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PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINATROPICAL
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FARMACOCINÉTICA DE TUBERCULOSTÁTICOS DE PRIMEIRA LINHA EM PACIENTES CRÍTICOS

FRANCISCO BERALDI DE MAGALHÃES

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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 grau de *Doutor em Doenças Tropicais e Infecciosas*

Orientador: **Prof.** Dr. Marcelo Cordeiro dos Santos

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FRANCISCO BERALDI DE MAGALHÃES

"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".

Banca Julgadora:

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Prof. Dr. Marcelo Cordeiro dos Santos
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Prof. Dr. Afrânio Lineu Kritski
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Prof.ª Dra. Anna Cristina Calçada Carvallho
Fiocruz – RJ

A todos os pacientes e seus familiares que sofrem com a tuberculose ao redor do mundo.

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Todas as pessoas que me conhecem sabem o tamanho do significado que esse trabalho tem para mim e para a minha vida. Sem dúvida nenhuma, essa não é uma conquista individual e sim de muitas pessoas que estiveram ao meu lado ao longo dos meus 34 anos.

Na matemática, existem três noções primitivas: o ponto, a reta e o plano. Esses objetos não possuem definição, mas precisam existir para dar base às demais definições geométricas. Da mesma forma, os conceitos de pai e mãe podem ser facilmente definidos como genitor ou genitora de um indivíduo. Entretanto, o exercício diário da paternidade e da maternidade são muito maiores do que isso. Acredito que pai e mãe são noções primitivas, como o ponto, a reta e o plano. Não possuem definição, mas são a base para o nosso conceito de pessoa e o significado do universo em que habitamos. Agradeço ao meu pai, Fábio Januário de Magalhães, que sempre me apoiou, criticou, ensinou e buscou fazer de mim uma pessoa melhor a cada dia. Ao longo dos 33 anos e 3 meses em que convivemos neste mundo, meu pai despertou na minha criança a paixão pela ciência, pelos fenômenos da natureza e pela tentativa de explicá-los. Agradeço à minha mãe, Sonia Maria do Rocio Beraldi de Magalhães, que me ensina diariamente a importância do olhar para o próximo, da dignidade humana e da ética. Mostra-me, pelo exemplo, que o trabalho é fundamental para a transformação da sociedade, que o reconhecimento não é o objetivo, mas a consequência natural de um trabalho dedicado à melhoria da sociedade e de todos os que nela vivem. Ambos serão sempre minhas noções primitivas, impossíveis de serem definidos, mas fundamentais e imprescindíveis na minha existência.

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Minha vida não estaria completa se, em 2014, não tivesse conhecido aquela com o maior poder transformador na minha vida. Aproximadamente dois meses depois da pergunta que esta tese busca responder ter sido formulada na minha mente, conheci aquela com quem minha alma se conectou desde o primeiro instante. Tornou-se paixão, namorada, noiva, esposa e, por fim, mãe da nossa pequena Manuela, quem ainda carrega no ventre. Agradeço à minha esposa, sócia e parceira, Raquel Monteiro de Moraes Magalhães que, mais de perto do que qualquer um, esteve comigo nas glórias e decepções, saltos e tropeços, risos e choros. Raquel faz de mim um ser humano melhor a cada dia. Não me deixou desistir frente aos piores desafios e me apoiou, incondicionalmente, em toda a construção deste e de outros projetos. Não posso deixar também de agradecer ao Darwin, não o Charles Darwin que certamente merece todos os agradecimentos por lançar as bases de praticamente tudo o que fazemos na infectologia, mas ao cão Darwin, nosso pequeno *dachshund* que, pacientemente, participou de vários cursos, aulas, seminários e apresentações sobre tuberculose nos últimos anos e conseguiu me trazer alegria nos momentos mais tristes que precisei atravessar.

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Acredito que a construção do saber é um processo contínuo, que a ampliação de conhecimentos gerais é a base para podermos nos aprofundar em uma determinada área de maneira questionadora. A informação e o desenvolvimento de raciocínio crítico são as chaves para a produção de mais conhecimento. Agradeço a todos os meus professores de ensino infantil, fundamental e médio que me ensinaram e me inspiraram ao longo da minha formação. Aos Irmãos Maristas, que se mantêm fiéis à missão proposta por São Marcelino Champagnat: “ser farol que orienta e promove a vivência dos valores do Evangelho, do jeito de Maria, contribuindo para a formação de cidadãos éticos e solidários para a transformação da sociedade, com foco nos direitos de crianças e jovens”. Ao Colégio Marista Santa Maria, onde estudei por 13 anos e construí as bases que possibilitaram a minha formação como cidadão e cognição para ingressar no ensino superior.

Essa base possibilitou minha entrada no curso de Medicina da primeira universidade federal do Brasil, a Universidade Federal do Paraná (UFPR), instituição pela qual tenho uma gratidão imensurável e um carinho único. Agradeço imensamente à instituição e seus servidores e aos professores que guiaram minha formação médica. Foi por inspiração deles e por meio deles que escolhi a infectologia como especialidade.

Foi durante a residência médica em infectologia no, hoje, Complexo do Hospital de Clínicas da Universidade Federal do Paraná (CHC-UFPR), que desenvolvi o olhar crítico e comecei a formular questionamentos cujas respostas dependiam do método científico. Em 2014, no segundo ano de residência, atendendo a um paciente jovem com tuberculose anérgica, clinicamente grave e com contexto social complexo, o universo das micobactérias abriu-se para mim. Foi com esse paciente que questionei a conduta convencional. Com uma abordagem diferente, obtive sucesso no tratamento e proporcionei a cura desse jovem, que hoje leva uma vida normal. Surgiu assim a dúvida clínica que me motivou a buscar respostas e desenvolver o projeto apresentado nesta tese. Agradeço a esse paciente, que me deu a oportunidade de lhe prestar assistência e confiou nas propostas de tratamento e no trabalho da equipe assistente. Sem dúvida nenhuma, nada disso seria possível sem o auxílio dos meus preceptores. Agradeço especialmente ao Dr. João César Beenke França, que me ensinou algo muito mais complexo do que doenças ou tratamentos – o raciocínio clínico. Com ele aprendi a investigar minuciosamente cada sintoma, cada queixa,

cada exame e correlacioná-los para buscar um diagnóstico. Aprendi a buscar, incessantemente, respostas na literatura para os questionamentos do dia a dia. Foi com o Dr. João que atendi o paciente a quem me referi anteriormente e foi com ele que formulei a dúvida clínica a ser respondida nesta tese. Agradeço, também, ao Dr. Maurício de Carvalho e à Dra. Mônica Maria Gomes da Silva pela participação fundamental na minha vida e na minha formação médica.

Ainda na residência, tive meu primeiro contato com as doenças tropicais em um estágio em Manaus, na Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (FMT-HVD), uma instituição vocacionada para ensino e pesquisa. Nesse contexto, agradeço ao Dr. Marcus Barros, médico e pesquisador que em curto intervalo de tempo me ensinou muito sobre medicina tropical, antropologia e aspectos culturais da região amazônica. Foi ele que, mais tarde, me colocaria em contato com o meu orientador, o que viabilizou este projeto.

Interessado nos estudos de sepse, nos avanços da farmacocinética e fascinado com o estudo *Defining Antibiotic Levels in Intensive Care Unit Patients* (DALI), busquei um estágio na unidade de terapia intensiva do Royal Brisbane and Women's Hospital em Queensland, na Austrália. Foi o Professor Dr. Jeffrey Lipman, idealizador do estudo DALI, que me concedeu o estágio, foi meu tutor e muito me ensinou sobre o manejo de pacientes críticos e otimização do uso de antimicrobianos. Quando conversamos sobre este projeto, me auxiliou no raciocínio metodológico e me colocou em contato com pesquisadores magníficos para a execução. Também por ele, tive contato com o Professor Dr. Jason Roberts que, durante um café, ouviu atentamente a minha ideia e disse: *"This is new, this is game changer, we need to go for it!"*. Ambos foram, e continuam sendo, meus grandes inspiradores. Agradeço aos dois, em especial ao Prof. Dr. Jason Roberts, pelo apoio técnico, dedicação e pela simplicidade e modéstia com que sempre me atendeu e auxiliou.

Conhecendo a vocação de pesquisa da FMT-HVD e a alta incidência de tuberculose na região amazônica, ficou fácil entender que o caminho para o projeto seria Manaus. Busquei o Dr. Marcus Barros, que indicou meu orientador, Professor Dr. Marcelo Cordeiro dos Santos. Conversamos em uma mesa de almoço no XIX Congresso Brasileiro de Infectologia em 2015. Apertamos as mãos e iniciamos esta jornada. Sou imensamente grato ao Professor Marcelo pela oportunidade que me deu na pós-

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O estudo envolveu diversas coletas de amostras de cada paciente. Para isso, era fundamental que alguém hábil em punção venosa fizesse os acessos periféricos e auxiliasse nas coletas. Nesse contexto, Leandro de Sousa Garcia, técnico de enfermagem da UTI da FMT-HVD e aluno do 4º período de enfermagem, estava em busca de iniciação científica e acreditou na ideia do projeto. Agradeço ao, agora enfermeiro, Leandro pela dedicação, comprometimento, honestidade e lealdade. Por ter se entregado completamente a este projeto, estando ao meu lado em absolutamente todos os momentos.

Passada a etapa das coletas de amostras dos pacientes, era necessário realizar as dosagens dos fármacos. Nesse momento, busquei um antigo conhecido do Departamento de Farmácia da UFPR, o Professor Dr. Roberto Pontarolo. O professor Pontarolo colocou à disposição o seu laboratório, uma de suas alunas de doutorado, a farmacêutica Mariana Millan Fachi, que junto com o Professor Dr. Marcus Vinicius de Liz, do Departamento de Química e Biologia da UTFPR, trabalharam dias e noites para conseguir realizar a aferição dos quatro fármacos em cada uma das 540 amostras analisadas. Sem a entrega, competência e solidariedade desses pesquisadores esse trabalho não teria sido executado.

De posse desses dados, era necessário realizar as análises matemáticas e construir os modelos farmacocinéticos, um tema até então novo e desconhecido para mim. Voltei para a Austrália, dessa vez no Centro de Pesquisa Clínica da Universidade de Queensland, para trabalhar nessas análises junto com o Professor Dr. Jason Roberts e a Dra. Suzanne Parker. Essa, provavelmente, foi a etapa tecnicamente mais difícil de ser executada. As dificuldades só foram vencidas graças à dedicação da Professora Dra. Cristina Sanches, do Departamento de Farmácia da UFSJ. Fui apresentado à professora Cristina pelo próprio professor Jason em 2015 e, a partir daí, dialogamos, trocamos ideias e colaboramos um com o outro, mesmo tendo nos visto apenas uma vez. Farmacêutica, com vasta experiência em farmacocinética e modelagem matemática, a professora Cristina e a Dra. Suzanne foram as grandes responsáveis por encontrar as saídas para os entraves do modelo matemático. Foram meses de dedicação, reuniões e conversas para as soluções do modelo e depois para a redação do artigo. A professora Cristina e a Dra. Suzanne foram os anjos da guarda deste projeto. Incansáveis, generosas, dedicadas e compreensivas. Jamais conseguirei retribuir o que fizeram para que este sonho fosse realizado.

