não são capazes de identificar quais são os genes envolvidos e suas variantes genéticas na manifestação das diferentes formas clínicas da hanseníase.

Estudos moleculares são necessários e um grande número deles foi conduzido recentemente, evidenciando regiões cromossômicas e genes candidatos na manifestação da doença. Uma hipótese, amplamente aceita, é a de que um conjunto de genes influencia em três diferentes etapas da doença: 1) no controle da infecção *per se*, ou seja, a doença independente de sua forma clínica; 2) na definição da forma clínica e 3) na ocorrência dos estados reacionais (Figura 2).

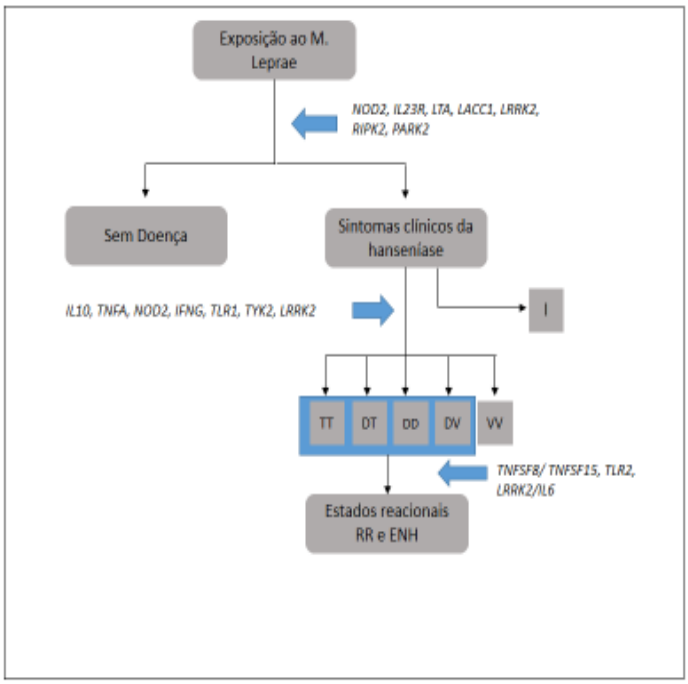


Figura 2 - Patogênese da hanseníase, com genes selecionados, e impacto em seus fenótipos. Adaptado de Cambri & Mira (2018)(178). Abreviações: I, indeterminado; TT, tuberculoide; DT, dimorfa tuberculoide; DD, dimorfa dimorfa; DV, dimorfa virchowiana; VV, virchowiana

Os estudos de ligação e de associação, em diferentes grupos populacionais, levaram a avanços importantes de regiões genômicas e genes associados à hanseníase. Porém, poucos genes candidatos têm sido replicados em diferentes populações e/ou com ensaios funcionais. Continua como um grande desafio da genética moderna desvendar quais são esses genes e suas variações, que estão envolvidos numa doença complexa, como a hanseníase. Provavelmente, centenas de genes contribuem nas diferentes etapas e formas clínicas da doença, junto com fatores não genéticos.

O GWLS, de Siddiqui e colaboradores(179), utilizando microssatélites como marcadores genéticos, em famílias indianas, identificaram a região cromossômica 10p13 associada com a forma paucibacilar. Enquanto que os estudos de associação identificaram três genes da região cromossômica 10p13 (*MRC1, CUBN e NEBL*) (180,181) associados com a suscetibilidade a forma multibacilar. Um outro gene denominado de *GATA3*, distante 6.5 Mb do pico de ligação do cromossomo 10p13, foi associado com a resistência à hanseníase em pacientes brasileiros(182). A falta de associação de variantes genéticas com a forma paucibacilar se deve

provavelmente a variantes raras dentro desta região. Porém, até o momento não existe nenhum estudo que possa confirmar essa hipótese(181).

O importante estudo de Mira e colaboradores(39), com a população vietnamita, também detectou sinal sugestivo de ligação no cromossomo 6p21 e na identificação de um *locus* principal de suscetibilidade à hanseníase na região cromossômica 6q25-q27. A região 6p21 abriga o complexo MHC/HLA (*Major Histocompatibility Complex/Human Leukocyte Antigens*). O *locus* HLA contém um grande número de genes altamente polimórficos, contidos em um intervalo de 3.6 Mb no *locus* 6p21. Estão envolvidos no reconhecimento, processamento e apresentação de antígenos, mecanismos importantes que regulam as respostas para as células T CD4+ e T CD8+(183). Em estudos com diferentes populações, haplótipos tem sido associado ao risco e proteção a hanseníase, em genes localizados nas três classes de HLA(183,184). Dois genes HLA, de classe III, vêm sendo constantemente investigados nos estudos de associação com a hanseníase: *TNF* (Fator de Necrose Tumoral) e o *LTA (*Linfotoxina alfa*)*. As variantes localizadas na região promotora de ambos os genes, conferem proteção e risco, respectivamente à hanseníase(170,185). No entanto, devido esse *locus* apresentar uma alta densidade de genes e com diversidade alélica, há uma dificuldade na interpretação de uma associação positiva com a doença, devido a um fenômeno denominado Desequilíbrio de Ligação (LD)(186).

Em relação à região cromossômica 6q25-q27, dois genes que compartilham vários polimorfismos presentes na região promotora, mostraram associação com a hanseníase *per se*: a *PRKN*, antes denominado *PARK2*, e a *PACRG*(36). Mais recentemente outro novo gene foi encontrado nesse *locus*, o *SOD2*, como fator de suscetibilidade em duas amostras independentes da população brasileira(187). Além dessas importantes regiões cromossômicas associadas aos diferentes fenótipos da doença, outros *locus* demostraram evidências sugestivas de ligação em vários GWLS, como as regiões 6p21.32, 17q22 e 20p13(188) em famílias brasileiras e 4q22, 8q24 e 16q24 em chinesas(189).

O primeiro GWAS realizado com hanseníase foi em 2009, por Zhang e colaboradores(190), envolvendo quatro amostras populacionais chinesas, independentes. O estudo inicialmente testou em torno de 500.000 SNPs e na análise

final evidenciou seis *loci* associados com hanseníase, localizados em cinco genes: *RIPK2*, *TNFSF15*, *NOD2*, *CCDC122* e *LACC1*; e no locus HLA-DR–DQ. Outros GWAS seguiram descobrindo novos *loci* em diferentes genes. Numa análise expandida do primeiro GWAS, Zhang e colaboradores(191) identificaram, na amostra chinesa, dois novos genes, *IL23* e *RAB32,* envolvidos com a suscetibilidade a hanseníase. Mais recentemente, o GWAS de Liu e colaboradores (192) também na população chinesa, identificaram seis novos *loci* associados com a hanseníase *per se*, envolvendo novos genes: *BATF3*, *CCDC88B* e *CIITA-SOCS1.* No ano seguinte, o GWAS de Wang e colaboradores(193), evidenciaram mais quatro novos *loci* nos genes: *SYN2*, *BBS9*, *CTSB*, *MED30*. Por último, um único GWAS com foco somente nas variantes de genes que codificam proteínas e com frequência do alelo menor (MAF) superior a 0,1%, foi realizado na população chinesa(169). Nesse estudo, 40.491 variantes codificantes foram testadas e replicadas em quatro populações chinesas independentes. Sete variantes não sinônimas foram associadas com a suscetibilidade a hanseníase, incluindo duas variantes raras nos genes C*ARD9* e *NCKIPSD*; três variantes de baixa frequência nos genes *FLG, IL23R* e *TYK2*; e duas variantes comuns em *SLC29A3* e *IL27.* Este estudo também confirmou a associação do *IL23R* com a doença, como previamente identificado do GWAS de Zhang e colaboradores(191).

Até o momento, somente um GWAS foi direcionado para detectar variantes associadas com alterações inflamatórias agudas da hanseníase(194), decorrentes de mecanismos imunológicos. Neste estudo, foram genotipadas 6.3 milhões de variantes, buscando identificar associação com a Reação do Tipo I (RT1). Após a replicação em duas populações independentes, do Brasil e do Vietnã, em estudos de caso *versus* controle, somente um SNP no gene *lncRNA* foi associado com o risco para a RT1.

Nosso estudo genético replicou diferentes SNPs de genes candidatos, que foram validados em vários estudos do tipo caso-controle, alguns deles, representado na (Tabela 2).

Tabela 2 - Associação de SNPs de genes candidatos com a hanseníase, nos estudos de caso-controle.

Gene	SNPs	Associação Sim	Associação Não	Associação	População	Referências
NOD2	**rs8057341**	**Sim**		**Hanseníase**	**Chinesa**	(190)
		Sim		**Hanseníase**	**Brasileira**	(171)
			Não	**Hanseníase**	**Vietnamita**	(195)
			Não	**Hanseníase**	**Indiana /Africana**	(196)
NOD2	**rs751271**	**Sim**		**Reação**	**Brasileira**	(197)
		Sim		**Hanseníase**	**Nepalesa**	(198)
TLR1	**rs4833095**	**Sim**		**Hanseníase**	**Brasileira**	(199)
		Sim		**Hanseníase**	**Bengalesa**	(200)
		Sim		**Reação**	**Bengalesa**	(200)
TNF	**rs1800629**	**Sim**		**Hanseníase**	**Brasileira**	(201)
IL-10	**rs1800871**	**Sim**		**Hanseníase**	**Brasileira/Indiana/ Chinesa/Malawianos**	(202)
		Sim		**PB**	**Brasileira**	(38)
IFNG	**rs2430561**	**Sim**		**Hanseníase**	**Brasileira /Chinesa**	(203)
				PB	**Brasileira**	(204)
PACRG / PRKN	**rs9356058**	**Sim**		**Hanseníase**	**Brasileira**	(168)
		Sim		**Hanseníase**	**Vietnamita**	(168)
		Sim		**Hanseníase**	**Vietnamita**	(205)
		Sim		**Hanseníase**	**Indiana**	(205)
			Não	**Hanseníase**	**Indiana**	(206)
			Não	**Hanseníase**	**Chinesa**	(207)
			Não	**Hanseníase**	**Indiana**	(208)
PACRG / PRKN	**rs1040079**	**Sim**		**Hanseníase**	**Brasileira**	(168)
		Sim		**Hanseníase**	**Vietnamita**	(168)
		Sim		**Hanseníase**	**Vietnamita**	(205)
			Não	**Hanseníase**	**Indiana**	(205)
			Não	**Hanseníase**	**Indiana**	(206)
			Não	**Hanseníase**	**Chinesa**	(207)
			Não	**Hanseníase**	**Indiana**	(208)
CCDC122/ LACC1	**rs4942254**	**Sim**		**Hanseníase**	**Brasileira**	(171)
IL-6	**rs2069845**	**Sim**		**Reação**	**Brasileira**	(197)

1.7.3 Genes candidatos no estudo dos biomarcadores

TLRs (toll-like receptors)

É uma família de glicoproteínas integrais de membrana, cujo domínio extracelular é caracterizado pela presença de um variado número de repetições do aminoácido leucina (*leucine-rich repeats – LRRs*), em forma de ferradura(209). Funcionam como sensores, conhecidos como receptores de reconhecimento padrão (*patttern recognition receptors* - PRRs), capaz de identificar padrões moleculares conservados (*pathogen-associated molecular patterns* - PAMPs) de ligantes, categorizados como lipídeos, proteínas ou ácidos nucleicos de uma grande variedade de bactérias, vírus, fungos ou protozoários(10,210). Um total de 11 homológos do *TLR* foi descoberto nas células humanas, sendo 10 deles funcionais e seus ligantes identificados(210). São expressos nas células do sistema imune inato, principalmente nas células dendríticas (CD), monócitos e macrófagos(211). A maioria dos TLRs atua na forma de homodimerização e alguns deles atuam em complexos heterodiméricos que reconhecem um amplo leque de moléculas microbianas. Na hanseníase, os PAMPs microbianos são as lipoproteínas di ou tri-aciladas que se ligam a complexos heterodiméricos TLR2/6 e TLR1/2, respectivamente. Mas é o complexo TLR1/2 o principal sensor dos PAMPs micobacterianos, devido à grande quantidade de lipoproteínas triaciladas, na parede celular e membrana desses patógenos(76,211). A ligação de agonistas com os TLRs desencadeia uma cascata sinalizadora intracelular, ativando genes regulados pelo Fator Nuclear Kappa B (NF-kβ), principalmente genes que codificam diferentes citocinas pro-inflamatórias, quimiocinas e moléculas coestimulatórias requeridas na ativação de células T(212,213), cujo objetivo final é a eliminação do patógeno invasor.

Variações genéticas, como os polimorfismos de base única (SNPs) que alteram o aminoácido (SNPs não sinônimos), afetam a estrutura da proteína ou a sua função, podem influenciar na resposta imune inata ou adaptativa do hospedeiro, diante de um microorganismo patogênico, e favorecendo um maior risco ou proteção para infecções(210,214). Polimorfismos presentes nos *TLRs* estão associados com doenças em humanos, como a tuberculose, hanseníase, malária, HSV-1 e HCV, por

exemplo(210). Na hanseníase, a maneira como os SNPs se agrupam em haplótipos e a influencia da regulação epigenética são decisivos na dinâmica de resposta do sistema imune(215). Portanto, mais estudos funcionais de variantes alélicas desses receptores podem providenciar um melhor entendimento da patofisiologia das doenças infecciosas(210).

A contribuição dos polimorfismos genéticos dos genes *TLR1*, *TLR2* e *TLR4* na suscetibilidade ou proteção à hanseníase *per se* e nas reações hansênicas, tem sido investigada em diferentes populações étnicas, tais como, a indiana, a turca, a bengalesa, a etíope e a brasileira(199,200,216,217). O estudo de Sales-Marques(199) evidenciou associação estatística do gene *TLR1* 248S (rs4833095) com o risco de desenvolver a hanseníase, tanto no estudo baseado em famílias (z=2.02; p=0.05), como no estudo caso-controle, combinando populações de diferentes cidades brasileiras, como Bauru, Rio de Janeiro e Rondonópolis (OR= 1.51; p=0.001). No entanto, na população de Salvador, esse mesmo SNP não mostrou associação com a doença, apesar dos diferentes genótipos de SNPs dos genes *TLR1*, *TLR2* e *TLR4* demonstrarem um papel regulatório na produção de níveis séricos alterados de citocinas, como *IL-12* (rs4833095 e rs5743551), *IL-17*(rs4833095, rs3804099 e rs1927914), *MCP-1* (rs4833095 e rs5743551), *IL-6* (rs3804099), *CXCL-10* (rs7656411) e *IL-1β* (rs1927914), durante a infecção por *M. leprae*(215).

NOD (*nucleotide-binding and oligomerization domain*)

Além de alguns TLRs intracelulares, outra família de PRRs, porém somente citosólicos, são os receptores semelhantes ao NOD (*NLRs - Nucleotide-binding oligomerization domain – like receptors*). São 23 NLRs conhecidos(218), com a maioria deles atuando como PRRs. Sua interação com ligantes como os PAMPs ou DAMPs, próprios ou de moléculas ambientais, desencadeia distintos processos biológicos, como da diferenciação da resposta imune adaptativa, ativação do complexo proteico conhecido como inflamassoma, a sinalização da transdução, a ativação da transcrição e a autofagia(218–220). Os inúmeros agonistas reconhecidos por esses PRRs podem ser provenientes de patógenos microbianos, como, o DNA viral, hifas de fungos, flagelina, ou componentes das paredes

bacterianas, como o ácido D-glutamil-meso-diaminopimélico e o muramil dipeptídeo (MDP, *muramyl dipeptide*), moléculas de células do hospedeiro (ATP, cristais de colesterol, ácido úrico, etc.) e fontes ambientais como o asbesto, a sílica e o amianto, por exemplo(218). Na hanseníase, o reconhecimento do MDP pelo NOD2 de monócitos humanos, induz a produção de IL-32, que leva a diferenciação de monócitos em células dendríticas (221).

A autofagia é um importante mecanismo homeostático responsável pela degradação de proteínas defeituosas e organelas celulares danificadas, como, as mitocôndrias e retículo endoplasmático, conhecido como mitofagia e reticulofagia, respectivamente. Também pode ocorrer com microorganismos, principalmente bactérias, denominado de xenofagia(222). A autofagia é mediada por estruturas esféricas com dupla membrana, os autofagossomos, que se fundem com lissomomos e promovem a degradação do material citoplasmático sequestrado e de microorganismos(223). O início da autofagia começa pelo reconhecimento do peptidoglicano, liberado da parede bacteriana, e não requer o adaptor de proteína RIP2 ou ativação do NF-κB(223). A formação do autofagossomo envolve inúmeras proteínas ATG (*Autophagy-related Genes*)(222), como a ATG16L1, recrutado pelo NOD1 e NOD2 no local de entrada da bactéria, na membrana plasmática(223).

Polimorfismos no gene *NOD2* têm revelado associação significativa com doenças inflamatórias ou crônicas, como a doença de Crohn e a hanseníase. Essas duas doenças podem compartilhar bases genéticas comuns(190,195). Vários estudos têm correlacionado SNPs no gene *NOD2* com o risco ou a proteção à hanseníase ou às reações hansênicas, em populações como, a chinesa(190), a vietnamita(195), a nepalesa(198) e a brasileira(171). No estudo brasileiro, Marques e colaboradores(171) validaram o estudo de GWAS realizado por Zhang e colaboradores(190) na população chinesa. Confirmaram que o alelo A (rs8057341), do gene *NOD2*, está associado com resistência à hanseníase, tanto numa estimativa global, envolvendo dois estudos baseados em famílias, quanto em três replicações do tipo caso-controle (OR=0.80; p=0.0001).

PRKN/PACRG (parkin RBR E3 ubiquitin protein ligase/parkin coregulated)

São genes que compartilham uma região promotora bidimensional(224), com a função específica do gene *PACRG* (*parkin coregulated*) menos conhecida. Já o gene *PRKN* (*parkin RBR E3 ubiquitin protein ligase*) codifica uma proteína denominada de parquina, uma E3 Ubiquitina ligase, última enzima de uma reação em cascata, responsável pela ligação de moléculas de ubiquitina em alvos específicos, conhecido como ubiquitinização(225). Essa sinalização induz a um processo de autofagia, com a formação do autofagossoma, que após a fusão com os lisossomos, desencadeia a degradação molecular(178). É um processo de renovação intracelular de biomoléculas (lipídeos e proteínas) e organelas, como o retículo endoplasmático e a mitocôndria, conhecido como reticulofagia e mitofagia, respectivamente. As proteínas codificadas do gene *PRKN* junto com o gene *PINK1* (*PTEN-induced putative kinase protein 1* ou *PARK6*), compartilham a mesma via de controle de qualidade das mitocôndrias. Esse controle é perdido quando mutações em um desses dois genes são encontradas, resultando no acúmulo de mitocôndrias danificadas e um aumento de espécies reativas de oxigênio tóxico (*Reactive Oxygen Species - ROS*), levando a morte de células neuronais e o risco de desenvolver a Doença de Parkinson(225). A parquina também está envolvida no processo de destruição de patógenos intracelulares, conhecido como xenofagia (226), atuando como um mecanismo da resposta imune inata, contra a infecção bacteriana, como acontece com a *Mycobacterium tuberculosis*(227), a *Chlamydia trachomatis*(228) e o *Mycobacterium leprae*(229). Porém, nas formas multibacilares da hanseníase, a via da autofagia falha na destruição do bacilo, favorecendo a sua sobrevivência dentro da célula do hospedeiro(229).

Os estudos genéticos, para verificar associação com a hanseníase, são direcionados, principalmente, para os polimorfismos presentes na região reguladora comum, dos genes *PRKN* e *PACRG*. Mira e colaboradores(36) encontraram vários SNPs nestes genes associados com a doença, em famílias vietnamitas e confirmadas na população brasileira, destacando uma forte associação nos SNPs rs9356058, rs1040079 e rs1333955. Alter e colaboradores(205), confirmaram que SNPs nos genes *PRKN* e *PACRG* estão associados com a doença, em populações etnicamente distintas. Os autores observaram que diferenças no padrão do Desequilíbrio de Ligação e da idade no diagnóstico, podem explicar a

heterogeneidade de resultados, entre os recentes estudos, deste *loci* com a hanseníase *per se*. O dado interessante foi a idade no diagnóstico, ou seja, o efeito genético foi mais pronunciado em pacientes mais jovens. No entanto, o estudo de 11 SNPs, na população chinesa,(207) e seis SNPs, na população indiana(208), ambos na região intergênica do *PRKN/PACRG*, não mostraram associação estatística com a hanseníase, inclusive do rs9356058.

IFNG (*Interferon-gama*)

O interferon gama (*IFNG*) humano faz parte de uma grande família de interferons e único membro da classe do tipo II. Está localizado no cromossomo 12 (12q14) e tem quatro exons, abrangendo aproximadamente 6kb(230). É uma das mais relevantes citocinas envolvidas na resposta imune e nos processos inflamatórios, atuando em diferentes frentes como, na ativação de macrófagos, na defesa do hospedeiro contra patógenos intracelulares, na resposta Th1, na regulação da imunidade adaptativa pela diferenciação das Th e Treg(231). É secretada principalmente pelas células CD4+ Th1, células T citotóxicas CD8+ e células *natural killer*(232). Em sinergia com o TNF, ativa mecanismos microbicidas efetores, em macrófagos humanos(233). Muitos SNPs são encontrados no gene *IFNG*, no entanto, apenas o SNP (+ 874T/A, rs2430561) é amplamente estudado. A expressão desse gene pode ser influenciada por esse polimorfismo, localizado no seu primeiro intron, provavelmente devido a este *locus* coincidir com o sítio de ligação do fator de transcrição nuclear kappa B (*nuclear factor- κB*)(234). Esse SNP tem sido associado em estudos de metanálise com a hanseníase(203), a tuberculose(230), a hepatite (232) e as leucemias(235). Nos estudos que mostraram associação, a presença do alelo T correlaciona-se com alta expressão do *IFNG* e aumento da resistência à infecção e ao câncer, enquanto o alelo A se correlaciona com a baixa expressão. No entanto, a associação do SNP (+874T/A) com a hanseníase, ainda não é consistente, tendo em vista a falta de associação, inclusive, em algumas amostras da população brasileira, como por exemplo: a do sul [OR= 0.79; 95% IC (0.51 – 1.25)](236), a de Bauru-SP [OR= 0.83; 95% IC (0.61 – 1.12)](35) e a de Manaus-Am [OR= 0.78; 95% IC (0.44 – 1.37)](204). O mesmo resultado foi encontrado na população chinesa [OR=0.65; 95% IC (0.32 – 1.32)](237). Entretanto, para a população do Rio de Janeiro(35), a associação foi

significativa [OR= 0.71; 95% IC (0.54 – 0.92)]. A metanálise realizada por Silva e colaboradores(203) , com os estudos das populações brasileiras e chinesa, confirmou associação do alelo T com a proteção à hanseníase, sob o modelo dominante [OR= 0.83; 95% CI (0.72 – 0.96)](203).

TNF (*tumor necrosis factor*)

O gene *TNF* está localizado no cromossomo 6p21.3, compreendendo quatro exons e três introns, com comprimento de aproximadamente 3 kb, e está localizado na região do complexo maior de histocompatibilidade (MHC) classe III(201). É uma citocina pleiotrófica que desempenha importante papel na ativação de macrófagos, na formação e manutenção do granuloma e na eficiência da resposta imune celular(238). O granuloma serve não apenas para conter a bactéria, mas também na resposta inflamatória à bactéria(238).

Variantes genéticas, localizadas na região promotora do *TNF*, foram associadas com níveis alterados da expressão de citocina, influenciando na suscetibilidade a várias doenças humanas, como o câncer, doenças infecciosas, neurodegenerativas e autoimunes(239). Dentre os polimorfismos genéticos estudados, na região promotora (-238, -308, -863), o *loci* -308 é o mais frequentemente associado com as doenças infecciosas(239). O alelo mais comum desse polimorfismo tem um resíduo de Guanina (G), enquanto que o menos comum tem um resíduo de Adenina (A). Estudos *in vitro*, com monócitos humanos de sangue periférico, verificaram que a presença do alelo “A”, na posição -308, do gene *TNF*, induziu maiores níveis de produção da citocina(240). Diversos estudos de associação entre a hanseníase *per se* e/ou as formas clínicas e à variante funcional *TNF* -308G/A já foram realizados em populações etnicamente distintas, porém, apresentando resultados contraditórios. Em um trabalho de meta-análise, realizado por Areeshi e colaboradores(201), envolvendo 3.327 casos de hanseníase e 3.203 voluntários saudáveis, com as populações asiática e latino-americana, não evidenciou associação do *TNF* -308G/A com a hanseníase, tanto alélica, quanto genotípica. Mas quando a análise foi realizada em subgrupos populacionais, como entre as populações brasileira e mexicana, houve evidência de associação do alelo A [OR= 0.83; 95% IC (0.72 – 0.96)] e de carreador do alelo A [OR= 0.79; 95% IC

(0.67–0.93)]. Já no subgrupo de asiáticos, não houve associação com a doença. Provavelmente, a composição étnica distinta entre as populações latinas e asiáticas esteja favorecendo resultados divergentes. Recentemente, um estudo realizado por Silva e colaboradores(241), com voluntários do Amazonas, não validaram associação do SNP TNF (-308G>A) com a hanseníase.

CCDC122-LACC1 (*coiled-coil domain containing 122 ge*ne - *laccase multicopper oxidoreductase domain containing 1 gen*e)

A função do gene *CCDC122* ainda é desconhecida, mas o *LACC1* está envolvido na oxidação dos ácidos graxos, produção de espécies de oxigênio reativas e controle da atividade microbicida em macrófagos(178). Há evidências científicas que variantes desses genes estejam associadas com o risco aumentado de manifestação de doenças, como, a hanseníase e a Doença de Crohn (195,242). Polimorfismos, nos genes *CCDC122-LACC1*, foram associados com hanseníase, no primeiro GWAS realizado em chineses(190). Isso estimulou estudos de replicação e validação em populações, etnicamente distintas. Em um segundo estudo subsequente, realizado em uma amostra populacional da Índia (Nova Deli e Kolkata) e de Mali (África ocidental), foi confirmado a associação de variantes dos genes *CCDC122* e *LACC1* e a hanseníase(196). Grant e colaboradores(195), investigaram famílias Vietnamitas e analisaram os 16 SNPs associados ä hanseníase, do GWAS em chinenes(190), e confirmaram associação com hanseníase em seis variantes, incluindo os genes *CCDC122-LACC1*.

Em um estudo de meta-análise, de Sales-Marques(171), realizado com a população brasileira, um polimorfismo no gene *CCDC122* (rs4942254), não identificado nos estudos prévios, mostrou associação com a hanseníase. A análise envolveu dois desenhos de estudo: um baseado em família (Vila da Prata-PA e Almenara-MG) e outro do tipo caso-controle, com três populações (Rio de Janeiro-RJ, Bauru-SP e Rondonópolis-MT), totalizando 3.614 voluntários. O resultado indicou uma OR (*odds ratio*) global, consistente com o efeito de proteção à hanseníase, para o alelo C (OR= 0,86; p= 0,003) no *CCDC122*. Porém, nesse mesmo estudo, os autores não descartaram a possibilidade que o gene vizinho (*LACC1*) possa ser o verdadeiro responsável pela associação capturada, devido ao

extenso padrão de desequilíbrio de ligação (*linkage disequilibrium*) encontrado entre os SNPs desses dois genes.

IL10 (interleucina 10)

É uma citocina com atividade imunoregulatória, com potente efeito imunossupressivo, essencial na regulação da resposta imune(243). É codificado pelo gene *IL10*, localizada no *locus* 1q31-32 e sua síntese ocorre principalmente em células da linhagem dos monócitos, macrófagos e linfócitos(243). Nos macrófagos, a IL-10 e a IL-15 são citocinas chave na indução diferenciada da programação de duas atividades essenciais, a fagocitose e a atividade microbicida(244). No entanto, a expressão dessas citocinas é dada de forma diferente, em pacientes com hanseníase, dependendo da forma clínica apresentada. A IL-15 e a IL-10 são mais expressas nas lesões das formas clínicas tuberculoides e virchovianas, respectivamente(97,244,245). A IL-15 atua estimulando a atividade microbicida, dependente da vitamina D, e a fagocitose pelo CD209, no limite da efetividade do macrófago. Enquanto que a IL-10 exacerba a ingestão da fagocitose de micobactérias, e a ingestão de lipídeos oxidados, através de receptores CD209 e de receptores LDL, respectivamente. A IL-10 não tem a capacidade de induzir a CYP27b1, enzima responsável pela conversão da 25D3 para 1,25D3, a forma ativa da vitamina D. Consequentemente, a via microbicida dependente da vitamina D não é ativada, resultando em uma capacidade de destruição do patógeno praticamente inexistente, contribuindo para a progressão da doença(244).

Os estudos do tipo caso-controle, dos polimorfismos do gene *IL10*, foram direcionados para a sua região promotora, localizados tanto na parte distal (-3575 T>A (rs1800890), -2849 G>A (rs6703630), -2763 C>A (rs6693899), quanto na parte proximal (-1082 G>A (rs1800896), -819 C>T (rs1800871) e -592 C>A (rs1800872) (246). Na primeira meta-análise envolvendo os polimorfismos do gene *IL10* e a hanseníase(246), a análise combinada de 5 estudos (2.702 indivíduos), com populações do Brasil, Malawi e da Índia, o SNP (rs1800871) -819 C>T esteve associado ao risco, nas três combinações testadas (alélica, genotípica e carreadora). Na recente meta-análise de Alvarado-Arnez(247), envolvendo as populações do Brasil, da China, do Malawi, do México, da Índia e da Colômbia,

reforçou o papel do alelo T (-819) rs1800871, ao risco à hanseníase [OR = 1.20, (95% CI = 1.10–1.31); p=0,00002]. Apesar da modesta chance de risco de 20%, o estudo incluiu populações de diferentes grupos étnicos, validando esse SNP como um importante tag snp.

IL6 (interleucina 6)

Diferentes tipos celulares sintetizam a Interleucina 6, como os macrófagos, os neutrófilos, os eusinófilos, e as células T e B. É uma citocina com efeito pleiotrópico na inflamação, na resposta imune e na hematopoiese(248). Desempenha um importante papel na resposta inflamatória aguda, como na resposta à uma infecção ou dano tecidual, auxilia induzindo a diferenciação das células B em plasmócitos, produtoras de anticorpos; nas células T CD4+, imaturas em efetoras Th17 ou T reg e nas T CD8+, em células T citotóxicas(248). Variantes genéticas desta citocina também podem estar associadas com inúmeras doenças, como, o Diabetes Mellitus tipo 2(249), o câncer(250), o Lúpus Eritematoso Sistêmico(251), a artrite idiopática juvenil(252) e a hanseníase(197). Níveis aumentados de Interleucina 6 estão associados com as reações inflamatórias na hanseníase, classificadas como Tipo 1 ou Reação Reversa, e, Tipo 2 ou Eritema Nodoso Hansênico. Esses episódios são a principal causa de danos neurais, podendo levar a incapacidades físicas, muitas vezes irreversíveis. Sales-Marques e colaboradores(197) verificaram que pacientes que carreavam o alelo G, do SNP rs2069845, do gene *IL6*, desenvolveram reações mais rápido do que os portadores dos outros genótipos ou alelos, servindo assim, como um bom marcador de prognóstico para os episódios reacionais.

Apesar dos polimorfismos no gene *IL6* estarem associados com os estados reacionais da hanseníase, a variante rs2069845 foi incluída no presente estudo, para avaliar se há alguma associação com a hanseníase *per se* em uma população etnicamente distinta de outras regiões brasileiras.

Os estudos de ligação e associação (GWLS e GWAS) e os diversos estudos de caso-controle, replicados em diferentes populações, têm associado vários genes e vias metabólicas, com a suscetibilidade à hanseníase. Interessante que muitas dessas variantes são também compartilhadas com desordens inflamatórias comuns

e neurodegenerativas, como a Doença de Crohn e Parkinson, respectivamente. Entretanto, esses estudos genéticos têm identificado covariáveis que podem dificultar a análise genética de doenças complexas, como é o caso da hanseníase. Algumas dessas covariáveis podem ser a idade no diagnóstico, o tamanho amostral, as diferenças no padrão do Desequilíbrio de Ligação, a homogeneidade fenotípica e o efeito de endofenótipos, e que podem impactar na interpretação dos achados genéticos(253).

Na era da genômica, o estudo e caracterização de um painel de marcadores genéticos, como os SNPs, em uma determinada população, permite conhecer o perfil genético de suscetibilidade à doença. Isto pode auxiliar no conhecimento dos candidatos prováveis em desenvolver a doença (entre os contatos domiciliares, por exemplo). Com isso, pode-se traçar alternativas de prevenção da hanseníase, profilaxia e diagnóstico precoce nesses pacientes, e, consequentemente, um grande impacto na diminuição de novos casos de hanseníase e das sequelas causadas pelo bacilo. Entretanto, para isso, os achados originais de associação, entre os marcadores moleculares e os diferentes fenótipos apresentados na hanseníase, precisam ser validados por replicação, em diferentes grupos étnicos. Neste contexto, é importante lembrar que a população do estado do Amazonas é miscigenada, como nos outros estados brasileiros, entretanto, com uma fração de ancestralidade genética mais acentuada de indígenas. Até hoje, poucos estudos do tipo caso-controle, com um grande número amostral, foram realizados nestra amostra populacional. Assim, dessa forma, a identificação de marcadores genéticos majoritários, nos indivíduos do estado do Amazonas, é essencial para definição de políticas de saúde, associadas ao monitoramento de contatos.

1.8 Relevância do estudo

A Organização Mundial de Saúde tem adotado estratégias quinquenais, com prioridades de ações, para que os países endêmicos possam adotar intervenções de combate a hanseníase. A recente Estratégia Global para a Hanseníase 2016-2020, baseado em estratégias quinquenais anteriores, tem como propósito, a detecção precoce e o tratamento imediato da doença, visando reduzir as incapacidades físicas e a transmissão da infecção na comunidade. A Parceria Global para a Erradicação da Hanseníase, recomenda o desenvolvimento de um teste diagnóstico que possa identificar casos de hanseníase, tão logo quanto possível após a sua infecção. Vários estudos têm focado na identificação de biomarcadores, tanto em humanos como no bacilo, em diferentes populações, visando qualificá-los para o uso no diagnóstico precoce da hanseníase.

O conhecimento de variações genéticas e do perfil de resposta imunológica do hospedeiro, podem ajudar a subsidiar um melhor entendimento da patogênese da hanseníase e talvez, levar a novos desafios de diagnóstico precoce, tratamento imediato ou quimioprofilaxia.

Na presente pesquisa de doutoramento foram propostos dois estudos: - No primeiro foi avaliada a performance de dois testes sorológicos rápidos (IgG/IgM), visando conhecer a acurácia desses testes e sua utilização como suporte ao diagnóstico clínico; - O segundo foi a análise de polimorfismos genéticos (SNPs) que influenciam na suscetibilidade à hanseníase. A validação desses polimorfismos visa colaborar com a formação de um futuro painel de SNPs que estejam associados com a doença. Esse painel contribuirá nas estratégias de vigilância, entre os contatos, por exemplo, permitindo identificar quais indivíduos, desse grupo de risco, possuem maior probabilidade em desenvolver a hanseníase.

2 OBJETIVOS

2.1 Objetivo Geral

Avaliar a associação de biomarcadores com a hanseníase em uma população miscigenada do Amazonas.

2.2 Objetivos Específicos

a) Analisar a performance de dois testes sorológicos rápido (PGL1 e NDO-LID), na discriminação de casos de hanseníase (paucibacilares e multibacilares) e indivíduos saudáveis;

b) Verificar a concordância entre os testes rápidos (PGL1 e NDO-LID), no soro, em pacientes e grupo controle;

c) Verificar associação do teste sorológico de melhor acurácia com parâmetros clínico-laboratoriais, de pacientes com hanseníase;

d) Relacionar os polimorfismos de base única (SNPs), nos genes, *TLR1*, *NOD2*, *PACRG/PRKN*, *IL6*, *IL10*, *TNF*, *IFNG* e CCDC122/LACC1 e a suscetibilidade à hanseníase, em um estudo de caso x controle;

e) Verificar associação dos polimorfismos de base única (SNPs), nos genes, *TLR1*, *NOD2*, *PACRG/PRKN*, *IL6*, *IL10*, *TNF*, *IFNG* e CCDC122/LACC1 entre a classificação operacional (PB x MB) e entre controles x MB.

3 SUJEITOS, MATERIAL E MÉTODOS

3.1 População de estudo

O estudo foi realizado na Fundação Alfredo da Matta (FUAM), Manaus-AM, em pacientes com hanseníase, contatos domiciliares e indivíduos saudáveis (grupo controle) que compareceram na Instituição por demanda espontânea e aceitaram participar do projeto, no período de março de 2014 a abril de 2018.

Pacientes com hanseníase foram classificados de acordo com os achados clínicos e laboratoriais (baciloscopia e exame histopatológico) e foram tratados como paucibacilares ou multibacilares, de acordo com o manual da OMS e Ministério da Saúde.

O grupo controle foi composto de indivíduos saudáveis que moravam na mesma área endêmica que os casos de hanseníase. Esse grupo de pessoas compareceu na Fundação Alfredo da Matta (FUAM) para receber um atestado dermato-neurológico, que é um dos pré-requisitos documentais exigidos pela indústria, comércio ou setor de entretenimento a fim de conseguirem uma colocação no mercado de trabalho. Foram convidados a participar da pesquisa somente após um exame dermatológico que confirmou a ausência de lesões características ou suspeitas de hanseníase, e da confirmação individual de que não teve contato com doentes ou de não haver casos da doença na família.

Esse estudo foi aprovado pelo Comitê de Ética em Pesquisa (555.620 de 13/03/2013), (ANEXO C) da FUAM. Todos os participantes assinaram o Termo de Consentimento Livre e Esclarecido (TCLE), (APÊNDICES B, C e D). Participantes com idade inferior a 18 anos tiveram o TCLE assinado por um dos pais ou por um representante legal.

3.2 Detecção pelo NDO-LID e PGL1

Os testes rápidos de fluxo lateral, NDO-LID e o PGL1, são testes imunocromatográficos que detectam IgM/IgG e IgM, respectivamente, contra o *M. leprae*. O NDO-LID (Orange Life, Rio de Janeiro, Brazil) usa como antígeno um

dissacarideo sintético (ND), que mimetiza o PGL1, ligado a um radical octil (O), conjugado com duas proteínas de fusão, o ML 0304 e o ML 2331, formando o LID.

O antígeno imobilizado na membrana de nitrocelulose do teste rápido PGL1, é o glicolipidio fenólico nativo, que detecta anticorpos específicos IgM. Esse teste é confeccionado no IPTSP-UFG, sob a coordenação da Dra. Samira Bührer-Sekula.

Cada dispositivo de teste rápido consiste de um cassete de plástico que contem um *strip* de membrana de nitrocelulose, impregnada com um *spot* de teste (contendo antígenos do *M. leprae*) e um *spot* de controle.

O teste é considerado positivo quando uma gota de sangue, soro ou plasma do paciente, junto com duas gotas de tampão de diluente, adicionado na linha de teste, apresentar mudança de cor após dez minutos.

Dois técnicos realizaram a mesma leitura para evitar inconsistências. Um terceiro leitor deu sua opinião final quando os resultados foram discordantes.

3.3 Detecção de DNA do *M. leprae* pela técnica de qPCR

A extração de DNA (ANEXO A) foi realizada a partir do raspado dérmico de contatos domiciliares de pacientes com hanseníase, utilizando o *kit DNeasy Blood & Tissue (Qiagen)* e seguindo as instruções do fabricante. Os métodos de qPCR para detecção, utilizando como alvo o *16S* rRNA de *M. leprae*, foram descritos anteriormente em vários trabalhos de Martinez e colaboradores(30–32) e operacionalizados de acordo com as indicações do Ministério da Saúde para o diagnóstico de hanseníase. As reações de q-PCR foram preparadas, com reagentes da *Applied Biosystems*, para um volume final de 10 µL, e realizados no equipamento *StepOne Plus real-time PCR system* (*Applied BioSystems*), seguindo as condições de ciclagem padrão. As concentrações finais da reação foram: oligonucleotídeos [300 nM], sonda *16S* rRNA [100 nM], Master Mix da Applied BioSystem [1x] e DNA da amostra extraída de raspado dérmico [1 – 5 ng]. Para descartar qualquer possibilidade de inibidores da PCR, a amplificação de um fragmento do gene da β-actina humana foi realizada.

3.4 Genotipagem dos SNPs

O presente estudo foi desenhado para investigar a associação de SNPs nos genes *TLR1* (rs4833095), *NOD2* (rs751271, rs8057341), *TNF* (rs1800629), *IL10* (rs1800871), *CCDC122/LACC1* (rs4942254), *PACRG/PRKN* (rs9356058, rs1040079), *IFNG* (rs2430561) e *IL6* (rs2069845) com a hanseníase. A seleção dos SNPs se baseou em recentes estudos publicados, que mostraram associação com a hanseníase *per se*(36,171,199,201,203,247) ou reação(197) na população brasileira.

Os SNPs escolhidos foram baseados no seu papel funcional, na região promotora, exônica ou intrônica do gene e incluídos com desvio do equilíbrio de Hardy–Weinberg (P>0.01) nos controles e amplificação acima de 95%.

O DNA foi extraído de amostras de sangue (ANEXO B), utilizando o *kit DNeasy Blood & Tissue (Qiagen)* e seguindo as instruções do fabricante. Em seguida foi quantificado pelo *Qubit® 3.0 Fluorometer* e diluído (1/10) para genotipagem por PCR em tempo real (q-PCR). Os ensaios de genotipagem com sondas *TaqMan* (*Life Technologies*, EUA) foram realizados no equipamento *StepOne Plus real-time PCR system* (*Applied BioSystems*), pela técnica de discriminação alélica baseado na fluorescência e seguindo as condições de ciclagem padrão.

Na técnica de discriminação alélica, a obtenção dos genótipos é fornecida através da intensidade de fluorescência, gerada por sondas marcadas com fluoróforos específicos para cada alelo, por meio do sistema de ensaios (*assays*, sondas *TaqMan MGB)* (*Life Technologies*). Cada ensaio contém um par de *primers* e um par de sondas *TaqMan MGB* (*Life Technologies*) marcadas com fluorescência, VIC (verde), para o alelo selvagem, e FAM (azul), para o alelo mutado ou menos frequente, na região de interesse. A sonda é marcada duplamente, em uma extremidade com um fluoróforo repórter (*reporter*) e em outra extremidade com o fluoróforo silenciador (*quencher*).

Os ensaios consistiram de 5 µL de reação, contendo 2,5 µL de *TaqMan Universal PCR Master Mix No AmpErase UNG (Applied Biosystems)* [concentração final de 1X], 1,375 µL de água, 0,125 µL de sondas*TaqMan assay* [concentração final de 1X], e 1 µL de DNA diluído (10–40 ng).

3.5 Análise Estatística

Para avaliar a performance dos testes rápidos foi utilizado o *software* estatístico *MedCalc* para Windows, versão 17.0.4 (*MedCalc Software*, Ostend, Belgium; https://www.medcalc.org; 2016), na caracterização da sensibilidade, especificidade, valor preditivo positivo (VPP), valor preditivo negativo (VPN) e acurácia, com 95% de nível de confiança. Na identificação do grau de concordância entre os testes foi utilizado o índice kappa (κ)(254): baixo (0–0.5), moderado (0.51–0.75) e excelente (0.76–1). O teste qui-quadrado foi usado para verificar associação de desempenho entre os lotes do NDO-LID, amostras biológicas (soro e sangue) e associação de variáveis clínico-laboratoriais com o resultado do teste NDO-LID. O P-valor <0.05 foi considerado estatisticamente significativo.

No estudo genético, o desequilíbrio de ligação (DL) para os dois SNPs do gene *NOD2* foram estimados, em indivíduos controles, usando o parâmetro r^2, através do *software Haploview*, versão 4.2(255). O equilíbrio de *Hardy-Weiberg* foi estimado por meio do *software* R versão 3.4.3 para *Windows*. As análises das frequencias alélicas, genotípicas e de carreador foram realizadas em casos e controles e comparadas entre esses dois grupos. Através do modelo de regressão logística, foi calculada a OR (*odds ratio*) que foi usada como estimativa de associação, com intervalo de confiança (IC) de 95% e p-valor significativo (≤0.05). Levando-se em conta o valor do IC, de p-valor e de OR, os modelos indicaram suscetibilidade ao risco ou à proteção. Sendo: OR>1, risco; OR=1, não há associação com a doença e OR<1 proteção.

As análises de regressão logística foram obtidas por meio do *software* R, versão 3.4.3 para *Windows* e foram corrigidas para as covariáveis de sexo, idade e ancestralidade, usando os pacotes “*genetics*” e “*rms*” (https://www.r-project.org)(256).

4 PRODUTOS DA TESE

4.1 Artigo 1

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RESEARCH ARTICLE Open Access

Performance of serological tests PGL1 and NDO-LID in the diagnosis of leprosy in a reference Center in Brazil

André Luiz Leturiondo[1,2*], Ariani Batista Noronha[2], Monik Oney Oliveira do Nascimento[2], Cynthia de Oliveira Ferreira[2], Fabíola da Costa Rodrigues[2], Milton Ozório Moraes[3] and Carolina Talhari[1,2,4]

Abstract

Background: Early detection of leprosy and multidrug therapy are crucial to achieve zero transmission and zero grade II incapacities goals of World Health Organization. Leprosy is difficult to diagnose because clinical forms vary and there are no gold standard methods to guide clinicians. The serological rapid tests aid the clinical diagnosis and are available for field use. They are easy to perform, do not require special equipment or refrigeration and are cheaper than the molecular tests.

Methods: We evaluated the performance of two rapid serological tests (PGL1 and NDO-LID) in the discrimination of leprosy cases from healthy individuals at the Alfredo da Matta Foundation, a reference center for the disease in Manaus, Amazonas, Brazil. PGL1 and NDO-LID rapid tests are capable of detecting specific antibodies of *M. leprae*, IgM and IgM/IgG, respectively. A total of 530 healthy subjects and 171 patients (50 with paucibacillary and 121 multibacillary leprosy) were included in the study.

Results: Among the paucibacillary leprosy patients, the sensitivity was 34.0 and 32.0% for the NDO-LID and PGL1, respectively. In multibacillary leprosy patients, the NDO-LID sensitivity was 73.6% and the PGL1 was 81.0%. Serological tests demonstrated specificities of 75.9% for PGL-1 and 81.7% for NDO-LID. The positive predictive value (PPV), negative predictive value (NPV) and accuracy in multibacillary patients were 47.9, 93.1, and 80.2% respectively for the NDO-LID, and 43.4, 94.6 76.8% for PGL1.

Conclusions: The tests showed limited capacity in the diagnosis of the disease, however, the high negative predictive value of the tests indicates a greater chance of true negatives in this group favoring exclusion of leprosy. This characteristic of the ML flow test is important in aiding clinical Diagnosis, especially in a region endemic to the disease and with other confounding skin conditions.

Keywords: Leprosy, Accuracy, Serologic tests, PGL1, NDO-LID

Background

Leprosy is chronic infectious diseases that show a long incubation period. The disease is caused by *Mycobacterium leprae* or *M. lepromatosis* that affect the skin and peripheral nerves. If not early detected and adequately treated, the disease may lead to physical incapacities and irreversible deformities [1]. The introduction of multidrug therapy (MDT), in the early 1980s, had a huge impact in the prevalence of leprosy; more than 16 million patients were cured in the last 30 years [2]. However, the incidence is still high where approximately 200,000 new cases are diagnosed each year. Probably, MDT has a modest impact on incidence because transmission occurs prior to diagnosis. Recent strategies to stop leprosy transmission rely on prophylactic protocols using rifampicin and/or BCG [3, 4]. The World Health Organization (WHO) developed a strategy to achieve zero transmission, zero cases among children with grade II incapacities by 2020 [5]. In Brazil, there are still difficulties in achieving this goal, due not

* Correspondence: andre_leturiondo@yahoo.com.br
[1]Programa de Pós-Graduação em Medicina Tropical, Universidade do Estado do Amazonas, Manaus, Amazonas, Brazil
[2]Fundação Alfredo da Matta, Av Codajás 24, Cachoeirinha, Manaus, Amazonas, Brazil
Full list of author information is available at the end of the article

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only to the lack of professionals experienced in diagnosing leprosy, but also to the inherent issues of the disease. Some paucibacillary (PB) and neural forms may be easily confused with other common dermatoses such as granuloma annulare, sarcoidosis or pityriasis alba [6]. In addition, about 30% of patients, many of them multibacillary (MB), do not present cardinal symptoms such as loss of sensitivity, favoring the active transmission of the disease [7].

The development of a simple and practical test, able to confirm the clinical decision, is of vital importance, especially in the field, where there are few specialists to diagnose and treat the disease. Slit skin smear and histopathological examinations, despite high specificity, have low sensitivity [8]; those techniques are also invasive and require trained professionals. Molecular techniques such as PCR and qPCR are promising because of their high accuracy, however, these tools are still costly and require skilled technicians [9]. Serological tests, although not stand-alone diagnosis tools, are point-of-care in the early identification of leprosy, even before the initial lesions appearance [10, 11]. Moreover, these tests present a lower cost when compared to molecular assays, are of easy execution, suitable for field diagnosis and require no special equipment or refrigeration [12, 13].

However, the sensitivity of the serological tests varies, depending on the population studied, in order to have a real specific profile of each locality [14]. In this context, this study analyzed the performance of two rapid serological tests for identification of patients with leprosy, at a REFERENCE Center for the disease in the north of Brazil. The two tests exhibited a high negative predictive value (NPV), useful to exclude leprosy supporting clinical diagnosis in endemic regions.

Subjects, materials and methods

Design and study population

Evaluation of two serological tests, immunochromatographic, with untreated leprosy patients and healthy individuals who attended by spontaneous demand at the Alfredo da Matta Foundation (Manaus-AM, Brazil). All included patients and controls were 10 years-old or older and were recruited, from March 2014 to March 2016. Patients with leprosy were classified according to the clinical, slit skin smears and histopathological findings [15] and were treated as paucibacillary or multibacillary, according to the number of skin lesions, slit skin smear result and the compromised nerve [16]. The endemic control group was among individuals who lived in the same endemic area as the cases. These endemic controls seek a medical certificate for employment purposes. These individuals were subjected to a dermatoneurological examination and had no suspected leprosy lesions and declared no information concerning contact with leprosy or tuberculosis patients. The study did not include volunteers on corticosteroid treatment, cancer chemotherapy or HIV. Samples that presented hemolyzed serum were also excluded.

This study was approved by the Research Ethical Committee (555.620–13/03/2013) of the "Alfredo da Matta Foundation". All participants signed an informed consent before enrolment. Participants under the age of 16 have had the consent provided by a parent or legal guardian.

Detection by NDO-LID and PGL1

The NDO-LID (Orange Life, Rio de Janeiro, Brazil) rapid test uses as antigens a semi-synthetic disaccharide (ND) attached to the octyl (O) radical, which mimics PGL1, conjugated with two fusion proteins, ML 0304 and ML 0331, forming the LID, capable of recognizing, respectively, IgM and IgG antibodies against *M. leprae*. The antigen immobilized on the nitrocellulose membrane, from the PGL1 rapid test (IPTSP/UFG), was the native phenol-1 glycolipid, which detects specific IgM antibodies. Rapid tests followed the instructions recommended by the manufacturer. Two independent technicians performed reading to avoid inconsistencies. A third reader gave his final opinion on discordant results.

Statistical analysis

For the calculation of sensitivity, specificity, positive predictive value, negative predictive value, with 95% confidence level, MedCalc statistical software for Windows, version 17.0.4 (MedCalc Software, Ostend, Belgium; https://www.medcalc.org; 2016) was used. To evaluate the level of concordance of the trials we used Kappa concordance test (κ) [17]: low (0–0.5), moderate (0.51–0.75) and excellent (0.76–1). The chi-square test was used to verify the association between batches, biological samples and the frequencies of the rapid test result and the clinical-laboratory variables. P-value < 0.05 was considered statistically significant.

Results

A total of 530 healthy subjects and 250 leprosy patients, ranging in age from 10 to 77 years-old (mean of 41.24 years-old) were enrolled in the study. The group most affected by the disease was between 21 to 60 years-old (64.8%), mostly men (66.0%), mixed (85.0%) and had finished elementary school (47%) (Table 1).

Out of 250 patients with leprosy, 250 blood and 171 serum samples were collected. First, we compared the performance of the test NDO-LID between biological samples from serum and blood of the same patient and between two different commercial batches (L-1 and L-2). Thirty samples (13 PB and 17 MB) were randomly selected to verify the sensitivity of the rapid test. Among the 13 PB patients tested, we detected 2 (15.4%) in L-1 and 1 (7.7%)

Table 1 Distribution of leprosy cases according to socio-demographic variables

Characteristics	Number	Percentage (%)
Gender		
Male	165	66.0%
Female	85	34.0%
Total	250	100.0%
Age group (years)		
10–20	44	17.6%
21–40	78	31.2%
41–60	84	33.6%
> 60	44	17.6%
Total	250	100.0%
Education		
Illiterate	11	6.1%
Elementary School	85	47.0%
High school	56	30.9%
Higher education	15	8.3%
Ignored	14	7.7%
Total	181	100.0%
Ethnicity[a]		
Caucasian	9	6.5%
African	8	5.7%
Asian	3	2.1
Mixed	119	85.0%
Indigenous	1	0.7%
Total	140	100.0%

[a]self-reported Ethnicity

in L-2 when whole blood samples were probed. The serum from the same patients was tested and 6 (46.2%) in L-1 and 2 (15.4%) in L-2 were detected, demonstrating better sensitivity for serum as compared to blood. Among the 17 MB patients, positivity was higher, as expected: 9 (52.9%) and 8 (47.1%) for L-1 and L-2 batches, respectively. While in serum, results were far more sensitive and 14 (82.4%) in L-1 and 11 (64.7%) in L-2 (data not shown).

Then, 171 patients (50 PB and 121 MB) were tested for PGL1 and NDO-LID in serum since results suggested that this sample was the best. The specificity was evaluated in 530 healthy volunteers. The Kappa value was used to compare the agreement rates between samples in both tests. Paucibacillary patients exhibited the excellent concordance and demonstrating that there was no significant difference between them. For MB leprosy patients and healthy controls, the tests had an excellent agreement, with significant differences between them. PGL1 was superior to NDO-LID in detecting MB patients. However, this test also detected a higher number of healthy volunteers indicating lower specificity (Table 2).

Among the PB leprosy patients, sensitivity was 34.0 and 32.0% for NDO-LID and PGL1, respectively (Table 3). In MB patients, NDO-LID sensitivity was 73.6% and PGL1 was 81.0% (Table 4). Among healthy individuals, specificity was 81.7 and 75.9% for NDO-LID and PGL1 respectively, indicating that both tests have a high percentage of false positives. The positive predictive value (PPV), negative predictive value (NPV), and accuracy in MB leprosy patients were 47.9, 93.1 and 80.2%, respectively for the NDO-LID, and 43.4, 94.6, and 76.8%, for PGL1 (Table 4).

We decided to test clinical-laboratory parameters evaluating only NDO-LID, because this test exhibited the highest accuracy. One may observe that there was a significant difference in the rapid test for MB leprosy patients with positive skin smear, lepromatous lepromatous leprosy and with more than five lesions ($p < 0.05$) (Additional file 1: Table S1).

Discussion

Although we know the limited capacity of serological tests in the diagnosis of leprosy, they are still important as a tool to aid clinical diagnosis. This study allowed evaluating the performance of two tests in a region of high endemicity for leprosy and with that to draw a better alternative to their use in the clinical practice. NDO-LID and PGL1 tests may be useful as a support tool for clinical diagnosis. Furthermore, they could be employed for excluding leprosy as a possible cause of a skin lesion, especially in endemic areas where other common dermatological conditions are detected.

When we assessed the best biological samples to be used in the study, no statistical association was found between them or between batches (data not shown), despite the better serum sensitivity than blood. A

Table 2 Agreement between NDO-LID and PGL-I rapid tests in serum

	NDO-LID+PGL1-(%)	NDO-LID-PGL1+(%)	AGR (%)	K (IC)	*p*-value*
MB	0.0%	7.4%	92.6%	0.79 (0.66 - 0.92)	0.0024
PB	2.0%	0.0%	98.0%	0.95 (0.87 - 1.04)	0.8324
EC	0.0%	5.8%	94.2%	0.83 (0.77 - 0.89)	< 0.0001

MB multibacillary leprosy patients, *PB* paucibacillary leprosy patients, *EC* endemic control, *AGR* agreement, *k* kappa value, + = positive; − = negative. *Chi-square test

Table 3 ML Flow performance test in paucibacillary leprosy patients and healthy volunteers

ML Flow	Sensitivity	Specificity	PPV	NPV	Accuracy
NDO-LID	34% (17/50)	81.7% (433/530)	14.9%	92.9%	77.6%
PGL1	32% (16/50)	75.9%(402/530)	11.1%	92.2%	72.1%

PPV Positive Predictive Value, *NPV* Negative Predictive Value

similar result was also found in other studies, showing a strong correlation between the results from whole blood and serum [13, 18].

Although the evaluation of the tests presented excellent agreement, they did not have the same performance in the identification of patients with leprosy. Both tests had a better capacity to detect individuals with MB leprosy, but were inefficient for the diagnosis of PB forms of leprosy, thus confirming that serological tests could be considered effective tools for the diagnosis of MB leprosy [12, 14, 19, 20]. However, we found a lower sensitivity for MB forms of the disease than the previously reported. In other studies, the sensitivity of NDO-LID antigens in MB patients ranged from 87.0 to 95% [14, 19-22]. This trend has been also observed among leprosy patients classified according to the WHO operational classification, both for rapid tests using immunochromatography, and for those using ELISA methodology. The seropositivity found in the tests under analysis reflects the type of immune response developed by the host [14, 19, 20, 22, 23]. Regarding specificity, other studies found values higher than ours, of 88 and 96.1% for NDO-LID [19, 22]. This might probably indicate differences in the endemicity in the regions where the studies were performed [24]. In assessing the specificity of the rapid tests, we did not include other confounding dermatoses with leprosy, such as granuloma annulare, sarcoidosis, pityriasis alba or mycobacterial infections like tuberculosis, for example.

Both NDO-LID and PGL1 tests showed very low capacity to detect true positives in PB patients (14.9 and 11.1%, respectively) and in MB patients (47.9 and 43.4%, respectively). In fact, 93.1% of NPV is high demonstrating that a negative NDO-LID test could be employed in excluding leprosy as a possible cause of a skin patch or lesion.

Nevertheless, a positive result in the rapid test cannot be used to include patients since we found several false positive samples (20%) among controls. Our control group is composed of healthy individuals living in a high endemicity region. Thus, the elevated seropositivity in this group suggest that this population is regularly being exposed to *M. leprae*, and likely to suggest active transmission [25]. We suggest that ML flow tests (NDO-LID or PGL1) represent an important test as an indicator of *M. leprae* circulation for the surveillance evaluating whether it can be considered an active transmission area. Recently, in a household contact cohort in Bangladesh a positive anti-PGL-I was not a good predictive marker of leprosy outcome [26]. Here, our results of positive ML flow among healthy individuals are far more difficult to predict whether it could be used to estimate the risk of progression towards leprosy since anti-PGL-I could be a surrogate markers of infection. After the evaluation, all positive healthy individuals ($n = 128$) were contacted by telephone 3 years after serological testing and only one responded. In this preliminary analysis, we observed that the adherence is very low, and unfortunately follow up is difficult to achieve in a group of healthy individuals.

Table 4 ML Flow performance test in multibacillary leprosy patients and healthy volunteers

ML Flow	Sensitivity	Specificity	PPV	NPV	Accuracy
NDO-LID	73.6% (89/121)	81.7% (433/530)	47.9%	93.1%	80.2%
PGL1	81.0% (98/121)	75.9% (402/530)	43.4%	94.6%	76.8%

PPV Positive Predictive Value, NPV Negative Predictive Value

It is not yet possible to use any molecular, genetic or serological marker in the diagnosis or prognosis of leprosy. Infection can be demonstrated by PCR or ML Flow and ELISA (PGL-I) serological tests, but these tests are not able to predict, who will progress towards the disease among household contacts. Currently, the diagnosis of leprosy is still based on the appearance and recognition of clinical signs and symptoms. But, improvement of DNA-based detection of *M. leprae* or host targets by qPCR could be useful tools to aid clinical diagnosis.

Only the involvement of experienced physicians, government, population of hyperendemic area [27], along with novel technologies such as qPCR to diagnose [28] and large-scale public policies clinically screening household contacts with chemo- and immunoprophylaxis of healthy ones could halt *M. leprae* transmission in the community and reduce the number of cases of visible disability [29].

Conclusions

The tests showed limited capacity in the diagnosis of the disease. However, a negative result from the LID-1 or NDO-LID tests showed a greater probability of ruling out the diagnosis of leprosy as a possible cause of a skin lesion. This ML flow test feature is important, especially in an endemic region of the disease and other confusing skin conditions.

Additional files

Additional file 1: Table S1. Evaluation of NDO-LID performance in patients with leprosy. (DOC 36 kb)

Additional file 2: Raw database. Clinical, laboratory and demographic variables used in this study. (XLS 226 kb)

Abbreviations
AMF: Alfredo da Matta Foundation; ELISA: Enzyme-linked immunosorbent assay; IgG: Immunoglobulin G; IgM: Immunoglobulin M; IPTSP/UFG: Instituto de Patologia Tropical e Saúde Pública/ Universidade Federal de Goiás; MB: Multibacillary; MDT: Multidrug therapy; ML flow: Measurement lateral flow test; NDO-LID: Natural disaccharide octyl – leprosy IDRI diagnostic 1; NPV: Negative predictive value; PB: Paucibacillary; PCR: Polymerase chain reaction; PGL-1: Phenolic glycolipid 1; PPV: Positive predictive value; qPCR: quantitative polymerase chain reaction

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Availability of data and materials
All data generated or analysed during this study are included in this published article [and its Additional file 1: Table S1 and Additional file 2: Raw database].

Authors' contributions
All authors read and approved the final manuscript. ALL, study design, performed the experiments, analyzed and interpreted the data and wrote the manuscript; MOM, CT Study design, analyzed and interpreted the data and wrote the paper; ABN, MOON, performed the experiments, analyzed and interpreted the data; COF, FCR performed the experiments, analyzed and interpreted the data and drafted the paper.

Ethics approval and consent to participate
This study was approved by the Research Ethical Committee (555.620–13/03/2013) of the "Alfredo da Matta Foundation". All participants signed an informed consent before enrolment. Participants under the age of 16 have had the consent provided by a parent or legal guardian.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.
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Author details
[1]Programa de Pós-Graduação em Medicina Tropical, Universidade do Estado do Amazonas, Manaus, Amazonas, Brazil. [2]Fundação Alfredo da Matta, Av Codajás 24, Cachoeirinha, Manaus, Amazonas, Brazil. [3]Laboratório de Hanseníase, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, Rio de Janeiro, Brazil. [4]Universidade Nilton Lins, Manaus, Amazonas, Brazil.

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References
1. Scollard DM. The biology of nerve injury in leprosy. Lepr Rev. 2008;79(3):242–53.
2. Smith CS, Aerts A, Saunderson P, Kawuma J, Kita E, Virmond M. Multidrug therapy for leprosy: a game changer on the path to elimination. Lancet Infect Dis. 2017;17(9):e293–e297. https://doi.org/10.1016/S1473-3099(17)30418-8.
3. Duthie MS, Balagon MF. Combination chemoprophylaxis and immunoprophylaxis in reducing the incidence of leprosy. Risk Manag Healthc Policy. 2016;9:43–53. https://doi.org/10.2147/RMHP.S76058.
4. Moet FJ, Pahan D, Oskam L, Richardus JH, COLEP Study Group. Effectiveness of single dose rifampicin in preventing leprosy in close contacts of patients with newly diagnosed leprosy: cluster randomised controlled trial. BMJ. 2008; 336(7647):761–764. https://doi.org/10.1136/bmj.39500.885752.BE.
5. WHO. Global Leprosy Strategy 2016–2020. Accelerating towards a leprosy-free world. Geneva: World Health Organization; 2016. http://www.searo.who.int/entity/global_leprosy_programme/documents/global_leprosy_strategy_2020/en/. Accessed 28 Sept 2018.
6. Talhari S, Penna GO, Gonçalves HS, de Oliveira MLW. Hanseníase. In: Talhari S, de Andrade ARC, Ramos AMC, Talhari C, Braga B, Gonçalves HS. Manifestações cutâneas e diagnóstico diferencial. Manaus: DiLivros; 2015. p. 17-43.
7. Saunderson P, Groenen G. Which physical signs help most in the diagnosis of leprosy? A proposal based on experience in the AMFES project, ALERT, Ethiopia. Lepr Rev. 2000;71(1):34–42.
8. Cavalcanti AA, Lucena-Silva N, Montarroyos UR, Albuquerque PM. Concordance between expected and observed bacilloscopy results of clinical forms of leprosy: a 6-year retrospective study in Recife, State of Pernambuco, Brazil. Rev Soc Bras Med Trop. 2012;45(5):616–9.
9. Martinez AN, Talhari C, Moraes MO, Talhari S. PCR-Based Techniques for Leprosy Diagnosis: From the Laboratory to the Clinic. PLoS Negl Trop Dis. 2014;8(4):e2655. https://doi.org/10.1371/journal.pntd.0002655.
10. Geluk A, Klein MR, Franken KL, Van Meijgaarden KE, Wieles B, Pereira KC et al. Postgenomic approach to identify novel Mycobacterium leprae antigens with potential to improve immunodiagnosis of infection. Infect Immun. 2005;73(9):5636–44. https://doi.org/10.1128/IAI.73.9.5636-5644.2005.
11. Spencer JS, Duthie MS, Geluk A, Balagon MF, Kim HJ, Wheat WH, et al. Identification of serological biomarkers of infection, disease progression and treatment efficacy for leprosy. Mem Inst Oswaldo Cruz. 2012; 107(Suppl 1):79–89.
12. Oskam L, Slim E, Buhrer-Sekula S. Serology: recent developments, strengths, limitations and prospects: a state of the art overview. Lepr Rev. 2003;74(3):196–205.
13. Stefani MMA, Grassi AB, Sampaio LH, de Sousa ALOM, Costa MB, Scheelbeek P, et al. Comparison of two rapid tests for anti-phenolic glycolipid-I serology in Brazil and Nepal. Mem Inst Oswaldo Cruz. 2012;107(Suppl 1):124–31.
14. Duthie MS, Balagon MF, Maghanoy A, Orcullo FM, Cang M, Dias RF, et al. Rapid quantitative serological test for detection of infection with Mycobacterium leprae, the causative agent of leprosy. J Clin Microbiol. 2014;52(2):613–9. https://doi.org/10.1128/JCM.02085-13.
15. Ridley DS, Jopling WH. Classification of leprosy according to immunity. A five-group system. Int J Lepr Other Mycobact Dis. 1966;34(3):255–73.
16. MS/SVS - Ministério da Saúde/Secretaria de Vigilância em Saúde. Departamento de Vigilância das Doenças Transmissíveis. Diretrizes para vigilância, atenção e eliminação da Hanseníase como problema de saúde pública: manual técnico-operacional. Brasília: MS; 2016. p. 58. http://portalsaude.pe.gov.br/sites/portal.saude.pe.gov.br/files/diretrizes_para_eliminacao_hanseniase_-_manual_-_3fev16_isbn_nucom_final_2.pdf. Accessed 28 Sept 2018.
17. Svanholm H, Starklint H, Gundersen HJ, Fabricius J, Barlebo H, Olsen S. Reproducibility of histomorphologic diagnoses with special reference to the Kappa statistic. APMIS. 1989;97(8):689–98.
18. Bührer-Sékula S, Smits HL, Gussenhoven GC, Van Leeuwen J, Amador S, Fujiwara T, et al. Simple and fast lateral flow test for classification of leprosy patients and identification of contacts with high risk of developing leprosy. J Clin Microbiol. 2003;41(5):1991–5.
19. Cardoso LP, Dias RF, Freitas AA, Hungria EM, Oliveira RM, Collovati M, et al. Development of a quantitative rapid diagnostic test for multibacillary leprosy using smart phone technology. BMC Infect Dis. 2013;13:497. https://doi.org/10.1186/1471-2334-13-497.
20. Fabri ACOC, Carvalho APM, Araujo S, Goulart LR, de Mattos AM, Teixeira HC, et al. Antigen-specific assessment of the immunological status of various groups in a leprosy endemic region. BMC Infect Dis. 2015;15(218):1-9. https://doi.org/10.1186/s12879-015-0962-4.
21. Wen Y, You YG, Yuan LC, Yuan YH, Zhang Y, Duthie MS, et al. Evaluation of Novel Tools to Facilitate the Detection and Characterization of Leprosy Patients in China. BioMed Res Int. 2014. https://doi.org/10.1155/2014/371828.

22. Amorim FM, Nobre ML, Ferreira LC, Nascimento LS, Miranda AM, Monteiro GRG, et al. Identifying Leprosy and Those at Risk of Developing Leprosy by Detection of Antibodies against LID-1 and LID-NDO. PLoS Negl Trop Dis. 2016;10(9):e0004934. https://doi.org/10.1371/journal.pntd.0004934.
23. Duthie MS, Hay MN, Rada EM, Convit J, Ito L, Oyafuso LK, et al. Specific IgG antibody responses may be used to monitor leprosy treatment efficacy and as recurrence prognostic markers. Eur J Clin Microbiol Infect Dis. 2011; 30(10):1257–1265. https://doi.org/10.1007/s10096-011-1221-2.
24. Silva RC, Lyon S, Araos R, Lyon AC, Grossi MAF, Lyon SH, et al. The result patterns of ML Flow and ELISA (PGL-1) serological tests in leprosy-endemic and non-endemic areas. Rev Soc Bras Med Trop. 2008; 41 Suppl II:19-22. https://dx.doi.org/10.1590/S0037-86822008000700005
25. Moet F, Schuring R, Pahan D, Oskam L, Richardus J. The prevalence of previously undiagnosed leprosy in the general population of northwest bangladesh. PLoS Negl Trop Dis. 2008;2(2):e198.
26. Richardus RA, van der Zwet K, van Hooij A, Wilson L, Oskam L, Faber R, et al. Longitudinal assessment of anti-PGL-I serology in contacts of leprosy patients in Bangladesh. PLoS Negl Trop Dis. 2017;11(12):e0006083. https://doi.org/10.1371/journal.pntd.0006083.
27. Naafs B. World leprosy day 2018: How forward respecting the past? Indian J Med Res. 2018;147:1–3.
28. Barbieri RR, Sales AM, Illarramendi X, Moraes MO, Nery JAC, Moreira SJM, Sarno EN, Machado AM, Bozza FA. Diagnostic challenges of single plaque-like lesion paucibacillary leprosy. Mem Inst Oswaldo Cruz. 2014;109(7):944–7.
29. Barbieri RR, Sales AM, Hacker MA, Nery JAC, Duppre NC, Machado AM, et al. Impact of a Reference Center on Leprosy Control under a Decentralized Public Health Care Policy in Brazil. PLoS Negl Trop Dis. 2016;10(10): e0005059. https://doi.org/10.1371/journal.pntd.0005059.

4.2 Artigo 2

PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Association of *NOD2* and *IFNG* single nucleotide polymorphisms with leprosy in the Amazon ethnic admixed population

André Luiz Leturiondo[1,2☯*], Ariani Batista Noronha[2☯], Carla Yael Ribeiro Mendonça[2], Cynthia de Oliveira Ferreira[2], Lucia Elena Alvarado-Arnez[3,4], Fernanda Saloum de Neves Manta[4], Ohanna Cavalcanti de Lima Bezerra[4], Elizeu Fagundes de Carvalho[5], Milton Ozório Moraes[4], Fabiola da Costa Rodrigues[2], Carolina Talhari[1,2,6]

1 Programa de Pós-Graduação em Medicina Tropical, Universidade do Estado do Amazonas, Manaus, Brazil, **2** Laboratório de Biologia Molecular, Fundação Alfredo da Matta, Manaus, Brazil, **3** Coordinación de Investigación, Universidad Franz Tamayo/UNIFRANZ, La Paz, Bolivia, **4** Laboratório de Hanseníase, Instituto Oswaldo Cruz—Fiocruz, Rio de Janeiro, Brazil, **5** Laboratório de Diagnóstico por DNA, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil, **6** Curso de Medicina, Universidade Nilton Lins, Manaus, Brazil

☯ These authors contributed equally to this work.
* andre_leturiondo@yahoo.com.br

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Abstract

Leprosy is a chronic infectious disease, caused by *Mycobacterium leprae*, which affects skin and peripheral nerves. Polymorphisms in genes associated with autophagy, metabolism, innate and adaptive immunity confer susceptibility to leprosy. However, these associations need to be confirmed through independent replication studies in different ethnicities. The population from Amazon state (northern Brazil) is admixed and it contains the highest proportion of Native American genetic ancestry in Brazil. We conducted a case-control study for leprosy in which we tested fourteen previously associated SNPs in key immune response regulating genes: *TLR1* (rs4833095), *NOD2* (rs751271, rs8057341), *TNF* (rs1800629), *IL10* (rs1800871), *CCDC122/LACC1* (rs4942254), *PACRG/PRKN* (rs9356058, rs1040079), *IFNG* (rs2430561), *IL6* (rs2069845), *LRRK2* (rs7298930, rs3761863), *IL23R* (rs76418789) and *TYK2* (rs55882956). Genotyping was carried out by allelic discrimination in 967 controls and 412 leprosy patients. Association with susceptibility was assessed by logistic regression analyses adjusted for the following covariates: gender, age and ancestry. Genetic ancestry was similar in case and control groups. Statistically significant results were only found for *IFNG* and *NOD2*. The rs8057341 polymorphism within *NOD2* was identified as significant for the AA genotype (OR = 0.56; 95% CI, 0.37–0.84; P = 0.005) and borderline for the A allele (OR = 0.76; 95% CI, 0.58–1.00; P = 0.053) and carrier (OR = 0.76; 95% CI, 0.58–1.00; P = 0.051). The rs2430561 SNP in *IFNG* was associated with disease susceptibility for the AT genotype (OR = 1.40; 95% CI, 1.06–1.85; P = 0.018) and carrier (OR = 1.44; 95% CI, 1.10–1.88; P = 0.008). We confirmed that *NOD2* and *IFNG* are major players in immunity against *M.leprae* in the Amazon ethnic admixed population.

fapeam.am.gov.br/wp-content/uploads/2013/05/Chamada-Publica-001_2013-PPSUS-Decisao-CD-287-2013.pdf and PAPAC 005/2019. Programa de Pós-Graduação em Medicina Tropical da UEA/FMT-HVD. http://www.fapeam.am.gov.br/wp-content/uploads/2019/08/Decisao-CD-204-2019-Proc.-506.2019-Homologacao-do-Resultado-PAPAC-Edital-nA-005_2019.pdf. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Author summary

Leprosy is chronic infectious diseases caused by *Mycobacterium leprae* that affect the skin and peripheral nerves. The incidence is still high where approximately 200,000 new cases are diagnosed each year. There is no clear sign for early diagnosis and transmission is likely to occur before treatment, which, irrespective of its success, has not hampered stationary incidence in the past 20 years. Thus, there is pressing need for markers that discriminate exposure, infection and disease in order to better detect leprosy progression, control transmission and prevent disabilities. Here, we investigated whether polymorphisms located in eleven genes are associated to leprosy in a population from Amazon state (northern Brazil) which is admixed and it contains the highest proportion of Native American genetic ancestry in Brazil. We validated *NOD2* and *IFNG* associations with resistance and risk of leprosy, respectively, in the Amazon ethnic admixed population. Genetic patterns of leprosy susceptibility could have an impact on the prognosis of individuals that are more likely to develop the disease (among household contacts, for example). Therefore, this strategy could identify high-risk individuals prone for prophylactic measures such as treatment with single-dose rifampicin and BCG vaccination.

Introduction

Two hundred thousand new cases of Leprosy are continuously diagnosed every year. The disease is caused by *Mycobacterium leprae* that has a long incubation period, leads to nerve damage and development of physical disabilities [1]. There is no clear sign for early diagnosis and transmission is likely to occur before treatment, which, irrespective of its success, has not hampered stationary incidence in the past 20 years. Thus, there is pressing need for markers that discriminate exposure, infection and disease in order to better detect leprosy progression, control transmission and prevent disabilities. Genetic factors have proved to be key components for leprosy outcome. Studies with monozygotic/dizygotic twins, family, population-based designs, and more recently, genome-wide association studies (GWAS) and whole exome sequencing (WES) have pinpointed single nucleotide polymorphisms (SNPs) in genes that have been consistently replicated in different populations [2–6]. There is evidence that *NOD2*, *LRRK2*, *TLR1*, *TNF*, *IFNG*, *IL10*, *IL23R*, *TYK2* and *PACRG/PRKN* (formerly *PARK2*), which are genes that participate in autophagy and recognition pathways, regulating the host innate immune response are associated with leprosy susceptibility, reaction or its clinical forms [6–18]. However, genetic association of SNPs in major genes that modulate the immune response need independent replication in different ethnic groups to confirm leprosy outcome [19]. As observed in other regions, the Brazilian population from Amazon state is admixed, having three main ancestral contributions: Native Americans (NAM), Europeans (EUR) and Africans (AFR). Of these, the Native American contribution is highest, even within the urban populations of the region [20].

Genetic patterns of leprosy susceptibility could have an impact on the prognosis of individuals that are more likely to develop the disease (among household contacts, for example). Therefore, this strategy could identify high-risk individuals prone for prophylactic measures such as treatment with single-dose rifampicin and BCG vaccination [21–23].

Here, we investigated whether SNPs located in eleven genes: *CCDC122/LACC1*, *IFNG*, *IL6*, *IL10*, *IL23R*, *LRRK2*, *NOD2*, *PACRG/PRKN*, *TLR1*, *TNF* and *TYK2* are associated to leprosy susceptibility in a population in the North of Brazil. To avoid spurious associations due to

population stratification, as in the case of admixed populations such as Brazilians, we included genetic ancestry estimates, gender and age, as covariates in the logistic regression analysis.

Materials and methods

Ethical statement

This study was approved by the Research and Ethic Committee (N°555.620) of the Alfredo da Matta Foundation. All participants signed an informed consent before enrolment. Parents or legal guardians provided consent for participants under the age of 18. This study was performed in accordance with the guidelines strengthening the reporting of genetic association studies (STREGA) [24].

Design and study population

We performed a case-control study involving individuals from Manaus, a city in the Brazilian state of Amazonas, at the Alfredo da Matta Foundation (FUAM). Patients and controls were recruited from March 2014 to March 2017 by spontaneous demand at FUAM. Patients with leprosy were classified according to the clinical and laboratorial findings (slit skin smears and histopathological examination) and were treated as paucibacillary or multibacillary, according to World Health Organization guidelines. The control group was composed of individuals who lived in the same endemic area of the cases. They were subjected to a dermatoneurological examination, had no suspected leprosy lesions and declared not having contact with leprosy or tuberculosis patients.

Determination of SNP for genotypic analysis

Several genes have been tested associated in populations although few SNPs were already extensively evaluated and consensus estimates were calculated in meta-analysis (S1 Table). The present study was designed to investigate the association of *TLR1*, *NOD2*, *TNF*, *IL10*, *CCDC122/LACC1*, *PACRG/PRKN*, *IFNG*, *IL6*, *LRRK2*, *IL23R* and *TYK2* genes with leprosy. The SNPs *TLR1* (rs4833095), *NOD2* (rs751271, rs8057341), *TNF* (rs1800629), *IL10* (rs1800871), *CCDC122/LACC1* (rs4942254), *PACRG/PRKN* (rs9356058, rs1040079), *IFNG* (rs2430561) and *IL6* (rs2069845) were selected based on previously published data which showed their association in the Brazilian population to leprosy *per se* [3, 7, 10, 11, 13, 25] or reaction [14]. Based in the previous studies in the Chinese population, we also selected *LRRK2* (rs7298930, rs3761863) [8], *IL23* (rs76418789) and *TYK2* (rs55882956) [6]. SNPs were included from the promoter, exonic, intronic and chosen based on their functional role, as reported in literature. We excluded SNPs with a call rate < 95% or deviation from Hardy–Weinberg equilibrium proportions ($P<0.01$) in the controls.

DNA extraction, genotyping and genetic markers

DNA was extracted from frozen blood samples using DNeasy Blood & Tissue kit according to manufacturer's instructions (QIAGEN). DNA was quantified and diluted for genotyping by real-time PCR with TaqMan probe assays (Life Technologies, EUA) (S2 Table). Assays consisted of 5 μL reactions containing 2.5 μL of TaqMan Universal PCR Master Mix No AmpErase UNG (Applied Biosystems), 1.375 μL of water, 0.125 μL of the TaqMan assay (primers and probes), and 1 μL of DNA template (10–40 ng). Genotyping was carried out in the StepOne Plus real-time PCR system (Applied Biosystems), by fluorescence-based allelic discrimination following standard cycling conditions.

We used a panel of 46 ancestry-informative autosomal Indels (AIM-Indels) that were genotyped in a single multiplex PCR followed by capillary electrophoresis, as described by Pereira and coauthors (2012) [26], using an ABI 3500 Genetic Analyzer (Life Technologies). Alleles were conferred in GeneMapper v.4.1 software (Life Technologies). Ancestry estimates for each of the three main population components (EUR, NAM and AFR) were determined using STRUCTURE v2.3.3 software [27] and the HGDP-CEPH reference sample panel [28].

Statistical analysis

Linkage disequilibrium (LD) estimates for two SNPs in *NOD2* and two SNPs in *LRRK2* and deviations from Hardy–Weinberg equilibrium in the control group were performed using Haploview software, version 4.2 [29]. The estimated haplotypes was based on genotypes using an Expectation Maximization (EM) algorithm. Cases and controls were compared according to genotype, allele, and carrier frequencies, with and without adjustment for the variables: gender, age and ancestry (NAM and EUR, as continuous variables). Statistical analysis was performed using R software version 3.4.3 for Windows using "genetics" and "rms" packages (https://www.r-project.org) [30].

Results

Patients and controls were ten years of age or older. A total of 412 leprosy patients (284 males, 128 females) and 967 controls (526 males, 441 females) were recruited (Table 1). Mean age was significantly lower in control subjects than in cases (mean ± SD, 29.8 ± 9.94 vs. 43.3 ± 18.14 years; $P < 0.0001$). Likewise, the proportion of males and females was different between the two groups ($P < 0.001$), with significantly more males among leprosy patients (Table 1).

The analyzed population demonstrated typical ancestral characteristics of admixed populations for European, Native American and African ancestry (S1 Fig).

All SNPs in the control group were in Hardy–Weinberg equilibrium (HWE). The *PACRG/PRKN* SNP rs1040079 was excluded from the analysis because the call rate was <95%. There were no statistically significant differences between cases and controls for SNPs *PACRG/PRKN*, *IL10*, *TNF*, *TLR1*, *CCDC122/LACC1*, *IL6*, *LRRK2*, *TYK2* and *IL23R* in the three genetic models (genotypic, allelic and carriers) (Table 2).

The statistical power of the sample size from the present study was also evaluated considering the minor allele frequency (MAF) obtained from each of the SNPs and the Odds Ratio (OR) association effects ranging from 1.5 up to 2.5. For most of the SNPs the assessment of

Table 1. General characteristics of leprosy patients and controls.

Variables	Patients n = 412	Controls n = 967
Age[a]	43.3 ± 18.14	29.8 ± 9.94
Gender[b]		
Male	284 (68.9%)	526 (54.4%)
Female	128 (31.1%)	441 (45.6%)
WHO classification		
PB	133	-
MB	279	-

WHO, World Health Organization. Paucibacillary (PB); Multibacillary (MB)

Student's *t*-test for age between patients and control subjects $P < 0.0001$; Chi-squared test for gender $P < 0.0001$; Data shown as mean ± standard deviation.

[a]Student's t-test

[b]Chi-squared test

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Table 2. Association of Allele, Genotype and Carrier frequencies of candidate genes with Leprosy.

SNP	GenotypeN (%)	ControlsN (%)	LeprosyN (%)	OR(95% CI)	*P-value*	Adjusted OR[a](95% CI)	*P-value*
rs8057341 *NOD2*	AA	182 (19)	52 (13)	**0.56 (0.39–0.80)**	**0.0017**	**0.56 (0.37–0.84)**	**0.0052**
	GA	446 (47)	186 (46)	0.82 (0.64–1.05)	0.1220	0.85 (0.63–1.13)	0.2547
	GG[b]	328 (34)	167 (41)				
	Total	956	406				
Allele	A	0.42	0.36	**0.76 (0.60–0.96)**	**0.0243**	**0.76 (0.58–1.00)**	**0.0529**
	G[b]	0.58	0.64				
Carriers	AA/GA	628 (66)	239 (59)	**0.74 (0.59–0.95)**	**0.0153**	**0.76 (0.58–1.00)**	**0.0512**
HWE		0.1886					
rs751271 *NOD2*	TT	203 (21.0)	61 (15.0)	**0.63 (0.44–0.89)**	**0.0093**	0.67 (0.44–1.01)	0.0561
	GT	468 (49.0)	208 (51.0)	0.93 (0.71–1.20)	0.5738	0.93 (0.68–1.27)	0.6690
	GG[b]	286 (30.0)	137 (34.0)				
	Total	957	406				
Allele	T	0.46	0.41	0.81 (0.64–1.03)	0.0879	0.84 (0.63–1.10)	0.2092
	G[b]	0.54	0.59				
Carriers	TT/GT	671 (70.0)	269 (66.0)	0.84 (0.65–1.07)	0.1593	0.85 (0.64–1.15)	0.2976
HWE		0.7007					
rs2430561 *IFNG*	TT	55 (6.0)	29 (7.0)	1.39 (0.87–2.25)	0.1713	1.67 (0.97–2.89)	0.0641
	AT	341 (35.0)	164 (40.0)	1.27 (0.99–1.62)	0.0531	**1.40 (1.06–1.85)**	**0.0184**
	AA	566 (59.0)	214 (53.0)				
	Total	962	407				
Allele	T	0.23	0.27	1.22 (0.94–1.59)	0.1326	1.34 (0.99–1.81)	0.0584
	A[b]	0.77	0.73				
Carriers	TT/AT	396 (41)	193 (47)	1.29 (1.02–1.63)	0.0328	**1.44 (1.10–1.88)**	**0.0083**
HWE		0.7681					
rs9356058 *PACRG/ PRKN*	CC	89 (9)	43 (11)	1.32 (0.88–1.98)	0.1764	1.36 (0.86–2.15)	0.1878
	TC	382 (41)	193 (48)	**1.38 (1.08–1.77)**	**0.0101**	1.27 (0.96–1.69)	0.0880
	TT[b]	465 (50)	170 (42)				
	Total	936	406				
Allele	T	0.70	0.66	0.81 (0.64–1.04)	0.1069	0.83 (0.63–1.10)	0.2011
	C[b]	0.30	0.34				
Carriers	CC/TC	471 (50)	236 (58)	**1.37 (1.08–1.73)**	**0.0086**	1.29 (0.99–1.68)	0.0603
HWE		0.4600					
rs1800871 *IL10*	AA	143 (15.0)	60 (15.0)	1.05 (0.73–1.50)	0.7925	0.93 (0.62–1.39)	0.7317
	GA	443 (46.0)	196 (48.0)	1.11 (0.86–1.42)	0.4351	0.94 (0.70–1.25)	0.6551
	GG[b]	375 (39.0)	150 (37.0)				
	Total	961	406				
Allele	A	0.38	0.39	1.04 (0.82–1.32)	0.7315	0.96 (0.73–1.26)	0.7599
	G[b]	0.62	0.61				
Carriers	AA/AG	586 (61.0)	256 (63.0)	1.09 (0.86–1.39)	0.4709	0.93 (0.71–1.23)	0.6303
HWE		0.5605					
rs1800629 *TNF*	AA	7 (1.0)	1 (0.0)	0.33 (0.04–2.66)	0.2954	0.14 (0.01–1.79)	0.1291
	GA	135 (14.0)	50 (12.0)	0.84 (0.60–1.20)	0.3431	0.78 (0.53–1.16)	0.2226
	GG[b]	824 (85.0)	361(88.0)				
	Total	966	412				
Allele	A	0.08	0.06	0.81 (0.51–1.28)	0.3604	0.74 (0.43–1.25	0.2559
	G[b]	0.92	0.94				
Carriers	AA/GA	142 (15.0)	51 (12.0)	0.82 (0.58–1.15)	0.2562	0.75 (0.51–1.11)	0.1536

(*Continued*)

Table 2. (Continued)

SNP	GenotypeN (%)	ControlsN (%)	LeprosyN (%)	OR(95% CI)	*P-value*	Adjusted OR[a](95% CI)	*P-value*
HWE		0.7331					
rs4833095 *TLR1*	TT	213 (22.0)	102 (25.0)	1.21 (0.87–1.68)	0.2479	1.19 (0.77–1.62)	0.5553
	CT	482 (50.0)	207(50.0)	1.09 (0.82–1.44)	0.5542	1.00 (0.73–1.38)	0.9952
	CC[b]	261 (27.0)	103 (25.0)				
	Total	956	412				
Allele	T	0.47	0.50	1.10 (0.87–1.39)	0.4173	1.06 (0.81–1.37)	0,6841
	C[b]	0.53	0.50				
Carriers	TT/CT	695 (72.0)	30 (75.0)	1.13 (0.86–1.47)	0.3770	1.04 (0.77–1.40	0.8111
HWE		0.7851					
rs2069845 *IL6*	GG	94 (10.0)	38 (9.0)	1.03 (0.68–1.55)	O.8994	1.01 (0.63–1.60)	0.9751
	AG	400 (41.0)	182 (45.0)	1.16 (0.91–1.48)	0.2448	1.11 (0.84–1.47)	0.4676
	AA[b]	470 (49.0)	185 (46.0)				
	Total	964	405				
Allele	G	0.30	0.32	1.06 (0.83–1.37)	0.6208	1.04 (0.78–1.38)	0.7806
	A[b]	0.70	0.68				
Carriers	GG/AG	494 (51.0)	220 (54.0)	1.13 (0.90–1.43)	0.2985	1.09 (0.83–1.42)	0.5284
HWE		0.5600					
rs4942254 *CCDC122/ LACC1*	CC	126 (13.0)	55 (14.0)	1.09 (0.75–1.57)	0.6548	1.10 (0.72–1.66)	0.6682
	CT	412 (44.0)	187(47.0)	1.13 (0.88–1.45)	0.3412	1.21 (0.91–1.62)	0.1938
	TT[b]	396 (42.0)	159 (40.0)				
	Total	934	401				
Allele	C	0.36	0.37	1.07 (0.84–1.36)	0.6040	1.09 (0.82–1.44)	0.5486
	T[b]	0.64	0.63				
Carriers	CC/CT	538 (57.0)	242 (61.0)	1.12 (0.88–1.42)	0.3505	1.18 (0.90–1.56)	0.2276
HWE		0.2849					
rs7298930 *LRRK2*	AA	125 (13.0)	52 (13.0)	1.00 (0.69–1.46)	0.9830	1.07 (0.70–1.63)	0.7589
	AC	418 (45.0)	195 (48.0)	1.13 (0.88–1.45)	0.3521	1.14 (0.86–1.52)	0.3564
	CC[b]	391 (42.0)	162 (40.0)				
	Total	934	409				
Allele	A	0.36	0.37	1.03 (0.81–1.32)	0.7807	1.06 (0.81–1.40)	0.6604
	C[b]	0.64	0.63				
Carriers	AA/AC	543 (58.0)	247 (61.0)	1.10 (0.87–1.39)	0.4399	1.13 (0.86–1.47)	0.3863
HWE		0.471					
rs3761863 *LRRK2*	TT	218 (23.0)	76 (20.0)	0.78 (0.55–1.09)	0.1412	0.90 (0.61–1.32)	0.5856
	CT	457 (48.0)	189 (49.0)	0.92 (0.70–1.21)	0.5546	0.97 (0.71–1.33)	0.8690
	CC[b]	274 (29.0)	123 (32.0)				
	Total	949	388				
Allele	T	0.47	0.44	0.88 (0.70–1.12)	0.3012	0.95 (0.72–1.24)	0.7067
	C[b]	0.53	0.56				
Carriers	TT/CT	675 (71.0)	265 (69.0)	0.87 (0.68–1.13)	0.3044	0.95 (0.71–1.27)	0.7352
HWE		0.3334					
rs55882956 *TYK2*	AG	2 (0.21)	2 (0.49)	2.36 (0.33 16.8)	0.3903	4.71 (0.61–36.3)	0.1366
	GG[b]	960 (99.79)	406 (99.51)				
	Total	962	408				
Allele	A	0.001	0.002	2.36 (0.15–37.8)	0.5439	4.69 (0.26–83.5)	0.2932
	G[b]	0.999	0.998				
Carriers	AA/AG	2 (0.21)	2 (0.49)	2.36 (0.33–6.84)	0.3903	4.71 (0.61–36.3)	0.1366

(*Continued*)

Table 2. (Continued)

SNP	GenotypeN (%)	ControlsN (%)	LeprosyN (%)	OR(95% CI)	*P-value*	Adjusted OR[a](95% CI)	*P-value*
HWE		1					
rs76418789 *IL23R*	AG	2 (0.21)	1 (0.25)	1.17 (0.11–12.9)	0.8977	0.95 (0.01–60.5)	0.9804
	GG[b]	953 (99.79)	407 (99.75)				
	Total	955	408				
Allele	A	0.001	0.001	1.17 (0.04–34.9)	0.9276	0.95 (0.0–336.9)	0.9862
	G[b]	0.999	0.999				
Carriers	AA/AG	2 (0.21)	1 (0.25)	1.17 (0.11–12.9)	0.8977	0.95 (0.01–60.5)	0.9804
HWE		1					

Abbreviations: HWE, Hardy–Weinberg equilibrium; OR, odds ratio; SNP, single-nucleotide polymorphism. Bold values express statistically significant results.

[a]Results of logistic regression analyses adjusted for the covariates: gender, age and ancestry.

[b]Baseline. 95% CI, 95% confidence interval

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power attained 0.80 for the tested values, except for *TNF* SNP that was lower (0.52). The *TYK2* and *IL23R* SNPs had a rare frequency that resulted in power of less than 10%. Since no statistically significant associations were found for clinical forms comparison (PB vs MB and Controls vs MB) we did not include this power analysis.