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Galileu Galilei

RESUMO

Muito embora a tuberculose seja uma doença tratável e potencialmente curável, ainda é uma das maiores causas de mortalidade no mundo. Estima-se que entre 3% e 16% dos pacientes com tuberculose necessitarão de cuidados em uma unidade de terapia intensiva (UTI) devido a sepse causada pelo *Mycobacterium tuberculosis*. A letalidade dos pacientes que requerem cuidados intensivos chega a 78%, enquanto os pacientes em tratamento ambulatorial apresentam 85% de sucesso terapêutico utilizando os mesmos fármacos nas mesmas doses. Concentrações subterapêuticas de fármacos são uma provável causa da elevada letalidade. Dessa forma, este estudo tem como objetivo comparar a farmacocinética dos tuberculostáticos de primeira linha em pacientes em UTI com pacientes em tratamento ambulatorial e estabelecer se as doses atuais utilizadas atingem concentrações terapêuticas. Este é um estudo farmacocinético, prospectivo e aberto realizado na Fundação de Medicina Tropical Dr. Heitor Vieira Dourado, em Manaus, Amazonas, Brasil. Os pacientes estavam em uso de rifampicina, isoniazida, pirazinamida e etambutol, em comprimidos de dose fixa combinada conforme o peso. Analisaram-se como covariáveis, dados clínicos, sociodemográficos demográficos e os escores de gravidade SOFA e APACHE II. Todos os pacientes em UTI estavam em ventilação mecânica e receberam as medicações por sonda nasogástrica. Para o etambutol, foi desenvolvido um modelo farmacocinético populacional com alvos PK/PD a uma AUC/MIC maior que 11,9 mg.h/L e uma C_{max}/MIC maior que 0,48 mg/L. Para rifampicina, isoniazida e pirazinamida, foi realizada uma análise não compartimental comparando os parâmetros farmacocinéticos nos dois grupos. Nem os pacientes em UTI, nem os pacientes em tratamento ambulatorial atingiram as concentrações-alvo no sangue. A farmacocinética do etambutol é melhor descrita usando um modelo bicompartimental com uma absorção oral de primeira ordem. Os pacientes de UTI apresentaram uma taxa de absorção duas vezes maior ($P<0,05$) e a mediana de biodisponibilidade cinco vezes maior ($P<0,0001$) do que os pacientes ambulatoriais. Além disso, o clearance e o volume de distribuição foram, respectivamente, 93% e 53% menores no grupo de pacientes em terapia intensiva. Na análise não compartimental da isoniazida, não foi observada diferença entre os grupos. Já a rifampicina apresentou AUC/MIC 80% maior no grupo UTI. A pirazinamida apresentou clearance duas vezes e meia maior, T_{max} duas vezes maior e volume de distribuição duas vezes maior nos pacientes ambulatoriais. Observou-se diferença na farmacocinética da rifampicina, pirazinamida e etambutol entre pacientes admitidos em UTI e pacientes ambulatoriais. Muito embora essas diferenças garantam maior concentração de rifampicina e etambutol nos pacientes em UTI, em nenhum dos grupos os pacientes atingiram concentrações terapêuticas para os fármacos utilizados.

Palavras-chave: Antituberculosos, Tuberculose, Farmacocinética, Disponibilidade Biológica, Unidades de Terapia Intensiva, Cuidados Críticos.

ABSTRACT

Despite being a treatable and curable disease, tuberculosis is a leading cause of infectious diseases related deaths worldwide. It is estimated that 3–16% of TB patients will require admission to an intensive care unit (ICU) due to sepsis caused by *Mycobacterium tuberculosis*. While treatment success rates for outpatients reaches 85%, lethality rates in ICU could reach 78%. Subtherapeutic PK/PD index may be a cause of poor outcomes. The aim of this study was to compare the pharmacokinetics of first-line anti-tuberculosis drugs of patients with TB admitted to the ICU to outpatients and to establish whether contemporary dosing regimens using fixed-dose combination tablets achieved therapeutic exposures. A prospective population pharmacokinetic study of ethambutol was performed at Fundação de Medicina Tropical Dr. Heitor Vieira Dourado in Amazonas State, Brazil. Every patient was in directly observed treatment receiving a weight-based dose of rifampin, isoniazid, pyrazinamide, and ethambutol as fixed-dose-combination. For ethambutol, a population pharmacokinetic model was developed. Probability of target attainment was determined using AUC/MIC > 11.9 and C_{max}/MIC > 0.48 values. Optimized dosing regimens were simulated at steady state. Ethambutol pharmacokinetics were best described using a two-compartment model with first-order oral absorption. Neither ICU patients nor outpatients consistently achieved optimal ethambutol exposures. The absorption rate for ethambutol was 2-times higher in ICU patients ($p < 0.05$). Mean bioavailability for ICU patients was >5-times higher than outpatients ($p < 0.0001$). Clearance and volume of distribution were 93% ($p < 0.0001$) and 53% ($p = 0.002$) lower in ICU patients, respectively. No difference was observed between groups for isoniazid PK in a non-compartmental analysis. On the other hand, AUC/MIC of rifampin was 80% higher in ICU patients. Pyrazinamide presented more than two times higher clearance, half-life and T_{max} in outpatients. ICU patients displayed significantly different pharmacokinetics for an oral fixed-dose combination administration of rifampin, pyrazinamide and ethambutol compared to outpatients. Despite those differences granted higher concentrations in ICU patients, neither patient group consistently achieved pre-defined therapeutic exposures.

Keywords: Antitubercular Agents, Tuberculosis, Pharmacokinetics, Biological Availability, Intensive Care Units, Critical Care.

RESUMO LEIGO

A tuberculose é uma doença infecciosa que afeta principalmente os pulmões. Muito embora a maioria das pessoas a veja como uma doença do passado e que não existe mais, a tuberculose mata, em média, 1,5 milhão de pessoas ao redor do mundo. Dentre as doenças infecciosas perdeu apenas para a COVID-19 durante os anos de 2020 e 2021 como principal causa de morte. Entretanto, a doença pode ser tratada com uma combinação de quatro antibióticos por pelo menos seis meses: rifampicina, isoniazida, pirazinamida e etambutol. Para facilitar a tomada da medicação, esses antibióticos vêm combinados em um único comprimido e, de acordo com o peso do paciente, são utilizados de dois a cinco comprimidos por dia. Quando tratados corretamente, nove de cada dez pacientes se curam. Apesar disso, 9 em cada 100 doentes com tuberculose precisam ser internados em UTI e, quando isso ocorre, 8 em cada 10 acabam morrendo, apesar do tratamento. Pacientes em UTI não conseguem se alimentar e precisam da ajuda de uma sonda nasogástrica, um tubo que permite que suplementos alimentares e medicamentos sejam administrados diretamente no estômago do paciente. Dessa forma, os comprimidos também precisam ser macerados e passados pela sonda. Todavia, quando a condição de um paciente com tuberculose ou com qualquer outra infecção se agrava, os órgãos passam a ter dificuldade para funcionar. O sangue tem dificuldade para circular, o coração para bater, o pulmão para respirar e o intestino para absorver nutrientes e medicamentos. Assim, é possível que os antibióticos não atinjam a concentração necessária no sangue dos pacientes em UTI, impedindo o tratamento adequado da doença e causando o óbito. Neste estudo, analisou-se a concentração no sangue de rifampicina, isoniazida, pirazinamida e etambutol nos pacientes da UTI e comparou-se com os pacientes em tratamento ambulatorial. Os resultados mostraram que em nenhum dos grupos os pacientes atingiram concentrações sanguíneas necessárias para eliminar o bacilo causador da tuberculose. Foram observadas diferenças entre os dois grupos para três dos quatro antibióticos utilizados, rifampicina, pirazinamida e etambutol. Observou-se que a rifampicina e o etambutol no sangue dos pacientes em UTI atinge concentrações maiores do que nos pacientes ambulatoriais. No caso da pirazinamida, os parâmetros parecem favorecer os pacientes ambulatoriais. Apesar disso, 94% dos pacientes em tratamento ambulatorial apresentaram cura da doença, enquanto 77% dos pacientes internados em UTI morreram.

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LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES DE MEDIDA

AIDS	Síndrome da Imunodeficiência Adquirida
APACHE II	Acute Physiological and Chronic Health Evaluation II
ATP	Adenosina Trifosfato
AUC/MIC	Área Abaixo da Curva Sobre a MIC
AUC	Área Abaixo da Curva
BAAR	Bacilo Álcool-Ácido Resistente
BCG	Bacilo de Calmette-Guérin
BK	Bacilo de Koch
C	Concentração do fármaco
CL	Clearance
C_{max}/MIC	Concentração Máxima sobre a MIC
C_{max}	Concentração máxima
CR	Receptores do Complemento
CrCl	Clearance de Creatinina
DC-SIGN	*Dendritic Cell-specific Intercelular Adhesion Molecule-3-grabbing Nonintegrin*
DFC	Dose Fixa Combinada
dL	Decilitros
DTG	Dolutegravir
E	Etambutol
EFV	Efavirenz
EUCAST	Comitê Europeu de Testes de Susceptibilidade Antimicrobiana
F	Biodisponibilidade
FMT-HVD	Fundação de Medicina Tropical Dr. Heitor Vieira Dourado
FTA	*Fractional Target Attainment*
g	Grama
h	Hora
H	Isoniazida
HIV	Vírus da Imunodeficiência Humana
IDH	Índice de Desenvolvimento Humano
IL-10	Interleucina-10
IL-12	Interleucina-12

IL-18	Interleucina-18
ILTB	Infecção Latente por Tuberculose
IMC	Índice de Massa Corporal
INF-γ	Interferon-γ
IP	Inibidores de Protease
ITRNN	Inibidores de Transcriptase Reversa Não Nucleosídeos
irpm	Incursões Respiratórias Por Minuto
Ka	Constante de Absorção
Kcp	Constante de distribuição do compartimento central para o periférico
Ke	Constante de Eliminação
Kpc	Constante de distribuição do compartimento periférico para o central
L	Litro
LAM	Lipoarabinomanana
mg	Miligrama
MIC	Concentração Inibitória Mínima
min	Minuto
mL	Mililitro
mmHg	Milímetro de Mercúrio
mmol	Millimolar
Mtb	*Mycobacterium tuberculosis*
MTBC	Complexo *Mycobacterium tuberculosis*
NPAG	Grade Adaptativa Não Paramétrica
O_2	Oxigênio
OFV	Função Objetivo
OMS	Organização Mundial da Saúde
PaO_2	Pressão Arterial de Oxigênio
PAS	Ácido Para-Aminossalicílico
PD	Farmacodinâmica
pH	Potencial Hidrogeniônico
PK	Farmacocinética
PPD	Purified Protein Derivative
PTA	*Probability Target Attainment*
PVHIV	Pessoas Vivendo com HIV
Q	Depuração intercompartimental

qSOFA	Quick-SOFA
R	Rifampicina
RAL	Raltegravir
RT-PCR	Reação em Cadeia de Polimerase em Tempo Real
S	Estreptomicina
SDRA	Síndrome do Desconforto Respiratório Agudo
SIRS	Síndrome da Resposta Inflamatória Sistêmica
SOFA	Sequential Organ Failure Assessment
$T_{>MIC}$	Tempo acima da MIC
$T_{1/2}$	Tempo de Meia-Vida
TARV	Terapia Antirretroviral
TB	Tuberculose
TB-DR	Tuberculose Droga-Resistente
TDM	Monitoramento terapêutico de fármacos (*Therapeutic Drug Monitoring)*
Th1	T-helper 1
Th2	T-helper 2
T_{lag}	Tempo de Latência
TLR	*Toll-like Receptors*
T_{max}	Tempo máximo de absorção
TNF-α	Fator de Necrose Tumoral-α
µg	Micrograma
UTI	Unidade de Terapia Intensiva
Vc	Volume do Compartimento Central
Vd	Volume de Distribuição
Vp	Volume do Compartimento Periférico
Z	Pirazinamida

SUMÁRIO

1. INTRODUÇÃO

1.1 Tuberculose, epidemiologia e contexto histórico

A tuberculose (TB) é uma doença transmitida pelo ar e representa uma das maiores causas de mortalidade no mundo. Até a pandemia de COVID-19, era o agravo gerado por um agente infeccioso isolado que mais ocasionava óbitos na população mundial, superando até mesmo HIV/AIDS(1). O ano de 2020 foi marcado por uma redução expressiva de diagnósticos e notificações de tuberculose em todos os países, inclusive no Brasil(1,2). A Organização Mundial da Saúde (OMS) estima que aproximadamente 10 milhões de pessoas foram infectadas pela tuberculose, das quais 1,3 milhão morreram em decorrência da TB e mais 214 mil da coinfecção TB/HIV(1,3). Entretanto, apenas 5,8 milhões de casos foram notificados, 18% a menos que em 2019. Simultaneamente, a contagem de óbitos pela doença aumentou 7,6% em relação ao mesmo período(4). É a primeira vez em uma década que se registrou aumento no total de mortes por tuberculose(3). Esses números foram expressivos nos países mais acometidos pelo SARS-CoV-2, onde serviços de saúde com recursos limitados precisaram direcionar seus esforços para o atendimento dos casos de COVID-19. Por consequência, houve redução de 15% das pessoas que receberam tratamento para tuberculose droga resistente (TB-DR) e de 21% para o tratamento da infecção latente por tuberculose (ILTB), ações essas que deixarão sequelas em um futuro próximo(1,3).

No Brasil, embora tenha sido observada uma constante tendência de queda entre os anos de 2011 e 2016, o coeficiente de incidência da tuberculose aumentou entre os anos de 2017 e 2019(2). Em 2020, seguindo a tendência global, observou-se uma queda de 37,4 para 31,6 casos/100 mil habitantes(2). Provavelmente, a redução está associada a um menor número de diagnósticos no país. A contagem anual de óbitos por tuberculose no Brasil tem variado de 4.400 a 4.600 e o coeficiente de mortalidade, entre 2,2 e 2,3 óbitos/100 mil habitantes desde 2012(2).

O estado do Amazonas, localizado na região norte do Brasil, é composto por 62 municípios, com uma população estimada em 3.938.336 habitantes, sendo que 52% vivem na capital, Manaus(5). Em 2020, foi o estado que apresentou a maior taxa

de incidência de TB dentre as unidades da federação, com 64,8 casos/100 mil habitantes, mais que o dobro da média nacional(2). Manaus também se destaca com a maior incidência dentre as capitais – 90,1 casos/100 mil habitantes, tendo a letalidade superado em 86% a média brasileira, com 4,1 casos/100 mil habitantes(2).

A origem precisa da tuberculose não é clara(6–8). Ao que parece, o *Mycobacterium tuberculosis* (Mtb) e o *Mycobacterium bovis* coexistiram ao longo da história(7). Durante o período neolítico, a espécie humana começa a se organizar em comunidades maiores para cultivar plantações e criar animais. Isso resulta em maior convivência entre os humanos, o que potencializou a transmissão de Mtb(7). Do mesmo modo, a domesticação dos animais criou grupos maiores e mais aglomerados, o que intensificou a transmissão do *Mycobacterium bovis*(6,7).

No início do século XVIII, o médico francês René Laennec sugere que diferentes manifestações clínicas observadas nos pacientes, como tosse produtiva, sudorese noturna e a pectoriloquia, faziam parte de uma mesma patologia, que mais tarde receberia o nome de tuberculose. Essa doença ganha força em 1750 com o advento da Revolução Industrial na Europa(9). Más condições de trabalho e moradia associadas a aglomerações, desnutrição, falta de saneamento básico, cuidado médico, especialmente no início e no final da Revolução Industrial, foram fatores fundamentais para a progressão da epidemia(7,9). O número de óbitos por tuberculose apenas reduziu com a implementação de melhorias laborais e habitacionais para os cidadãos(7,9). No Brasil, a história da tuberculose abrange um contexto político, social e científico atingindo, majoritariamente, a população menos favorecida(6). Na região amazônica, os relatos da doença começaram a ser registrados nos anos de 1800(6,10). Considerada o “mal romântico” no século XIX, a doença passou a ser um problema de saúde no século XX(10).

Em 24 de março de 1882, Robert Koch apresenta seu trabalho em Berlim, mostrando o *Mycobacterium tuberculosis* como agente causador da tuberculose, o que possibilitou o avanço de estratégias para o combate à epidemia(7). Por tratar-se de uma doença infecciosa de transmissão aérea, fica claro que a separação entre doentes e não doentes e a identificação dos seus contatos são peças fundamentais no controle da propagação(6,7,11). Assim, em 25 de março de 1887, o primeiro dispensário de tuberculose foi inaugurado em Edimburgo, por Sir Robert William

Philip, com o objetivo de acompanhar e tratar os indivíduos doentes(6,7). Com o mesmo intuito, no final do século XIX e início do século XX, diversos dispensários foram construídos ao redor do mundo(6,7). Em 1890, Robert Koch tenta, sem sucesso, utilizar a tuberculina como tratamento para a tuberculose e, em 1921, Calmette e Guérin apresentam a vacina com o Bacilo de Calmette-Guérin (BCG)(7).

Na atualidade, segundo a OMS, os principais determinantes da tuberculose no mundo são a desnutrição – responsável por 1,9 milhão de casos/ano –, seguida por HIV, abuso de álcool, tabagismo – responsáveis cada um por 740 mil casos/ano – e, por fim, o diabetes mellitus – que responde por 370 mil novos casos/ano(1). Após o surgimento da Síndrome da Imunodeficiência Adquirida (AIDS), houve um aumento mundial no número de casos de TB(7). O risco de progressão da forma latente para a forma ativa é de 20 a 37 vezes maior em pessoas vivendo com HIV do que naquelas que não vivem com HIV(12).

O Brasil é um dos 30 países que concentram 80% dos casos de tuberculose, tendo a coinfecção TB/HIV como a principal determinante dessa doença(1). Por essa razão, o Programa Nacional de Controle da Tuberculose no Brasil recomenda a testagem para HIV em todos os pacientes com diagnóstico de tuberculose, pois frequentemente o diagnóstico da infecção pelo vírus é dado nesse momento(13). De 2011 a 2019, a proporção de casos novos de TB testados para HIV cresceu(2). Em 2020, 76,5% dos casos novos de TB foram testados para HIV, dos quais 8,4% eram positivos(2). Acre, Roraima e Paraná apresentaram os maiores percentuais de testagem para o HIV(2). As maiores proporções de coinfecção TB/HIV ocorrem no Distrito Federal (15,1%), no Rio Grande do Sul (15%), em Santa Catarina (13,1%) e no Amazonas (11,3%)(2). No estado do Amazonas, 74,7% dos casos novos de tuberculose foram testados para HIV, sendo 11,3% positivos(2). Chama atenção que, no Brasil, dentre as pessoas com coinfecção TB/HIV, em 2020, apenas 45,1% realizaram terapia antirretroviral (TARV) durante o tratamento da TB(2).

1.2 Tuberculose, agente causal e fisiopatologia

O *Mycobacterium tuberculosis*, também conhecido como Bacilo de Koch (BK), é um patógeno intracelular de virulência variável, aeróbio estrito, de crescimento lento,

que se multiplica a cada 18 a 36 horas dentro do macrófago(6–8). O Mtb é um bacilo aeróbio, imóvel, não esporulado, não encapsulado, reto ou ligeiramente curvo, medindo de 1 a 5 µm(6). Sua parede celular é complexa, formada por ácidos micólicos, o que a torna hidrofóbica(6,7). Uma vez corada com corantes básicos, não pode ser descorada com solução de álcool e ácido, caracterizando-se como um bacilo álcool-ácido resistente (BAAR) visto na técnica de coloração Ziehl-Neelsen(14).

Estudos genômicos mostraram grande variabilidade genética entre o Mtb ao redor do mundo, provavelmente associada a padrões de migração humana ou à própria patogenicidade de diferentes linhagens(7). Cerca de 200 dos seus genes parecem estar envolvidos na capacidade do bacilo de crescer dentro do hospedeiro por codificarem o metabolismo de ácidos graxos, a principal fonte de carbono do bacilo infectante(6,7). O complexo *Mycobacterium tuberculosis* (MTBC) consiste em 11 espécies que apresentam similaridade genética maior que 99%, mas diferem em distribuição geográfica, nicho ecológico e patogenicidade(7). A maior parte das infecções em humanos são causadas pelo Mtb *stricto sensu*, que correspondem a cinco linhagens do MTBC(7). Além desses, organismos mais próximos como *Mycobacterium africanum* (duas linhagens), espécies zoonóticas como *Mycobacterium bovis* e *Mycobacterium caprae* também podem causar doenças(7). Outras espécies infectam animais, como *Mycobacterium microti* (roedores), *Mycobacterium orygis* (antílopes), *Mycobacterium pinnipedii* (focas e leões marinhos), “dassie bacillus” (hyraxes), *Mycobacterium suricattae* (suricatos), *Mycobacterium mungi* (mangustos) e o “bacilo do chimpanzé” (chimpanzés)(7).

Uma infecção pelo Mtb pode ou não evoluir para uma doença tuberculosa(6,8,11,15). Observa-se que, além da variabilidade genética, fatores relacionados ao hospedeiro e ao meio são decisivos para o processo de adoecimento(6,8,11,15). A fórmula de Rich determina que a possibilidade da infecção evoluir para a enfermidade é diretamente proporcional ao número de bacilos infectantes, à sua virulência e à reação de hipersensibilidade ou resposta inflamatória provocada; e inversamente proporcional às resistências natural e adquirida pelo organismo(6).

$$L = \frac{N.V.H}{In.Ia}$$

L = lesão; *N* = número de bacilos; *V* = virulência; *H* = hipersensibilidade; *In* = imunidade natural; *Ia* = imunidade adquirida.

Praticamente todas as infecções tuberculosas se iniciam pela via inalatória(6,8,11,15). Um indivíduo portador de uma forma cavitária da doença, ao tossir ou falar, elimina no ar bacilos tuberculosos dentro de gotículas (gotículas de Flügge)(6,8). Pela ação dos raios solares e do vento, essas gotículas são ressecadas e passam a ter tamanho ainda menor (gotículas de Wells), podendo ser inaladas por outras pessoas(6,8). Penetrando o pulmão, esse microrganismo pode ter três destinos:

1. A resposta imunológica do hospedeiro pode ser capaz de eliminar completamente o bacilo, impedindo a progressão para a doença.
2. A população de bacilos começa a se multiplicar e cresce causando infecção conhecida como tuberculose primária ou primo-infecção.
3. Os bacilos tornam-se dormentes (latentes) e não causam doença no momento, mas podem, no futuro, iniciar o processo de multiplicação que leva à doença.

A partir do pulmão, os bacilos podem se dispersar pela circulação linfática, atingindo linfonodos e formando o complexo primário ou complexo de Gohn (lesão satélite e adenomegalia)(6,8,11,15). Também pode haver invasão da corrente sanguínea após a corrosão da parede de um vaso, disseminando-se pelo organismo e causando doença em qualquer órgão ou sistema(6). A tuberculose pulmonar responde por 80% a 90% dos casos, pois o pulmão é a porta de entrada para o bacilo e reúne as condições favoráveis para o seu desenvolvimento(6,15).

Os bacilos que permanecem em processo de latência são responsáveis por outro espectro de doença, a ILTB(6). Fatores que possam comprometer o sistema imunológico, como envelhecimento, desnutrição, comorbidades (diabetes, HIV, silicoses, neoplasias), uso de medicamentos imunossupressores ou ainda a exposição a novos bacilos que aumentam a carga bacilar favorecem a reativação desses microrganismos que podem causar o adoecimento(6,7,15). Estima-se que 10% dos indivíduos com ILTB podem desenvolver a doença ao longo dos 10 anos subsequentes(6,13).

A exposição ao bacilo, na ausência de doença ativa, pode ser comprovada pelo teste tuberculínico, utilizando o Purified Protein Derivative (PPD), que se torna positivo de 2 a 10 semanas após o contato do indivíduo com o Mtb(6,13). O líquido é aplicado por via intradérmica, no terço médio da face anterior do antebraço esquerdo, na dose de 0,1 ml (2 unidades de tuberculina PPD RT-23), gerando o surgimento de uma pequena área de limites precisos, pálida e de aspecto pontilhado como casca de laranja(13). A leitura deve ser realizada de 48 a 72 horas após a aplicação, medindo-se o maior diâmetro transverso da área do endurado palpável, com régua milimetrada transparente(13). Resultados acima de 5mm para a população geral ou acima de 10mm para profissionais de saúde indicam infecção e não doença por Mtb, sendo úteis para o diagnóstico de ILTB(13).

Para se instalar no organismo, o Mtb pode se ligar a muitos receptores(6). A falta de uma via preferencial sugere que as opções visam a maximizar a possibilidade de entrada no tecido(6). Dentre os principais receptores envolvidos no processo estão os receptores de surfactante, os toll-like receptors (TLR), os receptores do complemento (CR), os receptores de manose, os receptores removedores, os DC-SIGN (*Dendritic Cell-specific intercelular adhesion molecule-3-grabbing nonintegrin*) e os CD14(6,7). O microambiente também é fundamental nesse processo(7). Por exemplo, a proteína D do surfactante pode impedir a fagocitose do bacilo pelo macrófago, enquanto a proteína A facilita sua adesão(7).