The genotype, allele and carrier frequencies of *NOD2* rs8057341 confirmed association with protection from leprosy. The rs8057341 polymorphism was identified as significant for the AA genotype (OR = 0.56; 95% CI, 0.37–0.84; P = 0.005) and borderline for the A allele (OR = 0.76; 95% CI, 0.58–1.00; P = 0.053) and AA/GA carriers (OR = 0.76; 95% CI, 0.58–1.00; P = 0.051). The rs751271 polymorphism was identified as borderline for the TT genotype (OR = 0.67; 95% CI, 0.44–1.01; P = 0.056) (Table 2). High LD (r2 = 0.83) revealed dependent association for these two SNPs. Analysis of *NOD2* SNPs haplotypes (rs751271-rs8057341) were statistically significant for the T-A combination (OR = 0.79; 95% CI, 0.64–0.97; P = 0.0226), T-G combination (OR = 1.91; 95% CI, 1.21–3.01; P = 0.0055) and G-A (OR = 3.88; 95% CI, 1.50–10.04; P = 0.0052) (S3 Table).

SNP rs2430561 in the *IFNG* gene was associated with disease susceptibility in AT heterozygotes (OR = 1.40; 95% CI, 1.06–1.85; P = 0.018) and TT/AT carriers (OR = 1.44; 95% CI, 1.10–1.88; P = 0.008) (Table 2).

SNPs in the *LRRK2* gene showed a weak LD ($r^2 = 0.18$). Analysis of *LRRK2* SNPs haplotypes was not statistically significant. The C-T combination (rs7298930—rs3761863) was shown to be borderline (P = 0.07), but lost association when adjusted for co-variates (OR = 0.78; 95% CI, 0.58–1.06; P = 0.1095) (S4 Table). Also, we tested rs9356058 in the *PACCRG*/*PRKN* genes and observed association when TC/CC carriers were evaluated, however, this was not sustained following co-variate adjustment (OR = 1.29; 95% CI, 0.99–1.68; P = 0.0603) (Table 2).

Discussion

Genetic association studies in population designs, such as case-control, can lead to spurious associations, especially in admixed populations [31], which may confer false-positive results and the risk of developing a disease [32,33]. In our study, to reduce the chance of bias, we performed data adjustment by age, gender as well as ancestry. The current profile of the genetic ancestry of the Amazonas population began to form during the Portuguese colonization in the mid-16th century [34] and continued with the exploration of natural resources, primarily rubber [35]. Construction of the Transamazon Highway and the establishment of an Industrial Park (Manaus Duty Free Zone) in the 1960s also contributed to a huge migratory flow of

Brazilians from different regions of the country [36]. The populations analyzed in our study had similar ancestral contributions in both case and control groups, which is ideal in genetic association studies. Nevertheless, the data was highly impacted by age adjustment since cases and controls had very different mean ages.

We validated *NOD2* and *IFNG* associations with resistance and risk of leprosy, respectively, in the Amazon ethnic admixed population. The *NOD2* gene encodes an intracellular sensing molecule, which, along with *NOD1*, recruits ATG16L1 to the plasma membrane to initiate autophagy of bacteria entering the host cell [37]. Similarly, Sales-Marques and coauthors (2014) [7], in a meta-analysis with population samples from different Brazilian regions combining case-control and family-based studies, confirmed that the A allele of *NOD2* (rs8057341) is a genetic resistance factor for leprosy. On the other hand, in the first GWAS in the Chinese population [4], the G allele (MAF = 0.22) of SNP rs8057341 was associated with disease risk. However, two other studies failed to validate this SNP in three populations: Indian, African and Vietnamese [5,38].

The SNP rs9356058 in *PACRG/PRKN*, for the T allele, points to protection, although statistical significance did not reach the threshold level. Although the specific function of the *PACRG* gene has not been elucidated [9], together with *PRKN*, they share a regulatory region and participate in the proteolytic system mediated by ubiquitin for the clearance of damaged biomolecules (lipids and proteins) and organelles [9,39]. The *PRKN* gene encodes the Parkin protein, an E3 Ubiquitin Ligase, the last sequential enzyme in the ubiquitination process [40]. Recently, the relationship of this protein with the innate immune response of the host, known as xenophagy, which is the degradation of intracellular pathogens [41], as described for *M. tuberculosis* [42], Chlamydia [43] and *M. leprae* [44] has been identified. Polymorphic variants in the *PRKN* gene were initially associated with the autosomal recessive form of Parkinson's disease (AR-JP) [45]. In 2004, Mira and coauthors [3] identified polymorphisms in the promoter region shared by *PACRG/PRKN* genes associated with leprosy *per se* in Southern Brazilians and Vietnamese. In this same study, the common T allele of rs9356058 was associated with the risk of leprosy. Later, Alter and coauthors confirmed that this variant (T allele of rs9356058) is a risk factor for the disease in the Vietnamese and Indian populations [46]. In this study, *PRKN* analysis suggested that genetic association is higher in children/youths, when data is stratified for age. Since our population was older (40 years old) and the mean age of the control group varied, statistical adjustments might explain different results. The lack of consensus among ours and previous studies for the *PRKN* region is probably due to distinct patterns of LD in the analyzed populations, even within the same country, leading to differences in allele and haplotype frequencies of the studied SNPs, as previously observed for lymphotoxin-alpha [47]. It is likely that NAM ancestry could explain this pattern. Noteworthy, studies in Indian and Chinese populations failed to replicate the association of these *PRKN* polymorphisms with leprosy [48, 49].

Interferon gamma (*IFNG*) is one of the most significant cytokines involved in the protective immune response against mycobacterial infection and is secreted mainly by CD4+ Th1, CD8 + T cytotoxic and natural killer cells [50]. In synergy with TNF, it activates microbicide effector mechanisms in human macrophages [51]. IFNγ expression may be influenced by the polymorphism present in the first intron of IFNG +874 T>A rs2430561, probably since this locus coincides with the binding site of the NF-kB transcription factor [52]. This polymorphism has been associated in meta-analysis studies with leprosy [24], tuberculosis [53] and Hepatitis [50]. Our results indicated T allele carriers have an increased risk of leprosy (P = 0.0083). A new meta-analysis would indicate a consensus estimate for this SNP. In studies that show an association, the presence of the T allele correlates with high IFNG expression and increased resistance to infection whereas the A allele correlates with low expression [54–56]. On the other

hand, in vitro clinical trials showed that interferon levels were not statistically different between T carriers and AA genotype, in the presence of *M. leprae* antigens [57].

We did not validate associations for SNPs in *TLR1*, *TNF*, *PACRG/PRKN*, *IL10*, *CCDC122/LACC1*, *IL6*, *LRRK2*, *IL23R*, and *TYK2* genes. Nevertheless, some of these SNPs, such as rs3761863 in *LRRK2* had been associated with a type-1 reaction, which is considered an endophenotype of leprosy [58]. These SNPs could possibly play a role in the uncontrolled inflammatory phenotype throughout the natural course of the disease. Recently, rare *LRRK2* SNPs were confirmed as associated with either leprosy type-1 reactions or Parkinson´s Disease [59]. Hence, we also tested SNPs from *TYK2* and *IL23R*, both low-frequency variants that were described in the Chinese population [6]. In the present Amazon population sample, these variants were rare leading to a low detection power of association with leprosy. Although significance was not found, *TYK2* heterozygote frequency was two times higher among patients. However, we cannot rule out the possibility of other rare or common variants of *IL23R* or *TYK2* being related to leprosy susceptibility in the Amazon population.

The few common SNPs that showed association with leprosy and the modest odds ratios values presented demonstrate the difficulty of unraveling the major genes involved in leprosy. Genome-wide association studies and exome analysis could possibly improve the ability to describe novel rare SNPs and call on a combination of different genotypes to explain one complex phenotype. It is likely that this can help define genetic variants and understand their role in the pathophysiology of leprosy, contributing to either diagnosis or treatment [39].

Supporting information

S1 Fig. Individual ancestry estimates obtained for the HGDP-CEPH reference samples and individuals tested from CASES (Leprosy) and CONTROLS (Endemic Control) STUDY using 46 AIM-Indels (AFR: African; EUR: European; NAM: Native American).
(TIF)

S1 Table. Summary of candidate genes of immune response in the case-controls studies in Leprosy.
(DOC)

S2 Table. Genotyping assays used for allelic discrimination.
(DOC)

S3 Table. Haplotypes of the intron region of NOD2 present in the study population.
(DOC)

S4 Table. Haplotypes of the LRRK2 present in the study population.
(DOC)

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Author Contributions

Conceptualization: Milton Ozório Moraes, Carolina Talhari.

Data curation: André Luiz Leturiondo, Fabíola da Costa Rodrigues.

Formal analysis: André Luiz Leturiondo, Cynthia de Oliveira Ferreira, Lucia Elena Alvarado-Arnez, Milton Ozório Moraes, Fabíola da Costa Rodrigues.

Funding acquisition: André Luiz Leturiondo.

Methodology: André Luiz Leturiondo, Fernanda Saloum de Neves Manta, Ohanna Cavalcanti de Lima Bezerra, Elizeu Fagundes de Carvalho, Milton Ozório Moraes, Fabíola da Costa Rodrigues.

Supervision: André Luiz Leturiondo.

Validation: André Luiz Leturiondo, Ariani Batista Noronha, Carla Yael Ribeiro Mendonça.

Writing – original draft: André Luiz Leturiondo, Lucia Elena Alvarado-Arnez, Milton Ozório Moraes.

Writing – review & editing: Milton Ozório Moraes, Carolina Talhari.

References

1. Britton WJ, Lockwood DN. Leprosy. Lancet. 2004; 363:1209–1219. https://doi.org/10.1016/S0140-6736(04)15952-7 PMID: 15081655
2. Chakravartti MR, Vogel F. A twin study on leprosy. In: Becker PE, Lenz W, Vogel F, Wendt GG, editors. Topics in Human Genetics. Stuttgart: Thieme; 1973. pp. 1–29.
3. Mira MT, Alcais A, Nguyen VT, Moraes MO, Di Flumeri C, et al. Susceptibility to leprosy is associated with PARK2 and PACRG. Nature 2004; 427:636–640. https://doi.org/10.1038/nature02326 PMID: 14737177
4. Zhang FR, Huang W, Chen SM, Sun LD, Liu H, Li Y, et al. Genomewide association study of leprosy. N Engl J Med. 2009; 361(27):2609–2618. https://doi.org/10.1056/NEJMoa0903753 PMID: 20018961
5. Wong SH, Hill AV, Vannberg FO, India–Africa–United Kingdom Leprosy Genetics Consortium. Genomewide association study of leprosy. N Engl J Med. 2010; 362(15):1446–1447. https://doi.org/10.1056/NEJMc1001451 PMID: 20393182
6. Liu H, Wang Z, Li Y, Yu G, Fu X, et al. Genome-Wide Analysis of Protein-Coding Variants in Leprosy. J Invest Dermatol. 2017; 137(12): 2544–2551; https://doi.org/10.1016/j.jid.2017.08.004 PMID: 28842327
7. Sales- Marques C, Salomão H, Fava VM, Alvarado- Arnez LE, Pinheiro EP, et al. NOD2 and CCDC122- LACC1 genes are associated with leprosy susceptibility in Brazilians. Hum Genet. 2014; 133(12):1525–1532. https://doi.org/10.1007/s00439-014-1502-9 PMID: 25367361
8. Wang D, Xu L, Lv L, Su LY, Fan Y, Zhang DF, et al. Association of the LRRK2 genetic polymorphisms with leprosy in Han Chinese from Southwest China. Genes Immun. 2015; 16(2):112–119. https://doi.org/10.1038/gene.2014.72 PMID: 25521227
9. Chopra R, Ali S, Srivastava AK, Aggarwal S, Kumar B, Manvati S, et al. Mapping of PARK2 and PACRG overlapping regulatory region reveals LD structure and functional variants in association with leprosy in unrelated Indian population groups. PLoS Genet. 2013; 9(7). Available from: http://www.journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1003578
10. De Sales Marques C, Brito-De-Souza VN, Guerreiro LTA, Martins JH, Amaral EP, Cardoso CC, et al. Toll-like receptor 1 N248s single-nucleotide polymorphism is associated with leprosy risk and regulates immune activation during mycobacterial infection. J Infect Dis. 2013; 208(1):120–129. https://doi.org/10.1093/infdis/jit133 PMID: 23547143
11. Areeshi MY, Mandal RK, Dar SA, Jawed A, Wahid M, Lohani M, et al. Impact of TNF -308 G>A (rs1800629) gene polymorphism in modulation of leprosy risk: a reappraise meta-analysis of 14 case-control studies. Biosci Rep. 2017; 37(5). Available from: http://www.bioscirep.org/content/37/5/BSR20170806
12. Wang D, Feng JQ, Li YY, Zhang DF, Li XA, Li QW, et al. Genetic variants of the MRC1 gene and the IFNG gene are associated with leprosy in Han Chinese from Southwest China. Hum Genet. 2012; 131(7):1251–1260. https://doi.org/10.1007/s00439-012-1153-7 PMID: 22392581
13. Alvarado-Arnez LE, Amaral EP, Sales-Marques C, Durães SM, Cardoso CC, Nunes Sarno E, et al. Association of IL10 Polymorphisms and Leprosy: A Meta-Analysis. PLoS One. 2015; 10(9). Available from: http://www.journals.plos.org/plosone/article?id=10.1371/journal.pone.0136282
14. Sales-Marques C, Cardoso CC, Alvarado-Arnez LE, Illaramendi X, Sales AM, Hacker MdA, et al. Genetic polymorphisms of the *IL6* and *NOD2* genes are risk factors for inflammatory reactions in

leprosy. PLoS Negl Trop Dis. 2017; 11(7): e0005754. https://doi.org/10.1371/journal.pntd.0005754 PMID: 28715406

15. Berrington WR, Macdonald M, Khadge S, Sapkota BR, Janer M, Hagge DA, Kaplan G, and Hawn TR. Common polymorphisms in the *NOD2* gene region are associated with leprosy and its reactive states. J Infect Dis. 2010; 201(9): 1422–1435. https://doi.org/10.1086/651559 PMID: 20350193
16. Schuring RP, Hamann L, Faber WR, Pahan D, Richardus JH, Schumann RR, et al. Polymorphism N248S in the human Toll-like receptor 1 gene is related to leprosy and leprosy reactions. J Infect Dis. 2009; 199(12):1816–1819. https://doi.org/10.1086/599121 PMID: 19456232
17. Santos AR, Suffys PN, Vanderborght PR, Moraes MO, Vieira LM, Cabello PH, et al. Role of tumor necrosis factor-alpha and interleukin-10 promoter gene polymorphisms in leprosy. J Infect Dis. 2002; 186(11):1687–1691. https://doi.org/10.1086/345366 PMID: 12447749
18. Silva GAV, Santos MP, Mota-Passos I, et al. IFN-gamma +875 microsatellite polymorphism as a potential protection marker for leprosy patients from Amazonas state, Brazil. Cytokine. 2012; 60(2):493–497. https://doi.org/10.1016/j.cyto.2012.04.043 PMID: 22683002
19. Cardoso CC, Pereira AC, de Sales Marques C, Moraes MO. Leprosy susceptibility: genetic variations regulate innate and adaptive immunity, and disease outcome. Future Microbiol. 2011; 6(5):533–549. https://doi.org/10.2217/fmb.11.39 PMID: 21585261
20. Manta FSN, Pereira R, Vianna R, Araújo ARB, Gitaí DLG, et al. Revisiting the genetic ancestry of Brazilians using autosomal AIM-Indels. PloS One. 2013; 8(9). Available from: http://www.journals.plos.org/plosone/article?id=10.1371/journal.pone.0075145
21. Moet FJ, Pahan D, Oskam L, Richardus JH, COLEP Study Group. Effectiveness of single dose rifampicin in preventing leprosy in close contacts of patients with newly diagnosed leprosy: cluster randomised controlled trial. BMJ. 2008; 336(7647):761–764. https://doi.org/10.1136/bmj.39500.885752.BE PMID: 18332051
22. Duthie MS, Balagon MF. Combination chemoprophylaxis and immunoprophylaxis in reducing the incidence of leprosy. Risk Manag Healthc Policy. 2016; 9:43–53. https://doi.org/10.2147/RMHP.S76058 PMID: 27175099
23. Guidelines for the diagnosis, treatment and prevention of leprosy. New Delhi: World Health Organization, Regional Office for South-East Asia; 2017 https://www.who.int/lep/resources/9789290226383/en/
24. Little J, Higgins JP, Ioannidis JP, Moher D, Gagnon F, et al. STrengthening the REporting of Genetic Association Studies (STREGA): an extension of the STROBE Statement. PLoS Med. 2009; 6(2):151–163. https://doi.org/10.1371/journal.pmed.1000022 PMID: 19192942
25. Silva GAV, Naveca FG, Ramasawmy R, Boechat AL. Association between the IFNG +874A/T gene polymorphism and leprosy resistance: A meta-analysis. Cyto. 2014; 65(2):130–133. https://doi.org/10.1016/j.cyto.2013.12.002 PMID: 24389160
26. Pereira R, Philips C, Pinto N, Santos C, dos Santos SE, et al. Straightforward Inference of Ancestry and Admixture Proportions through Ancestry-Informative Insertion Deletion Multiplexing. PloS One. 2012; 7 (1). Available from: https://www.journals.plos.org/plosone/article?id=10.1371/journal.pone.0029684
27. Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. Genetics. 2000; 155(2): 945–959. PMID: 10835412
28. Cann HM, de Toma C, Cazes L, Legrand MF, Morel V, et al. A human genome diversity cell line panel. Science. 2002; 296(5566): 261–262. https://doi.org/10.1126/science.296.5566.261b PMID: 11954565
29. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics. 2005; 21:263–265. https://doi.org/10.1093/bioinformatics/bth457 PMID: 15297300
30. R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.r-project.org/.
31. Bhaskar A, Javanmard A, Courtade T, Ade D. Novel probabilistic models of spatial genetic ancestry with applications to stratification correction in genome-wide association studies. Bioinformatics. 2017; 33(6):879–885. https://doi.org/10.1093/bioinformatics/btw720 PMID: 28025204
32. Kittles RA, Chen W, Panguluri RK, Ahaghotu C, Jackson A, et al. CYP3A4-V and prostate cancer in African Americans: causal or confounding association because of population stratification? Hum Genet. 2002; 110(6):553–560. https://doi.org/10.1007/s00439-002-0731-5 PMID: 12107441
33. Santos NP, Ribeiro-Rodrigues EM, Ribeiro do Santos AK, Pereira R, Gusmão L, et al. Assessing Individual Interethnic Admixture and Population Substructure Using a 48-Insertion-Deletion (INDEL) Ancestry-Informative Marker (AIM) Panel. Hum Mutat. 2010; (2): 184–190. https://doi.org/10.1002/humu.21159 PMID: 19953531
34. Mendes CMM. A questão da colonização do Brasil: Historiografia e documentos. Imagens Educ. 2012; 2(2): 1–13. https://doi.org/10.4025/imagenseduc.v2i2.17292

35. Afonso L. Panorama da cidade de Manaus: crise, progresso e cultura na década de 1960. SOMANLU. 2010; 10(2): 45–60. https://doi.org/10.17563/somanlu.v10i2.491

36. Murrie ZDF. História e Geografia: livro do estudante: ensino fundamental. In: Da Costa GP, editors. Mudanças no espaço geográfico do Brasil. Brasília: MEC: INEP; 2006. pp 33–40.

37. Travassos LH, Carneiro LAM, Ramjeet M, Husseym S, Kim Y-G, Magalhães JG, et al. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. Nat Immunol. 2010; (1):55–62. https://doi.org/10.1038/ni.1823 PMID: 19898471

38. Grant AV, Alter A, Huong NT, et al. Crohn's disease susceptibility genes are associated with leprosy in the Vietnamese population. J Infect Dis. 2012; 206(11):1763–1767. https://doi.org/10.1093/infdis/jis588 PMID: 22984114

39. Cambri G and Mira MT. Genetic Susceptibility to Leprosy—From Classic Immune-Related Candidate Genes to Hypothesis- Free, Whole Genome Approaches. Front Immunol. 2018; 9:1674. Available from: https://www.frontiersin.org/articles/10.3389/fimmu.2018.01674/full PMID: 30079069

40. Seirafi M, Kozlov G, Gehring K. Parkin structure and function. FEBS J. 2015; 282(11): 2076–2088. https://doi.org/10.1111/febs.13249 PMID: 25712550

41. Huang J, Brumell JH. Bacteria-autophagy interplay: a battle for survival. Nat Rev Microbiol. 2014; 12 (2):101–114. https://doi.org/10.1038/nrmicro3160 PMID: 24384599

42. Watson RO, Manzanillo PS, Cox JS. Extracellular M. tuberculosis DNA targets bacteria for autophagy by activating the host DNA-sensing pathway. Cell. 2012; 150(4):803–815. https://doi.org/10.1016/j.cell.2012.06.040 PMID: 22901810

43. Radomski N, Kagebein D, Liebler-Tenorio E, Karger A, Rufer E, Tews BA, et al. Mito-xenophagic killing of bacteria is coordinated by a metabolic switch in dendritic cells. Sci Rep. 2017; 7(1):3923. Available from: https://www.nature.com/articles/s41598-017-04142-5 https://doi.org/10.1038/s41598-017-04142-5 PMID: 28634388

44. Silva BJ, Barbosa MG, Andrade PR, Ferreira H, Nery JA, Côrte-Real S, et al. Autophagy is an innate mechanism associated with leprosy polarization. PLoS Pathog. 2017; 13(1). Available from: https://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat.1006103

45. Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature. 1998; 392(6676): 605–608. https://doi.org/10.1038/33416 PMID: 9560156

46. Alter A, Fava VM, Huong NT, Singh M, Orlova M, Van Thuc N, et al. Linkage disequilibrium pattern and age-at-diagnosis are critical for replicating genetic associations across ethnic groups in leprosy. Hum Genet. 2013; 132(1):107–116. https://doi.org/10.1007/s00439-012-1227-6 PMID: 23052943

47. Alcais A, Alter A, Antoni G, Orlova M, Nguyen VT, et al. Stepwise replication identifies a low-producing lymphotoxin-alpha allele as a major risk factor for early-onset leprosy. Nat Genet. 2007; 39(4):517–522. https://doi.org/10.1038/ng2000 PMID: 17353895

48. Malhotra D, Darvishi K, Lohra M, Kumar H, Grover C, et al., Association study of major risk single nucleotide polymorphisms in the common regulatory region of PARK2 and PACRG genes with leprosy in an Indian population. Eur. J. Hum. Genet. 2006; 14(4): 438–442. https://doi.org/10.1038/sj.ejhg.5201563 PMID: 16391553

49. Li J, Liu H, Liu J, Fu X, Yu Y, et al. Association study of the single nucleotide polymorphisms of PARK2 and PACRG with leprosy susceptibility in Chinese population. Eur J Hum Genet. 2012; 20(5): 488–489. https://doi.org/10.1038/ejhg.2011.190 PMID: 22009144

50. Sun Y, Lu Y, Li T, Xie L, Deng Y, Li S, et al. Interferon Gamma +874T/A Polymorphism Increases the Risk of Hepatitis Virus-Related Diseases: Evidence from a Meta-Analysis. PLoS One. 2015; 10(5). Available from: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0121168

51. Lin PL, Flynn JL. Understanding latent tuberculosis: a moving target. J Immunol. 2010; 185 (1):15–22. https://doi.org/10.4049/jimmunol.0903856 PMID: 20562268

52. Heinmeyer T, Wingender E, Reuter I, Hermjakob H, Kel AE, Kel OV, Ignatieva EV, Anako EA, Podkolodnaya OA, Kolpakov FA, Podkolodny NL, Kolchanov NA: Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. Nucleic Acids Res. 1998; 26(1):362–367. https://doi.org/10.1093/nar/26.1.362 PMID: 9399875

53. Wei Z, Wenhao S, Yuanyuan M, Yang L, Daming Z, Jiangchun X, Jijun J. A single nucleotide polymorphism in the interferon-γ gene (*IFNG* +874 T/A) is associated with susceptibility to tuberculosis. Oncotarget. 2017; 8(31): 50415–50429. https://doi.org/10.18632/oncotarget.17304 PMID: 28881572

54. Vallinoto ACR, Graca ES, Araujo MS, Azevedo VN, Cayres-Vallinoto I, Machado LFA, et al. IFNG +874T/A polymorphism and cytokine plasma levels are associated with susceptibility to mycobacterium tuberculosis infection and clinical manifestation of tuberculosis. Hum Immunol. 2010; 71(7):692–696. https://doi.org/10.1016/j.humimm.2010.03.008 PMID: 20353805

55. López-Maderuelo D, Arnalich F, Serrantes R, González A, Codoceo R, Madero R, et al. Interferon-gamma and Interleukin-10 gene polymorphisms in pulmonary tuberculosis. Am J Respir Crit Care Med. 2003; 167(7):970–975. https://doi.org/10.1164/rccm.200205-438BC PMID: 12531774

56. Sallakci N, Coskun M, Berber Z, Gurkan F, Kocamaz H, Uysal G,Bhuju S, Yavuzer U, Singh M, Yegin O. Interferon- gamma gene +874T-A polymorphism is associated with tuberculosis and gamma interferon response. Tuberculosis (Edinb). 2007; 87(3):225–230. https://doi.org/10.1016/j.tube.2006.10.002 PMID: 17276141

57. Cardoso CC, Pereira AC, Brito-de-Souza VN, Dias-Baptista IM, Maniero VC, Venturini J, et al. IFNG +874 T>A single nucleotide polymorphism is associated with leprosy among Brazilians. Hum Genet. 2010; 128(5):481–490. https://doi.org/10.1007/s00439-010-0872-x PMID: 20714752

58. Fava VM, Manry J, Cobat A, Orlova M, Van Thuc N, Ba NN, Thai VH, Abel L, Alcais A, Schurr E. A Missense LRRK2 Variant Is a Risk Factor for Excessive Inflammatory Responses in Leprosy. PLoS Negl Trop Dis. 2016; 10(2). Available from: https://journals.plos.org/plosntds/article?id=10.1371/journal.pntd.0004412

59. Fava VM, Xu YZ, Lettre G, Van Thuc N, Orlova M, Thai VH, Tao S, Croteau N, Eldeeb MA, MacDougall EJ, Cambri G, Lahiri R, Adams L, Fon EA, Trempe JF, Cobat A, Alcais A, Abel L, Schurr E. Pleiotropic effects for Parkin and LRRK2 in leprosy type-1 reactions and Parkinson's disease. Proc Natl Acad Sci U S A. 2019; 116(31):15616–15624. https://doi.org/10.1073/pnas.1901805116 PMID: 31308240

5 LIMITAÇÕES DA PESQUISA E PERSPECTIVAS

As limitações da pesquisa ocorreram no estudo molecular (Coorte), com a reduzida captação de contatos. No início do estudo, em 2015, projetávamos a coleta de aproximadamente 1200 contatos nos primeiros anos da pesquisa. No entanto, nos dois anos seguintes, o país passou por uma recessão econômica que teve impacto no desemprego e redução de recursos financeiros, levando a diminuição da demanda espontânea e a redução da busca ativa nesse grupo de risco.

Devido ao longo tempo de incubação do bacilo (média de 3 a 5 anos) e a coleta dos últimos contatos ter finalizado em abril de 2018, faremos uma consulta no SINAN em abril de 2021 para verificar se houve algum contato que adoeceu com hanseníase. Somente após esse período em que todos os contatos passarão com o mínimo de três anos de acompanhamento, é que teremos a capacidade de avaliar se o marcador molecular (16S rRNA) do *M. leprae* será um bom marcador de adoecimento.

Com o estudo genético será dado seguimento, com a aprovação de um recente projeto (Edital do Pró-estado - 2019, FAPEAM), coordenado pela Dra Carolina Talhari. A investigação de novos polimorfismos de base única (SNPs), em genes candidatos, terá início durante o ano de 2021. Serão avaliados SNPs de genes, isoladamente ou em haplótipos, de importantes vias metabólicas associadas com a resposta imune do hospedeiro. Os achados significativos desse estudo farão parte do painel de marcadores genéticos de suscetibilidade à hanseníase, com o objetivo de ser utilizado na identificação dos prováveis candidatos ao adoecimento, como nos contatos domiciliares, por exemplo.

6 CONCLUSÃO

a) Os testes rápidos mostraram limitada capacidade no diagnóstico da doença, contudo apresentaram alto valor preditivo negativo favorecendo a exclusão de hanseníase. Esta característica dos testes é importante no auxílio ao diagnóstico clínico, especialmente em uma região endêmica para a doença e com outras dermatoses confundidoras;

b) A concordância entre os testes rápidos (PGL1 e NDO-LID) foi excelente, tanto para os pacientes, paucibacilares e multibacilares, quanto para o grupo controle;

c) A positividade do teste rápido NDO-LID foi estatisticamente significativa com a classificação operacional, formas clínicas, índice baciloscópio e número de lesões;

d) Somente os SNPs dos genes *NOD2 e IFNG* apresentaram associação estatisticamente significativa com a proteção e risco a hanseníase, respectivamente, no estudo de caso x controle;

e) Não houve associação estatística dos polimorfismos de base única (SNPs), dos genes *TLR1*, *NOD2*, *PACRG/PRKN*, *IL6*, *IL10*, *TNF*, *IFNG* e *CCDC122/LACC1*, com as formas operacionais (PB x MB) e entre controles x MB.

7 REFERÊNCIAS

1. Robbins G, Mushrif Tripathy V, Misra VN, Mohanty RK, Shinde VS, Gray KM, et al. Ancient skeletal evidence for leprosy in India (2000 B.C.). PLoS One. 2009;4(5):1–8.
2. Souza-Araújo HC. História de lepra no Brasil: período republicano: 1889-1946: álbum das organizações antileprosas. Rio de Janeiro; 1948.
3. Sarno EN, Duppre NC, Sales AM, Hacker MA, Nery JA, de Matos HJ. Leprosy exposure, infection and disease: A 25-year surveillance study of leprosy patient contacts. Mem Inst Oswaldo Cruz. 2012;107(8):1054–9.
4. Fine PEM. Natural history of leprosy - aspects relevant to a leprosy vaccine. Int J Lepr [Internet]. 1983;553–5. Available from: https://pubmed.ncbi.nlm.nih.gov/6368419/
5. Cury MR de CO, Paschoal VDA, Nardi SMT, Chierotti AP, Júnior ALR, Chiaravalloti-Neto F. Spatial analysis of leprosy incidence and associated socioeconomic factors. Rev Saude Publica. 2012;46(1):110–8.
6. Schuenemann VJ, Singh P, Mendum TA, Krause-Kyora B, Jäger G, Bos KI, et al. Genome-wide comparison of medieval and modern Mycobacterium leprae. Science (80-). 2013;341(6142):179–83.
7. Francheschi DSA, Sacramento WSD, Mazini PS, Visentainer JEL. Hanseníase no Mundo Moderno. Arq Med. 2009;23(4):159–65.
8. World Health Organization. Global leprosy (Hansen disease) update, 2019: time to step-up prevention initiatives. Wkly Epidemiol Rec. 2020;95(36):417–440.
9. Geluk A. Challenges in immunodiagnostic tests for leprosy. Expert Opin Med Diagn. 2013;7(3):265–74.
10. Talhari S, Penna GO, Gonçalves HS OM. Hanseníase. 5ª ed. DiLivros, editor. 2015.
11. Saunderson P, Groenen G. Which physical signs help most in the diagnosis of leprosy? A proposal based on experience in the AMFES project, ALERT, Ethiopia. Lepr Rev. 2000;71:34–42.
12. Ministério da Saúde. Diretrizes para a vigilância, atenção e eliminação da Hanseníase como problema de saúde pública: manual técnico-operacional. [Internet]. Ministério da Saúde. 2016. 58 p. Available from:

http://lproweb.procempa.com.br/pmpa/prefpoa/cgvs/usu_doc/diretrizes_hanseniase.pdf

13. Pedrosa VL, Dias LC, Galban E, Leturiondo A, Palheta J, Santos M, et al. Leprosy among schoolchildren in the Amazon region: A cross-sectional study of active search and possible source of infection by contact tracing. PLoS Negl Trop Dis. 2018;12(2):1–12.
14. Hacker MDA, Duppre NC, Nery JAC, Sales AM, Sarno EN. Characteristics of leprosy diagnosed through the surveillance of contacts: A comparison with index cases in Rio de Janeiro, 1987-2010. Mem Inst Oswaldo Cruz. 2012;107(SUPPL.1):49–54.
15. Fine P, Sterne J, Ponnighaus J, Bliss L, Saul J, Chihana A, et al. Household and dwelling contact as risk factors for leprosy in northern Malawi. Am J Epidemiol [Internet]. 1997;146(1):91–102. Available from: http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=emed7&NEWS=N&AN=27291370
16. Moet FJ, Meima A, Oskam L, Richardus JH. Risk factors for the development of clinical leprosy among contacts, and their relevance for targeted interventions. Lepr Rev. 2004;75(4):310–26.
17. Teixeira CSS, Pescarini JM, Alves FJO, Nery JS, Sanchez MN, Teles C, et al. Incidence of and Factors Associated with Leprosy among Household Contacts of Patients with Leprosy in Brazil. JAMA Dermatology. 2020;1–10.
18. Centro de Integração de Dados e Conhecimentos para a Saúde-Cidacs [Internet]. Available from: http://cidacs.bahia.fiocruz.br/
19. Brennan P, Barrow W. Evidence for Species-Specific Lipid Antigens in Mycobacterium Leprae. Int J Lepr Other Mycobact Dis [Internet]. 1980;382–7. Available from: https://pubmed.ncbi.nlm.nih.gov/7009448/
20. Van Hooij A, Tjon Kon Fat EM, Richardus R, Van Den Eeden SJF, Wilson L, De Dood CJ, et al. Quantitative lateral flow strip assays as user-friendly tools to detect biomarker profiles for leprosy. Sci Rep. 2016;6(September):2–11.
21. Duthie MS, Truman RW, Goto W, O'Donnell J, Hay MN, Spencer JS, et al. Insight toward early diagnosis of leprosy through analysis of the developing antibody responses of Mycobacterium leprae-infected armadillos. Clin Vaccine Immunol. 2011;18(2):254–9.
22. Spencer JS, Kim HJ, Wheat WH, Chatterjee D, Balagon M V., Cellona R V., et

al. Analysis of antibody responses to Mycobacterium leprae phenolic glycolipid I, lipoarabinomannan, and recombinant proteins to define disease subtype-specific antigenic profiles in leprosy. Clin Vaccine Immunol. 2011;18(2):260–7.

23. Spencer JS, Dockrell HM, Kim HJ, Marques MAM, Williams DL, Martins MVSB, et al. Identification of Specific Proteins and Peptides in Mycobacterium leprae Suitable for the Selective Diagnosis of Leprosy . J Immunol. 2005;175(12):7930–8.
24. Sengupta U. Recent Laboratory Advances in Diagnostics and Monitoring Response to Treatment in Leprosy. Indian Dermatol Online J. 2019;10(2):106–14.
25. Carvalho APM, Coelho A da CO, Correa-Oliveira R, Lana FCF. Specific antigen serologic tests in leprosy: Implications for epidemiological surveillance of leprosy cases and household contacts. Mem Inst Oswaldo Cruz. 2017;112(9):609–16.
26. Zenha EMR, Ferreira MAN, Foss NT. Use of anti-PGL-1 antibodies to monitor therapy regimes in leprosy patients. Brazilian J Med Biol Res. 2009;42(10):968–72.
27. Oskam L, Slim E, Bührer-Sékula S. Serology: Recent developments, strengths, limitations and prospects: A state of the art overview. Lepr Rev. 2003;74(3):196–205.
28. Stefani MM de A, Grassi AB, Sampaio LH, de Sousa ALOM, Costa MB, Scheelbeek P, et al. Comparison of two rapid tests for anti-phenolic glycolipid-I serology in Brazil and Nepal. Mem Inst Oswaldo Cruz. 2012;107(1):124–31.
29. Almeida EC de, Martinez AN, Maniero VC, Sales AM, Duppre NC, Sarno EN, et al. Detection of Mycobacterium leprae DNA by polymerase chain reaction in the blood and nasal secretion of Brazilian household contacts. Mem Inst Oswaldo Cruz. 2004;99(5):509–11.
30. Martinez AN, Britto CFPC, Nery JAC, Sampaio EP, Jardim MR, Sarno EN, et al. Evaluation of real-time and conventional PCR targeting complex 85 genes for detection of Mycobacterium leprae DNA in skin biopsy samples from patients diagnosed with leprosy. J Clin Microbiol. 2006;44(9):3154–9.
31. Martinez AN, Lahiri R, Pittman TL, Scollard D, Truman R, Moraes MO, et al. Molecular determination of Mycobacterium leprae viability by use of real-time PCR. J Clin Microbiol. 2009;47(7):2124–30.

32. Martinez AN, Ribeiro-Alves M, Sarno EN, Moraes MO. Evaluation of qPCR-Based Assays for Leprosy Diagnosis Directly in Clinical Specimens. PLoS Negl Trop Dis. 2011;5(10):1–8.
33. Bang PD, Suzuki K, Phuong LT, Chu TM, Ishii N, Khang TH. Evaluation of polymerase chain reaction-based detection of Mycobacterium leprae for the diagnosis of leprosy. J Dermatol. 2009;36(5):269–76.
34. Martinez AN, Talhari C, Moraes MO, Talhari S. PCR-Based Techniques for Leprosy Diagnosis: From the Laboratory to the Clinic. PLoS Negl Trop Dis. 2014;8(4):1–8.
35. Cardoso CC, Pereira a. C, Brito-De-Souza VN, Dias-Baptista IM, Maniero VC, Venturini J, et al. IFNG +874 T>A single nucleotide polymorphism is associated with leprosy among Brazilians. Hum Genet. 2010;128(5):481–90.
36. Mira MT, Alcaı A, Moraes MO, Flumeri C Di, Thai VH, Khoa PX, et al. Susceptibility to leprosy is associated with PARK2 and PACRG. Lett to Nat. 2004;427(2):636–40.
37. Alter A, Alcaïs A, Abel L. Leprosy as a genetic model for susceptibility to common infectious diseases. Hum Genet. 2008;123:227–35.
38. Santos AR, Suffys PN, Vanderborght R, Moraes MO, Vieira LMM, Cabello PH, et al. Role of Tumor Necrosis Factor – a and Interleukin-10 Promoter Gene Polymorphisms in Leprosy. J Infect Dis. 2002;186:1687–91.
39. Mira MT, Alcaïs A, Thuc N Van, Thai VH, Huong NT, Ba N, et al. Chromosome 6q25 is linked to susceptibility to leprosy in a Vietnamese population. Letter. 2003;33(3):412–5.
40. Misch EA, Hawn TR. Toll-like receptor polymorphisms and susceptibility to human disease. Clin Sci. 2008;114:347–60.
41. Nath I, Saini C, Valluri VL. Immunology of leprosy and diagnostic challenges. Clin Dermatol [Internet]. 2015;33(1):90–8. Available from: http://dx.doi.org/10.1016/j.clindermatol.2014.07.005
42. Han XY, Seo YH, Sizer KC, Schoberle T, May GS, Spencer JS, et al. A new Mycobacterium species causing diffuse lepromatous leprosy. Am J Clin Pathol. 2008;130(6):856–64.
43. Han XY, Sizer KC, Thompson EJ, Kabanja J, Li J, Hu P, et al. Comparative sequence analysis of Mycobacterium leprae and the new leprosy-causing Mycobacterium lepromatosis. J Bacteriol. 2009;191(19):6067–74.

44. Han XY, Sizer KC TH-H. Identification of the leprosy agent Mycobacterium lepromatosis in Singapore. J Drugs Dermatol. 2012;11(2):168–72.
45. Jessamine PG, Desjardins M, Gillis T, Scollard D, Jamieson F, Broukhanski G et al. Leprosy-like illness in a patient with Mycobacterium lepromatosis from Ontario, Canada. J Drugs Dermatol. 2012;11:229–33.
46. Singh P, Benjak A, Schuenemann VJ, Herbig A, Avanzi C, Busso P, et al. Insight into the evolution and origin of leprosy bacilli from the genome sequence of Mycobacterium lepromatosis. Proc Natl Acad Sci U S A. 2015;112(14):4459–64.
47. Meredith A, Del Pozo J, Smith S, Milne E, Stevenson K M. Leprosy in red squirrels in Scotland. Vet Rec. 2014;285–6.
48. Desikan K, Iyer C. The distribuition of Mycobacterium leprae in different structures of the skin. Lepr Rev. 1972;43(1):30–7.
49. Klioze AM, Ramos-Caro FA. Visceral leprosy. Int J Dermatol. 2000;39(9):641–58.
50. Lastória JC, de Abreu MAMM. Leprosy: Review of the epidemiological, clinical, and etiopathogenic aspects - Part 1. An Bras Dermatol. 2014;89(2):205–18.
51. Desikan K. Viability of Mycobacterium leprae outside the human body. Lepr Rev. 1977;48(4):231–5.
52. Scollard DM, Adams LB, Gillis TP, Krahenbuhl JL, Truman RW, Williams DL. The continuing challenges of leprosy. Clin Microbiol Rev. 2006;19(2):338–81.
53. De Lima CS, Marques MAM, Debrie AS, Almeida ECC, Silva CAM, Brennan PJ, et al. Heparin-binding hemagglutinin (HBHA) of Mycobacterium leprae is expressed during infection and enhances bacterial adherence to epithelial cells. FEMS Microbiol Lett. 2009;292(2):162–9.
54. Silva CAM, Danelishvili L, McNamara M, Berredo-Pinho M, Bildfell R, Biet F, et al. Interaction of Mycobacterium leprae with human airway epithelial cells: Adherence, entry, survival, and identification of potential adhesins by surface proteome analysis. Infect Immun. 2013;81(7):2645–59.
55. Alter A, Grant A, Abel L, Alcaïs A, Schurr E. Leprosy as a genetic disease. Mamm Genome. 2011;22(1–2):19–31.
56. World Health Organization. Leprosy [Internet]. 2017 [cited 2020 May 7]. Available from: htpp://www.who.int/mediacentre/factsheets/fs101/en/.
57. Martin W Bratschi, Peter Steinmann, Anna Wickenden TPG. Current

Knowledge on Mycobacterium Leprae Transmission: A Systematic Literature Review. Lepr Rev [Internet]. 2015;86(2):142–55. Available from: https://pubmed.ncbi.nlm.nih.gov/26502685/

58. Barreto JG, Frade MAC, Bernardes Filho F, da Silva MB, Spencer JS, Salgado CG. Leprosy in Children. Curr Infect Dis Rep. 2017;19(6).
59. Ploemacher T, Faber WR, Menke H, Rutten V, Pieters T. Reservoirs and transmission routes of leprosy; A systematic review. PLoS Negl Trop Dis [Internet]. 2020;14(4):e0008276. Available from: https://dx.plos.org/10.1371/journal.pntd.0008276
60. Cole ST, Eiglmeier K, Parkhill J, James KD, Thomson NR, Wheeler PR, et al. Massive gene decay in the leprosy bacillus. Nature. 2001;409(6823):1007–11.
61. Lastória JC, de Abreu MAMM. Leprosy: A review of laboratory and therapeutic aspects - Part 2. An Bras Dermatol. 2014;89(3):389–403.
62. Organization WH. Guidelines for the diagnosis, treatment and prevention of leprosy [Internet]. 2018. Available from: https://www.who.int/lep/resources/9789290226383/en/.
63. Ridley DS, Jopling W. Classification of leprosy according to immunity. A five-group system. Int J Lepr. 1966;255–73.
64. Organization WH. Enhace global strategy for further reducing the disease burden due to leprosy [Internet]. 2010. Available from: https://www.searo.who.int/entity/leprosy/documents/SEA_GLP_2009_3/en/index.html.
65. Ministério da Saúde. Aprova as diretrizes para vigilência, atenção e controle da hanseníase [Internet]. 2010. Available from: https://bvsms.saude.gov.br/bvs/saudelegis/gm/2010/prt3125_07_10_2020.html.
66. Palit A, HK Kar. Prevention of transmission of leprosy: The current Scenario. Indian J Dermatol Venereol Leprol. 2020;115–23.
67. Moet FJ, Pahan D, Oskam L, Richardus JH. Effectiveness of single dose rifampicin in preventing leprosy in close contacts of patients with newly diagnosed leprosy: Cluster randomised controlled trial. Bmj. 2008;336(7647):761–4.
68. Richardus R, Alam K, Kundu K, Chandra Roy J, Zafar T, Chowdhury AS, et al. Effectiveness of single-dose rifampicin after BCG vaccination to prevent

leprosy in close contacts of patients with newly diagnosed leprosy: A cluster randomized controlled trial. Int J Infect Dis. 2019;88:65–72.

69. Mieras LF, Taal AT, Brakel WH Van, Cambau E, Saunderson PR, Smith WCS, et al. An enhanced regimen as post-exposure chemoprophylaxis for leprosy : PEP ++. BMC Infect Dis. 2018;18(506):1–8.
70. Klatser PR, Van Beers S, Madjid B, Day R, De Wit MYL. Detection of Mycobacterium leprae nasal carriers in populations for which leprosy is endemic. J Clin Microbiol. 1993;31(11):2947–51.
71. Van Beers SM, Izumi S, Madjid B, Maeda Y, Day R, Klatser PR. An epidemiological study of leprosy infection by serology and polymerase chain reaction. Int J Lepr. 1994;62(1):1–9.
72. Job CK, Jayakumar J, Kearney M, Gillis TP. Transmission of leprosy: A study of skin and nasal secretions of household contacts of leprosy patients using PCR. Am J Trop Med Hyg. 2008;78(3):518–21.
73. Modlin RL. The innate immune response in leprosy. Curr Opin Immunol. 2010;22(1):48–54.
74. Girardin SE, Boneca IG, Thomas G, Philpott DJ, Sansonetti PJ. Nod2 Is a General Sensor of Peptidoglycan through Muramyl Dipeptide (MDP) Detection *. J Biol Chem. 2003;278(11):8869–73.
75. Takeuchi O, Akira S. Pattern Recognition Receptors and Inflammation. Cell [Internet]. 2010;140(6):805–20. Available from: http://dx.doi.org/10.1016/j.cell.2010.01.022
76. Krutzik SR, Ochoa MT, Sieling PA, Uematsu S, Ng YW, Legaspi A, et al. Activation and regulation of Toll-like receptors 2 and 1 in human leprosy. Nat Med. 2003;9(5):525–32.
77. Krutzik SR, Tan B, Li H, Ochoa MT, Liu PT, Sharfstein SE, et al. TLR activation triggers the rapid differentiation of monocytes into macrophages and dendritic cells. Nat Med. 2005;11(6):653–60.
78. Hunger RE, Porcelli SA, Modlin RL, Hunger RE, Sieling PA, Ochoa MT, et al. Langerhans cells utilize CD1a and langerin to efficiently present nonpeptide antigens to T cells Find the latest version : Langerhans cells utilize CD1a and langerin to efficiently present nonpeptide antigens to T cells. J Clin Invest [Internet]. 2004;113(5):701–8. Available from: https://www.jci.org/articles/view/19655

79. Inkeles MS, Teles RMB, Pouldar D, Andrade PR, Madigan CA, Lopez D, et al. Cell-type deconvolution with immune pathways identifies gene networks of host defense and immunopathology in leprosy. JCL insight. 2016;1(15):1–16.
80. Ochoa MT, Loncaric A, Krutzik SR, Becker TC, Modlin RL. ‘“ Dermal Dendritic Cells ”’ Comprise Two Distinct Populations : CD1 þ Dendritic Cells and CD209 þ Macrophages. J Invest Dermatol. 2008;128:2225–31.
81. Pinheiro RO, Schmitz V, Jorge B, Silva DA, Sánchez-garcía FJ. Innate Immune Responses in Leprosy. Front Immunol. 2018;9(518):1–15.
82. Sica A, Erreni M, Allavena P, Porta C. Macrophage polarization in pathology. Cell Mol Life Sci. 2015;72(21):4111–26.
83. Gordon S, Plüddemann A. Tissue macrophages : heterogeneity and functions. BMC Biol. 2017;15(53):1–18.
84. Ka MB, Daumas A, Textoris J, Mege JL. Phenotypic diversity and emerging new tools to study macrophage activation in bacterial infectious diseases. Front Immunol. 2014;5(OCT):1–7.
85. de Sousa JR, de Sousa RPM, de Souza Aarão TL, Dias LB, Carneiro FRO, Fuzii HT, et al. In situ expression of M2 macrophage subpopulation in leprosy skin lesions. Acta Trop [Internet]. 2016;157:108–14. Available from: http://dx.doi.org/10.1016/j.actatropica.2016.01.008
86. Fallows D, Peixoto B, Kaplan G, Manca C. Mycobacterium leprae alters classical activation of human monocytes in vitro. J Inflamm [Internet]. 2016;13(8):4–8. Available from: http://dx.doi.org/10.1186/s12950-016-0117-4
87. Fulco T de O, Andrade PR, Barbosa MG de M, Pinto TGT, Ferreira PF, Ferreira H, et al. Effect of apoptotic cell recognition on macrophage polarization and mycobacterial persistence. Infect Immun. 2014;82(9):3968–78.
88. Teles RMB, Kelly-scumpia KM, Sarno EN, Rea TH, Ochoa MT, Cheng G, et al. IL-27 suppresses antimicrobial activity in human leprosy. J Invest Dermatol. 2016;135(10):2410–7.
89. Teles RMB, Graeber TG, Krutzik SR, Montoya D, Lee DJ, Komisopoulou E, et al. Type I Interferon Suppresses Type II Interferon–Triggered Human Anti-Mycobacterial Responses. Science (80-). 2014;339(6126):1448–53.
90. Barbosa A, Fonseca DL, Simon V, Cazzaniga RA, Moura TR De, Almeida RP De, et al. The influence of innate and adaptative immune responses on the differential clinical outcomes of leprosy. Infect Dis Poverty [Internet].