Os bacilos são fagocitados pelos macrófagos e pelos pneumócitos tipo II(6). Após a fagocitose, ocorre a fusão do fagossomo com o lisossomo, repleto de substâncias para destruir o germe invasor(6). Todavia, o Mtb bloqueia o lisossomo e modifica o microambiente do fagossomo para garantir sua sobrevivência(6). Na sequência, destrói o fagossomo e libera proteínas e DNA do bacilo no citoplasma do macrófago(6). Após esse processo, o Mtb fica ligado a um fagossomo por meio de receptores do complemento (CR1, CR2 e CR4) e, principalmente, ao receptor de manose(6). O macrófago é utilizado pelo Mtb como seu habitat natural, tendo papel importante na sua sobrevivência(6). O macrófago é dotado de diversos receptores que facilitam a ligação do antígeno e permitem a invasão. As interações do bacilo com receptores de superfície podem influenciar no destino do Mtb(6). A interação com receptores TLR ou receptores de imunoglobulinas (FcR) estimulam as defesas

humanas, enquanto a interação com receptores do complemento promove a sobrevivência da micobactéria(6).

A proteína surfactante D, por sua vez, inibe a fagocitose do Mtb, evitando a maturação do fagossomo desencadeada pelo interferon-γ (INF-γ), o qual estimula mecanismos antimicrobianos nos macrófagos mediados pelos reativos de oxigênio e de nitrogênio(6,7). Esse mecanismo parece dificultar o acesso do bacilo ao ferro, mineral fundamental para sua sobrevivência no meio intracelular(6,7). O Mtb dispõe de sideróforos, moléculas especializadas em captar ferro(6–8). Além disso, ao impedir a maturação do fagossomo, o bacilo garante acesso à transferrina(6–8).

Quando ocorre a fusão dos lisossomos com os fagossomos, o ambiente passa a ser hostil para o bacilo devido ao pH ácido, a ação de reativos de oxigênio e de nitrogênio(6). A ação dos reativos de nitrogênio parece ser a estratégia mais eficaz do macrófago e, a resistência do Mtb a esse mecanismo pode estar relacionada a sua virulência(6). Com este mecanismo, parte dos bacilos é destruída dentro do macrófago e parte delas entra em processo de latência, em que a redução da atividade metabólica facilita a sobrevivência em condições de carência de nutrientes e de oxigênio(6,15).

A resposta adaptativa ao Mtb envolve tanto a resposta humoral quanto a resposta celular(7,8). Após a fagocitose do Mtb, ocorre a apresentação do antígeno para os linfócitos T $CD4^+$(6). Consequentemente, os linfócitos são ativados e proliferam(6,7). Devido à presença de citocinas, uma resposta Th1 pode ser estabelecida com a produção das citocinas pró-inflamatórias IL-12, IL-18 e IFN-γ, potencializando a liberação de espécies reativas de oxigênio e nitrogênio(6,15). O processo de produção de IFN-γ pelos linfócitos T $CD4^+$ é fundamental para a contenção do Mtb(6). As células $CD8^+$ citotóxicas também têm papel importante por lizarem macrófagos infectados(8).

A parede do Mtb tem produtos quimiotáticos para as células T, particularmente a glicoproteína lipoarabinomanana (LAM)(6,7). A apresentação de antígeno aos linfócitos T os ativa, levando à produção de anticorpos pelos plasmócitos(6–8). Esses anticorpos não são capazes de destruir o bacilo, porém, sua detecção auxilia alguns testes diagnósticos(6–8). Embora muitos linfócitos T estejam presentes na defesa do

hospedeiro, o linfócito T $CD4^+$ parece ter um papel primordial: são da linhagem Th1 e possuem alta capacidade de produção de INF-γ(6–8). A queda do número de linfócitos T $CD4^+$ no adoecimento por tuberculose pode ser resultado do consumo dessas células na formação do granuloma(6). O linfócito T $CD8^+$ também produz INF-γ(7,8). Porém, quando o linfócito T $CD4^+$ amplifica a resposta imune pela ativação de células efetoras, o linfócito T $CD8^+$ torna-se citotóxico, lizando os macrófagos infectados pelo Mtb e forçando a translocação do bacilo para outras células, o que permite a destruição do agente agressor(6). A ação destrutiva ocorre por ação da granulisina e da perforina(6).

Por outro lado, a resposta Th2 promove a ativação de linfócitos B, a produção de anticorpos e as respostas antiinflamatórias para os macrófagos(6). As células B são abundantes nos granulomas, agindo como células apresentadoras de antígenos e modulando a resposta inflamatória por meio da secreção de IL-10(7,8,11,16). Pacientes com TB apresentam reduzida quantidade de células B, além de algum grau de disfunção celular nessa linhagem(6–8).

A formação do granuloma é a marca registrada da tuberculose, resultado do acúmulo de células imunes que cercam os macrófagos infectados pelo Mtb para conter a infecção(6–8). A estrutura básica de um granuloma inclui um centro necrótico cercado por macrófagos, seguidos por um anel de células T $CD4^+$ e B $CD8^+$(17). As células apresentadoras de antígeno produzem TNF-α que age de maneira sinérgica ao INF-γ para controlar o bacilo(6–8). Modelos animais demonstram que a supressão do TNF-α faz com que os bacilos voltem a se multiplicar, desencadeando tuberculose ativa(6,7). Esse fenômeno é de suma importância uma vez que, na atualidade, diversas medicações anti-TNF-α são utilizadas para tratamentos de doenças autoimunes(6,8,15). Sendo assim, o uso dessas medicações, sem o prévio conhecimento da presença de ILTB, expõe o paciente a um alto risco de desenvolvimento de uma forma agressiva de tuberculose ativa(7).

1.3 Tuberculose, apresentação clínica e diagnóstico

A TB pode acometer uma série de órgãos ou sistemas, mimetizando outras patologias infecciosas ou não infecciosas(18). Sua apresentação clínica depende do local de acometimento(8,15). A apresentação da forma pulmonar, além de ser a mais

frequente, é também a mais relevante para a saúde pública, pois é responsável pela manutenção da cadeia de transmissão da doença(6,11). Em 2021, segundo a OMS, 82% dos casos notificados no mundo foram de tuberculose pulmonar(1). No Brasil, a mesma forma pulmonar corresponde a 70% dos casos de tuberculose confirmados laboratorialmente (2). Sendo assim, define-se o conceito de sintomático respiratório:

> *Pessoa que, durante a estratégia programática de busca ativa, apresenta tosse por 3 semanas ou mais. Essa pessoa deve ser investigada para tuberculose através de exames bacteriológicos.* (13)

Os sintomas clássicos da TB pulmonar são tosse persistente seca ou produtiva, febre vespertina, sudorese noturna e emagrecimento(19,20). A TB pulmonar primária normalmente ocorre em seguida ao primeiro contato do indivíduo com o bacilo e, por isso, é mais comum em crianças(6). As manifestações clínicas podem ser insidiosas, com o paciente demonstrando-se irritadiço, com febre baixa, sudorese noturna e inapetência. Nem sempre a tosse está presente(6,11). O exame físico pode ser inexpressivo(13).

A tuberculose pleural é a segunda forma mais comum de apresentação da doença(6,8,13,15,18,21). Na literatura, há controvérsia sobre tratá-la como forma pulmonar ou extrapulmonar. Por vezes, a lesão pleural ocorre por contiguidade, como consequência da ruptura de uma cavidade tuberculosa para o espaço pleural, sugerindo doença pulmonar(6–8). Esse é o processo de formação do empiema pleural tuberculoso. Por isso, além do líquido nesse espaço, em muitas circunstâncias ocorre também pneumotórax secundário à fístula broncopleural(6). Por outro lado, frequentemente, observa-se derrame pleural bilateral, que sugere disseminação hematogênica e, portanto, doença extrapulmonar(6,8). Clinicamente, o empiema pleural tuberculoso é indistinguível de um empiema pleural por bactéria comum.

O derrame pleural na TB apresenta-se com padrão exsudativo, predominantemente linfocítico, com proteínas geralmente maiores que 2,5 mg/dL, glicose moderadamente reduzida em relação ao seu nível sérico e pH 7,3 ou menor(8). Essa forma ocorre mais em pacientes jovens e cursa com dor torácica do tipo pleurítica(8). A tríade, astenia, emagrecimento e anorexia, ocorre em 70% dos pacientes. Já a febre com tosse seca, em 60%. Eventualmente, simula pneumonia bacteriana aguda. Nos pacientes com maior tempo de evolução dos sintomas pode ocorrer dispneia(13).

O termo tuberculose miliar foi inicialmente utilizado para descrever um achado patológico e radiológico característico: a presença de pequenas imagens que lembravam grãos de milho espalhadas pela circulação pulmonar(6). Atualmente, tuberculose miliar representa qualquer tuberculose disseminada progressivamente por via hematogênica, apresentando dois espectros: TB miliar aguda e TB miliar não reativa(6). A TB miliar aguda apresenta progressão grave, rápida e, frequentemente, seguida da primo-infecção em adultos jovens. Caracteriza-se por criar granulomas com poucos microrganismos que, habitualmente, resulta em baciloscopias e culturas negativas(8). A TB miliar não reativa constantemente ocorre em adultos na reativação da doença, com baciloscopias e culturas positivas(7). A característica principal desse quadro é o surgimento de abscessos neutrofílicos com grande quantidade de bacilos. Também é chamada de TB criptogênica por apresentar formas oligossintomáticas e 80% dos diagnósticos são feitos *post mortem*(18).

A tuberculose ganglionar é a forma extrapulmonar mais frequente de tuberculose(8,13). Em indivíduos não coinfectados pelo HIV, com frequência, é cervical e unilateral(6,7). Em geral, apresenta linfonodos com características inflamatórias, móveis, fibroelásticos e com sinais de vermelhidão ao seu redor(22). Em pessoas vivendo com HIV, a linfonodomegalia pode acometer outras cadeias, proporcionando sintomas sistêmicos mais intensos(8). Para o diagnóstico, é necessária a identificação do bacilo no linfonodo, a qual pode ser feita por biópsia excisional ou por punção com agulha fina(6).

A meningoencefalite tuberculosa apresenta um espectro clínico que varia desde um quadro que mimetiza uma meningite bacteriana até uma doença subaguda que cursa com cefaleia e febre(23,24). É responsável por 3% dos casos de TB em pacientes não infectados pelo HIV e por até 10% em pessoas vivendo com HIV/AIDS (PVHIV)(7,15,25,26). Pode ser causada por disseminação hematogênica ou pela ruptura do tubérculo subependimário no espaço subaracnóideo(6,7). Vasculites podem levar a trombose, infarto e formação de aneurismas no local(8). É considerada forma grave pela alta morbidade e mortalidade(6,8,18). Clinicamente, pode ser subaguda ou crônica, apresentando sinais e sintomas com duração superior a quatro semanas(6,7,11). Na forma subaguda, cursa com cefaleia holocraniana, irritabilidade, alterações de comportamento, sonolência, anorexia, vômitos e dor abdominal associados a febre, fotofobia e rigidez de nuca por tempo superior a duas semanas(8).

Eventualmente, apresenta sinais focais relacionados a síndromes isquêmicas locais ou ao envolvimento de pares cranianos (pares II, III, IV, VI e VII), podendo evidenciar sinais de hipertensão intracraniana(6). Na forma crônica, o paciente evolui várias semanas com cefaleia até que o acometimento de pares cranianos traz à tona a suspeita de meningite crônica(6,8). Ocorre doença pulmonar concomitante em até 59% dos casos(18). Outra forma de TB do sistema nervoso central é a localizada – os tuberculomas. Nessa apresentação, o quadro clínico é de um processo expansivo intracraniano de crescimento lento, com sinais e sintomas de hipertensão intracraniana, sendo que a febre pode não estar presente(6).

A pesquisa bacteriológica é de importância fundamental em adultos, tanto para o diagnóstico quanto para o controle de tratamento da tuberculose(6,8,15,27). Resultados bacteriológicos positivos confirmam a doença ativa em pacientes com quadro clínico sugestivo de TB(28). Cada vez mais, se destaca a importância da confirmação pelo diagnóstico bacteriológico. Humphrey et al. associaram o tratamento empírico de tuberculose com o aumento da mortalidade em PVHIV(29). Em 2021, segundo a OMS, apenas 59% dos pacientes diagnosticados com TB tiveram confirmação bacteriológica(1). Esse percentual mantém-se desde 2005.

Dentre as ferramentas utilizadas para o diagnóstico bacteriológico da tuberculose destacam-se três testes(13,14,27,30,31):

1. baciloscopia;
2. teste Rápido Molecular; e
3. cultura para micobactérias.

1.4 Baciloscopia

A baciloscopia baseia-se no fato de que o Mtb apresenta uma parede celular rica em lipídios, o que impede o acesso aos corantes de anilina(8). Contudo, quando fixados com carbol-fucsina ou fluorocromos em condições especiais para fixação, estes bacilos não são facilmente descorados, mesmo quando expostos a soluções álcool-ácidas(8,14,32). Por esse motivo, todos os membros do gênero *Mycobacterium* spp. são chamados bacilos álcool-ácido resistentes (BAAR)(8).

O método de Ziehl-Nielsen consiste na adição de fucsina à lâmina que contém o esfregaço(14,32). Ela é aquecida em uma chama por cinco minutos e depois lavada com água(14,32). A seguir, é aplicado o substrato com ácido clorídrico 3% e álcool etílico, descolorindo toda a lâmina, exceto os bacilos álcool-ácido resistentes que permanecerão vermelhos(14,32). Ao final, adiciona-se azul de metileno à lâmina a fim de corar as demais substâncias de azul, o que gera contraste para facilitar a leitura(14,32).

A baciloscopia é um método barato e amplamente disponível. Porém, suas limitações são o alto número de bacilos necessário para um teste positivo (5.000 a 50.000/mL) e a sua baixa sensibilidade, que varia entre 20% e 60% quando comparada com a cultura(28). Além disso, a sensibilidade pode oscilar de acordo com a gravidade da doença, o tamanho da lesão, o tipo, o número e a qualidade das amostras, a atenção e a persistência do microscopista no preparo e na avaliação da lâmina(14). A coleta de uma segunda amostra pode acrescentar 11,9% na sensibilidade, e uma eventual terceira amostra para pesquisa de BAAR, mais 3,1%(33). Os resultados são apresentados conforme a tabela a seguir(13):

Leitura	Resultado
Não são encontrados BAAR em 100 campos observados	NEGATIVO
1 a 9 BAAR em 100 campos observados	Relata-se a quantidade de bacilos encontrada
10 a 99 BAAR em 100 campos observados	POSITIVO +
1 a 10 BAAR por campo em 50 campos observados	POSITIVO ++
Em média, mais de 10 BAAR por campo em 20 campos observados	POSITIVO +++

Tabela 1: Resultado de BAAR de acordo com a quantidade de bacilos álcool-ácido resistentes encontrados por campo

1.5 Teste Rápido Molecular (GeneXpert©MTB/RIF Ultra)

A biologia molecular tem se mostrado o método mais promissor no diagnóstico de Mtb. Por esse motivo, em 2020, a OMS indicou que o GeneXpert© MTB/RIF Ultra seja o teste inicial para diagnóstico de tuberculose(28,34–38). O ensaio combina a preparação de amostras com funções de detecção e amplificação de PCR em tempo real (RT-PCR), totalmente automatizadas(39). Ele pode ser operado por pessoas sem grande experiência em laboratório e ocupa em torno de 15 minutos de trabalho do

profissional(39). É composto por um cartucho que incorpora a tecnologia de microfluídica e de detecção do DNA que, automaticamente, purifica, concentra, detecta e identifica as sequências-alvo de DNA das amostras clínicas(39). Utiliza tecnologia de RT-PCR para diagnosticar TB e detectar resistência à rifampicina simultaneamente(39).

O cartucho é inserido em um equipamento conectado a um computador(39). O teste detecta sete diferentes sequências de ácidos nucleicos por meio de sondas marcadas em uma reação(39). Cada sonda é complementar a uma sequência-alvo diferente dentro do gene *rpoB*, que determina a resistência à rifampicina(39). O limite inferior para detecção é de 16 bacilos/mL, muito próximo do limite inferior da cultura em meio líquido, 10 bacilos/mL(31,40,41). A sensibilidade varia de 87,5% a 100%, dependendo dos estudos e da condição de realização dos testes(38,42–44). Outro fator relevante é a sensibilidade em pacientes com baciloscopia negativa, que varia de 78,9% a 96,7%(41,42). Para a detecção de resistência à rifampicina, a sensibilidade é de 95,1% e a especificidade, 98,9%(41,45).

1.6 Cultura para micobactérias

Culturas fornecem um diagnóstico definitivo de tuberculose. A principal vantagem dos testes de cultura sobre a microscopia de escarro é sua maior sensibilidade(14,46). As culturas permitem a detecção de números muito baixos de bacilos (aproximadamente 10 bacilos/mL de escarro em comparação com pelo menos 5 mil bacilos/mL de escarro para baciloscopia), o que aumenta o potencial de diagnóstico de TB em estágios iniciais da doença(14). O uso da cultura aumenta de 30% a 50% a identificação dos casos de TB(14). Além disso, as culturas são usadas para identificação de espécies do gênero *Mycobacterium* sp. e para a realização de testes de sensibilidade a medicamentos(14).

Os métodos para cultura de micobactérias utilizam a semeadura da amostra em meios sólidos e líquidos(13,14,32). Os meios de cultura mais comumente utilizados são os sólidos à base de ovo, Löwenstein-Jensen e Ogawa-Kudoh(32). Eles têm a vantagem de apresentar menor custo e um baixo índice de contaminação(32). A desvantagem do meio sólido é o tempo de detecção do crescimento bacteriano, que varia de 14 a 30 dias, podendo estender-se por até 8 semanas(14,32,33). O meio

líquido é utilizado nos métodos automatizados disponíveis no Brasil, entre eles o MGIT®, no qual o tempo de resultado, quando positivo, varia entre 5 e 12 dias e, quando negativo, é de 42 dias.(13). O resultado da cultura confirma o diagnóstico de micobacteriose, sendo necessária a identificação de espécie para caracterizar se é um caso de TB ou de outra micobactéria(6,8,11).

1.7 Tuberculose, tratamento

Na década de 1940, Waksman demonstrou a ação de alguns fungos contra o Mtb e desenvolveu a actinomicina e a estreptomicina (S), mas ambas com diversos efeitos tóxicos. Em novembro de 1944, a estreptomicina foi utilizada pela primeira vez em humanos, demonstrando seu poder terapêutico(47). Contudo, no ano seguinte, já se observou o surgimento de bacilos resistentes, indicando a importância da associação de fármacos(48). Em 1946, foi demonstrada a ação do ácido para-aminossalicílico (PAS) sobre o BK e, em 1948, iniciado o uso de ambos (S + PAS) em associação. Os demais fármacos surgiram na sequência: isoniazida em 1951, pirazinamida em 1954, ciclosserina em 1955, etambutol em 1962 e rifampicina em 1963(49).

Três características do Mtb são essenciais na fundamentação do tratamento: a aerobiose estrita, a multiplicação lenta e a alta proporção de mutantes resistentes(6,19).

Por ser totalmente dependente de oxigênio (O_2) para o seu metabolismo, o Mtb tem seu comportamento modulado pela concentração do gás no ambiente. No interior dos macrófagos, onde a concentração de O_2 é baixa e o pH é ácido, ele apresenta multiplicação lenta(6). Na lesão caseosa fechada, a concentração do gás é muito baixa e o bacilo apresenta multiplicação intermitente(6). Os bacilos de multiplicação lenta ou intermitente são denominados persistentes e, por isso, responsáveis pela manutenção da doença e eventuais recaídas(6).

Na população intramacrofágica, agem os fármacos que melhor se difundem no meio intracelular e atuam em pH ácido, como a rifampicina, a pirazinamida e o etambutol(6,8). Nas lesões caseosas fechadas, a maior ação é da rifampicina. Já nas

lesões cavitárias, a rifampicina, a isoniazida e a estreptomicina são muito efetivas(6,8).

Pelas condições ideais, nas lesões cavitárias, formam-se grandes populações bacilares, com frequência variável de subpopulações de bacilos com mutações genéticas que conferem resistência natural aos medicamentos usados no tratamento da TB(6). Se o esquema terapêutico é equivocado, realizado de maneira irregular, com doses inadequadas ou interrompido precocemente, cepas resistentes aos medicamentos podem ser selecionadas, caracterizando-se a resistência adquirida(6,50,51).

Levando-se em consideração o comportamento metabólico e a localização do bacilo, o esquema terapêutico anti-TB, para ser mais efetivo, deve atender a três grandes objetivos(6,8,11,19,52):

- ter atividade bactericida precoce;
- ser capaz de prevenir a emergência de bacilos resistentes; e
- ter atividade esterilizante.

A atividade bactericida precoce é a capacidade de matar a maior quantidade de bacilos, o mais rapidamente possível, mensurada pela velocidade com que são mortos(6). Essa velocidade é identificada pela conversão da cultura de escarro no final da fase intensiva do tratamento (segundo mês)(6). Em geral, após 2 a 3 semanas de tratamento com esquema anti-TB que contenha fármacos com atividade bactericida precoce, ocorre significativa diminuição da capacidade de transmissão de bacilos pelos indivíduos doentes(13). Os medicamentos com maior atividade bactericida precoce são isoniazida, estreptomicina e rifampicina(13).

Para prevenir a seleção de bacilos resistentes e a efetiva cura da doença, é necessária a utilização de esquemas terapêuticos com a associação de diferentes medicamentos(6,8,19,51). Eles agem sobre os bacilos sensíveis e nas diversas populações de bacilos naturalmente resistentes, pois bacilos resistentes a um medicamento podem ser sensíveis a outro(6).

A atividade esterilizante é a capacidade de eliminar todos os bacilos presentes no indivíduo, seja nas cavidades pulmonares, no interior das lesões caseosas

fechadas ou no interior dos macrófagos(6,8,53). A falha na fase esterilizante é definida pela proporção de recidivas que ocorrem após o término do tratamento(6,13).

No Brasil, o esquema de tratamento da TB é padronizado, realizado de acordo com as recomendações do Ministério da Saúde e compreende duas fases: a intensiva ou de ataque e a de manutenção ou esterilizante(13). A fase intensiva tem o objetivo de reduzir rapidamente a população bacilar e a eliminação dos bacilos com resistência natural a algum medicamento(13,54). Uma consequência da redução rápida da população bacilar é a diminuição da contagiosidade(6). Para tal, são associados medicamentos com alto poder bactericida(6,19). Já a fase de manutenção tem o objetivo de eliminar os bacilos latentes ou persistentes e reduzir a possibilidade de recidiva da doença(6,13,19,54). Nessa fase, são associados dois medicamentos com maior poder bactericida e esterilizante, ou seja, com boa atuação em todas as populações bacilares(6).

Também no Brasil, o esquema básico para tratamento da TB em adultos e adolescentes é composto por quatro fármacos na fase intensiva e dois na fase de manutenção(13). O Ministério da Saúde recomenda que os pacientes sejam tratados inicialmente por via oral com rifampicina (R), isoniazida (H), pirazinamida (Z) e etambutol (E). Esse esquema está disponível em comprimidos de doses fixas combinadas (DFC), com apresentação 4 em 1 (RHZE) contendo as seguintes doses: R 150 mg, H 75 mg, Z 400 mg e E 275 mg(13). A fase intensiva é concluída após dois meses, desde que o paciente apresente evolução clínica satisfatória e baciloscopia negativa(13). A fase de manutenção tem início imediatamente após a fase de ataque e tem duração de quatro meses(13). São utilizados comprimidos DFC do tipo 2 em 1 (RH) contendo R 150 mg e H 75 mg(13). A fase de manutenção deve ser prolongada para dez meses nos casos de TB meningoencefálica e osteoarticular(13).

Até 2018, o tratamento na fase intensiva ou de ataque era proposto conforme o peso do paciente, da seguinte forma(55):

- < 36kg – 2 comprimidos por dia
- 36kg a 50kg – 3 comprimidos por dia
- > 50kg – 4 comprimidos por dia

A partir de 2018, foi realizado um ajuste em que pacientes de 51 a 70 kg utilizam 4 comprimidos por dia e pacientes acima de 70 kg, 5 comprimidos por dia(13).