2017;6(5):1–8. Available from: http://dx.doi.org/10.1186/s40249-016-0229-3

91. Soilleux EJ, Sarno EN, Hernandez MO, Moseley E, Horsley J, Lopes UG, et al. DC-SIGN association with the Th2 environment of lepromatous lesions: Cause or effect? J Pathol. 2006;209(2):182–9.
92. Philippidis P, Mason JC, Evans BJ, Nadra I, Taylor KM, Haskard DO, et al. Hemoglobin Scavenger Receptor CD163 Mediates Interleukin-10 Release and Heme Oxygenase-1 Synthesis: Antiinflammatory Monocyte-Macrophage Responses In Vitro, in Resolving Skin Blisters In Vivo, and after Cardiopulmonary Bypass Surgery. Circ Res. 2004;94(1):119–26.
93. Platt N, Suzukit H, Kuriharat Y, Kodamat T, Gordon S. Role for the class A macrophage scavenger receptor in the phagocytosis of apoptotic thymocytes in vitro. Proc Natl Acad Sci U S A. 1996;93(October):12456–60.
94. Parthasarathy S, Fong LG, Otero D, Steinberg D. Recognition of solubilized apoproteins from delipidated , oxidized low density lipoprotein (LDL) by the acetyl-LDL receptor. Proc Natl Acad Sci U S A. 1987;84(January):537–40.
95. Cruz D, Watson AD, Miller CS, Montoya D, Ochoa M, Sieling PA, et al. Host-derived oxidized phospholipids and HDL regulate innate immunity in human leprosy. J Clin Invest. 2008;118(8):2917–28.
96. Tripp CS, Beckerman KP, Unanue ER. Immune complexes inhibit antimicrobial responses through interleukin-10 production. Effects in severe combined immunodeficient mice during Listeria infection. J Clin Invest. 1995;95(4):1628–34.
97. Jullien D, Sieling PA, Uyemura K, Mar ND, Rea TH MR. IL-15, an immunomodulator of T cell responses in intracellular infection. J immunol. 1997;158(2):800–6.
98. Zhou W, Zhang F, Aune TM, Alerts E. Either IL-2 or IL-12 Is Sufficient to Direct Th1 Differentiation by Nonobese Diabetic T Cells. J Immunol. 2003;170:735–40.
99. Cho SN, Cellona R V., Villahermosa LG, Fajardo J, Balagon MVF, Abalos RM, et al. Detection of phenolic glycolipid I of Mycobacterium leprae in sera from leprosy patients before and after start of multidrug therapy. Clin Diagn Lab Immunol. 2001;8(1):138–42.
100. Bhat RM, Prakash C. Leprosy: An overview of pathophysiology. Interdiscip Perspect Infect Dis. 2012;2012:1–6.

101. Salgame P, Yamamura M, Bloom BR MR. Evidence for functional subsets of CD4+ and CD8+ T cells in human disease: lymphokine patterns in leprosy. Chem Immunol. 1992;44–59.
102. Sadhu S, Khaitan BK, Joshi B, Sengupta U, Nautiyal AK, Mitra DK. Reciprocity between Regulatory T Cells and Th17 Cells: Relevance to Polarized Immunity in Leprosy. PLoS Negl Trop Dis. 2016;10(1):1–16.
103. Bettelli E, Korn T, Oukka M KV. Induction and effector functions of TH17 cells. Nature. 2008;176(1):139–48.
104. Ministério da Saúde. Boletim epidemiológico de hanseníase [Internet]. 2020. Available from: https://www.saude.gov.br/images/pdf/2020/janeiro/31/Boletim-hanseniase-2020-web.pdf.
105. Fundação Alfredo da Matta - Departamento de controle de doenças e epidemiologia. Dados epidemiológicos de hanseníase - Amazonas 2018. 2018.
106. Koczula KM, Gallotta A. Lateral flow assays. Essays Biochem. 2016;60(1):111–20.
107. Van Der Pol B, Taylor SN, Mena L, Lebed J, McNeil CJ, Crane LS, et al. Evaluation of the Performance of a Point-of-Care Test for Chlamydia and Gonorrhea. JAMA Netw open. 2020;3(5):e204819.
108. Woloshin, S; Patel NKA. False Negative Tests for SARS-CoV-2 Infection - Challenges and Implications. N Engl J Med [Internet]. 2020;1–2. Available from: nejm.org
109. García-Basteiro AL, DiNardo A, Saavedra B, Silva DR, Palmero D, Gegia M, et al. Point of care diagnostics for tuberculosis. Rev Port Pneumol (English Ed [Internet]. 2018;24(2):73–85. Available from: http://dx.doi.org/10.1016/j.rppnen.2017.12.002
110. Hunter SW, Fujiwara T, Brennan PJ. Structure and antigenicity of the major specific glycolipid antigen of Mycobacterium leprae. J Biol Chem. 1982;257(24):15072–8.
111. Brett SJ, Payne SN, Draper P, Gigg R. Analysis of the major antigenic determinants of the characteristic phenolic glycolipid from Mycobacterium leprae. Clin Exp Immunol. 1984;56(1):89–96.
112. Fujiwara T, Hunter SW, Cho SN, Aspinall GO, Brennan PJ. Chemical synthesis and serology of disaccharides and trisaccharides of phenolic glycolipid antigens from the leprosy bacillus and preparation of a disaccharide protein

conjugate for serodiagnosis of leprosy. Infect Immun. 1984;43(1):245–52.

113. Chatterjee D, Cho SN, Brennan PJ, Aspinall GO. Chemical synthesis and seroreactivity of O-(3,6-di-O-methyl-β-d-glucopyranosyl)-(1→4)-O-(2,3-di-O-methyl-α-lrhamnopyranosyl)-(1→9)-oxynonanoyl-bovine serum albumin-the leprosy-specific, natural disaccharide-octyl-neoglycoprotein. Carbohydr Res. 1986;156(C):39–56.
114. Fujiwara T, Aspinall GO, Hunter SW, Brennan PJ. Chemical synthesis of the trisaccharide unit of the species-specific phenolic glycolipid from Mycobacterium leprae. Carbohydr Res. 1987;163(1):41–52.
115. Bührer-Sékula S. Sorologia PGL-I na hanseníase. Rev Soc Bras Med Trop. 2008;41(2):3–5.
116. Duthie MS, Goto W, Ireton GC, Reece ST, Cardoso LP V, Martelli CMT, et al. Use of Protein Antigens for Early Serological Diagnosis of Leprosy □. Clin Vaccine Immunol. 2007;14(11):1400–8.
117. Rada E, Duthie MS, Reed SG, Aranzazu N, Convit J. Serologic follow-up of IgG responses against recombinant mycobacterial proteins ML0405, ML2331 and LID-1 in a leprosy hyperendemic area in Venezuela. Mem Inst Oswaldo Cruz. 2012;107(SUPPL.1):90–4.
118. Buhrer-Sékula S, Smits HL, Gussenhoven GC, Leeuwen J Van, Amador S, Fujiwara T, et al. Simple and Fast Lateral Flow Test for Classification of Leprosy Patients and Identification of Contacts with High Risk of Developing Leprosy Downloaded from http://jcm.asm.org/ on June 5 , 2016 by Staats-und Universitaetsbibliothek Bremen. 2003;41(5):1991–5.
119. Lobato J, Costa MP, Reis EM, Gonçalves MA, Spencer JS, Brennan PJ, Goulart LR GI. Comparison of three immunological tests for leprosy diagnosis and detection of subclinical infection. Lepr Rev [Internet]. 2011;389–401. Available from: https://pubmed.ncbi.nlm.nih.gov/22439279/
120. Cardoso LPV, Dias RF, Freitas AA, Hungria EM, Oliveira RM, Collovati M, et al. Development of a quantitative rapid diagnostic test for multibacillary leprosy using smart phone technology. BMC Infect Dis. 2013;13(497):1–10.
121. Duthie MS, Raychaudhuri R, Tutterrow YL, Misquith A, Bowman J, Casey A, et al. A rapid ELISA for the diagnosis of MB leprosy based on complementary detection of antibodies against a novel protein-glycolipid conjugate. Diagn Microbiol Infect Dis [Internet]. 2014;79(2):233–9. Available from:

http://dx.doi.org/10.1016/j.diagmicrobio.2014.02.006

122. Duthie MS, Balagon MF, Maghanoy A, Orcullo FM, Cang M, Dias RF, et al. Rapid quantitative serological test for detection of infection with Mycobacterium leprae, the causative agent of leprosy. J Clin Microbiol. 2014;52(2):613–9.
123. Moura RS, Penna GO, Fujiwara T, Pontes MA de A, Cruz R, Gonçalves H de S, et al. Evaluation of a rapid serological test for leprosy classification using human serum albumin as the antigen carrier. J Immunol Methods [Internet]. 2014;412:35–41. Available from: http://dx.doi.org/10.1016/j.jim.2014.06.014
124. Fabri A da COC, Carvalho APM, Araujo S, Goulart LR, de Mattos AMM, Teixeira HC, et al. Antigen-specific assessment of the immunological status of various groups in a leprosy endemic region. BMC Infect Dis [Internet]. 2015;15(1):1–9. Available from: ???
125. Amorim FM, Nobre ML, Ferreira LC, Nascimento LS, Miranda AM, Monteiro GRG, et al. Identifying Leprosy and Those at Risk of Developing Leprosy by Detection of Antibodies against LID-1 and LID-NDO. PLoS Negl Trop Dis. 2016;10(9):1–17.
126. Duthie MS, Orcullo FM, Abbelana J, Maghanoy A, Balagon MF. Comparative evaluation of antibody detection tests to facilitate the diagnosis of multibacillary leprosy. Appl Microbiol Biotechnol. 2016;100(7):3267–75.
127. Frade MAC, de Paula NA, Gomes CM, Vernal S, Bernardes Filho F, Lugão HB, et al. Unexpectedly high leprosy seroprevalence detected using a random surveillance strategy in midwestern Brazil: A comparison of ELISA and a rapid diagnostic test. PLoS Negl Trop Dis. 2017;11(2):1–12.
128. Penna MLF, Penna GO, Iglesias PC, Natal S. Anti-PGL-1 Positivity as a Risk Marker for the Development of Leprosy among Contacts of Leprosy Cases : Systematic Review and Meta- analysis. PLoS Negl Trop Dis. 2016;10(5):1–11.
129. Barreto JG, Bisanzio D, Frade MAC, Moraes TMP, Gobbo AR, Guimarães L de S, et al. Spatial epidemiology and serologic cohorts increase the early detection of leprosy. BMC Infect Dis [Internet]. 2015;15(1):1–9. Available from: http://dx.doi.org/10.1186/s12879-015-1254-8
130. Richardus RA, Zwet K Van Der, Hooij A Van, Wilson L, Oskam L, Faber R, et al. Longitudinal assessment of anti-PGL-I serology in contacts of leprosy patients in Bangladesh. PLoS Negl Trop Dis. 2017;11(12):1–13.
131. Martins MVSB, Guimarães MMDS, Spencer JS, Hacker M a VB, Costa LS,

Carvalho FM, et al. Pathogen-specific epitopes as epidemiological tools for defining the magnitude of Mycobacterium leprae transmission in areas endemic for leprosy. PLoS Negl Trop Dis. 2012;6(4):1–12.

132. Geluk A, Bobosha K, van der Ploeg-van Schip JJ, Spencer JS, Banu S, V. S. B. Martins M, et al. New Biomarkers with Relevance to Leprosy Diagnosis Applicable in Areas Hyperendemic for Leprosy. J Immunol. 2012;188(10):4782–91.
133. Corstjens PLAM, Hooij A Van, Tjon EM, Fat K, Wilson L, Geluk A. Field-Friendly Test for Monitoring Multiple Immune Response. Clin Vaccine Immunol. 2016;23(6):515–9.
134. Charles B, Shepard C. The experimental disease that follows the injection of human leprosy bacilli into foot-pads of mice. Hum Lepr bacili Inject into mice. 1960;(1):445–59.
135. Kirchheimer WF, Storrs EE, Binford CH. Attempts to establish the armadillo (Dasypus novemcinctus Linn.) as a model for the study of leprosy. Int J Lepr. 1972;39(3):229–42.
136. Woods SA, Cole ST. A rapid method for the detection of potentially viable Mycobacterium leprae in human biopsies: a novel application of PCR. FEMS Microbiol Lett. 1989;65(3):305–9.
137. Cole S, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, et al. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature. 1998;396:537–44.
138. Santos AR, Miranda ABDE, Sarno EN, Suffys PN, Degrave WM. Use of PCR-mediated amplification of Mycobacterium leprae DNA in different types of clinical samples for the diagnosis of leprosy. J Med Microbiol. 1993;39:298–304.
139. Caleffi KR, Hirata RDC, Hirata MH, Caleffi ER, Siqueira VLD, Cardoso RF. Use of the polymerase chain reaction to detect Mycobacterium leprae in urine. brazilian J Med Biol Res. 2012;45(2):93–178.
140. Jardim MR, Antunes SLG, Santos AR, Nascimento OJM, Nery JAC, Sales AM, et al. Criteria for diagnosis of pure neural leprosy. J Neurol. 2003;250:806–9.
141. Shamsi FA, Chaudhry IA, Moraes MO, Martinez AN, Riley FC. Detection of Mycobacterium leprae in ocular tissues by histopathology and real-time polymerase chain reaction. Ophthalmic Res. 2007;39(2):63–8.

142. Reis EM, Araujo S, Lobato J, Neves AF, Costa A V, Gonc MA. Mycobacterium leprae DNA in peripheral blood may indicate a bacilli migration route and high-risk for leprosy onset. Clin Microbiol Infect. 2013;20:447–52.
143. Kampirapap K, Singtham N, Klatser PR WS. DNA amplification for detection of leprosy and assessment of efficacy of leprosy chemotherapy. Int J Lepr Other Mycobact Dis [Internet]. 1998;16–21. Available from: https://pubmed.ncbi.nlm.nih.gov/9614835/
144. Scollard DM, Gillis TP, Williams DL. Polymerase chain reaction assay for the detection and identification of Mycobacterium leprae in patients in the United States. Am J Clin Pathol. 1998;109(5):642–6.
145. Kent L, Hugh TDMC, Billington O, Dale JW. Demonstration of Homology between IS 6110 of Mycobacterium tuberculosis and DNAs of Other Mycobacterium spp . J Clin Microbiol. 1995;33(9):2290–3.
146. Barbieri RR, Sales AM, Illarramendi X, Moraes MO, Augusto J, Justo S, et al. Diagnostic challenges of single plaque-like lesion paucibacillary leprosy. Mem Inst Oswaldo Cruz. 2014;109(7):944–7.
147. Da Cunha FMB, Werneck MCM, Scola RH, Werneck LC. Pure neural leprosy: Diagnostic value of the polymerase chain reaction. Muscle and Nerve. 2006;33(3):409–14.
148. Jardim MR, Antunes SL, Simons B, Wildenbeest JG, Nery JA et al. Role of PGL-I antibody detection in the diagnosis of pure neural leprosy. Lepr Rev [Internet]. 2005;232–40. Available from: https://pubmed.ncbi.nlm.nih.gov/16248210/
149. Barbieri RR, Manta FSN, Moreira SJM, Sales AM, Nery JAC, Nascimento LPR, et al. Quantitative polymerase chain reaction in paucibacillary leprosy diagnosis: A follow-up study. PLoS Negl Trop Dis. 2019;13(3):1–12.
150. Pathak VK, Singh I, Turankar RP, Lavania M, Ahuja M, Singh V, et al. Utility of multiplex PCR for early diagnosis and household contact surveillance for leprosy. Diagn Microbiol Infect Dis. 2019;95(3).
151. Davis GL, Ray NA, Lahiri R, Gillis TP, Krahenbuhl JL, Williams DL, et al. Molecular Assays for Determining Mycobacterium leprae Viability in Tissues of Experimentally Infected Mice. PLoS Negl Trop Dis. 2013;7(8):1–9.
152. Lini N, Shankernarayan NP, Dharmalingam K. Quantitative real-time PCR analysis of Mycobacterium leprae DNA and mRNA in human biopsy material

from leprosy and reactional cases. J Med Microbiol. 2009;58(6):753–9.

153. Manta FSN, Barbieri RR, Moreira SJM, Santos PTS, Nery JAC, Duppre NC, et al. Quantitative PCR for leprosy diagnosis and monitoring in household contacts : A follow-up study , 2011 – 2018. Sci Rep. 2019;9(16675):1–8.
154. Araújo S, Lobato J, Reis ÉDM, Souza DOB, Gonçalves MA, Costa AV, et al. Unveiling healthy carriers and subclinical infections among household contacts of leprosy patients who play potential roles in the disease chain of transmission. Mem Inst Oswaldo Cruz. 2012;107(1):55–9.
155. Matsuoka M. Drug Resistance in Leprosy. Jpn J Infect Dis [Internet]. 2010;1–7. Available from: https://pubmed.ncbi.nlm.nih.gov/20093754/
156. Casanova JL, Abel L. Inborn errors of immunity to infection: The rule rather than the exception. J Exp Med. 2005;202(2):197–201.
157. Chapman SJ, Hill AVS. Human genetic susceptibility to infectious disease. Nat Rev Genet [Internet]. 2012;13(3):175–88. Available from: http://dx.doi.org/10.1038/nrg3114
158. Alcaïs A, Abel L, Casanova J. Human genetics of infectious diseases : between proof of principle and paradigm. J Clin Invest. 2009;119(9):2506–14.
159. Picard C, Casanova J, Abel L. Mendelian traits that confer predisposition or resistance to specific infections in humans. Mendelian Genet Infect. 2006;18:383–90.
160. Abel L, Casanova JL. Human genetics of infectious diseases: Fundamental insights from clinical studies. Semin Immunol. 2006;18(6):327–9.
161. Quintana-Murci L, Alcaïs A, Abel L, Casanova JL. Immunology in natura: Clinical, epidemiological and evolutionary genetics of infectious diseases. Nat Immunol. 2007;8(11):1165–71.
162. Herndon C, Jennings R. A twin-family study of susceptibility to poliomyelitis. Am J Hum Genet. 1951;3(1):17–46.
163. Comstock GW. Tuberculosis in Twins : A Re-Analysis of the Prophit Survey. 1978;117(6):621–4.
164. Chakravartti MR VF. A twin study on leprosy. In: Topics in Human Genetics. Stuttgart: Georg Thieme Publishers; 1973. p. 1–29.
165. Akcay IM, Katrinli S, Ozdil K, Doganay GD, Doganay L. Host genetic factors affecting hepatitis B infection outcomes: Insights from genome-wide association studies. World J Gastroenterol. 2018;24(30):3347–60.

166. Harishankar M, Selvaraj P, Bethunaickan R. Influence of Genetic Polymorphism Towards Pulmonary Tuberculosis Susceptibility. Front Med. 2018;5(213):1–18.
167. Klebanov N. Genetic Predisposition to Infectious Disease. Cureus. 2018;10(8):1–8.
168. Mira MT, Alcaïs A, Van Thuc H, Moraes MO, Di Flumeri C, Thai VI, et al. Susceptibility to leprosy is associated with PARK2 and PACRG. Nature. 2004;427(6975):636–40.
169. Liu H, Wang Z, Li Y, Yu G, Fu X, Wang C, et al. Genome-Wide Analysis of Protein-Coding Variants in Leprosy. J Invest Dermatol. 2017;137(7):2544–51.
170. Alcaïs A, Alter A, Antoni G, Orlova M, Van Thuc N, Singh M, et al. Stepwise replication identifies a low-producing lymphotoxin-α allele as a major risk factor for early-onset leprosy. Nat Genet. 2007;39(4):517–22.
171. Sales C, Heloisa M, Vinicius S, Fava M, Elena L, Arnez A, et al. NOD2 and CCDC122 - LACC1 genes are associated with leprosy susceptibility in Brazilians. Hum Genet. 2014;133:1525–32.
172. Orlova M, Pietrantonio T Di, Schurr E. Genetics of infectious diseases: Hidden etiologies and common pathways. Clin Chem Lab Med. 2011;49(9):1427–37.
173. Jean- Laurent Casanova and Laurent Abel. The Genetic Theory of Infectious Diseases: A Brief History and Selected Illustrations. Annu Rev Genomics Hum Genet. 2016;14:215–43.
174. Monot M, Honoré N, Garnier T, Zidane N, Sherafi D, Paniz-Mondolfi A, et al. Comparative genomic and phylogeographic analysis of Mycobacterium leprae. Nat Genet. 2009;41(12):1282–9.
175. Schurr E, Alcaïs A, de Léséleuc L, Abel L. Genetic predisposition to leprosy: A major gene reveals novel pathways of immunity to Mycobacterium leprae. Semin Immunol. 2006;18(6):404–10.
176. Lázaro FP, Werneck RI, Mackert CCO, Vilar G, Abel L, Xavier B, et al. A Major Gene Controls Leprosy Susceptibility in a Hyperendemic Isolated Population from North of Brazil. J Infect Dis. 2010;201(10):1598–605.
177. Abel L, Demenais F. Detection of Major Genes for Susceptibility to Leprosy and Its Subtypes in a Caribbean Island : Desirade Island. Am J Hum Genet. 1988;42:256–66.
178. Cambri G, Mira MT. Genetic Susceptibility to Leprosy — From Classic immune-

Related Candidate Genes to Hypothesis- Free , whole Genome Approaches. Front Immunol. 2018;9(7):1–9.

179. Siddiqui MR, Meisner S, Tosh K, Balakrishnan K, Ghei S, Fisher SE, et al. A major susceptibility locus for leprosy in India maps to chromosome 10p13. Nat Genet. 2001;27(4):439–41.
180. Alter A, De Léséleuc L, Van Thuc N, Thai VH, Huong NT, Ba NN, et al. Genetic and functional analysis of common MRC1 exon 7 polymorphisms in leprosy susceptibility. Hum Genet. 2010;127(3):337–48.
181. Grant A V, Cobat A, Thuc N Van, Orlova M, Hong V, Laurent T, et al. CUBN and NEBL common variants in the chromosome 10p13 linkage region are associated with multibacillary leprosy in Vietnam. Hum Genet. 2014;
182. Medeiros P, da Silva WL, de Oliveira Gimenez BB, Vallezi KB, Moraes MO, de Souza VNB, et al. The GATA3 gene is involved in leprosy susceptibility in Brazilian patients. Infect Genet Evol [Internet]. 2016;39:194–200. Available from: http://dx.doi.org/10.1016/j.meegid.2016.01.015
183. Sauer MED, Salomão H, Ramos GB, Espindula HRSD, Rodrigues RSA, Macedo WC, et al. Genetics of leprosy : Expected and unexpected developments and perspectives. Clin Dermatol [Internet]. 2015;33(1):99–107. Available from: http://dx.doi.org/10.1016/j.clindermatol.2014.10.001
184. Jarduli LR, Sell AM, Reis PG, Sippert EÂ, Ayo CM, Mazini PS, et al. Role of HLA, KIR, MICA, and cytokines genes in leprosy. Biomed Res Int. 2013;2013.
185. Cardoso CC, Pereira AC, Brito-de-Souza VN, Duraes SMB, Ribeiro-Alves M, Nery JAC, et al. TNF -308G > A Single Nucleotide Polymorphism Is Associated With Leprosy Among Brazilians : A Genetic Epidemiology Assessment , Meta-Analysis , and Functional Study. J Infect Dis. 2011;204:1256–63.
186. Mira MT. Genetic host resistance and susceptibility to leprosy. Microbes Infect. 2006;8(4):1124–31.
187. Ramos GB, Salomão H, Francio AS, Fava VM, Werneck RI, Mira MT. Association Analysis Suggests SOD2 as a Newly Identified Candidate Gene Associated with Leprosy Susceptibility. J Infect Dis. 2016;214(3):475–8.
188. Miller EN, Jamieson SE, Joberty C, Fakiola M, Hudson D, Peacock CS, et al. Genome-wide scans for leprosy and tuberculosis susceptibility genes in Brazilians. Genes Immun. 2004;20(5):63–7.
189. Yang Q, Liu H, Low HQ, Wang H, Yu Y, Fu X, et al. Chromosome 2p14 is

linked to susceptibility to leprosy. PLoS One. 2012;7(1):1–5.

190. Zhang F-R, Huang W, Chen S-M, Zhang C, Zhang L, Han J, et al. Genomewide Association Study of Leprosy. N Engl J Med. 2009;361:2609–18.
191. Zhang F, Liu H, Chen S, Low H, Sun L, Cui Y, et al. Identification of two new loci at IL23R and RAB32 that influence susceptibility to leprosy. Nat Genet. 2011;43(12):1247–51.
192. Liu H, Irwanto A, Fu X, Yu G, Yu Y, Sun Y, et al. Discovery of six new susceptibility loci and analysis of pleiotropic effects in leprosy. Nat Genet. 2015;47(3):267–71.
193. Wang Z, Sun Y, Fu X, Yu G, Wang C, Bao F, et al. A large-scale genome-wide association and meta- analysis identified four novel susceptibility loci for leprosy. Nat Commun. 2016;7(13760).
194. Fava VM, Manry J, Cobat A, Orlova M, Thuc N Van, Moraes MO, et al. A genome wide association study identifies a lncRna as risk factor for pathological inflammatory responses in leprosy. Plos Genet. 2017;13(2):1–16.
195. Grant A V., Alter A, Huong NT, Orlova M, Van Thuc N, Ba NN, et al. Crohn's disease susceptibility genes are associated with leprosy in the vietnamese population. J Infect Dis. 2012;206(11):1763–7.
196. Wong SH, Hill AVS, Vannberg FO., India-Africa-United Kingdom Leprosy Genetics Consortium. Genomewide association study of leprosy. N Engl J Med. 2010; 15;362(15):1446-7. doi: 10.1056/NEJMc1001451.
197. Sales-Marques C, Cardoso CC, Alvarado-Arnez LE, Illaramendi X, Sales AM, Hacker M de A, et al. Genetic polymorphisms of the IL6 and NOD2 genes are risk factors for inflammatory reactions in leprosy. PLoS Negl Trop Dis. 2017;11(7):1–16.
198. Berrington WR, Macdonald M, Khadge S, Raj B, Janer M, Hagge DA, et al. Common polymorphisms in the NOD2 gene region are associated with leprosy and its reactive states. J Infect Dis. 2010;201(9):1422–35.
199. Marques CDS, Brito-de-souza VN, Tatiana L, Guerreiro A, Martins JH, Amaral P, et al. Toll-like Receptor 1 N248S Single-Nucleotide Polymorphism Is Associated With Leprosy Risk and Regulates Immune Activation During Mycobacterial Infection. J Infect Dis. 2013;208(2013):120–9.
200. Schuring RP, Hamann L, Faber WR, Pahan D, Richardus JH, Schumann RR,

et al. Polymorphism N248S in the Human Toll-Like Receptor 1 Gene Is Related to Leprosy and Leprosy Reactions. Lancet Infect Dis. 2009;199:1816–9.

201. Areeshi MY, Mandal RK, Dar SA, Jawed A, Wahid M, Lohani M, et al. Impact of TNF -308 G>A (rs1800629) gene polymorphism in modulation of leprosy risk: A reappraise meta-analysis of 14 case–control studies. Biosci Rep. 2017;37(5).
202. Alvarado-Arnez LE, Amaral EP, Sales-Marques C, Durães SMB, Cardoso CC, Sarno EN, et al. Association of IL10 polymorphisms and leprosy: A meta-analysis. PLoS One. 2015;10(9):1–13.
203. Silva GA V, Naveca FG, Ramasawmy R, Boechat AL. Association between the IFNG + 874A / T gene polymorphism and leprosy resistance : A meta-analysis. Cytokine [Internet]. 2014;65(2):130–3. Available from: http://dx.doi.org/10.1016/j.cyto.2013.12.002
204. Silva GAV, Santos MP, Mota-Passos I, Boechat AL, Malheiro A, Naveca FG, et al. IFN-γ +875 microsatellite polymorphism as a potential protection marker for leprosy patients from Amazonas state, Brazil. Cytokine [Internet]. 2012;60(2):493–7. Available from: http://dx.doi.org/10.1016/j.cyto.2012.04.043
205. Alter A, Medeiros V, Nguyen F, Huong T, Singh M. Linkage disequilibrium pattern and age-at-diagnosis are critical for replicating genetic associations across ethnic groups in leprosy. Hum Genet. 2013;132:107–16.
206. Chopra R, Ali S, Srivastava AK, Aggarwal S, Kumar B, Manvati S, et al. Mapping of PARK2 and PACRG Overlapping Regulatory Region Reveals LD Structure and Functional Variants in Association with Leprosy in Unrelated Indian Population Groups. PLoS Genet. 2013;9(7).
207. Li J, Liu H, Liu J, Fu X, Yu Y, Yu G, et al. Association study of the single nucleotide polymorphisms of PARK2 and PACRG with leprosy susceptibility in Chinese population. Eur J Hum Genet. 2012;20(5):488–9.
208. Malhotra D, Darvishi K, Lohra M, Kumar H, Grover C, Sood S, et al. Association study of major risk single nucleotide polymorphisms in the common regulatory region of PARK2 and PACRG genes with leprosy in an Indian population. Eur J Hum Genet. 2006;14:438–42.
209. Kobe B, Deisenhofer J. A structural basis of the interactions between leucine-rich repeats and protein ligands. Lett to Nat. 1995;374:183–6.
210. Skevaki C, Pararas M, Kostelidou K, Tsakris A, Routsias JG. Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious diseases.

Clin Exp Immunol. 2015;180(2):165–77.

211. Hart BE, Tapping RI. Genetic Diversity of Toll-Like Receptors and Immunity to M . leprae Infection. J Trop Med. 2012;2012:1–12.
212. Medzhitov R, Preston-Hurlburt P, Janeway CA. A human homologue of the Drosophila toll protein signals activation of adaptive immunity. Nature. 1997;388(6640):394–7.
213. Kawai T, Akira S. The roles of TLRs , RLRs and NLRs in pathogen recognition. Int Immunol. 2009;21(4):317–37.
214. Hill AVS. The genomics and genetics of human infectious disease susceptibility. Annu Rev Genomics Hum Genet. 2001;2(33):373–400.
215. Santana N de L, Rêgo JL, Oliveira JM, de Almeida LF, Braz M, Machado LMM, et al. Polymorphisms in genes TLR1, 2 and 4 are associated with differential cytokine and chemokine serum production in patients with leprosy. Mem Inst Oswaldo Cruz. 2017;112(4):260–8.
216. Bochud P, Hawn TR, Siddiqui MR, Saunderson P, Abraham I, Argaw AT, et al. Toll-Like Receptor 2 (TLR2) Polymorphisms Are Associated with Reversal Reaction in Leprosy. J Infect Dis. 2011;197(2):253–61.
217. Bochud, P.-Y., Sinsimer, D., Aderem, A., Siddiqui, M. R., Saunderson, P, Britton, S. , Abraham, I., Tadesse Argaw, A. , Janer, M., Hawn, T. R., Kaplan G. Polymorphisms in Toll-like receptor 4 (TLR4) are associated with protection against leprosy Published in final edited form as. J Clin Microbiol Infect Disses. 2009;28(9):1055–65.
218. Negroni A, Pierdomenico M, Cucchiara S, Stronati L. NOD2 and inflammation: current insights. J Inflamm Res. 2018;11:49–60.
219. Jeong E, Lee JY. Intrinsic and Extrinsic Regulation of Innate Immune Receptors. Yonsei Med J. 2011;52(3):379–92.
220. Motta V, Soares F, Sun T, Philpott DJ. Nod-like receptors: Versatile cytosolic sentinels. Physiol Rev. 2015;95(1):149–78.
221. Schenk M, Mahapatra S, Le P, Kim HJ, Choi AW, Brennan PJ, et al. Human NOD2 recognizes structurally unique muramyl dipeptides from Mycobacterium leprae. Infect Immun. 2016;84(9):2429–38.
222. Mizushima N, Yoshimori T, Ohsumi Y. The Role of Atg Proteins in Autophagosome Formation. Annu Rev Cell Dev Biol. 2011;27(1):107–32.
223. Travassos LH, Carneiro LAM, Ramjeet M, Hussey S, Kim YG, Magalhes JG, et

al. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. Nat Immunol [Internet]. 2010;11(1):55–62. Available from: http://dx.doi.org/10.1038/ni.1823

224. West AB, Lockhart PJ, O'Farell C, Farrer MJ. Identification of a novel gene linked to parkin via a bi-directional promoter. J Mol Biol. 2003;326(1):11–9.
225. Seirafi M, Kozlov G, Gehring K. Parkin structure and function. FEBS J. 2015;282(11):2076–88.
226. Huang J, Brumell JH. Bacteria-autophagy interplay: A battle for survival. Nat Rev Microbiol. 2014;12(2):101–14.
227. Watson RO, Manzanillo PS, Cox JS. Extracellular M. tuberculosis DNA Targets Bacteria for Autophagy by Activating the Host DNA-Sensing Pathway. Cell. 2013;150(4):803–15.
228. Radomski N, Kägebein D, Liebler-Tenorio E, Karger A, Rufer E, Tews BA, et al. Mito-xenophagic killing of bacteria is coordinated by a metabolic switch in dendritic cells. Sci Rep. 2017;7(1):1–18.
229. Jorge B, Silva DA, Garcia M, Barbosa DM, Nery C, Co S, et al. Autophagy Is an Innate Mechanism Associated with Leprosy Polarization. Plos Pathog. 2017;13(1):1–29.
230. Wei Z, Wenhao S, Yuanyuan M, Yang L, Daming Z, Jiangchun X, et al. A single nucleotide polymorphism in the interferon-γ gene (IFNG +874 T/A) is associated with susceptibility to tuberculosis. Oncotarget. 2017;8(31):50415–29.
231. Hu X, Ivashkiv LB. Cross-regulation of Signaling and Immune Responses by IFN-γ and STAT1. Immunity. 2009;31(4):539–50.
232. Sun Y, Lu Y, Li T, Xie L, Deng Y, Li S, et al. Interferon Gamma + 874T / A Polymorphism Increases the Risk of Hepatitis Virus-Related Diseases : Evidence from a Meta-Analysis. PLoS One. 2015;10(5):1–15.
233. Lin PL, Flynn JL. Understanding Latent Tuberculosis: A Moving Target. J Immunol. 2010;185(1):15–22.
234. Heinemeyer T, Wingender E, Reuter I, Hermjakob H, Kel AE, Kel O V., et al. Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. Nucleic Acids Res. 1998;26(1):362–7.
235. Wu Z, Sun Y, Zhu S, Tang S, Liu C, Wenzhou Qin. Association of Interferon Gamma þ 874T / A Polymorphism and Leukemia Risk. Medicine (Baltimore).

2016;95(12):1–7.

236. Franceschi DSA, Mazini PS, Rudnick CCC, Sell AM, Tsuneto LT, Ribas ML, et al. Influence of TNF and IL10 gene polymorphisms in the immunopathogenesis of leprosy in the south of Brazil. Int J Infect Dis. 2009;13(4):493–8.
237. Wang D, Feng JQ, Li YY, Zhang DF, Li XA, Li QW, et al. Genetic variants of the MRC1 gene and the IFNG gene are associated with leprosy in Han Chinese from Southwest China. Hum Genet. 2012;131(7):1251–60.
238. Scott Algood HM, Lin PL, Flynn JL. Tumor Necrosis Factor and Chemokine Interactions in the Formation and Maintenance of Granulomas in Tuberculosis. Clin Infect Dis. 2005;41(Supplement_3):S189–93.
239. Qidwai T, Khan F. Tumour Necrosis Factor Gene Polymorphism and Disease Prevalence. Scand J Immunol. 2011;74:522–47.
240. Louis E, Franchimont D, Piron A, Gevaert Y, Schaaf-Lafontaine N, Roland S, et al. Tumour necrosis factor (TNF) gene polymorphism influences TNF-α production in lipopolysaccharide (LPS)-stimulated whole blood cell culture in healthy humans. Clin Exp Immunol. 1998;113(3):401–6.
241. Silva GA V, Ramasawmy R, Boechat AL, Morais AC, Carvalho BKS, Sousa KBA, et al. Association of TNF À 1031 C / C as a potential protection marker for leprosy development in Amazonas state patients , Brazil. Hum Immunol [Internet]. 2015;76(2–3):137–41. Available from: http://dx.doi.org/10.1016/j.humimm.2015.01.011
242. Schurr E, Gros P. A common genetic fingerprint in leprosy and Crohn's disease? N Engl J Med. 2009;361(27):2666–8.
243. Cyktor JC, Turner J. Interleukin-10 and immunity against prokaryotic and eukaryotic intracellular pathogens. Infect Immun. 2011;79(8):2964–73.
244. Montoya D, Cruz D, Teles RMB, Lee DJ, Teresa M, Krutzik SR, et al. Divergence of macrophage phagocytic and antimicrobial programs in leprosy. October. 2010;6(4):343–53.
245. Yamamura M, Uyemura K, Deans RJ, Modlin RL. Defining Protective Responses to Pathogens : Cytokine Profiles in Leprosy Lesions. Reports. 1991;387(1984):277–9.
246. Pereira AC, Brito-de-souza VN, Cardoso CC, Dias-baptista IMF, Parelli FPC, Venturini J. Genetic , epidemiological and biological analysis of interleukin-10 promoter single-nucleotide polymorphisms suggests a definitive role for - 819C

/ T in leprosy susceptibility. Genes Immun. 2009;10:174–80.

247. Alvarado-arnez LE, Amaral EP, Sales-marques C, Sandra M. Association of IL10 Polymorphisms and Leprosy : A Meta-Analysis. PLoS One. 2015;10:1–13.
248. Tanaka T, Narazaki M, Kishimoto T. IL-6 in Inflammation, Immunity, and Disease. Cold Spring Harb Perspect Biol. 2014;6(1989):1–16.
249. Plataki MN, Zervou MI, Samonis G, Daraki V, Goulielmos GN, Kofteridis DP. Association of the Interleukin-6 rs1800795 Polymorphism with Type 2 Diabetes Mellitus in the Population of the Island of Crete, Greece. Genet Test Mol Biomarkers. 2018;22(7):448–52.
250. Zhou L, Zheng Y, Tian T, Liu K, Wang M, Lin S, et al. Associations of interleukin-6 gene polymorphisms with cancer risk: Evidence based on 49,408 cancer cases and 61,790 controls. Gene [Internet]. 2018;670(2017):136–47. Available from: https://doi.org/10.1016/j.gene.2018.05.104
251. Cui YX, Fu CW, Jiang F, Ye LX, Meng W. Association of the interleukin-6 polymorphisms with systemic lupus erythematosus: A meta-analysis. Lupus. 2015;24(12):1308–17.
252. Ziaee V, Maddah M, Moradinejad MH, Rezaei A, Zoghi S, Sadr M, et al. Association of interleukin-6 single nucleotide polymorphisms with juvenile idiopathic arthritis. Clin Rheumatol [Internet]. 2017;36(1):77–81. Available from: http://dx.doi.org/10.1007/s10067-016-3407-6
253. Sauer MD, Macedo WC, Schurr E. Human genetics of mycobacterial disease. Mamm Genome [Internet]. 2018;29(7):523–38. Available from: http://dx.doi.org/10.1007/s00335-018-9765-4
254. Svanholm H, Starklint H, Gundersen HJG, Fabriciusj J, Barleb H, Olsen S. Reproducibility of histomorphologic diagnoses with special reference to the kappa statistic. APMIS. 1989;97:689–98.
255. Barrett JC, Fry B, Maller J, Daly MJ. Haploview : analysis and visualization of LD and haplotype maps. Bioinforma Appl Note. 2005;21(2):263–5.
256. R Core Team. A language and environment for statistical computing [Internet]. Vienna, Austria: Foundation for statistical computing; 2017. Available from: https://www.r-project.org/.

APÊNDICE A – EQUIPE DE TRABALHO

Seq	Nome	Categoria/ Função	Instituição	Formação
01	Carolina Talhari	Pesquisadora / Orientadora	FUAM	Médico
02	Milton Ozório Moraes	Pesquisador / Coorientador	FIOCRUZ-RJ	Biólogo
03	Fabíola da Costa Rodrigues	Pesquisadora / Colaboradora	FUAM	Biólogo
04	André Luiz Leturiondo	Aluno de Doutorado	FUAM	Farmacêutico-Bioquímico
05	Cynthia de Oliveira Ferreira	Pesquisadora / Colaboradora	FUAM	Farmacêutico-Bioquímico
06	Camila Gurgel	Pesquisadora / Colaboradora	FUAM	Biomédico
07	Ariani Batista Noronha	Pesquisadora / Colaboradora	FUAM	Biomédico
08	Carla Yael Ribeiro Mendonça	Pesquisadora / Colaboradora	FUAM	Biomédico
08	Lucia Elena Alvarado-Arnez	Pesquisadora / Colaboradora	FIOCRUZ-RJ	Biólogo
10	Fernanda Manta	Pesquisadora / Colaboradora	FIOCRUZ-RJ	Biólogo
11	Ohanna Cavalcanti de Lima Bezerra	Pesquisadora / Colaboradora	FIOCRUZ-RJ	Biomédico
12	Marlon Wendell Athaydes Kerr	Aluno de IC / Colaborador	FUAM	Biomédico
13	Karina Pinheiro Pessoa	Aluna de IC / Colaboradora	FUAM	Biomédico
14	Monik Oney Oliveira do Nascimento	Colaboradora	FUAM	Biomédico
15	Michelle Carvalho do Espírito Santo	Aluna de IC / Colaboradora	FUAM	Farmacêutico-Bioquímico

APÊNDICE B - TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (ADULTO)

Você está sendo convidado (a) a participar da pesquisa "**MARCADORES MOLECULARES, GENÉTICOS E SOROLÓGICOS NA HANSENÍASE: SUPORTE AO DIAGNÓSTICO CLÍNICO DE PACIENTES E VIGILÂNCIA DOS CONTATOS"** em pessoas com idade igual ou superior a 10 anos, desenvolvida pela Fundação de Dermatologia Tropical e Venereologia "Alfredo da Matta" – FUAM. Este estudo tem como objetivo identificar qual o melhor teste laboratorial que será capaz de identificar o contato domiciliar (pessoa que vive na mesma casa do doente) com maior risco de desenvolver a hanseníase ou as reações inflamatórias. Os resultados servirão para possível implantação do teste no Serviço Único de Saúde (SUS), contribuindo para uma vigilância mais eficaz da doença. Com isso pode-se traçar alternativas de prevenção da hanseníase, tratamento medicamentoso e diagnóstico precoce, com grande impacto na diminuição de novos casos de hanseníase e diminuição de sequelas causadas pelo bacilo.

Nesta pesquisa, haverá um questionário a ser respondido por você ou o fornecimento de informações mínimas as quais serão importantes para análises deste estudo. Após esta fase, você será examinado por um profissional treinado que colherá da sua orelha (lóbulo auricular) uma pequena quantidade de linfa (líquido transparente) e 5 Ml de sangue. Com este material serão realizados testes laboratoriais que não são feitos na rotina de atendimento e que servem para identificar uma doença que pode afetar a sua saúde: a Hanseníase. Todo material coletado será armazenado no Laboratório de Biologia Molecular da Fundação "Alfredo da Matta", com identificação para a confirmação de algum resultado duvidoso. Nós prestaremos todas as orientações necessárias caso queira conhecer o resultado. Se você não quiser conhecer o resultado nós o manteremos em sigilo. Todas as informações fornecidas serão confidenciais.

Você terá disponibilidade de novas consultas nesta unidade ou, se necessário, em outra de maior complexidade. O tratamento, quando indicado, seguirá as recomendações do Ministério da Saúde e os medicamentos lhe serão entregues gratuitamente. A sua participação neste estudo é voluntária e livre para recusar ou se retirar em qualquer momento da pesquisa, sem que isto possa prejudicar o seu atendimento.

Declaro ter entendido as explicações referentes ao estudo:

_____

Assinatura do voluntário da pesquisa

Manaus, _____de _____de 20

_____

Assinatura do profissional responsável pelo atendimento

Qualquer dúvida referente ao estudo, favor entrar em contato com o Pesquisador Responsável pela Pesquisa: André Luiz Leturiondo / Fone: (92) 9123-7625.

APÊNDICE C - TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (RESPONSÁVEL DO MENOR)

O menor _____, sob sua responsabilidade, está sendo convidado (a) como voluntário (a) a participar da pesquisa **"MARCADORES MOLECULARES, GENÉTICOS E SOROLÓGICOS NA HANSENÍASE: SUPORTE AO DIAGNÓSTICO CLÍNICO DE PACIENTES E VIGILÂNCIA DOS CONTATOS"**. Este estudo tem como objetivo avaliar testes laboratoriais capazes de identificar o contato domiciliar (pessoa que vive na mesma casa do doente) com maior risco de desenvolver a hanseníase ou o paciente com hanseníase com maior risco em desenvolver as reações inflamatórias. Os resultados servirão para possível implantação do teste no Serviço Único de Saúde (SUS), contribuindo para uma vigilância mais eficaz da doença. Com isso pode-se traçar alternativas de prevenção da hanseníase, tratamento medicamentoso e diagnóstico precoce, com grande impacto na diminuição de novos casos de hanseníase e diminuição de sequelas causadas pelo bacilo.