Pacientes coinfectados pelo HIV devem utilizar o mesmo esquema preconizado para os demais pacientes, exceto em algumas situações nas quais regimes que não incluem a rifampicina são indicados devido à interação do medicamento com algumas classes de antirretrovirais(13,54,56–58). A rifampicina é um indutor do citocromo P450 e da glicoproteína P que reduz drasticamente a concentração de antirretrovirais da classe dos inibidores de protease (IP), os inibidores de transcriptase reversa não nucleosídeos (ITRNN) e os antagonistas CCR5(8,13,54). Por isso, esquemas antirretrovirais compostos por dois ITRNN associados a efavirenz (EFV) em dose padrão, raltegravir (RAL) a cada 12 ou 24 horas(13,59,60) e o dolutegravir (DTG) em dose dobrada constituem opções de primeira escolha do TARV para pacientes em uso de rifampicina(13,61). Regimes que não incluem a rifampicina, quando indicados em PVHIV, são menos eficazes, resultam em retardo na negativação da baciloscopia, prolongam a duração do tratamento da TB, têm maiores taxas de recidiva, falência e letalidade, além de pior comodidade posológica, o que impacta diretamente na adesão ao tratamento(62,63).

1.8 Rifampicina

As rifamicinas constituem uma classe de antimicrobianos semissintéticos, derivados da rifamicina B, um antibiótico natural extraído, em 1959, de culturas de *Streptomyces mediterranei*, hoje chamado, *Amycolatopsis mediterranei*(8,21). Em 1968, a rifamicina SV, um dos primeiros fármacos utilizados dessa classe, foi substituída pela rifampicina devido à sua melhor biodisponibilidade e atividade contra bactérias gram-positivas e, principalmente, contra *Mycobacterium tuberculosis*(8).

A molécula apresenta uma massa molar de 822,94 g/mol e sua fórmula química é $C_{43}H_{58}N_4O_{12}$. A estrutura química da rifampicina corresponde a um 3-(4-metil-1-piperazinil)-iminometil derivado da rifamicina SV(8). Figura abaixo:

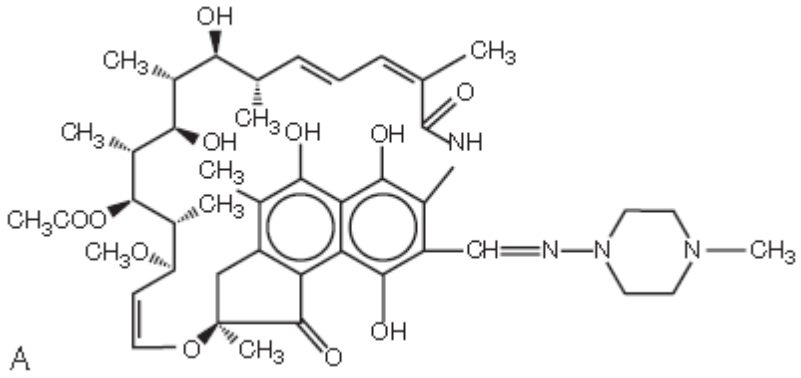


Figura 1: Estrutura molecular da rifampicina (8)

A rifampicina é considerada ativa contra o Mtb em concentrações superiores a 1 µg/mL(64). Seu mecanismo de ação consiste na inibição da síntese de RNA por meio da inibição da RNA polimerase(21,65). A RNA polimerase é uma enzima essencial no processo de síntese proteica, que permite a leitura da sequência de DNA alvo e catalisa a polimerização da cadeia complementar(8,21,65). O *core* da enzima RNA polimerase consiste em cinco subunidades α2, β, β', ω. Os genes que codificam essas subunidades foram denominados *rpoA, rpoB, rpoC e rpoD.* A rifampicina se liga à subunidade β da enzima, inibindo-a(65). Dessa forma, sabe-se também que mutações no gene *rpoB* têm um papel fundamental na resistência à rifampicina.

A droga é de ação rápida e completamente absorvida, o que é facilitado se tomada com estômago vazio(8,55,66,67). Apresenta biodisponibilidade de 68% e é amplamente distribuída pelo organismo, atingindo concentração média no líquido cefalorraquidiano de 2 µg/mL. A droga atinge concentrações ósseas iguais ou superiores às concentrações séricas. A rifampicina sofre metabolismo hepático a partir de desacetilação pelo citocromo P450 3A e é eliminada pela bile(8,21). Esse fármaco induz a própria produção de enzimas metabolizantes, aumentando a formação de metabólitos ao longo da terapêutica. A eliminação pela urina varia de 13% a 24%, não necessitando de ajuste de dose em caso de disfunção renal(8,21).

Como características farmacocinéticas apresenta: tempo máximo de absorção (T_{max}) de 1,5 a 2,5 horas, podendo levar até 4 horas quando administrada junto com alimentos; concentração máxima (C_{max}) de 8 a 20 µg/mL; tempo de meia vida ($T_{1/2}$) de 2 a 5 horas; ligação proteica de 80% e área abaixo da curva em 24 horas (AUC_{0-24})

de 55 a 59 mg.h/L(8,68,69). Sabe-se que a absorção da rifampicina não é afetada por antiácidos. Porém, a falta do jejum reduz o C_{max} e eleva o T_{max}, sem afetar a AUC(69). Estima-se ainda que a concentração da droga no fluido epitelial pulmonar seja de 34% da concentração sérica e na mucosa brônquica, de 50%(70,71). Entretanto, a concentração no interior do macrófago alveolar é 16 vezes superior à sérica, atingindo 251,8 mg/L(70).

1.9 Isoniazida

Em 1952, Bernstein e outros pesquisadores demonstraram que a hidrazida do ácido isonicotínico exercia potente ação contra o bacilo da tuberculose, o que deu ao fármaco o nome de isoniazida(6,21). Sua molécula apresenta uma massa molar de 137,14 g/mol e a fórmula química é $C_6H_7N_3O$, cuja estrutura é demonstrada na figura a seguir(8):

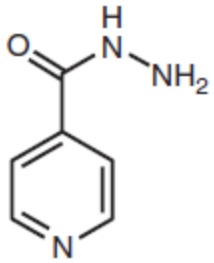


Figura 2: Estrutura molecular da isoniazida(8)

A isoniazida é um quimioterápico essencialmente bactericida contra bacilos de multiplicação rápida e bacteriostática contra bactérias de multiplicação intermitente ou latentes(6,21,72). Tem ação tanto em pH ácido como em pH alcalino, sendo capaz de exercer sua atividade tanto no meio intracelular como no meio extracelular(8,21). Atua inibindo os mecanismos de produção do ácido micólico, um constituinte importante da parede celular do Mtb(21). Também, é capaz de inibir a enzima catalase-peroxidase codificada pelo seu gene *katG*(8,21).

Concentrações maiores que 0,025 µg/mL a 0,05 µg/mL de isoniazida são consideradas bactericidas(64,73). Baixos níveis de resistência à isoniazida são definidos como concentrações inibitórias mínimas (MIC) entre 0,1 e 1,0 µg/mL e, em

geral, estão associados a mutações no gene *katG*(73–75). Resistências de alto nível são definidas como concentrações superiores a 1,0 µg/mL, associadas à perda completa da atividade sobre o gene envolvido na síntese do ácido micólico (*inhA*)(76,77).

A isoniazida é bem absorvida por via oral, com biodisponibilidade próxima a 100%, e pode sofrer interferência de substâncias alcalinas(6,8,21). Por esse motivo, não deve ser administrada com antiácidos(21). A droga concentra-se em material caseoso e mantém nível terapêutico por tempo maior do que sua circulação sérica(6,8,21). Cruza a barreira placentária e atinge concentração no feto e no líquido amniótico(8,21). É metabolizada no fígado por meio da enzima N-acetil-transferase, formando ácido nicotínico e acetilidrazina, substâncias sem ação tuberculostática e que são eliminadas pelo rim(21).

1.10 Pirazinamida

A pirazinamida é um análogo pirazínico da nicotinamida. Sua fórmula é $C_5H_5N_3O$, seu peso molar é 123,11 g/mol e sua estrutura é demonstrada na sequência(78):

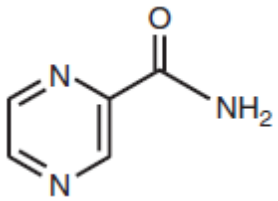


Figura 3: Estrutura molecular da pirazinamida(78)

É convertida no organismo em ácido pirazinóico, a substância ativa contra o Mtb(8,21). A conversão se faz pela enzima nicotinamidase produzida pelo bacilo da tuberculose(8,21). Uma vez convertido, o ácido pirazinóico sai do macrófago, recebe um próton e reentra na célula causando a ruptura da membrana do bacilo pelo seu acúmulo e mudança do potencial de membrana(21,78). Por esse motivo, a droga é pouco ativa no pH alcalino presente no *caseum* e mais ativa sobre os germes de localização intracelular(21,78). Dessa forma, a pirazinamida atua sobre os germes de localização intracelular no macrófago, uma vez que o meio ácido proporciona as melhores condições para a sua eficácia(6,21).

A concentração inibitória mínima é de 100 µg/mL(64,77). A resistência é observada em menos de 1% das tuberculoses primárias e está associada ao gene *pncA*, que codifica a enzima nicotinamidase(21,34,79). O medicamento é absorvido e se distribui facilmente por todo o organismo(21). A concentração plasmática atinge pico de 50 µg/mL com meia vida de 12 horas(8). É metabolizado no fígado e excretado pelos rins(8,21).

1.11 Etambutol

O etambutol foi descoberto em 1961(21). É uma poliamina do butanol e atua inibindo a síntese de ácidos nucleicos e enzimas envolvidas na biossíntese da arabinogalactana e lipoarabinomanana, que são parte da parede celular do bacilo(8,21). Sua fórmula química é $C_{10}H_{24}N_2O_2$, seu peso molar é de 204,31 g/mol e tem sua fórmula expressa na figura a seguir(8):

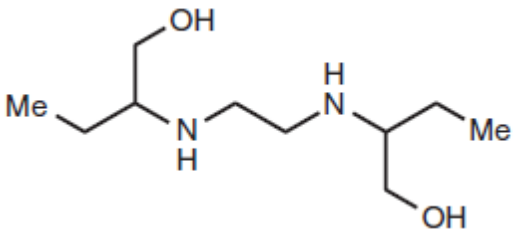


Figura 4: Estrutura molecular do etambutol(8)

Foi introduzido no tratamento da tuberculose em 1980, após uma série de estudos conduzidos pelo British Medical Council. Esses estudos demonstraram que o etambutol tem pouca atividade esterilizante, mas evita o surgimento de resistência contra rifampicina, isoniazida e pirazinamida(80). Por ser um inibidor de parede celular, tem ação contra bacilos de replicação rápida, sendo pouco efetivo contra bacilos de multiplicação intermitente ou latentes. Apresenta bom efeito bactericida, o segundo melhor após a isoniazida(80). Tem absorção oral de aproximadamente 80% e concentração inibitória mínima de 5,0 µg/mL(77,81).

1.12 Farmacocinética

Para atingir seu efeito terapêutico após ser administrado, um fármaco precisa ser absorvido e distribuído por corrente sanguínea, vasos linfáticos ou mesmo difusão para os órgãos e sistemas onde terá ação(82,83). A seguir, deverá ser metabolizado e eliminado, processo que, em geral, é feito pelo fígado, pelos rins e pelo trato gastrointestinal(82,83). A absorção, distribuição, metabolização e eliminação de medicamentos são processos farmacocinéticos(82,83). Mas as diversas barreiras que limitam a entrada de moléculas estranhas no organismo tornam esse processo complexo(82,83). Características físico-químicas das moléculas, como peso molecular, conformação estrutural, grau de ionização, lipossolubilidade e ligação proteica, bem como as características das membranas do organismo, influenciam diretamente esse processo(82).

Inicialmente, podemos considerar um modelo simples de ser humano, que consiste em um compartimento único, bem homogeneizado com um volume de distribuição (Vd), no qual certa quantidade de fármaco pode ser introduzida(83). Nesse caso, a concentração do fármaco (C) seria dada pela relação entre a quantidade desse fármaco (X) e o Vd, conforme a seguinte fórmula(82):

$$C = \frac{X}{Vd}$$

É importante compreender que esse volume vincula a quantidade de fármaco no organismo à sua concentração no próprio organismo(82). Ou seja, não se trata, necessariamente, de um volume fisiológico, mas sim do volume necessário para conter determinada concentração de fármaco no organismo(82).