Para esta pesquisa adotaremos os seguintes procedimentos: haverá um questionário a ser respondido pelo menor, com o fornecimento de informações mínimas as quais serão importantes para análises deste estudo. Após esta fase, ele (a) será examinado (a) por um profissional treinado que colherá da sua orelha (lóbulo auricular) uma pequena quantidade de linfa (líquido transparente), uma gota de sangue por punção digital (ponta do dedo) e 5 mL de sangue. Com este material serão realizados testes laboratoriais que não são feitos na rotina de atendimento e que servem para identificar uma doença que pode afetar a sua saúde: a Hanseníase. Todo material coletado será armazenado no Laboratório de Biologia Molecular da Fundação "Alfredo da Matta", com identificação para a confirmação de algum resultado duvidoso. Nós prestaremos todas as orientações necessárias caso queira conhecer o resultado. Se você não quiser conhecer o resultado nós o manteremos em sigilo. Todas as informações fornecidas serão confidenciais.

Riscos e Desconfortos: É mínimo o risco associado a sua participação neste estudo. O único desconforto será uma leve dor durante o raspado dérmico no lóbulo auricular, discreta dor na punção digital ou durante a coleta de sangue.

Benefícios: A participação neste estudo trará benefícios indiretos ao indivíduo, pois caso os resultados forem satisfatórios, será implantado na rotina da Fundação os testes diagnósticos em estudo, beneficiando todos os clientes atendidos pelo SUS com suspeita de estarem com hanseníase e seus respectivos contatos.

Para participar desta pesquisa, o menor sob sua responsabilidade não terá nenhum custo, nem receberá qualquer vantagem financeira. Ele (a) será esclarecido (a) em qualquer aspecto que desejar e estará livre para participar ou recusar-se a entrar no estudo. Você como responsável pelo menor, poderá retirar seu consentimento ou interromper a participação dele (a) a qualquer momento. A participação dele (a) é voluntária e a recusa em participar não acarretará qualquer penalidade ou modificação na forma como será atendido (a) na sua consulta de rotina. O menor não será

identificado em nenhuma publicação. O menor tem assegurado o direito a ressarcimento ou indenização no caso de quaisquer danos eventualmente produzidos pela pesquisa.

Os resultados estarão à sua disposição quando finalizada. O nome ou o material que indique a participação do menor não será liberado sem a sua permissão. Os dados e instrumentos utilizados na pesquisa ficarão arquivados com o pesquisador responsável por um período de 5 (cinco) anos, e após esse tempo serão destruídos. Este termo de consentimento encontra-se impresso em duas vias, sendo que uma cópia será arquivada pelo pesquisador responsável, e a outra será fornecida a você.

Solicito a sua autorização para que o material colhido seja armazenado e utilizado futuramente em outros estudos, respeitando sempre a resolução vigente e com a devida aprovação pelo Comitê de Ética em Pesquisa.

Eu, _____, portador (a) do documento de Identidade _____, responsável pelo menor _____, fui informado (a) dos objetivos do presente estudo de maneira clara e detalhada e esclareci minhas dúvidas. Sei que a qualquer momento poderei solicitar novas informações e modificar a decisão do menor sob minha responsabilidade de participar, se assim o desejar. Recebi uma cópia deste termo de consentimento livre e esclarecido e me foi dada a oportunidade de ler e esclarecer as minhas dúvidas.

Manaus, ____ de _____ de 20__.

_____

Assinatura do (a) responsável

_____

Assinatura do (a) Pesquisador (a)

APÊNDICE D - TERMO DE ASSENTIMENTO LIVRE E ESCLARECIDO (MENOR)

Você está sendo convidado (a) como voluntário (a) a participar da pesquisa **"MARCADORES MOLECULARES, GENÉTICOS E SOROLÓGICOS NA HANSENÍASE: SUPORTE AO DIAGNÓSTICO CLÍNICO DE PACIENTES E VIGILÂNCIA DOS CONTATOS"**. Este estudo tem como objetivo avaliar testes laboratoriais capazes de identificar o contato domiciliar (pessoa que vive na mesma casa do doente) com maior risco de desenvolver a hanseníase ou o paciente com hanseníase com maior risco em desenvolver as reações inflamatórias. Os resultados servirão para possível implantação dos testes no Serviço Único de Saúde (SUS), contribuindo para uma vigilância mais eficaz da doença ou seus processos inflamatórios. Com isso pode-se traçar alternativas de prevenção da hanseníase, tratamento medicamentoso e diagnóstico precoce, com grande impacto na diminuição de novos casos de hanseníase e diminuição de sequelas causadas pelo bacilo.

Para esta pesquisa adotaremos os seguintes procedimentos: haverá um questionário a ser respondido por você, com o fornecimento de informações mínimas as quais serão importantes para análises deste estudo. Após esta fase, você será examinado por um profissional treinado que colherá da sua orelha (lóbulo auricular) uma pequena quantidade de linfa (líquido transparente), uma gota de sangue por punção digital (ponta do dedo) e 5 mL de sangue. Com este material serão realizados testes laboratoriais que não são feitos na rotina de atendimento e que servem para identificar uma doença que pode afetar a sua saúde: a Hanseníase. Todo material coletado será armazenado no Laboratório de Biologia Molecular da Fundação "Alfredo da Matta", com identificação para a confirmação de algum resultado duvidoso. Nós prestaremos todas as orientações necessárias caso queira conhecer o resultado. Se você não quiser conhecer o resultado nós o manteremos em sigilo. Todas as informações fornecidas serão confidenciais.

Riscos e Desconfortos: É mínimo o risco associado a sua participação neste estudo. O único desconforto será uma leve dor durante o raspado dérmico no lóbulo auricular, discreta dor na punção digital ou durante a coleta de sangue.

Benefícios: A participação neste estudo trará benefícios indiretos ao indivíduo, pois caso os resultados forem satisfatórios, será implantado na rotina da Fundação os testes diagnósticos em estudo, beneficiando todos os clientes atendidos pelo SUS com suspeita de estarem com hanseníase e seus respectivos contatos.

Você poderá retirar o consentimento ou interromper a sua participação a qualquer momento. A sua participação é voluntária e a recusa em participar não acarretará qualquer penalidade ou modificação na forma como será atendido (a) na sua consulta de rotina. Você não será identificado em nenhuma publicação e tem assegurado o direito a ressarcimento ou indenização no caso de quaisquer danos eventualmente produzidos pela pesquisa.

Os resultados estarão à sua disposição quando finalizada. Seu nome ou o material que indique sua participação não será liberado sem a permissão do responsável por você. Os dados e

instrumentos utilizados na pesquisa ficarão arquivados com o pesquisador responsável por um período de 5 anos, e após esse tempo serão destruídos. Este termo de consentimento encontra-se impresso em duas vias, sendo que uma cópia será arquivada pelo pesquisador responsável, e a outra será fornecida a você.

Solicito a sua autorização para que o material colhido seja armazenado e utilizado futuramente em outros estudos, respeitando sempre a resolução vigente e com a devida aprovação pelo Comitê de Ética em Pesquisa.

Eu, _____, portador (a) do documento de Identidade _____ **(se já tiver documento),** fui informado (a) dos objetivos da presente pesquisa, de maneira clara e detalhada e esclareci minhas dúvidas. Sei que a qualquer momento poderei solicitar novas informações, e o meu responsável poderá modificar a decisão de participar se assim o desejar. Tendo o consentimento do meu responsável já assinado, declaro que concordo em participar dessa pesquisa. Recebi uma cópia deste termo de assentimento e me foi dada a oportunidade de ler e esclarecer as minhas dúvidas.

Manaus, ____ de _____ de 20_.

_____

Assinatura do (a) menor

_____

Assinatura do (a) pesquisador (a)

APÊNDICE E - PRODUÇÃO CIENTÍFICA EM OUTROS ESTUDOS NO PERÍODO DO DOUTORADO (ARTIGO 1)

Evaluation of screening for *Chlamydia trachomatis* among young women in primary health care services in Manaus, Amazonas State, Brazil

Avaliação de rastreamento de *Chlamydia trachomatis* em mulheres jovens em serviços de atenção primaria de Manaus, Amazonas, Brasil

Evaluación de los exámenes de control de *Chlamydia trachomatis* entre mujeres jóvenes en los servicios de salud de atención primaria en Manaus, Amazonas, Brasil

Dária Neves [1]
Meritxell Sabidó [2]
Camila Bótto-Menezes [1]
Nina Schwartz Benzaken [3]
Lucília Jardim [4]
Cynthia Ferreira [4]
André Leturiondo [4]
Camila Gurgel dos Santos [4]
Adele Schwartz Benzaken [4]

Abstract

Screening for Chlamydia trachomatis *is not routinely offered to young asymptomatic women in Brazil. This study evaluated the performance, usefulness, and operational suitability of the Digene Hybrid Capture II (HCII) CT-ID DNA-test as an opportunistic screening tool to detect* C. trachomatis *in the public health system in Manaus, Amazonas State. Women aged 14-25 years who attended primary health care services were interviewed and one cervical specimen was collected during cytological screening. The HCII CT test was evaluated for its ability to detect the presence of* C. trachomatis *and against real-time PCR (q-PCR) in a subset of samples. Operational performance was assessed through interviews with providers and patients. Overall, 1,187 women were screened, and 1,169 had a HCII CT-ID test result (292 of these were also tested by q-PCR). Of those, 13.1% (n = 153) were positive. The sensitivity, specificity, positive and negative predictive values of HCII CT were 72.3% (95%CI: 65.4-78.6), 91.3% (95%CI: 84.1-95.9), 93.8% (95%CI: 88.5-97.1), and 64.4% (95%CI: 56.0-72.1), respectively. Sample collection caused discomfort in 19.7% of women. Among health professionals (n = 52), the main barriers reported included positive cases who did not return for results (56.4%), unwillingness to screen without an appointment (45.1%), and increase in their workload (38.8%). HCII CT-ID identified a high proportion of* C. trachomatis *cases among young women in Manaus. However, its moderate sensitivity limits its use as an opportunistic screening tool in primary health care settings in Manaus. Screening was well accepted although the barriers we identified, especially among health professionals, challenge screening detection and treatment efforts.*

Chlamydia trachomatis: *Mass Screening; Women's Health*

[1] *Programa de Pós-graduação em Medicina Tropical, Universidade do Estado do Amazonas, Manaus, Brasil.*
[2] *Fundação de Medicina Tropical Doutor Heitor Vieira Dourado, Manaus, Brasil.*
[3] *Universidade Nilton Lins, Manaus, Brasil.*
[4] *Fundação Alfredo da Matta, Manaus, Brasil.*

Correspondence
M. Sabidó
Fundação de Medicina Tropical Doutor Heitor Vieira Dourado
Av. Pedro Teixeira 25, Manaus, AM 69040-000, Brasil.
mfisabido@gmail.com

Introduction

Chlamydia trachomatis is the most commonly diagnosed sexually transmitted bacterial infection (STI) worldwide [1,2]. Several countries such as The Netherlands and the United Kingdom have witnessed an increase in *C. trachomatis* case reports over the last decade [3,4], although the overall burden has decreased in other countries including the United States [5].

Genital infection with *C. trachomatis* is asymptomatic in 50-88% of Colombian women [6] and is most common in young women [7]. Untreated infections can cause pelvic inflammatory disease (PID) [8], ectopic pregnancy [9], and subfertility [10]. Risk factors for *C. trachomatis* infection include young age, having more than one sexual partner, and recent change of partner [11,12].

Given the frequent asymptomatic nature of the infection and the importance of early treatment to reduce transmission and prevent *C. trachomatis*-related morbidity, many developed countries are offering opportunistic screening to all sexually active people under the age of 25 years [1,13], except the US where screening focuses only on women younger than 25 years [14]. Evidence from clinical trials suggests that screening is effective in reducing the incidence of PID while a large nonrandomized cohort found no benefit of offering screening in reducing sequelae in women [15].

However, screening implementation might be hampered by operational and technical difficulties [16]. Barriers to health practitioners offering a test include lack of time and reluctance to raise sexual health issues within general practice [17]. Young people are willing to accept screening although low risk perception, poor health seeking behavior mainly due to the asymptomatic nature of the infection, poor understanding of what testing involves, and embarrassment remain important obstacles for screening uptake [18,19].

In Brazil, although data on *C. trachomatis* population prevalence is scarce, a study conducted in six cities estimated that 9.4% of women in the general population were infected [20] and nationwide, parturient women under 25 years of age showed a prevalence of infection at 9.8% [11]. *C. trachomatis* screening is not systematically offered at health services. The Digene Hybrid Capture II DNA test (Qiagen, Mississauga, Canada) to identify *C. trachomatis* (HCII CT-ID) is the only molecular test approved system for *C. trachomatis* screening in the Brazilian public health system. This nucleic acid hybridization assay is no longer recommended by the US Centers for Disease Control and Prevention (CDC) for routine use based on performance [21]. This test has shown sensitivity ranging from 93.8%-97.7% and specificity ranging from 95.9%-100.0% compared to those of culture [22,23,24,25]. Cell culture was considered the gold standard for detection of *Chlamydia* due to specificity as high as 100% [21]. However, culture presents disadvantages such as high-cost, technical complexity, labour intensity, and low sensitivity (50%) when compared to nucleic acid amplification testing (NAAT) [26]. Compared to culture, polymerase chain reaction (PCR) on cervical swab has shown very high specificity, usually ≥ 99% [21], while a meta-analysis reported a pooled sensitivity of 88.6% [26].

The aims of this study were to evaluate, in primary health care clinics in Manaus, Amazonas State, Brazil, the performance of the HCII CT-ID test using cervical specimens against the real-time PCR (q-PCR) assay as a gold standard; its usefulness to detect *C. trachomatis* cases among women under 25 years; and its operational suitability when used as an opportunistic screening tool from both the health professionals' and women's perspectives.

Material and methods

Study setting and population

Manaus is the capital city of Amazonas State (1,802,014 inhabitants) and has the second highest prevalence of *C. trachomatis* infection in Brazil [20]. The study was conducted in the Central-West Health District of the municipal public health system from the city of Manaus. This area, with two policlinics and 22 primary health care (PHC) services covering 14.1% of Manaus population, was selected because it presented a good health infrastructure system, PHC with equipped rooms for endocervical sample collection, and the health staff offered cervical cancer screening. Throughout the implementation of *C. trachomatis* screening, coordination between the Secretariat of Health in Manaus, the Central-West Health District, and its PHC services was established through regular meetings.

Between October 2012 and December 2013, we recruited women presenting at the two policlinics and 21 PHC services. Screening was offered during patient visits or when cytology to screen for cervical cancer was collected. We included asymptomatic women aged 14 to 25 years who agreed to sign the consent form. We excluded pregnant women and those who had used antibiotics during the previous 15 days.

Study and specimens processing

The target population were informed about the study aims during an advertising campaign that included displaying posters at health services, community health workers advertising during home visits, and educational talks at schools offered by medical students. The campaign was initiated shortly before recruitment and conducted throughout the screening program. Participants provided signed informed consent and completed a 10-minute questionnaire administered by trained health professionals who attended the services. The questionnaire included sections on sociodemographic indicators, sexual behavior, STI and HIV testing history, risk perception for STI, and substance abuse.

A nurse or doctor collected a single cervical specimen during the collection of cytology for cervical cancer screening. For *C. trachomatis* screening, we used a Digene cervical sampler brush (Qiagen, Mississauga, Canada) that was placed into a tube containing 1.0mL of Digene sample transport medium (STM). Samples were stored at room temperature until the end of the week, when they were transferred to the Fundação Alfredo da Matta (FUAM) laboratory and stored at -20ºC until processed.

Digene Hybrid Capture II DNA test procedure

A laboratory technician performed Digene HCII CT-ID according to the manufacturer's instructions as described previously [22]. Samples were processed in batches of up to 88 along with 8 control samples (4 positive, 4 negative). Test results were calculated by using assay-specific software accompanying the DML 2000 luminometer. Relative light units (RLUs) for the positive and negative controls were used to calculate the run-specific cutoff (CO). Specimen results were reported as RLU/CO ratios. Results were considered positive for RLU/CO values > 2.5. Samples with RLU/CO values ranging from 1.0 to 2.5 were considered equivocal and were retested. Test results were returned to the PHC.

Evaluation of the HCII CT-ID test against q-PCR

A subset of stored cervical specimens was used to evaluate the performance of HCII CT-ID against the Kit Artus CT Plus RG q-PCR 96 CE (Qiagen, Hilden, Germany) according to the assay package instructions. A laboratory technician, blind to the HCII CT results, tested all specimens that were positive by HCII CT test (n = 153) and the same number of those samples that tested negative. Negative samples were randomly selected from 15 storage boxes (ten per box) by another laboratory technician. A 300µL aliquot was transferred from each selected specimen to a 1.5mL tube before HCII CT-ID was performed. DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and the q-PCR performed on the Rotor-Gene 3000 instrument (Corbett Research, Australia). The interpretation of all q-PCR results followed the algorithms in the manufacturer's package insert. The only sample identifiers were specimen numbers.

Evaluation of operational characteristics

To identify factors that might influence test acceptability and operational performance, 52 health professionals attending the health services and all participants were interviewed. Information was gathered on service characteristics, opinions about *Chlamydia* screening, experience regarding STI, financial resources, time and other potential barriers to screening, and test's acceptability to participants. For participants, these questions were part of the initial interview and focused on reasons for *C. trachomatis* testing, STI knowledge, screening acceptability and its barriers.

Statistical analysis

Data were analyzed using Stata/SE version 11.2 (StataCorp LP, College Station, USA). Descriptive analyses were used to summarize participants' characteristics and the operational characteristics of screening to both participants and health professionals. The performance characteristics of HCII CT test compared to that of q-PCR (sensitivity, specificity, and positive and negative predictive values) were calculated by standard methods and are presented with 95% confidence intervals (95%CI).

Ethical statement

The study protocol was approved by the Ethics Research Committee of FUAM (approval number: 028/2011) and by the Ethics Committee of the Health Secretariat in Manaus. All participants signed a written consent form that was approved by the Ethics Committee. Testing with q-PCR was approved by the Ethics Research Committee of FUAM (approval number: 742.410). This study component had a waver for consent form because all samples were completely anonymized before being processed.

Results

Subjects' characteristics

A total of 1,187 consenting women were included in this study. The characteristics of study participants by age group are shown in Table 1. Mean age was 20.1 years (SD: 2.9). Most participants (82%) had at least completed primary school, and were single or did not live with a partner (85.9%). Almost half (47.4%) were evangelical christians, 20.2% earned less than minimum wage (< US$ 230.84/month), 35.4% had had an STI, 47.8% had been tested for HIV, and 57.4% had no or low risk perception for STIs. The majority (63.3%) had their first sexual intercourse at 15 years or older, 25.5% had had more than one partner during the preceding six months, 78.7% currently had a regular partner, and 16.1% had a new partner during the preceding 6 months. Among participants, 4.9% currently exchanged money for sex, 30.1% always used a condom during sexual intercourse in the preceding 6 months. During the preceding 6 months, recreational drug use was low (0.2%), 29.5% consumed alcohol, and 57.1% practised binge drinking which was defined as having at least 4 drinks in one occasion.

Performance of HCII CT-ID test

Among the 1,187 women screened, 10 were not tested with the HCII CT-ID test and were excluded from the analysis. Eight women had an indeterminate result and were also excluded from analysis. Therefore, 1,169 participants had a HCII CT-ID test result. Among those 13.1% (153/1,169) tested positive for *C. trachomatis*, including 16% (82/512) of women aged 14-19 years and 10.8% (71/657) of those aged 20-25 years. Overall, 292 specimens were tested by q-PCR. Of those, one resulted in no amplification and was excluded from the evaluation of the performance analysis. The sensitivity of HCII CT-ID compared to q-PCR was 72.3% (95%CI: 65.4-78.6) (136/188), specificity 91.3% (95%CI: 84.1-95.9) (94/103), positive predictive values 93.8% (95%CI: 88.5-97.1) (136/145), and negative predictive values 64.4% (95%CI: 56.0-72.1) (94/146). The HCII CT-ID test detected 72.3% (136/188) of *C. trachomatis* cases with a positive q-PCR result.

Operational characteristics

Table 2 presents reasons and potential barriers for *C. trachomatis* screening among young women. The mean time for patients to reach the PHC service was 15 minutes (IQR: 5-20), and most women (77.7%) reached it by walking. Only 27.9% received screening information during the advertisement campaign, mainly at the PHC service (38.6%). Only 40.6% were aware that testing for HIV and syphilis was offered at the PHC service. The main motivation for testing was that screening was offered during the campaign or at the PHC in 58.7% of the women. Overall, 80.8% women did not report problems with screening although 17.9% reported discomfort during endocervical sample collection. Screening acceptance was high (90.9%) and 98.1% were willing to test again. Overall, knowledge about STIs was high, with 92.5% of the women correctly identifying that condoms should be used before sexual intercourse starts, and 95.9% reporting that partners need treatment when someone has an STI.

The health professionals interviewed (n = 52) were 7 doctors (13.5%), 32 nurses or nurse technicians (61.5%), 8 community health agents (15.4%), and 5 social workers (9.7%). Health professionals (n = 52) found that screening for *C. trachomatis* is a priority (87.8%) and would support maintenance of the screening program (91.8%), although 38.8% believed that it would increase their work load. Most (76%) reported sufficient working experience to attend to STI patients, and 86% felt comfortable discussing patients' sexual health. Health professionals identified time pressure (27.4%), lack of human resources (28.5%), lack of privacy (55.8%), lack of sample collection material (26%), the existence of positive cases who did not return for their results (56.4%), lack of resources to perform active case finding of *Chlamydia* positive cases (53.9%), delays in delivery of testing results (46.8%), and lack of support from administrative staff (9.6%) as obstacles for offering *C. trachomatis* screening. Almost three-fourths (74.5%) correctly identified that screening was opportunistic (i.e. offered regardless of the women's reason for attending the PCH services), though only 45.1% reported that they would screen women with no appointment.

Discussion

This is the largest *C. trachomatis* screening study to date providing an evaluation of the introduction of HCII CT-ID test in PHC clinics in Brazil. The study results can help to revise the national recommendation of using HCII CT-ID, which is the only test available to detect *C. trachomatis* in the Brazilian public health system. The study results showed that the HCII CT-ID test had performed moderately well detecting *C. trachomatis* among asymptomatic women in Manaus. Although it had excellent specificity, its sensitivity was lower than that of q-PCR due in

Table 1

Sociodemographic characteristics, sexually transmitted infection (STI) and HIV testing history, STI risk perception, behavioral characteristics, and Digene Hybrid Capture II (HCII CT) test result in young women in Manaus, Amazonas State, Brazil, by age group.

Variables	Total sample (N = 1,187) n (%)	14-19 years (n = 520) n (%)	20-25 years (n = 667) n (%)
Education			
< Primary school	213 (18.0)	115 (22.2)	98 (14.7)
At least primary school	970 (82.0)	403 (77.8)	567 (85.3)
Marital status			
Married/Living with partner	167 (14.1)	39 (7.5)	128 (19.2)
Single/Not living with partner	1,019 (85.9)	481 (92.5)	538 (80.8)
Religion			
Evangelical christian	562 (47.4)	249 (47.9)	313 (46.9)
Others	625 (52.6)	271 (52.1)	354 (53.1)
Monthly income less than minimum wage (US$ *)			
Yes	222 (20.2)	97 (20.9)	125 (19.7)
No	878 (79.8)	368 (79.1)	510 (80.3)
Ever had an STI			
No	764 (64.6)	337 (65.1)	427 (64.3)
Yes	418 (35.4)	181 (34.9)	237 (35.7)
Ever tested for HIV			
Yes	561 (47.8)	155 (30.2)	406 (61.6)
No	612 (52.2)	359 (69.8)	253 (38.4)
Perceive themselves at risk for STIs			
Moderate/High	504 (42.6)	218 (42.1)	286 (42.9)
None/Low	680 (57.4)	300 (57.9)	380 (57.1)
Age at first sexual intercourse (years)			
< 15	435 (36.7)	251 (48.3)	184 (27.6)
≥ 15	751 (63.3)	269 (51.7)	482 (72.4)
Number of sexual partners, last 6 months			
0-1	884 (74.5)	364 (70.0)	520 (77.9)
> 1	303 (25.5)	156 (30.0)	147 (22.1)
Currently have a regular partner **			
No	236 (21.3)	118 (24.5)	118 (18.9)
Yes	872 (78.7)	364 (75.5)	508 (81.1)
Have a new partner, last 3 months			
No	961 (83.9)	408 (82.4)	553 (84.9)
Yes	185 (16.1)	87 (17.6)	98 (15.1)
Currently exchange money for sex			
No	1,108 (95.1)	484 (94.5)	624 (95.6)
Yes	57 (4.9)	28 (5.5)	29 (4.4)
Always used a condom with any partner, last 6 months			
Yes	357 (30.1)	176 (33.9)	181 (27.1)
No	830 (69.9)	344 (66.2)	486 (72.8)
Recreational drugs use, last 6 months			
No	1,180 (99.8)	515 (99.6)	665 (100.0)
Yes	2 (0.2)	2 (0.4)	0 (0.0)
Alcohol consumption, last 6 months			
No	830 (70.5)	370 (71.8)	460 (69.4)
Yes	348 (29.5)	145 (28.2)	203 (30.6)

(continues)

Table 1 (continued)

Variables	Total sample (N = 1,187) n (%)	14-19 years (n = 520) n (%)	20-25 years (n = 667) n (%)
Binge drinking (at least 4 drinks in one occasion), last 6 months			
No	144 (42.9)	69 (49.6)	75 (38.3)
Yes	191 (57.1)	70 (50.4)	121 (61.7)
HCII CT result			
Negative	1,016 (86.9)	430 (84.0)	586 (89.2)
Positive	153 (13.1)	82 (16.0)	71 (10.8)
Total ***	1,169	512	657

* US$ 230.84;

** Defined as a husband, steady partner, or boyfriend, regardless of living arrangements;

*** Total corresponds to number of patients with a HCII CT result. Of the total sample of 1,187 women, 18 were excluded from the performance analysis (10 were not tested, and 8 had an indeterminate HCII CT result).

Table 2

Reasons and potential barriers for *Chlamydia trachomatis* screening among 1,187 women. Manaus, Amazonas State, Brazil.

	Total sample (N = 1,187) n (%)
Mean time to reach the PHC service (IQR) in minutes	15 (5-20)
Mode of transport to the clinics	
Walking	918 (77.7)
Others (bus, taxi, own car)	264 (22.3)
Received screening information during the campaign	330 (27.9)
If so, where	
At school	29 (8.4)
Through a friend	35 (10.1)
Community health agent	119 (34.5)
PHC	133 (38.6)
Other places	29 (8.4)
Aware that HIV and syphilis tests are offered at the PHC service	472 (40.6)
Main reasons for testing	
To know their own health status	373 (31.4)
Perceive themselves at risk	131 (11.1)
It was offered during campaign or at the PHC service	697 (58.7)
Perceived problems with screening	
None	959 (80.8)
Discomfort	212 (17.9)
Waiting time in the clinic or for testing	16 (1.3)
Accept *Chlamydia* test as a screening test	897 (90.9)
Willing to test again	1,029 (98.1)
Correctly answered questions relating to STI topic	
Condom should always be put on before sexual intercourse	1,076 (92.5)
Having a regular partner does not reduce the risk of acquiring STIs	911 (78.1)
Need for partner treatment if either person has an STI	1,135 (95.9)
A person can be infected with STIs without showing any signs	992 (84.3)

IQR: interquartile range; PHC: primary health service; STI: sexually transmitted infection.

part to differences regarding the limit of detection for these assays (> 100 *C. trachomatis* copies for the HCII CT test compared to > 1 copy for q-PCR) [21]. Previous studies that compared the performance of HCII CT and Amplicor PCR for the diagnosis of genital *C. trachomatis* infection reported higher sensitivity, ranging from 93.3% to 95.4%, and specificity, ranging from 99% to 100% [22,24]. Interestingly, the HCII CT-ID-test identified a high proportion of *C. trachomatis* cases among young women in the 14-19 years of age group.

The majority of women attending PHC services accepted the test offered as a screening tool and were willing to test again, although low STI risk perception, low awareness that screening and other HIV/STI tests were offered in PHC, and discomfort caused by sample collection were identified as barriers to engage young people in screening. In fact, most women tested simply because it was offered, suggesting that the introduction of routine screening across all PHC may encourage testing among women who would otherwise not seek it, mainly because they are asymptomatic.

From a health professionals' perspective, *C. trachomatis* screening was considered a priority and was offered by staff with sufficient working experience to deal with STI cases and who felt comfortable discussing sexual health. However, the fact that less than half of health professionals interviewed would test without appointment – upon which routine offer of screening to control *C. trachomatis* infection hinges – is cause for concern. The increase in workload reported by health professionals, which has been identified as a barrier to screening delivery, is of additional concern [17]. Other barriers identified were poor supply chain management that leads to shortage of speculum and gloves to collect material by the municipality. Given that the objective of a *C. trachomatis* screening program is to decrease transmission and prevent sequelaes, the success of any program is dependent not only on the ability to screen women but also on the ability to ensure treatment of those who test positive. In our study, early treatment of infection faced challenges, including infected cases who did not return for their results, delays in delivering test results to patients, and shortage of health professionals to perform active case finding. In most of the PHCs (18/22, 85.7%), service providers took advantage of routine cytological examination to offer *C. trachomatis* screening. Offering both screenings together might be more convenient for both the patient and the health professional. However, restricting *C. trachomatis* screening to only women who seek cytology would miss opportunities to test women that attend the PHC for other reasons.

This study had a few limitations. The detection of *C. trachomatis* by use of the HCII CT DNA-test is dependent on the number of organisms present in the specimen and may be affected by patient factors, such as the presence of symptoms [27]. Although the q-PCR assay is highly sensitive and specific [28], it can be affected by contamination or inhibitors [29].

In conclusion, our results suggest that HCII CT-ID is not an appropriate test to identify chlamydial organisms in endocervical samples due to its moderate sensitivity compared to that of q-PCR. Screening for *Chlamydia* in PHC services was feasible and well accepted by participants and health professionals who considered it a priority. In light of this finding, we recommend offering *C. trachomatis* routine screening for young women at PHCs in Manaus but using a test with better performance, such as q-PCR. Evidence on cost-effectiveness will be needed to guide the decision on test selection and registration in the Brazilian public health system. High levels of coverage will be needed to ensure an effective screening programme. In fact, most screening programmes have experienced difficulties in achieving the 30-50% coverage among young people needed to reduce *C. trachomatis* prevalence [16].

We identified several operational barriers that may limit screening uptake. Health professionals struggled to deliver screening due to an increase in their workload and problems with supply chain management. They were reluctant to offer *C. trachomatis* screening without appointment and preferred to offer screening together with cytology for cervical cancer screening instead. Early treatment of positive cases faced challenges that might limit *Chlamydia* control efforts. Among young women, none or low risk perception of STIs, which was a risk factor for *C. trachomatis* infection, was also found to be a barrier to engaging them in screening. The successful implementation of screening programs for *Chlamydia* in similar settings will need to overcome such barriers. While still under debate, early *C. trachomatis* detection and treatment will produce the most benefits if opportunistic screening is offered as part of a national coordinated program.

Contributors

D. Neves and N. S. Benzaken contributed to the field work, interpreted data and reviewed the article. M. Sabidó contributed to the data analyses and wrote the article. C. Bôtto-Menezes contributed to the field work and data analysis and interpretation. L. Jarditm, C. Ferreira, A. Leturiondo, and C. G. Santos performed laboratory testing, interpreted results and reviewed the article. A. S. Benzaken conceived and coordinated the study, interpreted results and reviewed the article.

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Conflict of interests

The authors declare that they have no conflicts of interest.

References

1. European Centre for Disease Prevention and Control. *Chlamydia* control in Europe: literature review. Stockholm: European Centre for Disease Prevention and Control; 2014.
2. World Health Organization. Global prevalence and incidence of selected curable sexually transmitted infections. Geneva: World Health Organization; 2001.
3. van der Snoek EM, Gotz HM, Mulder PG, Verkooyen RP, van der Meijden WI. Prevalence of STD and HIV infections among attenders of the Erasmus MC STD clinic, Rotterdam, The Netherlands, during the years 1996 to 2000. Int J STD AIDS 2003; 14:119-24.
4. Health Protection Agency. Increasing rates of *Chlamydia trachomatis* and the role of screening. CDR Weekly 2006; 16(26). http://www.hpa.org.uk/cdr/archives/2006/cdr2606.pdf.
5. Datta SD, Torrone E, Kruszon-Moran D, Berman S, Johnson R, Satterwhite CL, et al. *Chlamydia trachomatis* trends in the United States among persons 14 to 39 years of age, 1999-2008. Sex Transm Dis 2012; 39:92-6.
6. Molano M, Meijer CJ, Weiderpass E, Arslan A, Posso H, Franceschi S, et al. The natural course of *Chlamydia trachomatis* infection in asymptomatic Colombian women: a 5-year follow-up study. J Infect Dis 2005; 191:907-16.

7. Adams EJ, Charlett A, Edmunds WJ, Hughes G. *Chlamydia trachomatis* in the United Kingdom: a systematic review and analysis of prevalence studies. Sex Transm Infect 2004; 80:354-62.
8. Risser WL, Risser JM. The incidence of pelvic inflammatory disease in untreated women infected with *Chlamydia trachomatis*: a structured review. Int J STD AIDS 2007; 18:727-31.
9. van Valkengoed IG, Morre SA, van den Brule AJ, Meijer CJ, Bouter LM, Boeke AJ. Overestimation of complication rates in evaluations of *Chlamydia trachomatis* screening programmes: implications for cost-effectiveness analyses. Int J Epidemiol 2004; 33:416-25.
10. Land JA, Van Bergen JE, Morre SA, Postma MJ. Epidemiology of *Chlamydia trachomatis* infection in women and the cost-effectiveness of screening. Hum Reprod Update 2010; 16:189-204.
11. Pinto VM, Szwarcwald CL, Baroni C, Stringari LL, Inocencio LA, Miranda AE. *Chlamydia trachomatis* prevalence and risk behaviors in parturient women aged 15 to 24 in Brazil. Sex Transm Dis 2011; 38:957-61.
12. Detels R, Green AM, Klausner JD, Katzenstein D, Gaydos C, Handsfield H, et al. The incidence and correlates of symptomatic and asymptomatic *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections in selected populations in five countries. Sex Transm Dis 2011; 38:503-9.
13. Public Health Agency of Canada. Canadian guidelines on sexually transmitted infections. Section 5 – management and treatment of specific infections, 2006. http://www.phac-aspc.gc.ca/std-mts/std-lts/.
14. Centers for Disease Control and Prevention. *Chlamydia* screening factsheet. http://www.cdc.gov/std/chlamydia/STDFact-Chlamydia.htm.
15. Gottlieb SL, Berman SM, Low N. Screening and treatment to prevent sequelae in women with *Chlamydia trachomatis* genital infection: how much do we know? J Infect Dis 2010; 201 Suppl 2:S156-67.
16. Baratiser P, Alexander S, Sheringham J. *Chlamydia trachomatis* screening in young women. Curr Opin Obstet Gynecol 2011; 23:315-20.
17. Hogan AH, Howell-Jones RS, Pottinger E, Wallace LM, McNulty CA. "...they should be offering it": a qualitative study to investigate young peoples' attitudes towards chlamydia screening in GP surgeries. BMC Public Health 2010; 10:616.
18. Richardson D, Maple K, Perry N, Ambler E, Jurd C, Fisher M. A pilot qualitative analysis of the psychosocial factors which drive young people to decline chlamydia testing in the UK: implications for health promotion and screening. Int J STD AIDS 2010; 21:187-90.
19. Balfe M, Brugha R, O'Donovan D, O'Connell E, Vaughan D. Young women's decisions to accept *Chlamydia* screening: influences of stigma and doctor-patient interactions. BMC Public Health 2010; 10:425.
20. Jalil EM, Pinto VM, Benzaken AS, Ribeiro D, Oliveira EC, Garcia EG, et al. Prevalência da infecção por clamídia e gonococo em gestantes de sete cidades brasileiras. Rev Bras Ginecol Obstet 2008; 30:614-9.
21. Centers for Disease Control and Prevention. Recommendations for the laboratory-based detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, 2014. MMWR Recomm Rep 2014; 63 (RR-02):1-19.
22. Girdner JL, Cullen AP, Salama TG, He L, Lorincz A, Quinn TC. Evaluation of the digene hybrid capture II CT-ID test for detection of *Chlamydia trachomatis* in endocervical specimens. J Clin Microbiol 1999; 37:1579-81.
23. Schachter J, Hook 3rd EW, McCormack WM, Quinn TC, Chernesky M, Chong S, et al. Ability of the digene hybrid capture II test to identify *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in cervical specimens. J Clin Microbiol 1999; 37:3668-71.
24. Van Der Pol B, Williams JA, Smith NJ, Batteiger BE, Cullen AP, Erdman H, et al. Evaluation of the Digene Hybrid Capture II Assay with the Rapid Capture System for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. J Clin Microbiol 2002; 40:3558-64.
25. Darwin LH, Cullen AP, Arthur PM, Long CD, Smith KR, Girdner JL, et al. Comparison of Digene hybrid capture 2 and conventional culture for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in cervical specimens. J Clin Microbiol 2002; 40:641-4.
26. Watson EJ, Templeton A, Russell I, Paavonen J, Mardh PA, Stary A, et al. The accuracy and efficacy of screening tests for *Chlamydia trachomatis*: a systematic review. J Med Microbiol 2002; 51:1021-31.
27. Michel CE, Sonnex C, Carne CA, White JA, Magbanua JP, Nadala Jr. EC, et al. *Chlamydia trachomatis* load at matched anatomic sites: implications for screening strategies. J Clin Microbiol 2007; 45:1395-402.
28. Bontin S. Advances in diagnostic testing of *Chlamydia trachomatis*. In: 14th European Congress of Clinical Microbiology and Infectious Diseases. http://www.blackwellpublishing.com/eccmid14/abstract.asp?id=15776.
29. Wilson IG. Inhibition and facilitation of nucleic acid amplification. Appl Environ Microbiol 1997; 63:3741-51.

Resumo

O rastreamento de Chlamydia trachomatis *não é feito de rotina em mulheres jovens assintomáticas no Brasil. O estudo avaliou o desempenho, utilidade e adequação operacional do teste de DNA Digene Hybrid Capture II (HCII) CT-ID como ferramenta de rastreamento oportunista para detectar* C. trachomatis *no sistema público de saúde em Manaus, Amazonas. Mulheres entre 14 e 25 anos de idade que frequentavam serviços de atenção básica foram entrevistadas, com a coleta de uma amostra cervicouterina durante o rastreamento citológico. O teste HCII CT foi avaliado em relação à capacidade de detectar a presença de* C. trachomatis, *e comparado à PCR em tempo real (q-PCR) em um subconjunto de amostras. O desempenho operacional foi avaliado através de entrevistas com profissionais e pacientes. Foram examinadas 1.187 mulheres, das quais 1.169 tiveram um resultado de teste HCII CT-ID (destas, 292 foram testadas também com q-PCR). Um total de 153 mulheres (13,1%) testaram positivas para* C. trachomatis. *A sensibilidade, especificidade e valores preditivos positivo e negativo do HCII CT foram 72,3% (IC95%: 65,4-78,6), 91,3% (IC95%: 84,1-95,9), 93,8% (IC95%: 88,5-97,1) e 64,4% (IC95%: 56,0-72,1), respectivamente. A coleta de amostras provocou desconforto em 19,7% das mulheres. As principais barreiras relatadas pelos profissionais de saúde (n = 52) eram casos positivos que não retornavam para os resultados (56,4%), falta de disponibilidade de realizar o rastreamento sem consulta agendada (45,1%) e aumento da carga de trabalho (38,8%). O HCII CT-ID identificou alta prevalência de* C. trachomatis *em mulheres jovens de Manaus. Entretanto, a sensibilidade moderada limita o uso como ferramenta de rastreamento oportunista em serviços de atenção básica naquela cidade. O rastreamento era bem-recebido, mas as barreiras identificadas, principalmente entre profissionais de saúde, limitam a detecção através do rastreamento e as iniciativas de tratamento.*

Chlamydia trachomatis; *Programas de Rastreamento; Saúde da Mulher*

Resumen

Los exámenes de control de Chlamydia trachomatis *no se ofrecen habitualmente a las mujeres jóvenes asintomáticas en Brasil. Este estudio evaluó los resultados, utilidad e idoneidad operativa del test Digene Hybrid Capture II (HCII) CT-ID DNA como una herramienta de examen apropiada para detectar la* C. trachomatis *en el sistema de salud público de Manaus, Amazonas. Las mujeres con una edad comprendida entre los 14-25 años que asistieron a un centro de atención primaria fueron entrevistadas, y se recogió una muestra cervical durante el examen citológico. Se evaluó el test HCII CT, debido a su habilidad para detectar la presencia de* C. trachomatis, *frente al realtime PCR (q-PCR) en un subconjunto de muestras. El resultado operativo fue evaluado mediante entrevistas con proveedores y pacientes. Globalmente, se examinaron a 1.187 mujeres, y 1.169 de ellas contaban con los resultados de la prueba HCII CT-ID (a 292 de las cuales también se les aplicó el test q-PCR). Entre ellas, un 13,1% (n = 153) eran positivo. La sensibilidad, especificidad, los valores predictivos positivos y negativos del HCII CT fueron 72,3% (IC95%: 65,4-78,6), 91,3% (IC95%: 84,1-95,9), 93,8% (IC95%: 88,5-97,1), y 64,4% (IC95%: 56,0-72,1), respectivamente. La toma de muestras resultó incómoda en un 19,7% de las mujeres. Entre los profesionales de la salud (n = 52), las barreras principales informadas incluyeron casos positivos que no volvieron a recoger los resultados (56,4%), reticencia a realizarse el examen sin cita previa (45,1%), e incremento en su carga laboral (38,8%). El HCII CT-ID identificó un alto porcentaje de casos de* C. trachomatis *entre mujeres jóvenes en Manaus. No obstante, su moderada sensibilidad limita su uso como una herramienta idónea en los centros de atención primaria en Manaus. El examen fue bien aceptado, pese a que identificamos obstáculos, especialmente entre los profesionales de salud, lo que supone un desafío para la detección de la enfermedad que requiere esfuerzos para su tratamiento.*

Chlamydia trachomatis; *Tamizaje Masivo; Salud de la Mujer*

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APÊNDICE F - PRODUÇÃO CIENTÍFICA EM OUTROS ESTUDOS NO PERÍODO DO DOUTORADO (ARTIGO 2)

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MICROBIOLOGY SOCIETY

Development, validation and testing costs of an in-house real-time PCR assay for the detection of *Chlamydia trachomatis*

Camila Gurgel dos Santos,[1,2,*] Meritxell Sabidó,[3,4,5] André Luiz Leturiondo,[2] Cynthia de Oliveira Ferreira,[2] Thielle Pereira da Cruz[2] and Adele Schwartz Benzaken[2,3,6]

Abstract

Purpose: To improve the screening of *Chlamydia trachomatis (C. trachomatis)* in Brazil, an accurate and affordable method is needed. The objective of this study was to develop and assess the performance and costs of a new in-house real-time PCR (qPCR) assay for the diagnosis of *C. trachomatis* infection. **Methodology:** Asymptomatic women aged 14–25 years who attended primary health services in Manaus, Brazil, were screened for *C. trachomatis* using the Digene Hybrid Capture II CT-ID (HCII CT-ID) DNA test. A subset of cervical specimens were tested using an in-house qPCR and a commercial qPCR, *Artus C. trachomatis* Plus RG PCR 96 CE (Artus qPCR) kit, as a reference test. A primer/probe based on the sequence of cryptic plasmid (CP) was designed. An economic evaluation was conducted from the provider's perspective. **Results:** The primers were considered specific for *C. trachomatis* because they did not amplify any product from non-sexually transmitted bacterial species tested. Overall, 292 specimens were tested by both the commercial kit (Artus qPCR) and the in-house qPCR. Of those, one resulted in no amplification and was excluded from the analysis. The sensitivity, specificity, and positive and negative predictive values of the in-house qPCR were 99.5 % [95 % confidence interval (CI): 97.1–100], 95.1 % (95 % CI: 89–98.4), 97.4 % (95 % CI: 94–99.1) and 99.0 % (95 % CI: 94.5–100), respectively. The cost per case of *C. trachomatis* was £0.44 ($0.55) for HCII CT-ID, £1.16 ($1.45) for Artus qPCR and £1.06 ($1.33) for in-house qPCR. **Conclusion:** We have standardized an in-house qPCR to detect cervical *C. trachomatis* targeting CP. The in-house qPCR showed excellent accuracy and was more affordable than the commercial qPCR kit.

INTRODUCTION

Chlamydia trachomatis is the most commonly diagnosed curable sexually transmitted infection worldwide [1, 2]. In Brazil, it is estimated that 9.4 % of women in the general population [3] and 9.8 % of parturient women under 25 years of age [4] are infected by *C. trachomatis*. Genital *C. trachomatis* infection remains unnoticed in 50–88 % of women [5]. Given the frequent asymptomatic nature of the infection, and the importance of early treatment to reduce transmission and prevent *C. trachomatis*-related morbidity, many developed countries are offering opportunistic screening to all sexually active people under the age of 25 years [1, 6, 7]. Screening has been shown to be effective for the identification of asymptomatic infected women, although there is some controversy about its impact to reduce the incidence of pelvic inflammatory disease [8]. Importantly, although the cost-effectiveness of screening is largely determined by the rates of complications prevented, studies indicate that shares in total savings and in quality-adjusted life-years (QALY) gains due to prevented cases of tubal infertility are 6–24 and 33 %, respectively [9].

In Brazil, the Digene Hybrid Capture II CT-ID (HCII CT-ID) DNA test (Qiagen) for identifying *C. trachomatis* is the only molecular test approved in the public health system for *C. trachomatis* screening. This nucleic acid hybridization assay is no longer recommended by the USA Centers for Disease Control and Prevention for routine use based on performance [10]. This test has shown sensitivity ranging from 93.8–97.7 % and specificity ranging from 95.9–100.0 % compared to that of culture [11–14]. However, up to 27.7 % of negative results generated by this test were reported to be false among asymptomatic young women in Manaus when compared with a commercial real-time PCR (qPCR) kit [15].

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Author affiliations: [1]Programa de Pós-graduação em Medicina Tropical, Universidade do Estado do Amazonas, Manaus, Amazonas, Brazil; [2]Fundação Alfredo da Matta, Manaus, Amazonas, Brazil; [3]Fundação de Medicina Tropical Doutor Heitor Vieira Dourado (FMT-HVD), Manaus, Amazonas, Brazil; [4]TransLab, Departament of Medical Sciences, Universitat de Girona, Catalunya, Spain; [5]CIBER de Epidemiología y Salud Pública, CIBERESP, Spain; [6]Departamento de IST, Aids e Hepatites Virais, Secretaria de Vigilância em Saúde, Ministério da Saúde, Brasília, Brazil.
***Correspondence:** Camila Gurgel dos Santos, milagurg@hotmail.com
Keywords: *Chlamydia trachomatis*; real-time polymerase chain reaction; validation studies; cost; Amazonas.
Abbreviations: Artus qPCR, *Artus C. trachomatis* Plus RG qPCR 96CE; CI, confidence interval; CP, cryptic plasmid; FUAM, Fundação Alfredo da Matta; HCII CT-ID, Digene Hybrid Capture II CT-ID; qPCR, real-time PCR.

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qPCR has been developed, and offers both high sensitivity and specificity when compared to traditional culture-based, commercial and in-house amplification methods [16, 17]. However, qPCR assays present disadvantages such as being relatively time consuming, having a high cost, and requiring specialized equipment and trained laboratory staff [18]. Therefore, a reliable and affordable new diagnostic method would be extremely valuable for clinicians and for the public health system. Importantly, the development of new in-house qPCR approaches that allow the minimization of the need for reagents, time and costs can help the scaling up of national *C. trachomatis* screening programmes in resource-constrained countries. Several studies have employed a composite reference standard, which combines the result of a more sensitive test with culture as a standard test in order to define a better standard against which a new test can be compared [19]. Culture of *Chlamydia* is not available routinely in diagnostic laboratories in Brazil and its use carries a high labour cost. Therefore, the *Artus C. trachomatis* Plus RG qPCR 96 CE (Artus qPCR) kit, which has shown high sensitivity and specificity [20], was used alone as a standard for evaluating the accuracy of the new in-house qPCR assay The objective of this study was to describe the development, and assess the performance and costs, of a new in-house qPCR assay for the diagnosis of genital chlamydial infection.

METHODS

Study population

The specimens were collected from 1187 women presenting at primary care services in Manaus for cervical cancer screening, between October 2012 and December 2013. We included asymptomatic women aged 14 to 25 years who agreed to sign the consent form. We excluded pregnant women and those who had used antibiotics during the previous 15 days.

For *C. trachomatis* screening, a single cervical specimen per participant was collected using the Digene cervical sampler brush (Qiagen) and was placed into a tube containing 1.0 ml Digene sample transport medium. Samples were stored at room temperature until the end of the week, when they were transferred to the Fundação Alfredo da Matta (FUAM) laboratory and stored at −20 °C until processed.

Specimens were tested using the HCII CT-ID according to the manufacturer's instructions [11]. Of the total 1187 women screened, 1169 participants had a HCII CT-ID test result (10 were not tested and 8 were excluded because they had an indeterminate test result). Among those, 153 tested positive for *C. trachomatis* [15].

Validation study

A subset of stored cervical specimens was used to evaluate the performance of the in-house qPCR assay against the Artus qPCR kit (Qiagen) according to the assay package instructions. A laboratory technician, blind to the HCII CT-ID results, tested specimens that were positive by HCII CT-ID and a randomly selected equal number of samples that tested negative. A total of 292 specimens were tested, given that 7 out of 153 HCII CT-ID specimens with a positive result could not be identified.

Artus qPCR

A 300 µl aliquot was transferred from each selected specimen to a 1.5 ml tube before the HCII CT-ID test was performed. DNA was extracted using a QIAamp DNA mini kit (Qiagen) and the Artus qPCR performed on the Rotor-Gene 3000 instrument (Corbett Research). The interpretation of all Artus qPCR results followed the algorithms in the manufacturer's package insert [21]. The only sample identifiers were specimen numbers.

In-house qPCR primer and probe design

Based on gene alignment of *omp1* (all genotypes) and cryptic plasmid (CP) conserved sequences available in the GenBank database (www.ncbi.nlm.nih.gov/GenBank/), specific primers and probes were designed using the Applications of Integrated DNA Technologies tool (IDT tools – www.idtdna.com/scitools/Applications/RealTimePCR/) (Table 1). OligoAnalyzer 3.1 (IDT tools – www.idtdna.com//calc/analyzer) was used to evaluate primer and probe parameters, including the melting temperature (T_m), difference in melting temperatures for primer pairs (ΔT_m), G+C content (G+C mol%), self-dimers, hairpins, hetero-dimer structures, repetitive sequence and Gibbs free energy (ΔG). Primers with acceptable parameters were selected and aligned with the sequences from the National Center for Biotechnology Information database using the Basic Local Alignment Search Tool (BLAST) to test for possible nonspecific interactions. The primers and probes selected did not have nonspecific interactions with the microorganisms present in the vaginal cavity or human genes. The designed primer set was a combination of two primer pairs that amplify CP target regions of *C. trachomatis*. The same methods described were used to design the primers and probe for the *omp1* gene. The names and sequences of all primers are shown in Table 2.

In-house qPCR assay

The in-house qPCR reaction was performed in a 10 µl volume, using 200 µM each primer and probe (Invitrogen), 1×

Table 1. Species identification and GenBank identification numbers for the strains used for primer and probe design for the CP target in this study

Strain	Genotype	GenBank ID*
L2b/CS784/08	L2b	NZ_CP009926.1
1a/CS190/96	1a	NZ_CP010572.1
Sweden2	E	FM865439.1
Sweden3	E	FM865440.1
Sweden4	F	FM865441.1
weden5	F	FM865442.1

*Sequence submission ID.

Table 2. In-house qPCR primer and probe sequences for the CP region

Primer/probe	Sequence
Forward CP	5′-CTAGGCGTTTGTACTCCGTG-3′
Probe CP	5′-FAM-TTGCAGCTTGTAGTCCTGCTTGAGA-3′
Reverse CP	5′-TGTCTTCGTAACTCGCTCC-3′

FAM, 6-Carboxyfluorescein.

TaqMan Universal PCR Master Mix NO UNG (Applied Biosystems) and 1 µl purified DNA [the same samples that were tested with Artus qPCR, which were extracted using a QIAamp DNA mini kit (Qiagen)]. The cycling conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and at 60 °C . This reaction was performed with the StepOnePlus real-time PCR system (Applied Biosystems). This reaction was used with the CP primer set and the *omp1* target.

All samples that showed no amplification for *C. trachomatis* were tested for the human β-actin constitutive gene to verify that DNA extraction had been performed satisfactorily and the presence of viable DNA. The reaction for β-actin was performed in a 5 µl volume, using 1× TaqMan Universal PCR Master Mix NO UNG (Applied Biosystems), 300 µM each primer (Invitrogen), 100 µM probe (Invitrogen) and 1 µl DNA. The cycling conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. This reaction was performed with the StepOnePlus real-time PCR system (Applied Biosystems). This reaction was also optimized in order to reduce an excessive waste of reagents, since the normal reaction was performed in 25 µl final volume, but was standardized for this project in a 5 µl volume. Both reaction volumes resulted in the same results. Specificity of the in-house qPCR was tested using DNA extracted from *Candida* sp. and *Neisseria gonorrhoeae*, as well as human DNA. All reactions were tested in triplicate, alongside positive and negative controls.

Economic analysis

We compared the costs of screening and managing *C. trachomatis* using the different tests. Incremental recurrent costs only were estimated, assuming all other costs would be equal. Costs of infrastructure, training and supervision were not included. Direct costs were labour, diagnostic supplies and drugs, which were estimated using the ingredients approach in which the total quantities of goods and services used were estimated and multiplied by their respective unit prices [22]. Cost of labour was determined through an observational time allocation study and time units were multiplied by the relevant salary units of staff (laboratory technicians) performing various tasks. The costing exercise was conducted from the provider's perspective, considering FUAM as the sole provider. Financial and economic costs were the same because there were no donated goods or services. With regards to HCII CT-ID, costs of tests included the costs when procured by the public Unified Health System (SUS) in Brazil for the year 2015, and converted into US dollars ($) using the fixed exchange rate of 3.49 reais per $. All research-related costs were excluded. Costs of diagnostic inputs, as well as formulas for economic evaluation can be found in Table 3. To calculate the cost per person screened, the retesting cost of indeterminate results was considered zero because in standard care such women are assumed to be positive and therefore treated. Total testing costs were divided by the number of people tested to obtain the cost per case identified by test. Cost values in the text are shown in pounds Sterling (£), using the exchange rate $1=£0.8.

Statistical analysis

Data were analysed using STATA/SE version 11.2 (Stata). The performance characteristics of the in-house qPCR test compared to that of Artus qPCR (sensitivity, specificity, and positive and negative predictive values) were calculated by standard methods, and are presented with the 95% confidence intervals (CI). To calculate the sample size of the validation study, we assumed a sensitivity of the in-house qPCR to be equal to that of the Artus qPCR (98 %) and a precision of 10 %. The number of infections needed to test the accuracy of the in-house qPCR was eight. Given that the prevalence of *C. trachomatis* in young women is approximately 9.6 %, the number of specimens to be tested was 83. However, it was further increased to obtain more precise estimates.

Ethical approval

The study protocol was approved by the Research Ethics Committee of FUAM (approval number 028/2011) and by the Ethics Committee of the Health Secretariat in Manaus. All participants signed a written consent form that was

Table 3. Economic evaluation of *C. trachomatis* screening testing using the HCII CT-ID assay, the Artus qPCR assay or the in-house qPCR in an asymptomatic young (14–25 years) female population in Manaus, Brazil

	HCII CT-ID	Artus qPCR	In-house qPCR
Test cost (*A*)	$8.98/£7.18	$17.15/£13.72	$5.11/£4.09
Testing time cost (*B*)	$94.32/£75.46	$254.66/£203.73	$245.23/£196.18
Total test and testing cost) (*C*)	$103.30/£82.64	$271.81/£217.45	$250.34/£200.27
Cost per person screened (*D*)	$0.36/£0.29	$0.93/£0.74	$0.86/£0.69
Cost per case identified by test	$0.55/£0.44	$1.45/£1.16	$1.33/£1.06

Test cost (*A*) = unit price of purchasing tests. Testing time cost (*B*) = (no. time units)×(cost per time unit) (time unit: monthly lab staff salary, expressed hourly, divided by 60 min). Testing cost (*C*) = unit price of purchasing test (*A*) + testing time cost (*B*). Cost per person screened (*D*) = testing cost of entire population + retesting cost of intermediate = *C**(*N* + *n*-retest)/*N*. Cost per case identified by test = *D* divided by number of tests positive.

approved by the Ethics Committee. Testing with qPCR was approved by the Research Ethics Committee of FUAM (approval number 742.410).

RESULTS

Primers and probes

Primers and probes for the CP and *ompl* targets were designed. The primers were tested at different concentrations (0.2, 0.5 and 0.9 μM) to identify the best candidate with the same probe concentration (0.2 μM). The best primer concentration was 0.2 μM, which was tested with CP and *ompl* gene primers and probes in separate reactions. Amplifications were only obtained with the CP primers and probe. Because *ompl* is a very polymorphic gene, we selected its most conserved part in order to detect all different *C. trachomatis* genotypes and used degenerate primers. With these *ompl* primers we achieved amplification in half of the specimens only, probably because of the presence of mutation(s) or a variable area in the remaining ones or, alternatively, a reaction inhibitor present in the in-house qPCR mix. Our reaction results are only using the CP target. The primers and probe designed for the CP target showed specificity, because no cross-reaction was observed for other sexually transmitted species tested or with the human DNA. We were able to standardize this reaction in 10 and 5 μl volumes for the CP and β-actin primer sets, respectively.

Performance of the in-house qPCR

Overall, 292 specimens were tested by Artus qPCR. Of those, one resulted in no amplification and was excluded from the evaluation of the performance analysis. The specimens were tested by the in-house qPCR. When compared to the commercial test, the sensitivity of the in-house qPCR was 99.5% (95% CI: 97.1–100) (187/188), the specificity was 95.1% (95% CI: 89–98.4) (98/103), the positive predictive value was 97.4% (95% CI: 94–99.1) (187/192) and the negative predictive value was 99.0% (95% CI: 94.5–100) (98/99). The in-house qPCR test detected 99.5% (187/188) of *C. trachomatis* cases with a positive Artus qPCR result. The in-house qPCR retrieved one out of 103 false-negative results and five out of 103 (4.9%) false-positive results (98/103).

Costs of *C. trachomatis* screening methods

Table 3 shows the costs of *C. trachomatis* detection using the three available screening tests. The cost per case of *C. trachomatis* was £0.44 ($0.55) for the HCII CT-ID, £1.16 ($1.45) for the Artus qPCR and £1.06 ($1.33) for the in-house qPCR.

DISCUSSION

Our evaluation of the in-house qPCR for *C. trachomatis* screening using cervical samples showed an excellent sensitivity and specificity compared to the commercial Artus qPCR kit in young women from the general population in Manaus. These results are consistent with previous studies in which in-house qPCR showed higher sensitivity and specificity versus Roche Cobas Amplicor [17, 23, 24]. Interestingly, the in-house qPCR identified almost all *C. trachomatis* cases among asymptomatic women, missing only one positive sample. A possible explanation for this false-negative result was a very small quantity of *C. trachomatis* DNA in that sample and/or some degree of degradation of the DNA during storage and freezing. In our study, 4.9% of the in-house qPCR samples had a false-positive result. This finding was lower than that reported in previous studies, in which between 5.3 and 8% of the in-house qPCR positive results were reported as false [23, 24]. The accuracy of a positive result is essential in order to avoid psychosexual issues, overtreatment and unnecessary partner notification. The HCII CT-ID described here showed a sensitivity of 72.3% and specificity of 91.3% compared to that of Artus qPCR [15].

Testing by qPCR allows simultaneous achievement of amplification, specific hybridization and detection. Specific and sensitive gene quantification occurs with a minimal contamination risk. A drawback of the study was that we were not able to standardize a reaction for the *ompl* gene and we could amplify half of the samples that were tested. This target was used by Jalal and colleagues [25], resulting in 96% of positive samples detected. However, six samples that were positive did not amplify *ompl*. They justified the absence of a positive result because the *ompl* gene needs at least there copies to be amplified *C. trachomatis* DNA. On the contrary, when CP is targeted, only one copy is needed to achieve amplification, which could reduce the sensitivity of the test. In our in-house qPCR, only the *C. trachomatis*-specific CP target was amplified in the reaction. However, all *C. trachomatis* strains are known to harbour a species-specific CP, and their DNA sequences are highly conserved, even between different strains [26]. By using a species-specific *C. trachomatis* sequencing primer and validating it, we could confirm the molecular diagnosis of *C. trachomatis* in the endocervical samples that showed a positive result in the in-house qPCR.

C. trachomatis screening is not routinely available in the primary health care system in Brazil, although the prevalence of *C. trachomatis* in the general population is high and screening has been claimed to be crucial for decreasing the burden of the infection. One of the main reasons is budget constraints. With a calculated cost of £1.38 ($1.72), the in-house qPCR test may represent a more affordable solution for *C. trachomatis* infection detection in Brazil than a commercial qPCR. Compared with the HCII CT-ID DNA test, it showed a better performance and cost effectiveness. In addition, one distinct advantage of the in-house qPCR versus the commercial qPCR from the laboratory technician perspective is a much simpler preparation step before amplification, as well as a shorter time needed thereafter. This difference becomes important for laboratories that need to process a large number of samples.

This study had a few limitations. The detection of *C. trachomatis* by use of the HCII CT-ID DNA test is dependent on the number of organisms present in the specimen and may be affected by patient factors, such as the presence of symptoms [27]. Although the qPCR assay is highly sensitive and specific [28], it can be affected by contamination or inhibitors [29]. Both Artus qPCR and the in-house qPCR were performed on the same source of stored DNA. The justification was to save costs and labour incurred to collect samples. As all samples were coded before the HCII CT-ID DNA test, the use of data and samples collected for a previous study was justified.

In conclusion, we have developed an in-house qPCR to detect cervical *C. trachomatis* targeting CP. The in-house qPCR showed excellent accuracy the commercial qPCR. An important additional advantage of the in-house qPCR method was its lower cost. Findings from this study can help to revise the national recommendation of using the HCII CT-ID test, which is the only test available to detect *C. trachomatis* in the Brazilian public health system. The in-house qPCR should be considered as a good candidate for a preferred diagnostic method for screening programmes in Brazil towards reducing the burden of *C. trachomatis* infection and its secondary transmission.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. **European Centre for Disease Prevention and Control.** *Chlamydia Control in Europe: Literature Review*. Stockholm: European Centre for Disease Prevention and Control; 2014.
2. **World Health Organization.** Global Prevalence and Incidence of Selected Curable Sexually Transmitted Infections. Geneva: World Health Organization; 2001. www.who.int/hiv/pub/sti/who_hiv_aids_2001.02.pdf.
3. **Jalil EM, Pinto VM, Benzaken AS, Ribeiro D, Oliveira EC *et al.*** [Prevalence of *Chlamydia* and *Neisseria gonorrhoeae* infections in pregnant women in six Brazilian cities]. *Rev Bras Ginecol Obstet* 2008;30:614–619 [in Portuguese].
4. **Pinto RM, Wall M, Yu G, Penido C, Schmidt C.** Primary care and public health services integration in Brazil's unified health system. *Am J Public Health* 2012;102:e69–e76.
5. **Molano M, Meijer CJ, Weiderpass E, Arslan A, Posso H *et al.*** The natural course of *Chlamydia trachomatis* infection in asymptomatic Colombian women: a 5-year follow-up study. *J Infect Dis* 2005; 191:907–916.
6. **Public Health Agency of Canada.** Management and treatment of specific infections (section 5). *Canadian Guidelines on Sexually Transmitted Infections*. Ottawa and Winnipeg: Public Health Agency of Canada; 2006. www.phac-aspc.gc.ca/std-mts/sti-its/.
7. **Centers for Disease Control and Prevention.** *Chlamydia – CDC Fact Sheet*. Atlanta, GA: Centers for Disease Control and Prevention; 2016. https://www.cdc.gov/std/chlamydia/stdfact-chlamydia.htm.
8. **Gottlieb SL, Berman SM, Low N.** Screening and treatment to prevent sequelae in women with *Chlamydia trachomatis* genital infection: how much do we know? *J Infect Dis* 2010;201:156–167.
9. **Land JA, van Bergen JE, Morré SA, Postma MJ.** Epidemiology of *Chlamydia trachomatis* infection in women and the cost-effectiveness of screening. *Hum Reprod Update* 2010;16:189–204.
10. **Centers for Disease Control and Prevention.** Recommendations for the laboratory-based detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* – 2014. *MMWR Recomm Rep* 2014;63:1–19.
11. **Girdner JL, Cullen AP, Salama TG, He L, Lorincz A *et al.*** Evaluation of the Digene Hybrid Capture II CT-ID test for detection of *Chlamydia trachomatis* in endocervical specimens. *J Clin Microbiol* 1999;37:1579–1581.
12. **Schachter J, Hook EW, Mccormack WM, Quinn TC, Chernesky M *et al.*** Ability of the Digene Hybrid Capture II test to identify *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in cervical specimens. *J Clin Microbiol* 1999;37:3668–3671.
13. **van der Pol B, Williams JA, Smith NJ, Batteiger BE, Cullen AP *et al.*** Evaluation of the Digene Hybrid Capture II assay with the rapid capture system for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. *J Clin Microbiol* 2002;40:3558–3564.
14. **Darwin LH, Cullen AP, Arthur PM, Long CD, Smith KR *et al.*** Comparison of Digene Hybrid Capture 2 and conventional culture for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in cervical specimens. *J Clin Microbiol* 2002;40:641–644.
15. **Neves D, Sabidó M, Bötto-Menezes C, Benzaken NS, Jardim L *et al.*** Evaluation of screening for *Chlamydia trachomatis* among young women in primary health care services in Manaus, Amazonas State, Brazil. *Cad Saude Publica* 2016;32:e00101015.
16. **Jespersen DJ, Flatten KS, Jones MF, Smith TF.** Prospective comparison of cell cultures and nucleic acid amplification tests for laboratory diagnosis of *Chlamydia trachomatis* infections. *J Clin Microbiol* 2005;43:5324–5326.
17. **Shattock RM, Patrizio C, Simmonds P, Sutherland S.** Detection of *Chlamydia trachomatis* in genital swabs: comparison of commercial and in house amplification methods with culture. *Sex Transm Infect* 1998;74:289–293.
18. **Bébéar C, de Barbeyrac B.** Genital *Chlamydia trachomatis* infections. *Clin Microbiol Infect* 2009;15:4–10.
19. **Alonzo TA, Pepe MS.** Using a combination of reference tests to assess the accuracy of a new diagnostic test. *Stat Med* 1999;18: 2987–3003.
20. **Eickhoff M, Cramer S, Grewing T.** Advances in diagnostic of *Chlamydia trachomatis*. *Clin Microbiol Infec* 2006;12:5.
21. **Qiagen.** artus® C. trachomatis Plus RG PCR Kit Handbook, version 1. Hilden, Germany: Qiagen; 2013. https://www.qiagen.com/fr/resources/resourcedetail?id=73116c26-8d9e-4120-9164-212c4f9e7cb9&lang=en&autoSuggest=true.
22. **Kumaranayake L, Watts C.** Economic costs of HIV/AIDS prevention activities in sub-Saharan Africa. *AIDS* 2000;14:S239–S252.
23. **Jalal H, Al-Suwaine A, Stephen H, Carne C, Sonnex C.** Comparative performance of the Roche COBAS Amplicor assay and an in-house real-time PCR assay for diagnosis of *Chlamydia trachomatis* infection. *J Med Microbiol* 2007;56:320–322.
24. **Sachdeva P, Patel AL, Sachdev D, Ali M, Mittal A *et al.*** Comparison of an in-house PCR assay, direct fluorescence assay and the Roche AMPLICOR *Chlamydia trachomatis* kit for detection of *C. trachomatis*. *J Med Microbiol* 2009;58:867–873.
25. **Jalal H, Stephen H, Curran MD, Burton J, Bradley M *et al.*** Development and validation of a rotor-gene real-time PCR assay for

detection, identification, and quantification of *Chlamydia trachomatis* in a single reaction. *J Clin Microbiol* 2006;44:206–213.

26. **Hatt C, Ward ME, Clarke IN.** Analysis of the entire nucleotide sequence of the cryptic plasmid of *Chlamydia trachomatis* serovar L1. Evidence for involvement in DNA replication. *Nucleic Acids Res* 1988;16:4053–4067.
27. **Michel CE, Sonnex C, Carne CA, White JA, Magbanua JP *et al.*** *Chlamydia trachomatis* load at matched anatomic sites: implications for screening strategies. *J Clin Microbiol* 2007;45:1395–1402.
28. **Bonin S,, Eickhoff MAdvances** in diagnostic testing of *Chlamydia trachomatis*. *Fourteenth European Congress of Clinical Microbiology and Infectious Diseases Prague, Czech Republic, May 1–4, 2004.* Poster number 903_r2134 http://www.blackwellpublishing.com/eccmid14/.
29. **Wilson IG.** Inhibition and facilitation of nucleic acid amplification. *Appl Environ Microbiol* 1997;63:3741–3751.

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APÊNDICE G - PRODUÇÃO CIENTÍFICA EM OUTROS ESTUDOS NO PERÍODO DO DOUTORADO (ARTIGO 3)

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Leprosy among schoolchildren in the Amazon region: A cross-sectional study of active search and possible source of infection by contact tracing

Valderiza Lourenço Pedrosa[1]*, Luiz Claudio Dias[1], Enrique Galban[2], André Leturiondo[1], Jamile Palheta, Jr[1], Monica Santos[1,3], Milton Ozório Moraes[4], Carolina Talhari[1,3]

1 Departamento de Ensino e Pesquisa, Fundação Alfredo da Matta, Manaus, Brazil, 2 Departamento de Epidemiologia, Facultad de Medicina Calixto Garcia, La Habana, Cuba, 3 Escola de Ciências da Saúde, Universidade do Estado do Amazonas, Manaus, Brazil, 4 Laboratório de Hanseníase, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil

* valpedrosa@fuam.am.gov.br

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Abstract

Background

The high rate of leprosy cases among children under 15 years of age in Brazil indicates ongoing transmission within the community. The identification of the new leprosy cases among contacts can help identify the source of infection and interrupt the transmission chain. This study aims to determine the detection rate of previously undiagnosed cases of leprosy among schoolchildren who are under 15 years of age living in Manaus, Amazonas, Brazil, and their possible source of infection by contact tracing.

Methodology/Principal findings

This was a school-based, cross-sectional study in which the identification of active leprosy cases was conducted in 277 out of 622 randomly selected public schools in Manaus, Amazonas, Brazil. Suspected cases of leprosy were referred to the Alfredo da Matta Foundation, a reference center for leprosy in Manaus. A total of 34,547 schoolchildren were examined, and 40 new leprosy cases were diagnosed. Among new cases, 57.5% were males, and 80.0% demonstrated paucibacillary leprosy. A total of 196 of 206 registered contacts were screened, and 52.5% of the newly diagnosed children's cases had at least one positive household contact. In these contacts, grandparents (52.4%) were the most common co-prevalent cases, while 14.3% were uncles, 9.5% were parents and 9.5% were granduncles. Seven contacts (5.0%), including four siblings of child patients were newly diagnosed. Our data indicate that the prevalence is 11.58 per 10,000, which is 17 times higher than the registered rate.

Conclusions/Significance

This study suggests that the detection rate of leprosy among schoolchildren may have remained unchanged over the past thirty years. It also indicates that that active case finding

Competing interests: The authors have declared that no competing interests exist.

is necessary for reaching the World Health Organization's goals of zero detection among children, especially in endemic areas where the prevalence of leprosy is obscure. Moreover, we assert that all children must have their household contacts examined in order to identify the possible source of infection and interrupt the disease's transmission. Novel strategies to reinforce contact tracing associated with large-scale strategies of chemo- and immune-prophylaxis should be expanded to prevent the perpetuation of the disease cycle.

Author summary

Leprosy is a disease that has long since been eradicated in the developed world, but it still affects poor people in developing countries, such as India, Brazil, and Indonesia. Because the causative agent of the disease may involve the skin and peripheral nerves, the disease can cause physical disabilities and deformities. Although leprosy affects all ages, children under 15 years of age are an important epidemiological marker because infection in that age group indicates active transmission within the community. In our work, we examined 34,547 children from public schools in Manaus, a city in the north of Brazil. In this population, we found 40 new cases of leprosy that were further confirmed by clinical and laboratorial tests. We also examined 196 people who had familiar or close non-familiar contact with the affected children. Among them, we identified the possible source of infection of 21 affected children and found seven new cases of leprosy. Overall, our findings revealed a detection rate of leprosy cases that was 17 times higher than the registered number. This indicates the necessity of identifying active cases of leprosy in order to improve case detection and effectively control the disease.

Introduction

Mycobacterium leprae, the causative agent of leprosy, is primarily transmitted person-to-person and through the air. People living in leprosy-endemic regions are at greater risk of being exposed to the infection. The risk of developing the disease among paucibacillary (PB) contacts is 2–3 times higher than that of the general population, while the risk increases to 5–10 times among multibacillary (MB) contacts[1–3]. Therefore, contact tracing not only results in the detection of additional cases but further offers several indirect advantages such as early diagnosis and reduced risk of transmission [4].

Familial leprosy distribution indicates a relationship between the clinical forms of the disease and kinship degree. Consanguineous relatives belonging to families whose fathers or mothers had lepromatous leprosy showed a higher risk of developing the same type of disease. On the other hand, non-consanguineous relatives were at a higher risk of contracting other clinical forms of the disease [5]. A study conducted in the Philippines showed that the risk of developing lepromatous leprosy was three times higher when one of the parents presented with this clinical form of the disease [6]. In leprosy hyperendemic areas, the risk of developing the disease may be elevated not only for household contacts but also for the residents in neighboring homes [7, 8]. Recently, a survey conducted in a hyperendemic Brazilian region demonstrated no significant difference in detection rates between household contacts and neighbors [8].

According to the World Health Organization (WHO), Brazil accounts for more than 80% of leprosy cases diagnosed in the Americas[9]. In 2016, 25,218 new leprosy cases were

diagnosed in Brazil, and 1,696 (6.7%) of those individuals were children, which corresponds to a diagnosis rate of 3.63 per 10,000 people. In the same year, Amazonas State reported 443 new cases of leprosy; the diagnosis rate was 1.10/10,000 inhabitants, and this was considered highly endemic by the Brazilian Ministry of Health (BMH) [10]. Although the introduction of multidrug therapy (MDT) in the beginning of the 1980s drastically influenced the total number of cases, there has been stagnation and a slight decrease in incidence over the past 10 years. This data suggests that it is likely that MDT has little impact on incidence because transmission occurs prior to diagnosis. Thus, strategies to prevent leprosy transmission indicate that contact tracing and post-exposure prophylactic protocols using rifampicin and/or BCG[11–13] should be successful. In this regard, the diagnosis of leprosy cases among children under 15 years of age can help provide estimates of ongoing transmission [14, 15] and the presence of active disease foci in the community[16]. In the early 1980s, an active case finding in Manaus indicated a detection rate of 10.6 cases of leprosy per 10,000 children [17].

This study was carried out to identify previously undiagnosed cases of leprosy among schoolchildren and their possible source of infection by contact tracing. Patterns of family contact with leprosy are demonstrated through genograms.

Methods

Population and study design

This was a school-based, cross-sectional study. Active case finding of leprosy in children under 15 years of age was conducted from March 2014 to December 2016 in 277 of the 626 public schools in Manaus, Amazonas, Brazil. Manaus is one of the major cities in the north of Brazil and has approximately 2,800,000 inhabitants [18]. The metropolitan area of Manaus has 626 public schools that enroll approximately 250,000 children [19]. Target schools were randomly chosen through a lottery method using Open Source Epidemiologic Statistics for Public Health software to obtain the study population. The probabilistic sample of 30,352 students was calculated based on the target population of students; the sampling error was 0.03%, and the confidence interval was 95%.

Eligible participants and recruitment process

Children from randomly selected public schools in Manaus, Amazonas, were eligible to participate in the study. The recruitment process started with an open seminar on leprosy and the purpose of the study. After written informed consent was obtained from parents or legal guardians, children received an initial physical examination conducted by trained and experienced leprosy and skin nursing technicians. The initial physical examination took place at school. Suspected cases of leprosy and other skin diseases, along with their legal guardians, were referred to the Alfredo da Matta Foundation (AMF), a referral center for leprosy and other skin diseases in Manaus.

Three dermatologists and laboratory tests confirmed the diagnosis of leprosy, which was initially based on the presence of leprosy's cardinal signs, i.e., if the patient had one or more lesions with a definite loss of sensation and/or peripheral nerve thickening. If these signs were evident, diagnosis was confirmed by histopathological changes and analysis of bacillary loads in a slit skin smear test (SSS). Classification was performed according to Ridley and Jopling [20, 21]. In cases in which there was no confirmation through the previously mentioned routine tests, a polymerase chain reaction (PCR) was performed to detect *M. lepraе* DNA, as previously described [22]. This technique has been used in patients who have clinical signs of leprosy but no confirmation through routine tests and histopathology, in difficult-to-diagnose cases, and in early detection in household contacts [22].

For confirmed cases of leprosy, a standardized questionnaire was administered to gather past medical history and social and demographic information, such as BCG scar status, data on household and dwelling contacts, race—white, black, yellow, brown or indigenous -, etc.) was applied. For treatment purposes, leprosy cases were classified as PB or MB, as recommended by the Brazilian Ministry of Health (BMZ) [14] and the WHO [23].

Household contacts were defined as a group of people who lives or have lived with a leprosy patient within the past five years. Direct and next-door neighbors, when indicated by legal guardians, were also considered contacts. All contacts were initially examined by the nursing technicians for clinical evidence of leprosy; the diagnosis of leprosy was also confirmed by three dermatologists and the aforementioned laboratory tests.

Statistical analysis

Data were analyzed using Epi Info 7 software. Initial descriptive studies were performed through frequency tables, position measurements and variability. Pearson's chi-square test or Fisher's exact tests were used to analyze the categorical variables. The significance level was 0.05, and the confidence interval was 95%. The GenoPro version 3.0.0.7 software was used to create genograms in order to identify the probable source of infection of new leprosy cases.

Ethical statement

Ethical approval was granted by the AMF Research and Ethics Committee. Written informed consent was obtained from parents or guardians of children enrolled in the study. Parents or guardians disclosed the diagnosis of leprosy to the respective contacts.

Results

School epidemiological survey

This study was conducted in 277 randomly selected public schools located in various districts of Manaus. In total, 34,547 children under 15 years of age were enrolled in the study. Overall, 18,770 (54.3%) were females and 15,777 (45.7%) were males. The mean age was 9.6 years (standard deviation [SD] = 2.58). Regarding the distribution by self-reported ethnicity, the majority (90.0%) of schoolchildren examined were brown with similar results obtained for both sexes. According to the Brazilian Institute of Geography and Census, 69% of the Amazonas State's population is brown [19].The analysis for different proportions between sex and ethnicity did not show a statistical significance between browns and whites ($p = 0.16$).

Overall, 8.2% of the 34,547 schoolchildren examined had skin diseases. The most common skin disease was fungal ($n = 955$; 33.8%), followed by eczema/dermatitis ($n = 725$, 25.6%), viral diseases ($n = 153$, 5.4%), and leprosy ($n = 40$, 1.4%). A total of 40 leprosy cases were identified out of the total number of schoolchildren that were examined, resulting in a prevalence of 11.58 per 10,000 people. Among them, 23 (57.5%) patients were males, and 17 (42.5%) were females. Regarding leprosy classification, 32 (80%) and 8 (20%) patients had PB and MB forms of leprosy, respectively; among the PB patients, 24 (60%) presented with one lesion. The analysis for different proportions between sex and leprosy classification did not show a statistical significance between PB and MB leprosy ($p = 0.43$). The mean age was 10.6 years (range 4–13) (Table 1).

Among the total cases of leprosy, 23 (59.0%) were 11 to 14 years of age; a similar pattern was found in both genders. According to data from the Municipal and State Education Departments of the State of Amazonas, 334,228 (68%) of the students are within this age range [19]. The analysis of the distribution among these three age groups showed statistically significant

Table 1. Clinical and epidemiological aspects of the 40 newly diagnosed cases of leprosy among schoolchildren in Manaus, Amazonas, Brazil.

Characteristics	Paucibacillary		Multibacillary		Total		P
	n = 32	%	n = 8	%	n = 40	%	
Sex							
Male	17	53,1	6	75,0	23	57,5	
Female	15	46,9	2	25,0	17	42,5	
Age Range							
5–7	1	3,1	2	25,0	3	7,5	
8–10	9	28,1	5	62,5	14	35,0	
11–14	22	68,8	1	12,5	23	57,5	0,004
Race/Color							
Brown	28	87,5	8	100,0	36	90,0	
White	4	12,5	0	0,0	4	10,0	0,38
Number of Lesions							
1	24	75,0	0	0,0	24	60,0	
2–5	8	25,0	0	0,0	8	20,0	
> 5	0	0,0	8	100,0	8	20,0	
Disability grade							
Grade 0	32	100,0	6	75,0	38	95,0	
Grade I	0	0,0	1	12,5	2	5,0	
Grade II	0	0,0	1	12,5	0	0,0	

This table displays sex, age, race, number of lesions and disability grade of the 40 newly diagnosed cases of leprosy.

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differences ($p < 0.01$). Notably, the majority of MB leprosy cases ($n = 7$) were less than 11 years of age. There was a predominance of the brown race among the cases (90.0%), and a similar result was obtained for both genders.

Laboratory confirmation of clinically diagnosed cases

An overview of the tests performed to support diagnosis is presented in Fig 1. Of the 40 leprosy cases, 32 (80.0%) were PB, and 8 (20.0%) were MB. A SSS was performed in 37 (92.5%) of the leprosy cases, histopathological examination was performed in 34 (84.6%) cases, and PCR was performed in 26 (65.0%) cases. Six had a negative SSS test, but did not have either a skin biopsy nor PCR examination. Because these patients had skin lesions that fulfilled WHO clinical criteria for leprosy (four had up to five lesions and two had more than five lesions), they were diagnosed and treated for PB and MB leprosy, respectively.

Of the 37 patients who received a SSS, 33 (89.2%) were negative, and four (10.8%) were positive. Three patients did not undergo SSS, but these patients had a skin biopsy taken and presented a negative result for PCR. Histopathological features of leprosy were seen in two cases, and the result was inconclusive in one case. Because the latter patient had one lesion in which leprosy was clinically confirmed, he was given PB treatment.

Leprosy was confirmed by histopathological examination in 14 children from the 34 histopathological slides analyzed. The results were as follows: indeterminate (three cases), tuberculoid-tuberculoid (four cases), borderline-tuberculoid (three cases), borderline-lepromatous (two cases) and lepromatous-lepromatous (two cases). In 20 patients, the histopathological examination yielded results that were inconclusive but did not exclude the diagnosis of leprosy. In this group, nine patients had a positive PCR result, as they exhibited fewer than five lesions

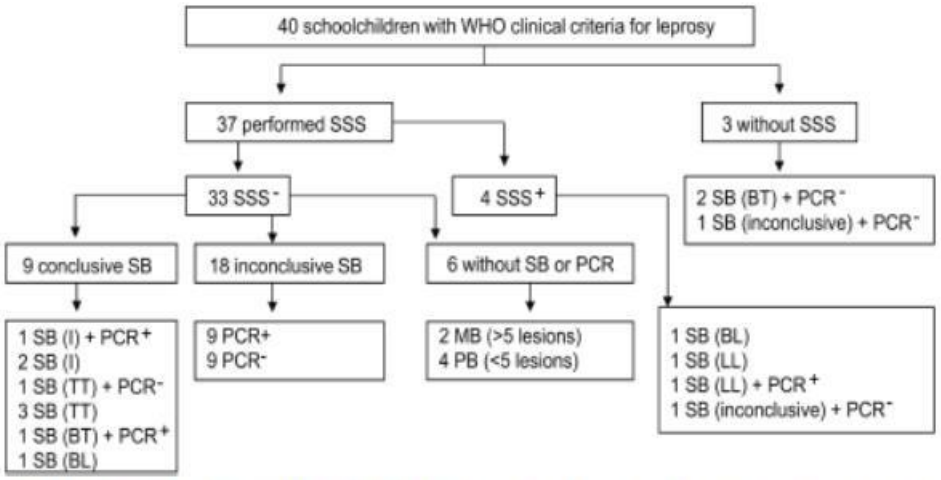

Fig 1. Scheme of laboratory tests performed to support clinical diagnosis. Of the 40 newly diagnosed cases of leprosy, 37 patients received a SSS test, 34 had histopathological examinations, and 26 had a PCR. SB: skin biopsy; SSS: slit skin smear; I: indeterminate; TT: tuberculoid-tuberculoid; BT: borderline-tuberculoid; BL: borderline-lepromatous; LL: lepromatous-lepromatous; MB: multibacillary; and PB: paucibacillary leprosy.

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that were being treated for PB leprosy, and 11 patients were negative for PCR examination. One out of the 11 had a positive skin smear, and 10 were clinically diagnosed as leprosy cases. The patient with the positive skin smear was treated for MB leprosy, and the other 10 who presented with fewer than three lesions, received PB treatment. In total, *M. leprae* DNA was detected in 12 (46.2%) out of 26 patients.

BCG scar status was recorded as positive in the majority ($n = 37$, 92.5%) of the patients. Thirty-eight (95.0%) children demonstrated a zero incapacity grade; one case of paresthesia (grade one disability) and one case of ulnar claw (grade two disability) were detected. Both patients had MB leprosy.

Contact tracing and diagnosis

A total of 206 people were registered as household contacts of the schoolchildren diagnosed with leprosy; only two direct neighbors, both with a past medical history of MB leprosy, were indicated as contacts by the legal guardians. Overall, 196 (95.1%) of the contacts were clinically examined. Among these contacts, we diagnosed seven new leprosy cases: five siblings, an uncle, and an aunt were detected as index case patients. Two of these contacts were also under 15 years of age. Three contacts had PB leprosy, and four, including the two children who were under 15 years of age, presented with MB leprosy.

Regarding the households, 21 (52.5%) patients lived with three to four people, whereas nine (22.5%) lived with more than seven people. More than 50% of the children lived with their families in households with up to four rooms, whereas 10 (25.0%) children lived in households with five or more rooms.

Among 40 schoolchildren diagnosed with leprosy, we were able to identify 21 (52.5%) who had or continued to have contact with patients within their household, familiar or not, who

had previously been treated for leprosy or were still under leprosy treatment. Of these, six (28.6%) children had contact with grandparents with a past medical history of leprosy. Three (14.3%) had contact with uncles; two (9.5%) had contact with parents; two (9.5%) had contact with their granduncles; one (4.8%) had contact with an aunt; one (4.8%) had contact with a great-grandfather; one (4.8%) had contact with a grandmother and two cousins; and one (4.8%) had close contact with a neighbor who was receiving leprosy treatment. Notably, the father of four sibling schoolchildren (19.0%) was receiving leprosy treatment, while a grandmother and a great-grandfather had already been treated for leprosy. All of them, including the recently diagnosed siblings, presented with MB leprosy. Nevertheless, we understand that we did not design the study to test the familial/genetic nature of the susceptibility. However, we were able to observe important clusters where the physical distance and familial distance were detected. This description reinforces the need for contact tracing to stop leprosy transmission. As for the other 19 schoolchildren diagnosed with leprosy during this survey, we were not able to identify the possible source of infection.

Fig 2 shows genograms of nine out of 40 schoolchildren with leprosy and their possible sources of infection. Children #1, #2, #3 and #4 belonged to the same family; during the investigation, it was found that the father, the maternal grandmother and the great-grandfather had been treated for leprosy, and the first two relatives were MB. Children #5, #6, #7, and #21 had a history of grandfathers treated for MB leprosy (Fig 2). The parents of children #8 and #20 were receiving treatment for MB and PB leprosy, respectively (Fig 2).

Discussion

This school-based, cross-sectional study found a higher leprosy prevalence among children than that registered in the official data. This result suggests that contact tracing is an important epidemiological tool in diagnosing new cases of the disease and possible sources of leprosy infection. In 2013, one year before we began enrolling children in our study, the prevalence of leprosy in this population in Manaus was 0.68 cases per 10,000 children (0.68/100,000 in Amazonas State and 0.50/100,000 in Brazil)[10,24]. Our study cannot estimate prevalence exactly. However, our data clearly indicate a hidden prevalence, since our data suggests that 11.58 per 10,000, which would be 17 times higher than that in the registered data. We detected 40 new cases of leprosy out of a total of 34,547 examined schoolchildren. New cases of leprosy diagnosed among screened contacts under 15 years of age were not included in the aforementioned prevalence. Overall, this data suggests the existence, in the city of Manaus, of a hidden prevalence of significant magnitude.

From 1979 to 1982, Talhari and co-authors performed an active case finding in Manaus and found a prevalence of 10.6 cases of leprosy per 10,000 children [17]. From 1991 to 2016, the birth rate decreased in Amazonas State, from 32.4 to 19.7 per 1,000, respectively [25, 26]. Accordingly, official data from BMH show that the leprosy prevalence among children has been declining for the past 25 years in Amazonas State and also in Brazil [10]. However, our data, if confirmed in a design to estimate the prevalence, it would likely to be even higher than that found over 30 years ago.

Recently, high rates of clinical[27] and subclinical leprosy have been reported in Brazil [28]. In both studies, the diagnosis of leprosy was based on clinical and serological results. In our study, the vast majority (85.0%) of new cases of leprosy were confirmed by at least one diagnostic method that combines classical and novel tools: SSS and/or skin biopsy and/or PCR test. Accurate diagnosis and careful description of previously undiagnosed leprosy cases are important to address the true prevalence of the disease in endemic countries.

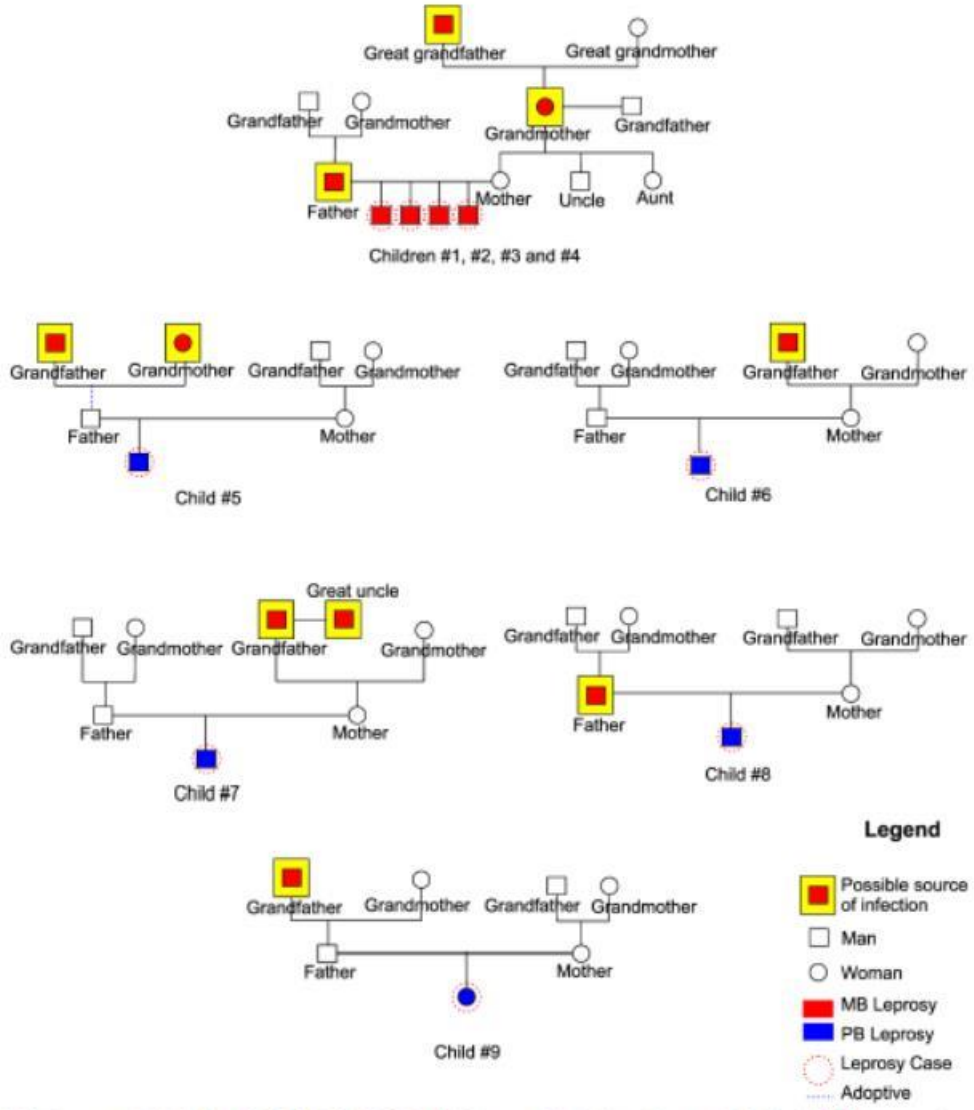

Fig 2. Genograms of schoolchildren #1, #2, #3, #4, #5, #6, #7, #8 and #9. Genograms showing the possible source of infection with *M. leprae* among nine newly diagnosed leprosy cases.

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In this study, 54.3% of screened schoolchildren were female, but the leprosy cases were male (57.5%); this data is in accordance to the official data from the BMZ [19, 24]. The majority of the leprosy cases were diagnosed in older children. This reinforces the need for active case finding and suggests that instituting an approach to contact tracing is probably a valuable policy.

The majority (80.0%) of the newly diagnosed cases of leprosy were PB with the presentation of a single lesion; this is similar to the rate found in other studies [27, 29, 30]. However, eight schoolchildren in addition to four contacts who were less than 15 years of age demonstrated MB leprosy. In endemic areas, the early exposure to *M. lepraе* and the presence of familial cases of the disease facilitate the greater frequency of contamination of children [17, 31–33].

Of the 21 schoolchildren with leprosy whose possible source of infection was identified among household contacts, 95.2% had contact with family members who previously had or were still receiving treatment for the disease. Contact with infected grandparents was found to be the most probable source of infection in our study. Notably, we found a cluster in which three generations had been diagnosed and treated for leprosy. High rates of consanguinity were found in other studies [30, 34], wherein parents and grandparents were the most likely source of infection [35–37]. Although household contact with an MB case is the strongest known determinant of leprosy risk, the vast majority of such contacts never manifest disease, which indicates the crucial role of genetic and/or environmental factors in the transmission of the *M. lepraе* infection and/or the pathogenesis of clinical leprosy [31].