Todavia, a introdução do fármaco pode levar determinado tempo(82). No caso da administração por via oral, o medicamento deverá ser absorvido para se distribuir no Vd(82). O tempo de absorção pode ser maior ou menor dependendo das características físico-químicas do medicamento e da membrana de absorção. Essa especificidade, é expressa através da constante de absorção (Ka)(82,83).

Esse modelo teórico de ser humano de compartimento único, bem homogeneizado, irá eliminar o fármaco gradativamente ao longo do tempo(82). Essa depuração (CL) poderá levar mais ou menos tempo, dependendo do fármaco e de sua

via de eliminação (renal, hepática ou gastrointestinal)(82,83). Esse tempo pode ser expresso através da constante de eliminação (Ke). Dessa forma, pode-se definir o modelo de compartimento único da seguinte forma:

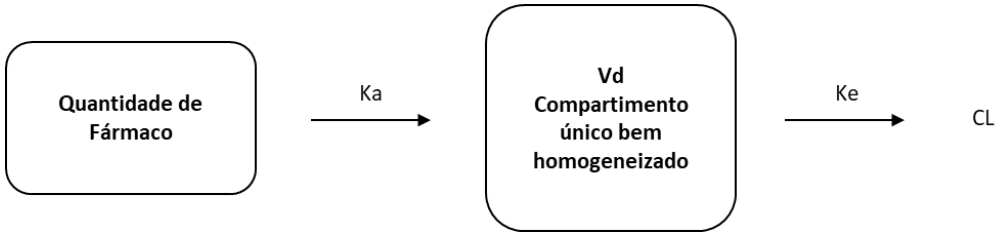


Figura 5: Representação esquemática de modelo farmacocinético de compartimento único

Uma vez que o fármaco é absorvido e eliminado, a concentração (C) varia de acordo com o tempo(82,83). Portanto, define-se que a maior quantidade de fármaco atingida no compartimento é chamada de concentração máxima (C_{max})(82,83). O ponto no tempo no qual o fármaco atinge C_{max} é o tempo máximo de absorção (T_{max})(82). Quanto mais rápida a absorção, menor o T_{max}. Da mesma forma, o CL ocorre ao longo do tempo, e chama-se de tempo de meia vida $T_{1/2}$ o tempo necessário para que o fármaco atinja 50% de C_{max}(82).

No entanto, as diversas partes do organismo, como cérebro, tecido adiposo, músculos, pulmões e etc., são muito diferentes entre si em termos de fluxo sanguíneo, permeabilidade capilar e partição de fármacos(82). Dessa forma, primeiro, o medicamento atinge o equilíbrio em um volume central (Vc), formado pelos reservatórios plasmáticos e teciduais(82). A seguir, é distribuído a um compartimento periférico, formando um volume de distribuição periférico (Vp)(82). A velocidade de passagem do fármaco entre esses dois compartimentos é variável(82,83). Por exemplo, quando o fluxo sanguíneo de alguns tecidos se altera no mesmo indivíduo, a taxa de distribuição do fármaco também é alterada(82). Dessa forma, o fator de distribuição pode ser definido por uma constante de transferência de Vc para Vp, chamada de Kcp(84). Pode-se imaginar o mesmo para a eliminação, na qual a droga deverá sair do compartimento periférico e voltar para o compartimento central antes de ser eliminada, transitando de Vp para Vc, expresso pela constante Kpc(84). Eventualmente, Kpc e Kcp podem estar representados pela depuração

intercompartimental (Q)(84). Assim, temos um modelo bicompartimental, expresso conforme figura que segue:

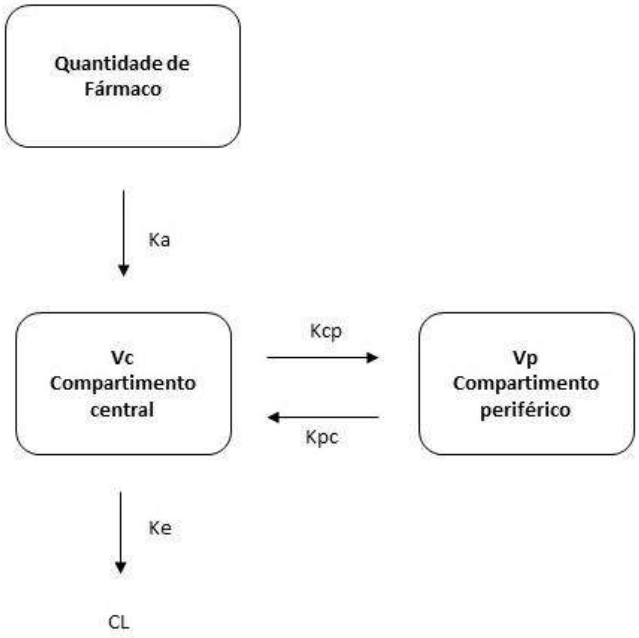


Figura 6: Representação esquemática de modelo farmacocinético bicompartimental

Diante disso, a depender do fármaco e do interesse na sua atividade, é possível ter modelos farmacocinéticos com diversos compartimentos, chamados de modelos multicompartimentais(83). Neles, o fármaco leva um tempo maior para atingir a C_{max} no compartimento de periférico (Vp), chamado de tempo de latência (T_{lag})(83).

Também pode ser compreendido que, a quantidade de fármaco administrada não necessariamente será a quantidade disponível no organismo(82). A dissolução e a absorção do medicamento podem ser parciais. Portanto, a fração (F) de uma dose que é absorvida reflete a biodisponibilidade de um medicamento e, portanto, $0 < F \leq 1$(82).

Ao reproduzir graficamente a concentração do fármaco ao longo do tempo, a área abaixo da curva da concentração versus tempo, "*area under the curve*" (AUC), representa a quantidade total de fármaco absorvido naquele compartimento, conforme modelo a seguir:

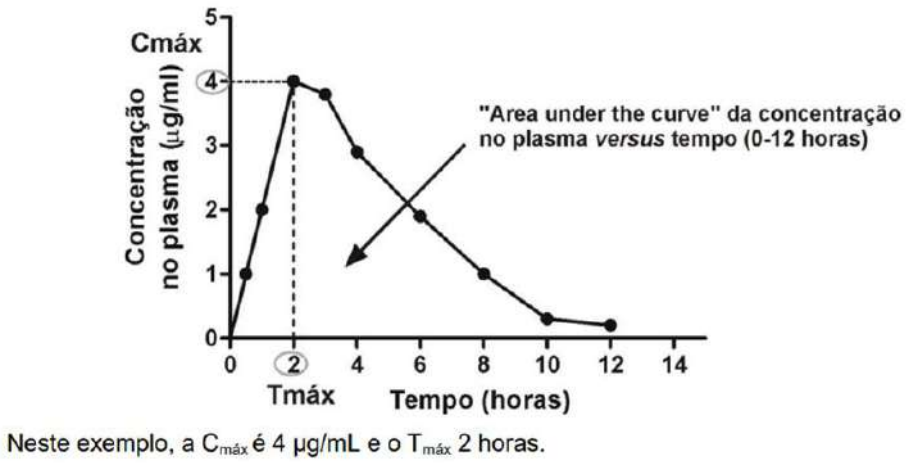


Figura 7: Concentração de fármaco ao longo do tempo, com representação de C_{max}, T_{max} e AUC(85)

Para que esses índices tenham aplicabilidade clínica, é importante que se conheça as faixas terapêuticas de cada fármaco(82). Abaixo dessa faixa, encontram-se as subdoses e acima, as doses tóxicas, conforme ilustrado abaixo:

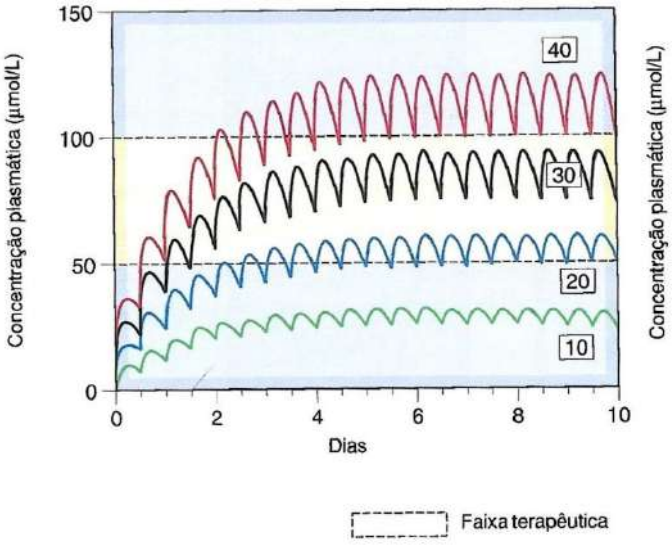


Figura 8: Identificação de níveis terapêuticos, tóxicos e subterapêuticos, a partir da concentração plasmática(83)

Após a introdução das sulfonamidas, em 1935, começa-se a estudar a eficácia e o uso seguro dos antimicrobianos(86). Em 1950, Eagle e colaboradores observaram que havia correlação entre a concentração sérica de penicilina e o seu efeito terapêutico. Abaixo de determinada concentração, posteriormente chamada de

concentração inibitória mínima (MIC), o efeito bactericida cessava por completo(87). Entretanto, esse efeito não melhorava com o aumento da concentração sérica, mas sim com o tempo que a penicilina permanecia acima da MIC no sangue(88). Esses estudos concluíram que a ação da penicilina é tempo dependente, formando as bases para o índice farmacológico de tempo acima da MIC ($T_{>MIC}$)(86).

Em 1956, Goodman & Gilman correlacionaram as concentrações de antimicrobianos com a eficácia terapêutica e postularam que, concentrações de 2 a 5 vezes maiores que a MIC definida *in vitro* deveriam ser atingidas para que houvesse eficácia terapêutica(86). Entre as décadas de 1970 e 1990, diversos autores estudaram a ligação entre as concentrações séricas e o efeito dos antimicrobianos, até que alguns conceitos fossem definidos(86,89):

1. Tempo acima da MIC ($T_{>MIC}$): o tempo percentual cumulativo ao longo de 24 horas que a concentração de um fármaco deve exceder a MIC(86).

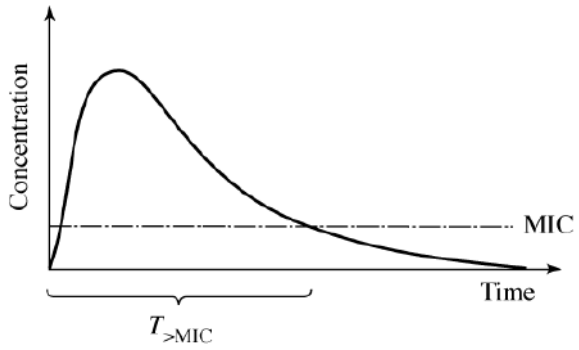


Figura 9: Representação de $T_{>MIC}$(86)

2. Concentração máxima sobre a MIC (C_{max}/MIC): também conhecida como pico sobre a MIC(86). Corresponde à divisão da concentração máxima do fármaco no sangue (C_{max}) pela concentração inibitória mínima (MIC)(86). Indica que o

efeito do antimicrobiano é concentração dependente e que um aumento na dose pode estar correlacionado com um aumento no efeito(86).

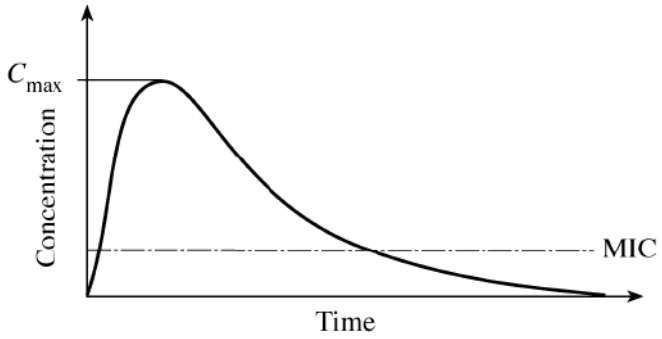


Figura 10: Representação de C_{max}(86)

3. Área abaixo da curva sobre a MIC (AUC/MIC): a área abaixo da curva de concentração ao longo do tempo dividido pela concentração inibitória mínima (MIC)(86). Esse índice se correlaciona com fármacos de concentração dependente.