It is worth highlighting that patients and families are frequently not aware of any contact they have had with the disease, and they are often unaware of leprosy patients in the family or in the nearby neighborhood. Patients with active disease and higher bacillary loads are considered the most important actors in transmitting and perpetuating the disease in a way that household contacts exhibit the highest risk of developing the disease. Therefore, screening family and non-family members in leprosy-affected households is mandatory. Also, chemo or immuno-prophylaxis has been shown to reduce the risk among the household contact population [11–13].

In addition to current leprosy cases in the family, housing in endemic areas, agglomerations of people living in a single household, family and social aggregation habits, household features, unfavorable conditions in the population and low educational level [35] are known risk factors for leprosy. In our study, conducted in an endemic area, more than 70% of the families lived in households with up to four rooms, and approximately 18 (45%) of the cases cohabited with more than five people.

Although controversial [13, 38], the administration of an additional dose of BCG to all healthy contacts is still recommended [11, 14]. Besides reducing clinical leprosy among vaccinees, mainly of the MB type, recent data suggested that BCG vaccination of household contacts of MB leprosy patients may induce activation of T cell clones that recognize *M. lepraе* specific antigens not shared with BCG [39]. The majority (92.5%) of schoolchildren diagnosed with leprosy in this study had one positive BCG scar and the PB form of the disease (80.0%). Perhaps, if these children had been examined and vaccinated when their relatives were diagnosed with leprosy, we would not have had them as patients. Furthermore, early diagnosis could have prevented the occurrence of disability as found in two of the newly diagnosed cases of leprosy in this study.

The frequency of leprosy occurrence in children is an important epidemiological indicator in determining the level of transmission of the disease. Recently, the WHO has published goals for the year 2020 suggesting that, among health control issues, leprosy should have zero cases in children and zero cases with incapacities [40]. Officially, a trend towards the decrease of leprosy among children under 15 years of age has been suggested. However, our

data indicates that the true prevalence of leprosy in this particular population may be slightly higher than that found over 30 years ago in the city of Manaus. To stop transmission, programs for the screening of household contacts should be improved and expanded, as screening has proven to be efficient for detecting early cases of leprosy [4]. These approaches associated with BCG vaccination and/or single dose rifampicin (SDR) reduce new cases in this household contact group [11, 41]. However, complementary approaches to improve surveillance and, thus, uncover hidden undiagnosed infectious cases that are actively transmitting leprosy are crucial to break the chain of transmission. Here, we provide evidence that screening of schoolchildren could be a valuable strategy to support leprosy control and achieve the goal of zero transmission.

This study shows that living with or in close proximity to leprosy patients and large family agglomerations in households with few rooms may be important risk factors for leprosy transmission among children. Moreover, this study highlights the value of contact screening of leprosy patients. There is a high level of family contact with leprosy in these cases, which gives support to the strategy of screening children in leprosy-affected households.

Supporting information

S1 Checklist. STROBE checklist.
(DOCX)

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Author Contributions

Conceptualization: Valderiza Lourenço Pedrosa, Milton Ozório Moraes, Carolina Talhari.

Data curation: Valderiza Lourenço Pedrosa, Jamile Palheta, Jr.

Formal analysis: Valderiza Lourenço Pedrosa, Enrique Galban.

Investigation: Valderiza Lourenço Pedrosa, Luiz Claudio Dias, André Leturiondo, Jamile Palheta, Jr, Monica Santos, Carolina Talhari.

Methodology: Valderiza Lourenço Pedrosa, Carolina Talhari.

Project administration: Valderiza Lourenço Pedrosa.

Resources: Valderiza Lourenço Pedrosa.

Software: Valderiza Lourenço Pedrosa, Enrique Galban, Jamile Palheta, Jr.

Supervision: Valderiza Lourenço Pedrosa, Enrique Galban.

Validation: Valderiza Lourenço Pedrosa, Luiz Claudio Dias, Carolina Talhari.

Visualization: Monica Santos.

Writing – original draft: Valderiza Lourenço Pedrosa, Monica Santos, Milton Ozório Moraes, Carolina Talhari.

Writing – review & editing: Valderiza Lourenço Pedrosa, Milton Ozório Moraes, Carolina Talhari.

References

1. Talhari S, Penna GO, Gonçalves HS, Oliveira MLW. Hanseníase. 5. ed. Manaus: Dilivros; 2015. pp. 1–6.
2. Sundar Rao PS, Jesudasan K, Mani K, Christian M. Impact of MDT on incidence rates of leprosy among household contacts. Part 1. Baseline data. Int J Lepr Other Mycobact Dis. 1989; 57: 647–51. PMID: 2778370
3. de Matos HJ, Duppre N, Alvim MFS, Vieira LMM, Sarno EN, Struchiner CJ. Epidemiologia da hanseníase em coorte de contatos intradomiciliares no Rio de Janeiro (1987–1991). Cad Saude Publica. Escola Nacional de Saúde Pública, Fundação Oswaldo Cruz; 1999; 15: 533–542. https://doi.org/10.1590/S0102-311X1999000300010
4. Hacker M de A, Duppre NC, Nery JAC, Sales AM, Sarno EN. Characteristics of leprosy diagnosed through the surveillance of contacts: a comparison with index cases in Rio de Janeiro, 1987–2010. Mem Inst Oswaldo Cruz. Fundação Oswaldo Cruz; 2012; 107: 49–54. https://doi.org/10.1590/S0074-02762012000900009
5. Beiguelman B. An Appraisal of Genetic Studies on Leprosy. Acta Genet Med Gemellol (Roma). Cambridge University Press; 1972; 21: 21–52. https://doi.org/10.1017/S1120962300011094
6. Smith DG. The genetic hypothesis for susceptibility to lepromatous leprosy. Hum Genet. 1979; 50: 163–77. Available: http://www.ncbi.nlm.nih.gov/pubmed/511131 PMID: 511131
7. Moet FJ, Pahan D, Schuring RP, Oskam L, Richardus JH. Physical Distance, Genetic Relationship, Age, and Leprosy Classification Are Independent Risk Factors for Leprosy in Contacts of Patients with Leprosy. J Infect Dis. 2006; 193: 346–353. https://doi.org/10.1086/499278 PMID: 16388481
8. Moura MLN, Dupnik KM, Sampaio GAA, Nóbrega PFC, Jeronimo AK, do Nascimento-Filho JM, et al. Active surveillance of Hansen's Disease (leprosy): importance for case finding among extra-domiciliary contacts. PLoS Negl Trop Dis. 2013; 7: e2093. https://doi.org/10.1371/journal.pntd.0002093 PMID: 23516645
9. World Health Organization. Global leprosy update, 2014: need for early case detection. Wkly Epidemiol Rec. 2015; No. 36, 2015, 90, 461–476.
10. Manaus, Fundação Alfredo da Matta. Boletim Epidemiologico, 2016 [Internet]. Manaus; 2016. http://www.fuam.am.gov.br/wp-content/uploads/2014/05/Boletim_2016.pdf
11. Richardus JH, Oskam L. Protecting people against leprosy: Chemoprophylaxis and immunoprophylaxis. Clin Dermatol. 2015; 33: 19–25. https://doi.org/10.1016/j.clindermatol.2014.07.009 PMID: 25432807
12. Sales AM, Ponce de Leon A, Düppre NC, Hacker MA, Nery JAC, Sarno EN, et al. Leprosy among patient contacts: A multilevel study of risk factors. PLoS Negl Trop Dis. 2011; 5.
13. Düppre NC, Camacho LAB, da Cunha SS, Struchiner CJ, Sales AM, Nery JAC, et al. Effectiveness of BCG vaccination among leprosy contacts: a cohort study. Trans R Soc Trop Med Hyg. 2008; 102: 631–638. https://doi.org/10.1016/j.trstmh.2008.04.015 PMID: 18514242
14. Brasil. Ministério da Saúde. Secretaria de Vigilância em Saúde. Diretrizes para vigilância, atenção e eliminacao da hanseníase como problema de saúde pública: manual técnico-operacional. Brasil, Saúde M da, Saúde S de V em, editors. Brasília: Editora MS; 2016.
15. Araújo MG, Lana FCF, Fonseca P de TS, Lanza FM. Leprosy incidence among children at Belo Horizonte city from 1992 to 1999: control implications. Rev méd Minas Gerais. 2016; 14: 78–83.
16. Ezenduka C, Post E, John S, Suraj A, Namadi A, Onwujekwe O. Cost-effectiveness analysis of three leprosy case detection methods in Northern Nigeria. PLoS Negl Trop Dis. 2012; 6: e1818. https://doi.org/10.1371/journal.pntd.0001818 PMID: 23029580
17. Talhari S, Torrecila MA, Talhari AC. A study of leprosy and other skin diseases in school children in the state of Amazonas, Brazil. Lepr Rev. 1987; 58: 233–7. PMID: 3669861
18. Brasil, Instituto Brasileiro de Geografia e Estatística. Estimativas da população residente no Brasil e Unidades da Federação com data de referencia em 1° de julho de 2017 [Internet]. 2017 [cited 2 Nov 2017]. ftp://ftp.ibge.gov.br/Estimativas_de_Populacao/Estimativas_2017/estimativa_tcu_2017.pdf
19. Instituto Brasileiro de Geografia e Estatística. In: Cidades [Internet]. 2017 [cited 4 May 2017]. http://www.cidades.ibge.gov.br/v4/brasil/am/manaus/panorama
20. Ridley DS, Jopling WH. A classification of leprosy for research purposes. Lepr Rev. 1962; 33: 119–28. Available: http://www.ncbi.nlm.nih.gov/pubmed/14492126 PMID: 14492126
21. Ridley DS. Histological classification and the immunological spectrum of leprosy. [Internet]. Bulletin of the World Health Organization. World Health Organization; 1974. http://www.ncbi.nlm.nih.gov/pubmed/4549496

22. Martínez AN, Talhari C, Moraes MO, Talhari S. PCR-based techniques for leprosy diagnosis: from the laboratory to the clinic. PLoS Negl Trop Dis. Public Library of Science; 2014; 8: e2655. https://doi.org/10.1371/journal.pntd.0002655 PMID: 24722358

23. World Health Organization. WHO | WHO recommended MDT regimens [Internet]. WHO. World Health Organization; 2016. http://www.who.int/lep/mdt/regimens/en/#.WgBWF-pr5U.mendeley

24. Brasil, Ministério da Saúde, Secretaria de Vigilância em Saúde. Indicadores epidemiológicos e operacionais de hanseníase [Internet]. Brasília; 2016. http://portalarquivos.saude.gov.br/images/pdf/2017/julho/10/Indicadores-epidemiol--gicos-e-operacionais-de-hansen--ase-Brasil-2001-.pdf

25. Brasil, Instituto Brasileiro de Geografia e Estatística. IBGE | Projeção da população [Internet]. 2013 [cited 7 Jan 2018]. https://ww2.ibge.gov.br/apps/populacao/projecao/

26. Ministério da Saúde, DATASUS, RIPSA. Indicadores Demográficos—Taxa de Natalidade [Internet]. 2012 [cited 9 Jan 2018]. http://tabnet.datasus.gov.br/cgi/idb2012/a07a.htm

27. Barreto JG, Bisanzio D, de Guimarães LS, Spencer JS, Vazquez-Prokopec GM, Kitron U, et al. Spatial Analysis Spotlighting Early Childhood Leprosy Transmission in a Hyperendemic Municipality of the Brazilian Amazon Region. PLoS Negl Trop Dis. Public Library of Science; 2014; 8.

28. Frade MAC, de Paula NA, Gomes CM, Vernal S, Bernardes Filho F, Lugão HB, et al. Unexpectedly high leprosy seroprevalence detected using a random surveillance strategy in midwestern Brazil: A comparison of ELISA and a rapid diagnostic test. Johnson C, editor. PLoS Negl Trop Dis. 2017; 11: e0005375. https://doi.org/10.1371/journal.pntd.0005375 PMID: 28231244

29. Wesley SR, Nair GT, Nair B. Leprosy among school children in Trivandrum city. Indian J Dermatol Venereol Leprol. Indian Journal of Dermatology, Venereology and Leprology (IJDVL); 1990; 56: 288.

30. Santos SD, Penna GO, Costa M da CN, Natividade MS, Teixeira MG. Leprosy in children and adolescents under 15 years old in an urban centre in Brazil. Mem Inst Oswaldo Cruz. Instituto Oswaldo Cruz; 2016; 111: 359–64. https://doi.org/10.1590/0074-02760160002 PMID: 27223655

31. Fine PE, Sterne JA, Pönnighaus JM, Bliss L, Saui J, Chihana A, et al. Household and dwelling contact as risk factors for leprosy in northern Malawi. Am J Epidemiol. 1997; 146: 91–102. PMID: 9215227

32. Lana FCF, Amaral EP, Lanza FM, Lima PL, de Carvalho ACN, Diniz LG. Hanseníase em menores de 15 anos no Vale do Jequitinhonha, Minas Gerais, Brasil. Rev Bras Enferm. 2007; 60: 696–700. https://doi.org/10.1590/S0034-71672007000600014 PMID: 18472544

33. Ferreira IN, Alvarez RRA. Hanseníase em menores de quinze anos no município de Pará de Minas, MG (1994 a 2001). Rev Bras Epidemiol. 2005; 8: 41–49. https://doi.org/10.1590/S1415-790X2005000100006

34. Franco MCA, Xavier MB, Franco ACA, Jucá Neto FOM, de Menezes BQ, Macedo GMM. Perfil de casos e fatores de risco para hanseníase, em menores de quinze anos, em município hiperendêmico da região norte do Brasil. Rev para med. 2014; 28.

35. Fernandes KB, Alves DM, de Mangueira JO. Fatores de risco para a transmissão da hanseníase. Rev Digit Buenos Aires. 2014; 195: 12. Available: http://www.efdeportes.com

36. Durães SMB, Guedes LS, da Cunha MD, Cavaliere FAM, de Oliveira MLWDR. Estudo de 20 focos familiares de hanseníase no município de Duque de Caxias, Rio de Janeiro. An Bras Dermatol. 2005; 80: S295–S300.

37. Madarasingha NP, Senavirathne JKK. A study of household contacts of children with leprosy. Ceylon Med J. 2011; 56: 112–4. Available: http://www.ncbi.nlm.nih.gov/pubmed/22164748 PMID: 22164748

38. Cunha SS, Alexander N, Barreto ML, Pereira ES, Dourado I, de Fátima Maroja M, et al. BCG Revaccination Does Not Protect Against Leprosy in the Brazilian Amazon: A Cluster Randomised Trial. Daumerie D, editor. PLoS Negl Trop Dis. 2008; 2: e167. https://doi.org/10.1371/journal.pntd.0000167 PMID: 18270542

39. de Carvalho FM, Rodrigues LS, Duppre NC, Alvim IMP, Ribeiro-Alves M, Pinheiro RO, et al. Interruption of persistent exposure to leprosy combined or not with recent BCG vaccination enhances the response to Mycobacterium leprae specific antigens. Azman AS, editor. PLoS Negl Trop Dis. 2017; 11: e0005560. https://doi.org/10.1371/journal.pntd.0005560 PMID: 28467415

40. World Health Organization. Global leprosy strategy 2016–2020: accelerating towards a leprosy-free world. [Internet]. New Delhi; 2016. 978-92-9022-509-6

41. Steinmann P, Reed SG, Mirza F, Hollingsworth TD, Richardus JH. Innovative tools and approaches to end the transmission of Mycobacterium leprae. Lancet Infect Dis. 2017; 17: e298–e305. https://doi.org/10.1016/S1473-3099(17)30314-6 PMID: 28693856

APÊNDICE H - PRODUÇÃO CIENTÍFICA EM OUTROS ESTUDOS NO PERÍODO DO DOUTORADO (ARTIGO 4)

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Virology Journal

RESEARCH Open Access

High risk human papillomavirus prevalence and genotype distribution among women infected with HIV in Manaus, Amazonas

Monique Figueiredo Teixeira[*†], Meritxell Sabidó[2,3†], André Luiz Leturiondo[4], Cynthia de Oliveira Ferreira[4], Kátia Luz Torres[5] and Adele Schwartz Benzaken[4,6,7†]

Abstract

Background: Human immunodeficiency virus (HIV)-positive women have a high prevalence of human papillomavirus (HPV), and are infected with a broader range of HPV types than HIV-negative women. We aimed to determine the prevalence of cervical cytologic abnormalities, high-risk (HR)-HPV prevalence, type distribution according to the severity of cervical lesions and CD4 cell count and identify factors associated with HR-HPV infection among women living with HIV in Manaus, Amazonas.

Methods: We enrolled 325 women living with HIV that attended an infectious diseases referral hospital. Each woman underwent a gynecological exam, cervical cytology, HR-HPV detection by Polymerase chain Reaction (PCR) using the BD Onclarity™ HPV Assay, colposcopy and biopsy, when necessary. We assessed the associations between potential risk factors and HR-HPV infection.

Results: Overall, 299 (92.0%) women had a PCR result. The prevalence of HR-HPV- infection was 31.1%. The most prevalent HR-HPV types were 56/59/66 (32.2%), 35/39/68 (28.0%), 52 (21.5%), 16 (19.4%), and 45 (12.9%). Among the women with HR-HPV infection ($n = 93$), 43.0% had multiple infections. Women with HPV infection showed higher prevalence of cervical abnormalities than that HPV-negative (LSIL: 22.6% vs. 1.5%; HSIL: 10.8% vs. 0.0%). The prevalence of HR-HPV among women with cytological abnormalities was 87.5% for LSIL and 100.0% for HSIL. Women with CD4 < 200 cell/mm^3 showed the highest HR-HPV prevalence (59.3%) although this trend was not statistically significant (p-value = 0.62). The mean CD4 cell count decreased with increasing severity of cervical lesions (p-value = 0.001). The multivariable analysis showed that increasing age was associated with a decreased risk of HR-HPV infection with an adjusted prevalence odds ratio of 0.9 (95.0% CI: 0.9–1.0, p-value 0.03) for each additional year. The only factor statistically significant associated with HR-HPV infection was CD4 cell count.

Conclusions: HR-HPV and abnormal cytology prevalence are high among women in the Amazonas. The low CD4 cell count was an important determinant of HPV infection and abnormal cytological findings. HPV quadrivalent vaccination used in Brazil might not offer protection for an important fraction of HPV-related disease burden in women living with HIV. This is partly explained by the high presence of non targeted vaccine HR-HPVs, such as the HPV genotype groups 56/59/66, 35/39/68 and individually HPV-52 and HPV-45, some of which contribute to high-grade lesion.

Keywords: Human papillomavirus, Human immunodeficiency virus, Prevalence, Cytology, Polymerase chain reaction

* Correspondence: monicdft@hotmail.com
†Equal contributors
[1]Universidade Federal do Amazonas (UFAM), 6200, Coronado I, General Rodrigo Octávio Ave, Manaus, Amazon 69080-900, Brazil
Full list of author information is available at the end of the article

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Background

Infection with human papilloma virus (HPV) is the main cause of cervical cancer [1]. In Brazil, it is estimated that approximately 10.7% of women in the general population with normal cytology have cervical HPV infection [2]. In the state of Amazonas, HPV has been shown to be the most prevalent sexually transmitted infection (STI) in the population [3].

Young women are the most affected by HPV and by multiple infections. The prevalence tends to decrease with increasing age [4]. A high viral load and the persistence of oncogenic HPV types are progression factors for precancerous lesions and cervical cancer [5]. Additional factors might influence the development of precursor lesions or cancer, such as those related to immunity, genetics and sexual behaviour. In women over 30 years, HPV infection tends to be more persistent than in younger women [6].

Women living with HIV have a higher prevalence of HPV infection with high-risk oncogenic (HR-HPV) multiple infections. Immunosuppression resulting from HIV increases the risk of developing squamous intraepithelial lesions when compared with the general population [7–10]. Patients more severely immunocompromised as a result of HIV infection might have a higher incidence and persistence of lesions caused by HPV [8].

It is also possible that adherence to highly active antiretroviral therapy (HAART) is associated with decreased development of precursor lesions of cervical cancer and improved clearance of HPV infection, increasing survival of women living with HIV with a consequent decrease in cases of cervical cancer [11].

In Amazonas, Brazil, there are few data on the epidemiology of HPV and related cancers and the impact of HIV on these conditions. The objective of this study was to estimate the prevalence of cervical HPV infection and the frequency of genotypes, according to the severity of cervical lesions and CD4 cell counts and identify factors associated with HPV infection in women living with HIV/AIDS that attended a reference hospital for HIV/AIDS in Manaus, Amazonas.

Methods

Study design

A cross-sectional study was conducted for HR-HPV screening in women living with HIV/AIDS that attended an outpatient HIV reference service within a tertiary care hospital (FMT-HVD). This teaching hospital attends most of the HIV/AIDS cases in the Amazon state (95.0%). This reference hospital is the unique ART provider in the Amazonas state. The study was performed from May 2014 to February 2015.

Study participants

Women who had a confirmed HIV diagnosis and consecutively sought a gynecological visit to perform routine cytology in the HIV outpatient service of the FMT-HVD hospital were eligible for the study. Women were included if older than 18 years of age, agreed to sign the consent form, were not pregnant and did not have a contraindication for Pap smear examination (i.e., current use of vaginal ovules, menstruation, vaginal clean-up during the last 24 h. In case of contraindication they were rescheduled after conditions were resolved. Hysterectomized women were excluded.

In this study, we aimed to include 323 women living with HIV, based on a prevalence of HPV infection in women living with HIV/AIDS of 65.2%, [12] with 80.0% power and assuming a 5.0% level of significance.

Data and sample collection

After signing the consent form, a nurse interviewed women using a structured questionnaire. The questionnaire included items on sociodemographic, clinical, behavioural, reproductive health and HIV history, including current antiretroviral therapy (ART) use and previous change in ART regimen, and current STI signs and symptoms. Data on CD4 cell counts (cells/mm^3) and detectable viral load (copies/mL), and nadir CD4 cell count (cells/mm^3) were obtained from electronical medical records of the hospital. However, when the last determination had been undertaken more than three months before enrolment, a blood sample was collected.

The participants underwent a gynecological evaluation and two samples of cervical cells were collected. The first sample was taken for conventional cytology using a long Ayres's spatula for subsequent processing at the hospital. The second sample was collected with a cervical brush (*Rovers Cervex-Brush Combi®*, Rovers Medical Devices B.V. Oss, the Netherlands) and introduced into *SUREPATH® Preservative Fluid* (TriPath Imaging, Burlington, NC). Samples were transported on the same day at room temperature to the Fundação Centro de Controle de Oncologia (FCECON) laboratory, where 1 mL of each sample was stored at − 80 °C until they were shipped for HPV determination.

Cervical cytology

Cervical cytology samples were processed at the Department of Pathology of the FMT-HVD. The smears were stained with the Papanicolaou stains and the 2001 Bethesda system was used for classification of cytology results [13]. Cytology examination was carried out under blinded conditions and independently of HPV detection results in PCR by two cytopathologists. A third

cytopathologist evaluated discordant results, and if discordancy persisted, agreement was reached between the three.

HPV detection and typing

Samples for HR-HPV determination were shipped to the Instituto de Câncer do Estado do São Paulo (ICESP). They were processed using the BD Onclarity™ HPV Assay (BD Diagnostics, Sparks, MD), which can detect 14 HR-HPV genotypes by simultaneous identification of the HR types 16, 18, 31, 45, 51, 52, and due to the limits of this test, other HR genotypes reported by genotype group (33/58; 56/59/66; 35/39/68). The BD Onclarity™ HPV Assay has shown good performance when compared with Hybrid Capture 2, with specificity ranging from 50.3 to 95.2% and sensitivity from 95.2 to 98.0% [14, 15]. Molecular testing was performed using the automated BD Viper™ LT System (BD Diagnostics, Sparks, MD). HR-HPV detection was carried out under blinded conditions with regard to subjects' characteristics and cytology results.

Data analysis

Data were analyzed using Stata 10.0 (StataCorp LP, College Station, TX). Data were described using percentages and medians with interquartile ranges (IQR), as appropriate. Prevalence and 95.0% confidence interval (CI) were calculated. The results were categorized according to CD4 cell counts (< 200; 200–499; ≥500 cells/mm^3). Comparisons between HR-HPV infected and non-infected women were formally carried out using for categorical variables the X^2 test, and for continuous variables, the student-t test or the Fisher exact test (when expected frequencies were less than 5), or the U-Mann Whitney test (for non-parametric variables). To ascertain associations between potential risk factors and HR-HPV infection, prevalence odds ratios (pOR) were calculated with their corresponding 95.0% CI. For the multivariate analysis, pOR were calculated by multiple logistic regression modelling that included covariates for potential confounders, and for factors that were statistically significant ($p < 0.1$) at univariate analysis. All tests were two-tailed and the p-value less than 0.05 was considered statistically significant.

The agreement for the blinded and independently cytology reading was measured through the percentage of overall agreement, the percentage of positive agreement, percentage of negative agreement, and the prevalence-adjusted bias-adjusted (PABA)-kappa coefficient, by lesion severity.

Ethics

The study was approved by the Ethical Institutional Review Board of FMT-HVD (number: 466/2012). Patients gave their signed consent to participate. All women were informed about conventional cytology and HR-HPV detection results. Colposcopy and biopsy were performed following recommendations of the Brazilian Ministry of Health [16]. The study is reported following the STROBE statement and using its checklist for cross-sectional studies [17].

Results

Study population description

A total of 331 women were pre-screened and all agreed to participate in the study. Among these, six were excluded because no HIV positive result could be documented in the medical record. Thus, the total number of participants available for analysis was 325. Their median age was 40.7 years (IQR: 33.1–46.2). A total of 299 women living with HIV had a valid PCR result and 324 a valid result in conventional cytology. The median CD4 cell count with IQR among ART users and ART-naïve when HIV was diagnosed was 321 (173–487) and 620 (422–739). The median (IQR) CD4+ cell count among ART users and patients not on ART was 257 (133–283) and 197.5 (88–314.5), respectively.

Table 1 shows the socio-demographic, risk behaviour, reproductive health and HIV history, and current sexually transmitted infections signs and symptoms. Women living with HIV with an HR-HPV-positive result were younger than those with HR-HPV-negative results (median in years: 38.8 vs. 41.1, p-value = 0.3). A higher proportion of those with an HR-HPV-positive result had not performed previously cervical cytologies than those with an HR-HPV-negative result (75.0% vs. 25.0%, p-value= 0.02), and most frequently had CD4 cells with counts< 200 cells/mm^3 than women with HP-HPV-negative result (40.7% vs. 59.3, p-value=0.001). In general, in each age group, women with a CD4 cell count < 200 cells/mm^3 had a higher HR-HPV prevalence than women with higher CD4 cell counts, although 95.0% CI was large, suggesting a small number In the CD4 cell count < 200 cells/mm^3 category.

HR-HPV prevalence, associated factors, and genotype distribution by age and CD4 cell count category

The results of the PCR screening showed that 93 out of 299 women were infected with HR-HPV, resulting in a prevalence of 31.1% (95.0% CI: 25.8–36.4). The distribution of age specific HR-HPV prevalence ranged from 25.4% in the age group 31–35 years to 43.5% in those aged > 50 years. The proportion seemed to increase from 36 years onwards, peaking at older women (Fig. 1) although this pattern was not statistically significant (p-value = 0.07). HR-HPV prevalence by CD4 cell group was 23.8% in those with CD4 > 500 cell/mm^3, 34.2% in 200–499 cell/mm3 and 59.3% in < 200 cell/mm^3. The proportion increased with decreasing CD4 cell count,

Table 1 Description of population characteristics and results of bivariable and multivariable analysis for risk factors related to HR-HPV infection among women living with HIV in Manaus, Amazonas

Variables	HR-HPV negative *n* = 206 N (%) Median (IQR)	HR-HPV positive *n* = 93 N (%) Median (IQR)	Crude pOR (95.0% CI)	*p*-value	Adjusted pOR (95.0% CI)	*p*-value
Sociodemographic						
Age in years (*N* = 299)	41.1 (45.9–33.5)	38.8 (31.2–44.4)	0.9 (0.9–1.0)	0.03	0.9 (0.9–1.0)	0.03
≤ 34	56 (27.2)	35 (37.6)	1			
35–39	30 (14.6)	18 (19.3)	1.0 (0.5–2.0)	0.91	...	...
40–44	55 (26.7)	18 (19.4)	0.5 (0.3–1.0)	0.27	...	...
≥ 45	65 (31.5)	22 (23.7)	0.5 (0.3–1.0)	0.06	...	...
Civil status (*N* = 299)						
Married/cohabitating	133 (64.6)	50 (53.8)	1			
Single/not cohabitating	73 (35.4)	43 (46.2)	1.5 (0.9–2.5)	0.08	...	...
Level of education (*N* = 299)						
< Primary school	42 (20.4)	25 (26.8)	1			
At least primary school	164 (79.6)	68 (73.2)	1.4 (0.8–2.5)	0.21	...	...
Currently working (*N* = 299)						
Yes	83 (40.3)	31 (33.3)	1			
No	123 (59.7)	62 (66.7)	1.3 (0.8–2.2)	0.25	...	...
Sexual behaviour and other risk behaviour						
Current smokers (*N* = 299)						
No	191 (92.7)	88 (94.6)	1			
Yes	15 (7.3)	5 (5.4)	0.7 (0.2–2.0)	0.54	...	...
Age at first sex (years) (*N* = 297)						
≤ 15	108 (52.4)	44 (48.4)	0.8 (0.5–1.3)	0.52	...	...
> 15	98 (47.6)	47 (51.6)	1			
Sexual partners in life to date (*N* = 297)						
< 4	60 (29.3)	28 (30.4)	1			
4 to 7	65 (31.7)	32 (34.8)	1.0 (0.5–1.9)	0.87	...	...
≥ 8	80 (39.0)	32 (34.6)	0.8 (0.4–1.5)	0.62	...	...
Regular partner currently (*N* = 299)						
Yes	148 (71.8)	62 (66.7)	1			
No	58 (28.2)	31 (33.3)	1.2 (0.7–2.1)	0.37	...	...
Condom use at last sex with regular partner (*N* = 210)						
Yes	107 (72.3)	45 (72.6)	1			
No	41 (27.7)	17 (27.4)	0.9 (0.5–1.9)	0.97	...	...
Occasional sex partner currently (*N* = 299)						
No	184 (89.3)	82 (88.2)	1			
Yes	22 (10.7)	11 (11.8)	1.1 (0.5–2.4)	0.78	...	...
Condom use at last sex with occasional partner (*N* = 33)						
Yes	16 (72.7)	8 (72.7)	1			
No	6 (27.3)	3 (27.3)	1.0 (0.2–5.0)	1.00	...	...
Reproductive and sexual health						
Current oral contraceptive use (*N* = 299)						
Yes	202 (98.1)	90 (96.7)	1			
No	4 (1.9)	3 (3.3)	1.6 (0.3–7.6)	0.50	...	...

Table 1 Description of population characteristics and results of bivariable and multivariable analysis for risk factors related to HR-HPV infection among women living with HIV in Manaus, Amazonas (Continued)

Variables	HR-HPV negative $n = 206$ N (%) Median (IQR)	HR-HPV positive $n = 93$ N (%) Median (IQR)	Crude pOR (95.0% CI)	*p*-value	Adjusted pOR (95.0% CI)	*p*-value
Previous cervical cytology ($N = 299$)						
Yes	204 (99.0)	87 (93.6)	1			
No	2 (1.0)	6 (6.4)	7.0 (1.3–35.5)	**0.02**	...	...
Ever had an STI ($N = 299$)						
No	146 (70.9)	65 (69.9)	1			
Yes	60 (29.1)	28 (30.1)	1.0 (0.6–1.7)	0.86	...	...
Parity ($N = 299$)						
Nulliparous	11 (5.3)	10 (10.8)	1			
1 to 3	97 (47.0)	39 (41.9)	0.4 (0.1–1.1)	0.09	...	...
≥ 4	98 (47.7)	44 (47.3)	0.4 (0.2–1.2)	0.14	...	...
Ever had an abortion ($N = 278$)						
No	90 (46.2)	44 (53.0)	1			
Yes	105 (53.8)	39 (47.0)	0.7 (0.4–1.2)	0.30	...	...
HIV history						
Time since HIV diagnosis in years (N = 298)	6 (3–10)	5 (2–10)	0.9 (0.9–1.0)	0.53	...	...
CD4 cell count nadir[b] ($N = 294$)	195 (83–317)	221 (86–357)	1.0 (0.9–1.0)	0.90	...	...
CD4 cells/mm^3[a] (N = 298)	348 (197–550)	322 (162–491)	0.9 (0.9–1.0)	0.30		
≥500	115 (56.1)	36 (38.7)	1		1	
200–499	79 (38.5)	41 (44.1)	1.6 (0.9–2.8)	0.06	1.6 (0.9–2.8)	0.06
< 200	11 (5.4)	16 (17.2)	4.7 (2.0–11.3)	**< 0.001**	4.7 (2.0–11.3)	**< 0.001**
Detectable viral load (copies/mL)[a] ($N = 298$)	6049 (390–32,000)	11,112.5 (1070–43,258.5)	1.0 (0.9–1.0)	0.60		
Current ART ($N = 299$)						
Yes	185 (89.8)	78 (83.9)	1			
No	21 (10.2)	15 (16.1)	1.6 (0.8–3.4)	0.14	...	...
Previous change in ART regimen ($N = 270$)						
No	119 (62.9)	44 (54.3)	1			
Yes	70 (37.1)	37 (45.7)	1.4 (0.8–2.4)	0.18	...	...

ART Antiretroviral therapy, *CI* Confidence interval, *IQR* Interquartile range, *pOR* Prevalence odds ratio, *SD* Standard deviation, *STI* Sexually transmitted infection, *p*-value < 0.05: statistically significant
[a]Most recent blood test collected within 3 months before enrolment
[b]It is the lowest CD4 cell count of the patient

although this pattern was not statistically significant (p-value = 0.62).

The most prevalent HR-HPV types among 93 HR-HPV-positive and HIV positive women were HPV pool-56/59/66 (32.3%), HPV-35/39/68 (28.0%) and isolated HPV-52 (21.5%), HPV-58 (20.4%), HPV-16 (19.4%), HPV-45 (12.9%), HPV-31 (11.8%), and HPV-18 (2.2%) (Fig. 2). Multiple infections (range: 2–4 types) were identified in 40 of 93 women (43.0%). The prevalence of women with multiple HR-HPV infections was 13.4% (40/299) (95.0% CI: 9.5%–17.3). Among cases of multiple infection ($N = 40$), the prevalence of HPV-16 was 27.5% ($n = 11$), of HPV-18 was 5.0% ($n = 2$) and of HPV-52 was 35.0% ($n = 14$). Multiple infections had a higher HR-HPV prevalence in older age groups (41–45 years: 18.9%; 46–50 years: 18.9%; > 50 years: 17.4%) than in younger age groups (< 26 years: 7.3%; 26–30 years: 13.2%; 31–35 years: 11.9%) but no trend with age was found (p-*value* = 0.10).

When compared with HR-HPV-negative women, those with a HR-HPV-positive result were younger, a lower proportion had undertaken a cervical cytology ever, had a lower median CD4 cell count at last determination, and a higher proportion had CD4 cell count < 200 cell/mm^3 (Table 1). Results from the multivariable analysis showed that increasing age was associated with a decreased risk of HR-HPV infection with an adjusted pOR of 0.9 (95.0% CI: 0.9–1.0, p-value= 0.03) for each additional year. The only

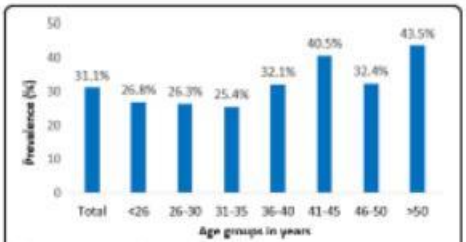

Fig. 1 Prevalence of HPV infection according to age among 299 women living with HIV in Manaus, Amazonas

factor statistically significant associated with HR-HPV infection was CD4 cell count: women with CD4 cell count < 200 cell/mm^3 had an pOR of having HR-HPV that was 4.7 (95.0% CI: 2.0–11.3, *p*-value < 0.001) times greater than that of women with CD4 cell count ≥500 cells/mm^3.

Relationship between HR-HPV infection, conventional cytology results, and CD4 cell counts

Overall, 84.2% (275) of women had a normal cytology, 2.7% (9) ASCUS, 1.7% (5) ASC-H, 8.1% (25) LSIL, 3.4% (10) HSIL, and no cancer cases. The proportion of cytological abnormalities was higher in women with HR-HPV-positive than in women with a negative HR-HPV-result. (Additional file 1: Table S1).

By cytological grade, the prevalence of HR-HPV was 37.5% in ASC-US, 80.0% in ASC-H, 87.5% in LSIL, and 100.0% in HSIL. The prevalence of HR-HPV increased with the degree of cytological abnormalities, ranging from 21.9% among women with normal cytology to 100.0% among women with HSIL (*p*-value for trend < 0.0001). The same pattern was observed by decreasing CD4 cell count groups (Table 2).

HPV- 56/59/66 (either 56, 59, 66 or any combination of these three types) was the most prevalent genotype (52.3%) among 21 HR-HPV-positive women with LSIL and the most prevalent one (60.0%) among 10 HR-HPV-positive women with HSIL in a total of 93 women co-infected with HR-HPV and HIV (Table 3). The second most common genotypes for LSIL were HPV-16 (23.8%) and HPV-35/39/68 (23.8%), whereas for HSIL HPV-16 was found in half (50.0%) of these women. None of the women with LSIL and HSIL were infected with HPV-18. The prevalence of HR HPV genotype increased by cytological grade in HPV-16, HPV-31, HPV-45, HPV-33/58, and HPV- single infection and multiple infection (*p*-value for trend <0.001), and in HPV-52 (*p*-value for trend = 0.04).

Compared to those with single infections, women with multiple HR-HPV infections had a higher prevalence of LSIL (42.9% vs. 57.1%, *p* < 0.001) and HSIL (20.0% vs. 80.0%, p < 0.001). In women with multiple HR-HPV infections, the degree of severity of cytological lesions was strongly correlated with a decreased CD4 cell count (Additional file 1: Table S2).

Figure 3 shows that in the HSIL category, women with CD4 counts ≥ 500 cell/mm^3 had a lower proportion of multiple HR-HPV-infections (50.0%) than those with CD4 200–499 cell/mm^3 (80.0%) and those with CD4 < 200 cell/mm^3 (100.0%).

The overall agreement for conventional cytology observed between the blinded first and second evaluators was 89.0%. By type of lesion, it was > 90.0% and the PABA-kappa statistic ranged from 0.82–0.97 (Additional file 1: Table S3).

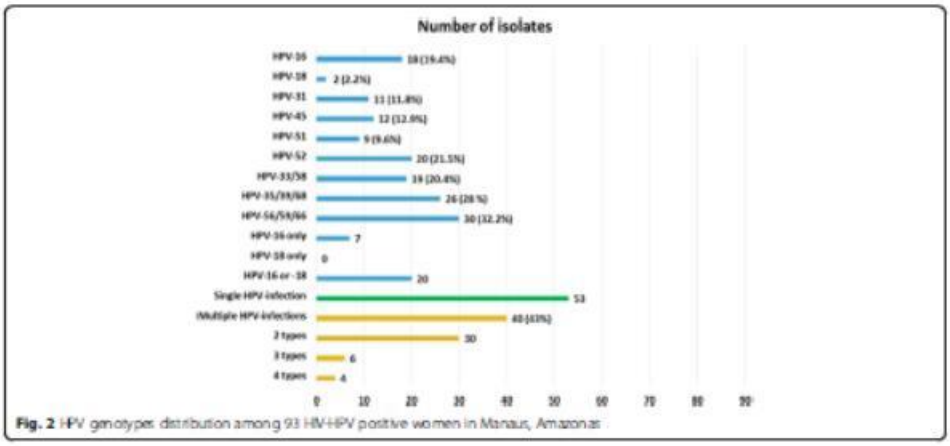

Fig. 2 HPV genotypes distribution among 93 HIV-HPV positive women in Manaus, Amazonas

Table 2 HR-HPV prevalence as determined by PCR, according to the cytological findings and HIV viral load among women living with HIV in Manaus, Amazonas

HR-HPV Prevalence				
Cytology	General	CD4 ≥ 500 cell/mm^3	CD4 200-499 cell/mm^3	CD4 < 200 cell/mm^3
Unsatisfactory	0	0	0	0
Normal	55/251 (21.9)	27/139 (19.4)	20/94 (21.3)	8/17 (47.1)
ASC-US	3/8 (37.5)	1/1 (100)	1/4 (25)	1/3 (33.3)
ASC-H	4/5 (80.0)	2/2 (100)	2/3 (66.7)	0
LSIL	21/24 (87.5)	4/6 (66.7)	13/14 (92.9)	4/4 (100)
HSIL	10/10 (100)	2/2 (100)	5/5 (100)	3/3 (100)

ASC-H Atypical Squamous Cells of Undetermined Significance, when it is not possible to disregard high degree lesions; *ASC-US* Atypical Squamous Cells of Undetermined Significance, *LSIL* Low-grade Squamous Intraepithelial Lesions, *HSIL* High-grade Squamous Intraepithelial Lesions, *HR-HPV* High Risk Human Papillomavirus

Discussion

This study provides evidence about the prevalence of HR-PV infection, their associations, and cervical lesions among women living with HIV, an area of research with scarce literature from the Amazonas.

The prevalence of HR-HPV infection found, 31.1%, is approximately half of that found in another study in HIV positive women in Amazonas (61.6%) [18]. This discrepancy could be explained because in the latter study women were younger (median age: 32 years [IQR]: 27–38, vs. 40.7 years [IQR]: 33.1–46.2) and a peak among younger women has been described in a number of countries [19]. In the same study, women had a lower median CD4 cell count at enrolment (338.5 [IQR: 211.5–513.3] cells/mm^3 vs. 504.0 [IQR: 321.0–676.5), which can limit the clearance of the virus, and 16.0% of women were commercial sex workers, which is a highly HPV exposed population [20]. Other studies in Brazil involving HIV positive women have found higher prevalence of HR-HPV from 35.7% to 98.0% [12, 21–24]. The differences with the results from other studies might be partly explained by information bias. Indeed, the study design used does not allow to differentiate between prevalent, incident and persistent HR-HPV infection leading to misclassification of the infection status and limiting comparability of our estimates with those from other studies.

We found that 43.0% of HR-HPV-infected women had multiple infections, which is in agreement with other Brazilian, ranging from 32.2% to 64.8% [24–27] and ranged from 52.0% to 64.3% in international studies [28, 29]. Women living with HIV are more likely to harbor multiple HPV infection than immunocompetent women [30] which is associated with an increased risk of intraepithelial neoplasia and of cancer [31, 32]. This probably reflects the effect of HIV-induced immunosuppression [33] rather than sexual risk behaviour [34].

In the present study, we found a wide diversity regarding HR-HPV types and their distribution, with HPV-52, HPV-16 and HPV-45 being the most frequent individual types. However, this needs to be taken with caution because the PCR method used did not allow the measurement for all individual genotypes and the various

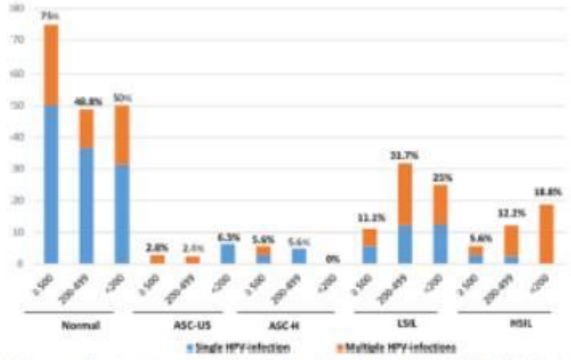

Fig. 3 Prevalence of HPV infection according to CD4 counts and cytology and distribution of multiple HPV infections from 93 HIV-HPV positive women in Manaus, Amazonas

Table 3 Distribution of HR-HPV genotype according to cytology results in 93 HPV+HIV positive women in Manaus, Amazonas

	Normal (*n* = 55)	ASC-US (*n* = 3)	ASC-H (*n* = 4)	LSIL (*n* = 21)	HSIL (*n* = 10)	*p*-test for trends
HPV-16	6 (10.9)	1 (33.3)	1 (25.0)	5 (23.8)	5 (50.0)	< 0.001
HPV-18	1 (1.8)	0 (0.0)	1 (25.0)	0 (0.0)	0 (0.0)	0.30
HPV-31	4 (7.3)	0 (0.0)	1 (25.0)	4 (19.0)	2 (20.0)	< 0.001
HPV-45	4 (7.3)	0 (0.0)	1 (25.0)	4 (19.0)	3 (30.0)	< 0.001
HPV-51	6 (10.9)	1 (33.3)	0 (0.0)	2 (9.5)	0 (0.0)	0.17
HPV-52	13 (23.6)	0 (0.0)	1 (25.0)	3 (14.3)	3 (30.0)	0.04
HPV-33/58	9 (16.4)	2 (66.7)	1 (25.0)	4 (19.0)	3 (30.0)	< 0.001
HPV-35/39/68	17 (30.9)	1 (33.3)	0 (0.0)	5 (23.8)	3 (30.0)	0.05
HPV-56/59/66	18 (32.7)	0 (0.0)	0 (0.0)	11 (52.3)	6 (60.0)	0.05
HPV-16 only	4 (7.3)	0 (0.0)	1 (25.0)	0 (0.0)	2 (20.0)	0.44
HPV-18 only	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.30
HPV-16 or − 18	7 (12.7)	1 (33.3)	2 (50.0)	5 (23.8)	5 (50.0)	0.40
HPV- single infection	38 (69.1)	1 (33.3)	3 (75.0)	9 (42.9)	2 (20.0)	< 0.001
HPV- multiple infection	17 (30.9)	2 (66.7)	1 (25.0)	12 (57.1)	8 (80.0)	< 0.001
2 types	13 (23.6)	2 (66.7)	1 (25.0)	9 (42.9)	5 (50.0)	...
3 types	2 (3.6)	0 (0.0)	0 (0.0)	1 (4.8)	3 (30.0)	...
4 types	2 (3.6)	0 (0.0)	0 (0.0)	2 (9.5)	0 (0.0)	...

p-value < 0.05: statistically significant

groupings of HPV types difficult the interpretation. As an example, the high prevalence of HPV-35/39/68 might be driven by HPV-35 alone and this type has been implicated in HSIL and invasive cervical cancer in women living with HIV [35, 36]. Women living with HIV are characterized by a wide variation of HPV genotypes, probably related to their sexual behaviour and the reactivation of latent infections, which can facilitate infection by different HPV genotypes. Contrary to what most studies suggest,[10, 12, 21, 28, 30, 37–39] HPV-16 was not the most common HPV type. This is in line with the results from other studies among indigenous populations in the Amazonas, [40] among women living with HIV in Brazil, [26] in the USA [29] and in Africa, [41] that corroborate our findings and place HPV-16 as not the most common type detected. It has been suggested that the contribution of HPV-16 correlates inversely with the overall HPV prevalence [42]. This pattern is explained by a higher prevalence of other HPV types in areas where HPV is extremely common, and the increase is not explained by the contribution of any other single type. Nevertheless, HPV-16 was the most prevalent individual HPV type among HSIL lesions, which is consistent with the results of a meta-analysis that included 19,883 women living with HIV from 86 studies worldwide [36]. This meta-analysis reported that HPV-16 positivity tended to increase with severity of cervical lesions. In Africa, HPV-16 accounts for 31.1% of HSIL and 46.6% of invasive cervical cancers. In Latin America, HPV-16 accounts for 37.5% of HSIL with no data available for invasive cervical cancers [36].

A low number of cases of HPV-18 were detected although it ranks amongst the top positions in most regions [37, 39]. In addition, HPV-18 accounts for a high proportion of HPV-positive in HSIL and invasive cervical cancers among women living with HIV [36]. Likewise, in other studies involving women living with HIV the HPV-18 contribution has been low [21, 24, 43–45]. It has either not been commonly detected in Brazilian studies [46, 47] or has shown a prevalence below 1.0% in asymptomatic women [48–50]. In two studies conducted in the Amazon region HPV-18 was not detected [37, 39]. It is unlikely that the low prevalence of HPV-18 found is related with the PCR method used. The BD Onclarity assay used showed good performance when compared with standard genotyping test [14, 15]. For HPV-18 (single or multiple infection), the agreement with the GP5+/6+ LMNX assay was high, with a kappa of 0.93 (95.0% CI: 0.87–0.99) [51].

Age-specific HR-HPV distribution presented as a unimodal distribution skewed to the left although this pattern was not statistically significant. Although there are studies that indicate a higher prevalence of HPV in younger women,[4, 52–55] we observed a higher prevalence of HR-HPV prevalence among older women (> 50 years) which has also been described in some other studies [10, 19, 32, 56–58]. It has been suggested that the increase in the perimenopause period may be due to higher rates of HPV persistence and recurrence at older ages rather than new HPV acquisition, [59] and that viral characteristics such as HPV type and

variants, [60] weakened immune system, changes in sexual behaviour during middle age (both for men and women [61]), or previous individual screening practices, may play a role [56]. The association between younger age and higher prevalence of HR-HPV is probably due to the presence of transient infections in this group of women; however, this association is generally observed in non-HIV infected women but is not consistent in women living with HIV/AIDS [62].

In multivariable analysis, increasing age presented only borderline association with an increasing risk of presenting HR-HPV. We did not observe a peak of prevalence among young women (< 26 years) which might be explained because only 13.0% in our sample were < 26 years and among them, only 11 (27.0%) were infected with HR-HPV.

In the present study we found a clear association between weakened immune status and infection by HR-HPV. In most studies of HIV and HPV, the magnitude of increased HPV prevalence was proportional to the severity of immunosuppression [10, 18, 38, 63–65]. While many HPV infections are transient, women living with HIV are more likely to have persistent HPV infections, [66] and in other studies the frequency of persistence varied inversely with CD4 cell count [62, 65]. These results suggest that HIV induced immunosuppression might cytological findings was 21.9%, higher than the global estimate of 16.1% reported a meta-analysis for women in the general population from Latin America [42]. We found a high prevalence of LSIL (8.1%) and HSIL (3.4%) that reflects long-term persistent infections, in concordance with the high rates of multiple infections observed among HPV infected with these lesions [10, 25, 55]. Our results are consistent with other studies in which the presence of HPV in cytology with abnormal results ranged from 71.0% to 90.0% for LSIL [10, 67], and between 80.6% to 100.0% for HSIL [10, 30, 67]. Worsening of immune status was correlated with severity of lesions, as previously described [68].

We found high diversity of HR-HPV types in women with abnormal cytology results. In our study, most cytological alterations were related to types 16, 31 and 45. HPV-16 was the most common type in HSIL, which has been reported in a meta-analysis of HIV positive women with HSIL, [30] and in women from the general population [32]. Half of the HSIL cases presented HPV-16, which has high oncogenic potential and its presence is affected by the immunology status of the patient. The high prevalence of HR-HPV non-targeted by current vaccines does not reduce the importance of vaccination against HPV-16 and -18, proven genotypes with the highest carcinogenic potential. Furthermore, cross-protection has been described for HPV-45 and HPV-31 [69]. However, newer vaccines such as the nonavalent HPV vaccine present the possibility of better coverage for women [70–72].

This study has some limitations. It did not include a truly population-based design, as study participants were recruited from a reference hospital. However, this hospital attends to 95.0% of HIV patients from the Amazonas. Our sample included women who had a prolonged history of HIV infection (median 6 years), most were taking HAART (87.9%), had previously cytology (97.2%), had a relatively immune competence status (median CD4 cell count 504.0 cell/mm^3), and median age was 40.7 years. These women could have a higher self-care standard, higher accessibility to health care and better health, which would result in underestimating the true HR-HPV prevalence in the population. In addition, we did not measure variables such as nutrition and behaviour of male partners that can influence HPV DNA detection [73–75]. The BD Onclarity ™ HPV Assay used for PCR does not allow a measurement for all individual genotypes. The various groupings of HPV types are difficult to interpret. It was not possible to investigate associations of duration of ART and the results of HR-HPV and cervical lesion, since the duration of ART would not be accurate given that treatment interruptions was not collected. Regarding the study size, the background estimate used was based on Brazilian studies [12, 22, 23] (ranging from 63.0% to 98.0%) but was higher than those reported in international studies. In addition, the statistical power was low at 80.0%. These reasons might have influenced the accuracy of the estimates measured and the strength of the association in the multivariable model. This study has a cross sectional design which allows only for presentation of baseline information.

Conclusions

In conclusion, we found a high prevalence of HR-HPV infection and cervical lesions among women living with HIV in Amazonas. We found a wide diversity of HR-HPV genotypes, being the most common ones individually HPV-52, HPV-16 and HPV-45, although the highest prevalence was found in the genotype groups 56/59/66 and 35/39/68. HPV-16 and HPV-18 were less common than other HPV types but 50.0% of women with HSIL had HPV-16. The most important determinant of HPV infection was a low CD4 cell count. Most abnormal cytological findings were observed in women with poor immunological status. HPV quadrivalent vaccination used in Brazil might not offer protection for an important fraction of HPV-related disease burden in women living with HIV given the high prevalence of non-targeted vaccine HR-HPV, some of which (eg. 35, 39, 45, 56) contribute to high-grade lesions. Newer vaccines such as the nonavalent HPV vaccine [70–72] present the possibility of better coverage for women and will need to be evaluated. Strengthening preventive efforts is necessary to improve early detection through increasing accessibility to screening programs, adherence to follow-up among those with lesions, and intensifying health education for women living with HIV.

Additional file

Additional file 1: Table S1. Prevalence of conventional cytology results according to HPV status and CD4 counts among 298 women living with HIV in Manaus, Amazonas. **Table S2** Association between cytological lesions and CD4 cell counts among women living with HIV in Manaus, Amazonas. **Table S3** Agreement between blinded observers in the independent reading of conventional cytology by type of lesion (DOCX 19 kb)

Abbreviations

AOR: Adjusted Odds Ratio; ASC-H: Atypical Squamous Cells of Undetermined Significance, when it is not possible to disregard high degree lesions; ASC-US: Atypical Squamous Cells of Undetermined Significance; CD4: Molecule that is expressed on the surface of some T cells; CI: Confidence Interval; FCECON: Fundação Centro de Controle de Oncologia; HAART: Highly Active Antiretroviral Therapy; HIV: Human Immunodeficiency Virus; HPV: Human Papillomavirus; HR-HPV: High Risk HPV; HSIL: High-grade Squamous Intraepithelial Lesions; ICESP: Instituto de Câncer do Estado do São Paulo; LSIL: Low-grade Squamous Intraepithelial Lesions; OR: Odds Ratio; PCR: Polymerase Chain Reaction; SD: Standard Deviation; STI: Sexually Transmitted Infection

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Availability of data and materials

Please contact author for data requests.

Authors' contributions

MFT participated in the drafting of the study, inclusion of subjects of the research and data collection, analysis and interpretation of the data and writing of the manuscript. MS participated in drafting the study, analysis and interpretation of data, writing of the manuscript and critical review of the manuscript. ALL participated in molecular biology data analysis and critical review of the manuscript. CDF participated in the analysis of molecular biology data and critical review of the manuscript. KLT participated in the analysis of molecular biology data and supervision in the preparation of slides and storage of samples and critical review of the manuscript. ASB participated in drafting the study, analysis and interpretation of the data, writing the manuscript and critical review of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

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Author details

[1]Universidade Federal do Amazonas (UFAM), 6200, Coronado I, General Rodrigo Octávio Ave, Manaus, Amazon 69080-900, Brazil. [2]TransLab. Departament de Ciències Mèdiques, Facultat de Medicina, Girona, Catalunya, Spain. [3]CIBER de Epidemiología y Salud Pública (CIBERESP), Madrid, Spain. [4]Fundação Alfredo da Matta (FUAM), Manaus, Amazonas, Brazil. [5]Fundação Centro de Controle de Oncologia do Amazonas (FCecon), Manaus, Amazonas, Brazil. [6]Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (FMT-HVD), Manaus, Amazonas, Brazil. [7]Departamento de IST, Aids, e Hepatites Virais, Ministério da Saúde, Brasília, DF, Brazil.

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References

1. Jemal A, Bray F, Ferlay J. Global cancer statistics. CA Cancer J Clin. 2011; 61(2):69–90.
2. ICO Information Centre on HPV and Cancer. Brazil: Human Papillomavirus and Related Cancers, Fact Sheet 2017 [Internet]. 19 april 2017. Available from: http://www.hpvcentre.net/statistics/reports/BRA_FS.pdf.
3. Pedrosa VL, Galban E, Benzaken A, Vasquez FG, Izan Jr. JL. DST e suas Determinantes: Quatro Anos de Vigilância em um Centro Sentinela no Estado do Amazonas – Brasil. J Bras Doenças Sex Transm [Internet]. 2011 [cited 2013 Oct 18];23(2):57–65. Available from http://www.dst.uff.br/revista23-2-2011/3-DST%20e%20suas%20Determinantes%20Quatro%20Anos%20de%20Vigilancia.pdf.
4. Argyri E, Papaspyridakos S, Tsimplaki E, Michala L, Myriokefalitaki E, Papasideri I, et al. A cross-sectional study of HPV type prevalence according to age and cytology. BMC Infect Dis [Internet]. 2013 Jan;13:53. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3575323&tool=pmcentrez&rendertype=abstract.
5. Kim YM, Park JY, Lee KM, Kong T-W, Yoo S-C, Kim WY, et al. Does pretreatment HPV viral load correlate with prognosis in patients with early stage cervical carcinoma? J Gynecol Oncol [Internet]. 2008 Jun [cited 2013 Oct 29];19(2):113–6. Available from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2676453&tool=pmcentrez&rendertype=abstract.
6. BRASIL. Instituto Nacional de Câncer José Alencar Gomes da Silva. Coordenação de Prevenção e Vigilância. Estimativa 2016: Incidência de câncer no Brasil [Internet]. Ministério da Saúde Instituto Nacional de Cancer José Alencar Gomes da Silva. 2016. 124 p. Available from: http://www.inca.gov.br/bvs/controledecancer/publicacoes/edicao/Estimativa_2016.pdf.
7. Kobayashi A, Greenblatt RM, Anastos K, Minkoff H, Massad LS, Young M, et al. Functional attributes of mucosal immunity in cervical intraepithelial neoplasia and effects of HIV infection: functional attributes of mucosal immunity in cervical intraepithelial neoplasia and effects of HIV infection. Am Assoc Cancer Res. 2004;6766–74.
8. Zimmermann JB, Melo VH, de Castro LPF, Alves MJM, Zimmermann SG, Del Castillo DM. Associação entre a contagem de linfócitos T CD4 + e a gravidade da neoplasia intra-epitelial cervical diagnosticada pela histopatologia em mulheres infectadas pelo HIV. Rev Bras Ginecol Obs. 2006;28(6):345–51.
9. Corrêa CM, Melo VH, Mendez D, Castillo D. Coinfecção HIV/HPV : prevalência e multiplicidade de genótipos do HPV no colo uterino co-infection of HIV-HPV : prevalence and multiplicity of HPV genotypes in the cervix uterine. Femina. 2009;37(6):3–7.
10. Stuardo V, Agustí C, Godínez JM, Montoliu A, Torné A, Tarrats A, et al. Human papillomavirus infection in HIV-1 infected women in Catalonia (Spain): implications for prevention of cervical cancer. PLoS One [Internet]. 2012 Jan [cited 2013 Oct 18];7(10):e47755. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3484159&tool=pmcentrez&rendertype=abstract.
11. Minkoff H, Zhong Y, Burk RD, Palefsky JM, Xue X, Watts DH, et al. Influence of adherent and effective antiretroviral therapy use on human papillomavirus infection and squamous intraepithelial lesions in human immunodeficiency

virus-positive women. J Infect Dis [Internet]. 2010 Mar [cited 2013 Oct 18]; 201(5):681–90. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2818607&tool=pmcentrez&rendertype=abstract.
12. Entiauspe LG, Teixeira LO, Mendoza-Sassi RA, Gonçalves CV, Gonçalves P, Barral M. Papilomavírus humano : prevalência e genótipos encontrados em mulheres HIV positivas e negativas, em um centro de referência no extremo Sul do Brasil. Rev Soc Bras Med Trop. 2010;43(3):260–3.
13. Solomon D, Davey D, Kurman R, Moriarty A, O'Connor D, Prey M, et al. The 2001 Bethesda System: terminology for reporting results of cervical cytology. JAMA [Internet]. 2002 Apr;24;287(16):2114–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11966386.
14. Bottari F, Sideri M, Guimini C, Iglidbashian S, Tticca A, Casadio C, et al. Comparison of ondarity human papillomavirus (HPV) assay with hybrid capture II HPV DNA assay for detection of cervical intraepithelial neoplasia grade 2 and 3 lesions. J Clin Microbiol [Internet]. 2011;53(7):2109–14. Available from: http://www.scopus.com/inward/record.url?eid=2-s2.0-84033600748&partnerID=40&md5=c270b30be7c44e44e960a0c7b01ee0.
15. Ejegod D, Bottari F, Pedersen H, Sandri MT, Bonde J. The BD Ondarity HPV assay on SurePath collected samples meets the International Guidelines for Human Papillomavirus Test Requirements for Cervical Screening. J Clin Microbiol. 2016; 54(9):2267–72. Available from: http://jcm.asm.org/content/54/9/2267.full.
16. Brasil. Ministério da Saúde. Instituto Nacional de Câncer. Coordenação Geral de Ações Estratégicas. Divisão de Apoio à Rede de Atenção Oncológica. Diretrizes Brasileiras para o Rastreamento do Câncer do Colo do Útero. INCA. 2016.
17. Elm, Von R. EM. Checklist of items that should be included in reports of cross-sectional studies [Internet]. Strobe statement. 2008 [cited 2014 Jun 3]. p. 3–4. Available from: https://www.strobe-statement.org/index.php?id=available-checklists.
18. Silva LCF, Miranda A, Batalha R, Ferreira L, Santos M, Talhari S. High-risk human papillomavirus and cervical lesions among women living with HIV/AIDS in Brazilian Amazon, Brazil. Brazilian J Infect Dis [Internet]. 2015;(x x):5–10. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1413867015001269.
19. Francoschi S, Herrero R, Clifford GM, Snijders PJF, Arslan A, Anh PTH, et al. Variations in the age-specific curves of human papillomavirus prevalence in women worldwide. Int J Cancer. 2006;119(11):2677–84.
20. Valéis X, Murga GB, Hernández G, Sabidó M, Chuy A, Lloveras B, et al. High prevalence of human papillomavirus infection in the female population of Guatemala. Int J Cancer. 2009;125(5):1161–7.
21. Coccato Junior BPV, Lopes APC, Nascimento LF, Novaes LM, Melo VH. Prevalência de infecção cervical por papilomavírus humano e neoplasia intraepitelial cervical em mulheres HIV-positivas e negativas. Rev Bras Ginecol e Obs [Internet]. 2015;37(4):178–85. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0100-72032015000400178&lng=pt&nrm=iso&tlng=en.
22. Vidila S, Darwich L, Cañadas MP, Paredes R, Tarrats A, Castilla E, et al. Epidemiological data of different human papillomavirus genotypes in cervical specimens of HIV-1-infected women without history of cervical pathology. J Acquir Immune Defic Syndr [Internet]. 2009;50(2):168–75. Available from: https://journals.lww.com/jaids/Fulltext/2009/02010/Epidemiological_Data_of_Different_Human.7.aspx.
23. Levi JE, Kleter B, Quint WG V, Fink MCS, Canto CLM, Matsubara R, et al. High Prevalence of Human Papillomavirus (HPV) Infections and High Frequency of Multiple HPV Genotypes in Human Immunodeficiency Virus-Infected Women in Brazil. J Clin Microbiol [Internet]. 2002 Sep 1 [cited 2013 Dec 17]; 40(9):3341–5. Available from: http://jcm.asm.org/content/40/9/3341.full.
24. Corrêa CM, Teixeira NCP, Araújo ACL. De, Carvalho NDO, Castillo DM del, Campos RR, et al. prevalence and multiplicity of HPV in HIV women in Minas Gerais, Brazil. Rev Assoc med bras [Internet], Available from: 2011; 57(4):425–30. http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0104-42302011000400017&lng=en&nrm=iso&tlng=en.
25. Campos RR, de Melo VH, del Castillo DM, Nogueira CPF. Prevalência do papilomavírus humano e seus genótipos em mulheres portadoras e não portadoras do vírus da imunodeficiência humana. Rev Bras Ginecol Obs. 2005;27(31):248–56.
26. Castilho JL, Levi JE, Luz PM, Cambou MC, Vanni T, de Andrade A, et al. A cross-sectional study of high-risk human papillomavirus clustering and cervical outcomes in HIV-infected women in Rio de Janeiro, Brazil. BMC Cancer [Internet]. 2015;15():478. Available from: http://www.biomedcentral.com/1471-2407/15/478.
27. Levi JE, Fernandes S, Tateno AF, Morta E, Lima LP, Eluf-Neto J, et al. Presence of multiple human papillomavirus types in cervical samples from HIV-infected women. Gynecol Oncol. 2004;92():225–31.
28. Anastos K, Hoover DR, Burk RD, Cajigas A, Shi Q, Singh DK, et al. Risk factors for cervical precancer and cancer in HIV-infected, HPV-positive Rwandan women. PLoS One [Internet]. 2010 Jan [cited 2013 Oct 18];5(10):e13525. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2958126&tool=pmcentrez&rendertype=abstract.
29. Luque AE, Jabeen M, Messing S, Lane C a, Demeter LM, Rose RC, et al. Prevalence of human papillomavirus genotypes and related abnormalities of cervical cytological results among HIV-1-infected women in Rochester, New York. J Infect Dis 2006;194(4):428–434.
30. Clifford GM, Gonçalves MAG, Franceschi S. Human papillomavirus types among women infected with HIV: a meta-analysis. AIDS [Internet]. 2006 Nov 28;20(may 2005):2337–44. Available from: https://journals.lww.com/aidsonline/fulltext/2006/11280/Human_papillomavirus_types_among_women_infected.10.aspx.
31. Mosicki A-B, Ellenberg JH, Crowley-Nowick P, Darragh TM, Xu J, Fahrat S. Risk of high-grade squamous intraepithelial lesion in HIV infected adolescents. J Infect Dis. 2004;190(8):1413–21.
32. Herrero R, Castle PE, Schiffman M, Bratti MC, Hildesheim A, Morales J, et al. Epidemiologic profile of type-specific human papillomavirus infection and cervical neoplasia in Guanacaste, Costa Rica. J Infect Dis. 2005;191(11):1796–807.
33. Palefsky J. Biology of HPV in HIV infection. Adv Dent Res. 2006;1999–105.
34. Strickler HD, Burk RD, Fazzari M, Anastos K, Minkoff H, Massad LS, et al. Natural history and possible reactivation of human papillomavirus in human immunodeficiency virus-positive women. J Natl Cancer Inst. 2005;97(8):577–86.
35. Kelly HA, Ngou J, Chikandiwa A, Sawadogo B, Gilham C, Omar T, et al. Associations of Human Papillomavirus (HPV) genotypes with high-grade cervical neoplasia (CIN2+) in a cohort of women living with HIV in Burkina Faso and South Africa. PLoS One [Internet]. 2017;12(3):1–14. Available from: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0174117.
36. Clifford GM, Tully S, Franceschi S. Carcinogenicity of human papillomavirus (HPV) types in HIV-positive women: a meta-analysis from HPV infection to cervical cancer. Clin Infect Dis. 2017;64(9):1228–35.
37. Rocha DAP, Barbosa Filho RAA, De Queiroz FA, Dos Santos CMB. High prevalence and genotypic diversity of the human papillomavirus in amazonian women, Brazil. Infect Dis Obstet Gynecol. 2013;(Article ID 514899):5.
38. Heard I, Tassie JM, Schmitz V, Mandelbrot L, Kazatchkine MD, Orth G. Increased risk of cervical disease among human immunodeficiency virus-infected women with severe immunosuppression and high human papillomavirus load(1). Obstet Gynecol. 2000;96(3):403–9.
39. Castro MM, Farias IP, Borborema-Santos CM, Gomila G, Astolfi-Filho S. Prevalence of human papillomavirus (HPV) type 16 variants and rare HPV types in the central Amazon region. Genet Mol Res. 2011; 10(1):186–96.
40. Fonseca AJ, Taoko D, Chaves TA, Amorim LDDC, Murad RSW, Miranda AE, et al. HPV infection and cervical screening in socially isolated indigenous women inhabitants of the Amazonian rainforest. PLoS One [Internet]. 2015;10(7): e0133635. Available from: http://dx.plos.org/10.1371/journal.pone.0133635.
41. Didelot-Rousseau MN, Nagot N, Costes-Martineau V, Valéis X, Cuedraogo A, Konate I, et al. Human papillomavirus genotype distribution and cervical squamous intraepithelial lesions among high-risk women with and without HIV-1 infection in Burkina Faso. Br J Cancer [Internet]. 2006;95(3):355–62. Available from: http://www.nature.com/doifinder/10.1038/sj.bjc.6603252.
42. Bruni L, Diaz M, Castellsagué X, Ferrer E, Bosch FX, de Sanjosé S. Cervical human papillomavirus prevalence in 5 continents: meta-analysis of 1 million women with normal cytological findings. J Infect Dis [Internet]. 2010 Dec 15 [cited 2013 Oct 18];202(12):1789–1799. Available from: https://academic.oup.com/jid/article/202/12/1789/2193082.
43. Davis AT, Chakraborty H, Flowers L, Mosunjac MB. Cervical dysplasia in women infected with the human immunodeficiency virus (HIV): a correlation with HIV viral load and CD4+ count. Gynecol Oncol [Internet]. 2001;80(3):350–4. Available from: http://www.gynecologiconcology-online.net/article/S0090-8258(00)96104-3/pdf.
44. Kjaer SK, van den Brule AJ, Bock JE, Poll PA, Engholm G, Sherman ME, et al. Human papillomavirus--the most significant risk determinant of cervical intraepithelial neoplasia. Int J Cancer. 1996;65(5):601–6.
45. Palefsky J. Human papillomavirus-related disease in people with HIV. Curr Opin HIV AIDS [Internet]. 2009;4(1):52–6. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2751707&tool=pmcentrez&rendertype=abstract.
46. Krambeck WM, Cadioli RM, Dalmarco EM, CMM DC. HPV Detection and genotyping as an earlier approach in cervical cancer screening of the female genital tract. Clin Exp Obstet Gynecol. 2008;35(3):175–8.

47. Ayres ARG, GA e S. Prevalência de infecção do colo do útero pelo HPV no Brasil: revisão sistemática. Rev Saúde Pública. 2010;44(5):963–74.
48. Da Fonseca AJ, Galvão RS, Miranda AE, LC de L F, Chen Z. Comparison of three human papillomavirus DNA detection methods: next generation sequencing, multiplex PCR and nested-PCR followed by sanger based sequencing. J Med Virol [Internet]. 2015;87(12):1–7. Available from: http://doi.wiley.com/10.1002/jmv.24413.
49. WHO/ICO. Human Papillomavirus and Related Diseases Report. Hum Papillomavirus Relat Dis Rep [Internet]. 2015;74. Available from: www.hpvcentre.net.
50. Eluf-Neto J, Booth M, Muñoz N, Bosch FX, Meijer CJ, Walboomers JM. Human papillomavirus and invasive cervical cancer in Brazil. Br J Cancer [Internet]. 1994;69(1):111–4. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1968795/.
51. Cuschieri K, Geraets DT, Moore C, Quint W, Duvall E, Arbyn M. Clinical and analytical performance of the onclarity HPV assay using the valgent framework. J Clin Microbiol. 2015;53(10):3272–9.
52. Cuzick J, Clavel C, Petry K-U, Meijer CJLM, Hoyer H, Ratnam S, et al. Overview of the European and North American studies on HPV testing in primary cervical cancer screening. Int J cancer [Internet]. 2006 Sep 1 [cited 2013 Oct 18];119(5):1095–1101. Available from: http://onlinelibrary.wiley.com/doi/10.1002/ijc.21955/full.
53. Coupé VMH, Berkhof J, Bulkmans NWJ, Snijders PJF, Meijer CJLM. Age-dependent prevalence of 14 high-risk HPV types in the Netherlands: implications for prophylactic vaccination and screening. Br J Cancer [Internet]. 2008;98(3):646–51. Available from: http://www.nature.com/doifinder/10.1038/sj.bjc.6604162.
54. Winer RL, Lee SK, Hughes JP, Adam DE, Kiviat NB, L A K. Genital human papillomavirus infection: incidence and risk factors in a cohort of female university students. Am J Epidemiol. 2003;157(3):218–26.
55. Manhart LE, Holmes KK, Koutsky L a, Wood TR, Kenney DL, Feng Q, et al. Human papillomavirus infection among sexually active young women in the United States: implications for developing a vaccination strategy. Sex Transm Dis 2006;33(8):502–508.
56. Sanjosé S, Díaz M, Castellsagué X, Clifford G, Bruni L. Worldwide prevalence and genotype distribution of cervical HPV in women with normal cytology: a meta-analysis. Lancet Infect Dis. 2007;7(July):453–9.
57. Smith JS, Melendy A, Rana RK, Pimenta JM. Age-Specific Prevalence of Infection with Human Papillomavirus in Females: A Global Review. J Adolesc Heal. 2008;43(4 SUPPL):
58. Lazcano-Ponce E, Herrero R, Muñoz N, Cruz A, Shah KV, Alonso P, et al. Epidemiology of HPV infection among Mexican women with normal cervical cytology. Int J Cancer [Internet]. 2001;91(3):412–20. Available from: http://onlinelibrary.wiley.com/doi/10.1002/1097-0215(200102)9999:9999<AID-IJC1071>3.0.CO;2-M/full.
59. Castle PE, Schiffman M, Herrero R, Hildesheim A, Rodriguez AC, Bratti MC, et al. A prospective study of age trends in cervical human papillomavirus acquisition and persistence in Guanacaste, Costa Rica. J Infect Dis. 2005;191(11):1808–16.
60. Schiffman M, Herrero R, Desalle R, Hildesheim A, Wacholder S, Rodriguez AC, et al. The carcinogenicity of human papillomavirus types reflects viral evolution. Virology. 2005;337(1):76–84.
61. Bosch FX, Burchell AN, Schiffman M, Giuliano AR, de Sanjose S, Bruni L, et al. Epidemiology and natural history of human papillomavirus infections and type-specific implications in cervical neoplasia. Vaccine. 2008;26(SUPPL. 10):K1–16.
62. Dilcher T, Ngo-Giang-Huong N, Rangdaeng S, Chotivanich N, Limtrakul A, Puttiyunh C, et al. Human papillomavirus infection and cervical lesions in HIV-infected women on antiretroviral treatment in Thailand. J Int Secur. 2017;74(5):501–11.
63. Djigma FW, Ouédraogo C, Karou DS, Sagna T, Bisseye C, Zoba M, et al. Prevalence and genotype characterization of Human Papillomaviruses among HIV-seropositive in Ouagadougou, Burkina Faso. Acta Trop [Internet]. Elsevier B.V. 2011;117(3):203–6. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0001706X10003013.
64. Kahn JA, Burk RD, Anastos K, Massad LS, Minkoff H, Watts DH, et al. Risk of cervical Precancer and cancer and no evidence of oncogenic HPV infection. JAMA. 2013;308(4):362–9.
65. Piper MA, Severin ST, Wilcox SZ, Unger ER, Ghys PD, Miller DL, et al. Association of human papillomavirus with HIV and CD4 cell count in women with high or low numbers of sex partners. Sex Transm Infect. 1999;75(4):253–7.
66. Sun XW, Elsterbrock TV, Lungu O, Chiasson MA, Bush TJ, Wright TC. Human papillomavirus infection in human immunodeficiency virus-seropositive women. Obstet Gynecol. 1995;85(5 I):680–6.
67. Ndil Sudi Tebeila de Souza, Victor Hugo do Melo LPF de C. Diagnóstico da Infecção pelo HPV em Lesões do Colo do Útero em Mulheres HIV +: Acuidade da Histopatologia Pacientes e Métodos. Rev Bras Ginecol Obs. 2001;23(9):555–61.
68. Clifford GM, Franceschi S, Kaiser O, Schoni-Affolter F, Lise M, Dehler S, et al. Immunodeficiency and the risk of cervical intra-epithelial neoplasia 2/3 and cervical cancer: a nested case-control study in the Swiss HIV cohort study. Int J Cancer. 2016;138(7):1732–40.
69. Bonanni P, Boccalini S, Bechini A. Efficacy, duration of immunity and cross protection after HPV vaccination: a review of the evidence. Vaccine. 2009;27(SUPPL. 1):46–53.
70. Riethmuller D, Jacquard A-C, Lacau St Guily J, Aubin F, Carcopino X, Pradat P, et al. Potential impact of a nonavalent HPV vaccine on the occurrence of HPV-related diseases in France. BMC Public Health [Internet]. 2015;15(1):453. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4433025&tool=pmcentrez&rendertype=abstract.
71. Van Damme P, Bonanni P, Bosch FX, Joura E, Krüger Kjaer S, Meijer CJLM, et al. Use of the nonavalent HPV vaccine in individuals previously fully or partially vaccinated with bivalent or quadrivalent HPV vaccines. Vaccine [Internet]. Elsevier Ltd. 2016;34(6):757–61. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0264410X15018745.
72. Van De Velde N, Boily MC, Drolet M, Franco EL, Mayrand MH, Kliewer EV, et al. Population-level impact of the bivalent, quadrivalent, and nonavalent human papillomavirus vaccines: a model-based analysis. J Natl Cancer Inst. 2012;104(22):1712–23.
73. Molano M, Posso H, Weiderpass E, AJC v d B, Ronderos M, Franceschi S, et al. Prevalence and determinants of HPV infection among Colombian women with normal cytology. Br J Cancer. 2002;87:324–33.
74. Winer RL, Hughes JP, Feng Q, O'Reilly S, Kiviat NB, Holmes KK, et al. Condom use and the risk of genital human papillomavirus infection in young women. N Engl J Med [Internet]. 2006;354(25):2645–54. Available from: http://www.nejm.org/doi/full/10.1056/NEJMoa053284.
75. Castellsagué X, Bosch FX, Muñoz N. The male role in cervical cancer. Salud Publica Mex. 2003;45(SUPPL. 3):345–53.

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ANEXO A - PROTOCOLO DE EXTRAÇÃO DNA (RASPADO DÉRMICO), KIT QIAGEN DNeasy Blood & Tissue

Preparo da amostra:

1- Centrifugar a amostra de Raspado Dérmico por 10 minutos a 13.300 RPM. Caso não apareça o *pellet*, centrifugar mais 5 minutos a 13.300 RPM;

2- Descartar o sobrenadante com cuidado para não perder o *pellet*. Deixar o tubo invertido por 10 minutos ou banho seco a 38^0C por 15 minutos. Se permanecer resto de álcool, deixar secando até sua COMPLETA EVAPORAÇÃO;

Reação de lise:

3- Pipetar 200 µl de buffer de lise (buffer AL, QIAGEN). Vortexar 15 segundos para soltar o *pellet*;

4- Pipetar 10 µl de Proteinase K;

5- Incubar a 56° C durante 3 a 5 horas. Vortexar a cada 30 minutos;

6- Pipetar 200 µl de etanol absoluto (96-100%) e fazer um spin;

Extração de DNA:

7- Pipetar (P1000) a mistura na coluna de extração QIAGEN e adicionar um tubo coletor.

8- Centrifugar a coluna com o tudo coletor a 8000 RPM por 1 minuto;

9- Descartar o tubo coletor com seu conteúdo;

10- Colocar a coluna em um novo tubo coletor e adicionar 500 µl de buffer AW1;

11- Centrifugar por 1 minuto a 8000 RPM;

12- Descartar o tubo coletor com seu conteúdo;

13- Colocar a coluna em um novo tubo e adicionar 500 µl de buffer AW2 (QIAGEN);

14- Centrifugar a 14000 RPM por 3 minutos para secar a membrana;

15- Descartar o tubo coletor.

Eluição do DNA:

16- Colocar a coluna em tubo coletor de 1,5 ml;

17- Adicionar **50 µl** de buffer AE na membrana;

18- Incubar 2 minutos a temperatura ambiente;

19- Centrifugar por 2 minutos a 8000 RPM para eluir o DNA;

20- Armazenar o DNA extraído a -20° C

ANEXO B - PROTOCOLO DE EXTRAÇÃO DNA (SANGUE TOTAL), KIT QIAGEN DNeasy Blood & Tissue

Reação de lise:

1.Pipetar 200 µL de sangue anticoagulado + 10 µL proteinase K dentro de um tubo de 1.5 mL. Ajustar o volume para 210 µl com PBS;

2. Adicionar 200 µL de tampão AL, misturar completamente por vortexação e incubar a 56°C por 10 min.

3. Adicionar 200 µL etanol (96–100%) na amostra e misturar completamente por vortexação.

Extração de DNA:

4. Pipetar a mistura da etapa 3 para a coluna Mini Spin DNeasy e encaixar um tubo coletor de 2 mL. Centrifugar a 8000 RPM por 1 min. Descartar o líquido drenado e o tubo coletor.

5. Colocar um novo tubo coletor de 2 mL na coluna Mini Spin DNeasy, adicionar 500 µL tampão AW1, e centrifugar por 1 min a 8.000 rpm. Descartar o líquido drenado e o tubo coletor.

6. Colocar um novo tubo coletor de 2 mL na coluna Mini Spin DNeasy, adicionar 500 µL tampão AW2, e centrifugar por 3 min a 14000 rpm para secar a membrana. Descartar o líquido drenado e o tubo coletor. Caso verifique que a membrana não esteja bem seca, faça outra centrifugação por 1 min a 14.000 rpm.

Eluição do DNA:

7. Coloque um microtubo limpo de 1.5 mL ou 2 mL na coluna de DNeasy Mini spin e pipete 75 µl Tampão AE diretamente na membrana do DNeasy. Incubar a temperatura ambiente por 1 min, e então centrifugar por 1 min a 8.000 rpm para eluir.

8. Recomendação: Para uma máxima concentração de DNA, repetir a eluição uma vez mais como descrito na etapa 7, isto é, eluindo mais 75 µL Tampão AE diretamente na membrana do DNeasy no mesmo tubo.

ANEXO C - PARECER CONSUBSTANCIADO DO COMITÊ DE ÉTICA EM PESQUISA

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PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: MARCADORES MOLECULARES, GENÉTICOS E SOROLÓGICOS NA HANSENÍASE: SUPORTE AO DIAGNÓSTICO CLÍNICO DE PACIENTES E VIGILÂNCIA DOS CONTATOS.

Pesquisador: André Luiz Leturiondo

Área Temática:

Versão: 2

CAAE: 27219514.3.0000.0002

Instituição Proponente: Fundação Alfredo da Matta

Patrocinador Principal: Fundação de Amparo à Pesquisa do Estado do Amazonas - FAPEAM

DADOS DO PARECER

Número do Parecer: 555.620

Data da Relatoria: 13/03/2014

Apresentação do Projeto:

Trata-se de Estudo de Coorte com seguimento de 2 anos, utilizando marcadores genéticos em casos novos de hanseníase para avaliar o risco de desenvolver episódios reacionais e seus contatos (estimativa de 1200) utilizando marcadores moleculares, genéticos e sorológicos para verificar o risco de adoecer.

O estudo incluira 150 pacientes em tratamento para servirem como controle positivo das reações de q-PCR e LID-1 para validação dos testes.

Objetivo da Pesquisa:

Objetivos Primários e Secundários:

1. Avaliar a introdução da tecnologia de qPCR para a identificação de DNA de Mycobacterium leprae em contatos, como ferramenta de atenção básica em saúde, visando a detecção precoce de hanseníase, bem como a suscetibilidade para a doença com o uso de marcadores sorológicos e genéticos (SNPs) que sejam capazes de definir estimativas de risco de adoecimento;

1.1- Adequar os métodos baseados na extração de DNA de amostras clínicas de pacientes e contatos domiciliares (raspado dérmico) destes, para sistemas comerciais implementando na rotina ambulatorial para monitoramento, vigilância de pacientes e seus contatos domiciliares;

1.2- Avaliar em uma população de pelo menos 600 contatos por ano (1200 indivíduos em 24

meses) a positividade de DNA de M. leprae por PCR em tempo real a partir do alvo 16S na tentativa de incrementar o diagnóstico precoce de hanseníase; Um grupo de pacientes paucibacilares e multibacilares será arrolado como controles positivos visando à validação do teste;

1.3- Relacionar a carga bacilar dos contatos de primeira vez pela técnica molecular (qPCR) com a evolução ou não para a doença após dois anos de acompanhamento;

1.4- Validar o teste sorológico LID-1 com os pacientespaucibacilares e multibacilares de primeira vez;

1.5- Verificar a soropositividade do teste sorológico (LID-1) entre os contatos. Um grupo de pacientes paucibacilares e multibacilares será arrolado como controles positivos visando à validação do teste;

1.6- Associar os resultados dos testes qPCR e soropositividade da LID-1 com dados clínico-laboratoriais (forma clínica do caso índice), status vacinal (BCG) e indicadores sociodemográficos após dois anos da primeira consulta;

2. Avaliar marcadores genéticos (SNPs) em um grupo de pacientes acompanhados por 2 anos para estimar risco de desenvolvimento de episódios reacionais.

2.2- Relacionar os polimorfismos de base única (SNPs) nos genes TLR1, NOD2 e PARK2/PACRG com os estados reacionais da hanseníase;

Avaliação dos Riscos e Benefícios:

- Riscos: É mínimo o risco associado a sua participação neste estudo. O único desconforto será uma leve dor durante o raspado dérmico no lóbulo auricular, discreta dor no dedo indicador(punção digital) ou durante a coleta de sangue.
- Benefícios: A participação neste estudo trará benefícios indiretos ao indivíduo, pois caso os resultados forem satisfatórios, será implantado os testes diagnósticos em estudo, na rotina da Fundação, beneficiando todos os clientes atendidos pelo SUS com suspeita de estarem com hanseníase e seus respectivos contatos.

Comentários e Considerações sobre a Pesquisa:

Considerações sobre algumas questões metodológicas:

- Formulário "Informações Básicas" refere 200 pacientes e 1000 contatos, mas o Projeto Básico cita e justifica 1200 contatos, sendo 600 por ano (calculando 4 contatos para cada caso), que eh o que esta sendo considerado, tendo previsto, então cerca de 150 casos novos de hanseníase por ano (detecção anual na Fundação Alfredo da Matta) ou 300 no estudo.
- Coleta de material biológico do 2o. ao 24o. mês do cronograma do projeto que sera executado

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no período de março de 2014 a dezembro de 2017.

- O armazenamento de material biológico esta sendo solicitado nos TCLEs para futuros estudos mediante aprovação do CEP, estando previsto armazenamento em Biobanco, contudo, na Fundação Alfredo da Matta (FUAM)até o presente não existe um Biobanco credenciado.

- Os dados e instrumentos utilizados na pesquisa ficarão arquivados com o pesquisador responsável por um período de 5 anos

Considerações sobre os Termos de apresentação obrigatória:

- Foi atendida a pendência da assinatura do patrocinador, FAPEAM, na Folha de Rosto e apresentado documento da mesma instituição que aprova o projeto para chamada pública no. 001\2013 PPSUS com o orçamento total de R$ 133.806,00, em anexo.

Recomendações:

- Apresentar relatórios parciais, a cada seis meses, ao CEP-FUAM conforme previsto na resolução 466/2012 de diretrizes e normas regulamentadoras das pesquisas envolvendo seres humanos

- As amostras de material biológico coletadas deverão ser desprezadas caso, ao término da pesquisa, a FUAM não tenha constituído um BIOBANCO, devidamente regulamentado e credenciado de acordo com as normas vigentes.

Conclusões ou Pendências e Lista de Inadequações:

Projeto APROVADO deverá seguir as orientações de envio de relatórios parciais e constituição de Biobanco.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

- Enviar relatórios semestrais ao CEP_FUAM demonstrando fatos relevantes e resultados parciais do desenvolvimento da pesquisa.

- Desprezar as amostras biológicas ao término da pesquisa caso a FUAM não tenha constituído e cadastrado um Biobanco.

Endereço: Rua Codajás,24
Bairro: Cachoeirinha **CEP:** 69.065-130
UF: AM **Município:** MANAUS
Telefone: (92)3212-8303 **Fax:** (92)3212-8302 **E-mail:** cep@fuam.am.gov.br

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Continuação do Parecer: 555.620

MANAUS, 13 de Março de 2014

Assinador por:
LÚCIO FIGUEIRA PIMENTEL
(Coordenador)

Endereço: Rua Codajás,24
Bairro: Cachoeirinha **CEP:** 69.065-130
UF: AM **Município:** MANAUS
Telefone: (92)3212-8303 **Fax:** (92)3212-8302 **E-mail:** cep@fuam.am.gov.br

ANEXO D - DECISÃO DO CONSELHO DIRETOR DA FAPEAM DO PROJETO APROVADO

GOVERNO DO ESTADO DO AMAZONAS

CONSELHO DIRETOR – DECISÃO 287/2013 – ANEXO ÚNICO

Programa Pesquisa para o SUS: Gestão Compartilhada em Saúde - PPSUS, Chamada Pública FAPEAM/SUSAM-SES-AM/MS/CNPq n.º 001/2013

	PROPONENTE	INSTITUIÇÃO DE VÍNCULO	TÍTULO DO PROJETO	DURAÇÃO DO PROJETO	BOLSAS					AUXÍLIO-PESQUISA			TOTAL (Aux.Pesq+Bolsas)
					MOD.	VALOR UNIT.	QTD.	MESES	TOTAL	CUSTEIO	CAPITAL	TOTAL	
1	Felipe Arley Costa Pessoa*	FIOCRUZ	Mansonelose em área urbana do município de São Gabriel da Cachoeira, Amazonas, Brasil.	24 meses	DCTA/C	R$ 1.234,00	1	24	R$ 29.616,00	R$ 109.302,00	R$ 53.500,00	R$ 162.802,00	R$ 192.418,00
2	André Luiz Leturiondo*	FUAM	Marcadores Moleculares, Genéticos e Sorológicos na Hanseníase: Suporte ao Diagnóstico Clínico de Pacientes e Vigilância dos Contatos	24 meses	DCTA/C	R$ 1.234,00	1	24	R$ 29.616,00	R$ 80.080,00	R$ 24.110,00	R$ 104.190,00	R$ 133.806,00
3	Felipe Gomes Naveca	FIOCRUZ	Epidemiologia molecular da Dengue no Estado do Amazonas: Filogeografia e fatores associados à falha na detecção sorológica do antígeno NS1.	24 meses	DCTA/C	R$ 1.234,00	1	24	R$ 29.616,00	R$ 109.939,50	R$ 60.000,00	R$ 169.939,50	R$ 199.555,50
4	Alex Panizza Jalkh*	FMT-HVD	Implantação de um Programa no SUS de Diagnóstico Rápido para Controle da Lobomicose.	24 meses	DCTA/C	R$ 1.234,00	1	24	R$ 29.616,00	R$ 66.945,00	R$ 29.250,00	R$ 96.195,00	R$ 125.811,00
5	Maurício Morishi Ogusku*	INPA	Testes rápidos para avaliação da resistência de Mycobacterium tuberculosis à Isoniazida e Rifampicina, a partir de amostras de escarros multibacilares de pacientes com Tuberculose pulmonar.	24 meses	DCTA/C	R$ 1.234,00	1	24	R$ 29.616,00	R$ 110.384,00	R$ 60.000,00	R$ 170.384,00	R$ 200.000,00
6	Celsa da Silva Moura Souza*	UFAM	Suplementação de Cálcio em baixa dose para prevenção de Pré-Eclampsia: Ensaio Clínico Randomizado	24 meses	DCTA/C	R$ 1.234,00	1	17	R$ 20.978,00	R$ 82.153,80	R$ 32.320,00	R$ 114.473,80	R$ 135.451,80
7	Wuelton Marcelo Monteiro*	FMT-HVD	Ivermectina para reduzir a transmissão de malária: avaliação de uma ferramenta inovadora para a Amazônia Brasileira (IVERMAL)	24 meses	DCTA/C	R$ 1.234,00	1	24	R$ 29.616,00	R$ 124.100,00	R$ 17.850,00	R$ 141.950,00	R$ 171.566,00
8	José Pereira de Moura Neto*	UFAM	Estudo das Hemoglobinopatias Estruturais e de Síntese na Região Metropolitana de Manaus-Amazonas	24 meses	–	–	–	–	–	R$ 117.311,00	R$ 27.770,00	R$ 145.081,00	R$ 145.081,00
9	Pritessh Jaychand Lalwani*	UFAM	Soroprevalência para Hantavirus em humanos e animais do Estado do Amazonas	24 meses	DCTA/C	R$ 1.234,00	1	24	R$ 29.616,00	R$ 133.840,00	R$ 27.000,00	R$ 160.840,00	R$ 190.456,00
10	Gisely Cardoso de Melo*	UEA	Busca de biomarcadores para detecção de resistência clínica a cloroquina em pacientes com malária por P. Vivax	24 meses	DCTA/C	R$ 1.234,00	1	24	R$ 29.616,00	R$ 78.000,00	R$ 2.500,00	R$ 80.500,00	R$ 110.116,00
11	Lucivana Prata de Souza *	UEA	Análise do gene FMR1 e ocorrência da Síndrome do X-frágil em pessoas com Transtorno do Espectro Autista.	24 meses	DCTA/C	R$ 1.234,00	1	24	R$ 29.616,00	R$ 83.862,00	R$ 35.780,00	R$ 119.642,00	R$ 149.258,00
12	Ormezinda Celeste Cristo Fernandes*	FIOCRUZ	Proteases Microbianas: Produção, Propriedades e Aplicação Biotecnológica à Saúde	24 meses	DCTA/C	R$ 1.234,00	1	24	R$ 29.616,00	R$ 57.301,65	R$ 60.000,00	R$ 117.301,65	R$ 146.917,65
13	Antônio Gelson de Oliveira Nascimento*	UEA	O impacto das mortes prematuras por violência sobre a expectativa de vida da população do Estado do Amazonas, 1991 - 2010	24 meses	DCTA/C	R$ 1.234,00	1	24	R$ 29.616,00	R$ 68.330,00	R$ 21.000,00	R$ 89.330,00	R$ 118.946,00
14	Patrícia Puccinelli Orlandi Nogueira*	FIOCRUZ	Identificação dos Fatores de Virulência associados a Shigelose severa em Modelo Murino.	24 meses	DCTA/C	R$ 1.234,00	1	24	R$ 29.616,00	R$ 99.110,49	R$ 53.100,00	R$ 152.210,49	R$ 181.826,49