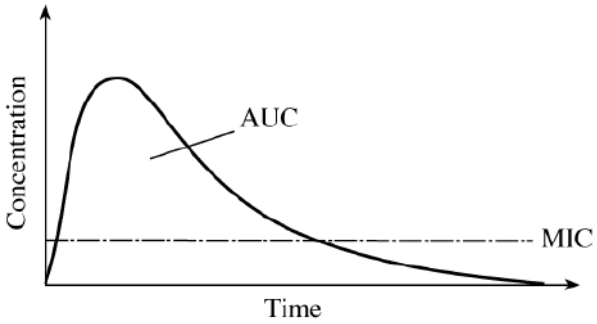


Figura 11: Representação de AUC(86)

1.13 Sepse

Em termos leigos, a sepse ocorre quando a resposta inflamatória contra um microrganismo agressor é tão intensa que causa lesão no próprio organismo do hospedeiro. Por definição, a sepse representa a resposta do organismo a um estímulo infeccioso. É caracterizada pela desregulação da resposta inflamatória, anti-inflamatória e de coagulação do organismo, levando a um estado pró-inflamatório

inicial, acompanhado de um estado pró-coagulante e antifibrinolítico(90). Como resultado, nos pacientes que evoluem com maior gravidade, as funções orgânicas começam a se deteriorar, dando origem a um quadro denominado síndrome da disfunção de múltiplos órgãos que, se não tratado, causa a morte do indivíduo(91).

A sepse acomete 48,9 milhões de pessoas por ano, com 11 milhões de mortes, uma letalidade estimada de 22,5%(92). Suas três principais causas no mundo são diarreia infecciosa, infecções respiratórias e tuberculose(93). Existe uma variação significativa da incidência e da letalidade de acordo com o Índice de Desenvolvimento Humano (IDH)(93). Boas condições sanitárias são peça fundamental na prevenção da sepse, uma vez que 50% dos casos de infecção podem ser evitados com água tratada e higiene de mãos(92). Além disso, recursos diagnósticos, acesso rápido ao serviço de saúde e capacidade de tratamento são essenciais para reduzir a letalidade do agravo(93). Por isso, regiões com menores IDH, como Ásia, África e América Latina, concentram 85% dos casos globais da doença(93).

As diferenças no IDH também impactam nos agentes etiológicos responsáveis pela sepse(93). Mtb disseminado é a principal causa na África subsaariana(94). Cummings e O'Donnell compararam as principais causas de sepse em países industrializados e em países de alta carga de coinfecção TB/HIV na África subsaariana. Nos países industrializados, a principal causa está nos cocos gram-positivos e nos bacilos gram-negativos, que representam, cada um deles, 34% das infecções de corrente sanguínea(94). Subsequentemente, encontram-se as infecções polimicrobianas, seguidas por aquelas causadas por germes anaeróbios e por fungos, com 25%, 4% e 2% das bacteremias, respectivamente(94). Mtb é o quinto agente com apenas 1% das infecções de corrente sanguínea(94). Já na África subsaariana há, como coloca o autor, uma inversão da pirâmide, na qual 30% dos casos de sepse têm o Mtb como agente etiológico(94). Bacilos gram-negativos, cocos gram-positivos, infecções polimicrobianas, micobatérias não-tuberculosas e fungos representam, respectivamente, 30%, 25%, 8%, 4%, 3%(94).

A disfunção orgânica na sepse está associada à resposta do hospedeiro ao agente agressor(91,95). Dessa forma, o conceito de sepse está intimamente ligado ao conceito de inflamação e de síndrome da resposta inflamatória sistêmica(95,96).

Inflamação aguda é uma resposta rápida a um agente nocivo, encarregada de levar mediadores de defesa do hospedeiro (leucócitos e proteínas) ao local da lesão(91). Para isso, existem três componentes básicos: i. alterações no calibre vascular (vasodilatação), que levam a um aumento no fluxo sanguíneo; ii. alterações estruturais na microcirculação (aumento da permeabilidade vascular), que permite que proteínas plasmáticas e leucócitos deixem a circulação e iii. migração de leucócitos dos vasos sanguíneos para o local acometido, o que favorece seu acúmulo e ativação para eliminar o agente nocivo e reparar o dano causado(17,91).

Quando o processo inflamatório é de caráter sistêmico, acometendo a corrente sanguínea, ou é perpetuado pelo agente infeccioso, o processo de vasodilatação e de aumento da permeabilidade vascular também afeta todo o organismo, levando à redução da pressão arterial, seja pela diminuição da resistência vascular periférica ou do volume plasmático causado pelo extravasamento de líquido(91,97).

No ciclo cardíaco, para que haja circulação sanguínea, é necessário que a pressão sistólica seja superior à pressão diastólica(98). Portanto, a redução da pressão arterial diminui a eficiência da circulação sanguínea, comprometendo órgãos e sistemas(97). Isso leva, por exemplo, a perda de consciência, insuficiência renal aguda, lesão hepática, íleo paralítico por má perfusão no sistema nervoso central, nos tecidos renal, hepático e no trato gastrointestinal, respectivamente(91,97). Nesse caso, ocorre a síndrome da resposta inflamatória sistêmica (SIRS). Uma vez que a pressão arterial é o produto do débito cardíaco e da resistência vascular periférica e que, por sua vez, o débito cardíaco é o produto da frequência cardíaca e do volume sistólico final, o coração aumenta a frequência cardíaca como mecanismo compensatório(91,98). Quando mesmo após a utilização de mecanismos compensatórios, o sistema circulatório não é capaz de fornecer O_2 para os tecidos periféricos, tem-se um quadro de choque circulatório(95,97,99,100). Nesse contexto, anormalidades circulatórias e metabólico-celulares são grandes o suficiente para elevar o risco de óbito em até 40%(91,97).

Além das vias inflamatórias, a cascata de coagulação também é ativada no processo. O estímulo inflamatório no endotélio vascular causa liberação de fator tecidual pelas células endoteliais, ativando a via extrínseca da cascata de coagulação e dando origem à trombina que, por sua vez, ativa a via intrínseca de coagulação e

permite a conversão de fibrinogênio em fibrina(91). Esse processo gera a formação de microtrombos que podem obstruir a microcirculação(91). A disfunção microcirculatória, por sua vez, leva à disfunção mitocondrial(91).

O objetivo principal da circulação sanguínea é o fornecimento de O_2 aos tecidos para que haja respiração celular, processo no qual a mitocôndria utiliza-o como substrato para a produção de adenosina trifosfato (ATP)(97,101). A falta de fornecimento de O_2 gera produção de ATP pela via anaeróbia com acúmulo de ácido lático e, consequente, acidose metabólica(101). Vale ressaltar que, além da restrição no fornecimento de O_2, há um aumento de seu consumo pelos tecidos uma vez que a atividade celular de defesa e reparação consome energia(97,102).

O choque séptico é um subgrupo da sepse no qual as alterações na circulação, microcirculação e no metabolismo celular são importantes o suficiente para elevar o risco de óbito(96,103). É definido clinicamente como hipotensão com necessidade de medicamentos vasopressores para manter PAM ≥ 65 mmHg com lactato > 2 mmol/L (18 mg/mL), apesar da ressuscitação volêmica adequada(96).

Diante disso, o diagnóstico da sepse passa, atualmente, pela identificação das disfunções orgânicas do paciente acometido. Com o objetivo de melhorar a compreensão da história natural da disfunção de órgãos e possibilitar sua mensuração, o escore SOFA (Sequential Organ Failure Assessment) foi desenvolvido pela European Society of Intensive Care Medicne em 1994 e publicado em 1996(91). Disfunções nos sistemas respiratório, de coagulação, hepático, cardiovascular, neurológico e renal são levados em conta conforme a tabela a seguir. Devem ser considerados os piores valores no período de 24 horas e cada variável oscila de 0 a 4 pontos.

Órgão ou Sistema	Variáveis	0	1	2	3	4
Respiratório	PaO2/FiO2	≥ 400 mmHg	< 400 mmHg	< 300 mmHg	< 200 mmHg (com suporte ventilatório)	< 100 mmHg (com suporte ventilatório)
Coagulação	Plaquetas	≥ 150 mil	< 150 mil	< 100 mil	< 50 mil	< 20 mil
Hepático	Bilirrubina	< 1,2 mg/dL	1,2-1,9 mg/dL	2,0-5,9 mg/dL	6,0-11,9 mg/dL	> 12 mg/dL
Cardiovascular	Pressão Arterial Média	≥ 70 mmHg	< 70 mmHg	Dopamina < 5 ou dobutamina	Noradrenalina ≤ 0,1 µg/kg/min	Noradrenalina > 0,1 µg/kg/min
Neurológico	Escala de Coma de Glasgow	15 pontos	13-14 pontos	10-12 pontos	6-9 pontos	< 6 pontos
Renal	Creatinina	<1,2 mg/dL	1,2-1,9 mg/dL	2,0-3,4 mg/dL	3,5-4,9 mg/dL	> 5,0 mg/dL
	Débito urinário				< 500 mL/24h	< 200 mL/24h

Tabela 2: Pontuação do escore SOFA(96)

Além do SOFA, diversos outros escores são utilizados nas unidades de terapia intensiva (UTI). Um deles é o Acute Physiological and Chronic Health Evaluation II (APACHE II)(104). No entanto, esse escore de gravidade não busca avaliar a disfunção orgânica, mas sim o risco de óbito de pacientes internados em UTI(91). É composto por 12 variáveis e o risco de óbito é apresentado em percentual, calculado a partir de uma equação de regressão logística que resulta em uma pontuação, conforme disposto na Tabela que segue(104).

Pontuação APACHE II	Risco de óbito
0 - 4 pontos	4% não cirúrgicos, 1% pós-cirúrgico
5 - 9 pontos	8% não cirúrgico, 3% pós-cirúrgico
10 - 14 pontos	15% não cirúrgico, 7% pós cirúrgico
15 - 19 pontos	24% não cirúrgico, 12% pós-cirúrgico
20 - 24 pontos	40% não cirúrgico, 30% pós-cirúrgico
25 - 29 pontos	55% não cirúrgico, 35% pós-cirúrgico
30 - 34 pontos	Aprox. 73% ambos
35 - 100 pontos	85% não cirúrgico, 88% pós-cirúrgico

Tabela 3: Correlação entre a pontuação no escore APACHE II e o risco de óbito(91)

São variáveis consideradas no APACHE II: temperatura, pressão arterial média, frequência cardíaca, frequência respiratória (em ventilação espontânea ou em ventilação mecânica), oxigenação (PaO2), pH arterial, sódio sérico, potássio sérico, creatinina sérica, hematócrito, contagem de leucócitos, escala de coma de Glasgow e bicarbonato sérico(91,104). As variáveis são pontuadas de 0 a 4 conforme o desvio da normalidade(91,104).

O Terceiro Consenso Internacional para Definição de Sepse considera que uma disfunção orgânica ameaçadora à vida corresponde a uma variação positiva de 2 pontos no SOFA subsequente a uma infecção(90). O SOFA ≥ 2 pontos traz um risco de óbito intra-hospitalar de aproximadamente 10%(90,105). O SOFA basal pode ser assumido como zero em paciente sem disfunções prévias conhecidas(90). No entanto, a avaliação do SOFA depende de dados laboratoriais que podem não estar disponíveis imediatamente, o que retarda a conduta terapêutica. Por isso, à beira-leito, sugere-se a utilização do *quick*-SOFA (qSOFA), avaliando alteração do estado mental, frequência respiratória maior que 22 irpm e pressão arterial sistólica menor ou igual a 100 mmHg(106). Cada item, quando positivo, soma 1 ponto(106). Deve-se suspeitar de sepse e iniciar medidas terapêuticas quando a variação no qSOFA for maior ou igual a 2 pontos(90). Após a obtenção de demais informações por meio de exames laboratoriais, o quadro de sepse poderá ser confirmado caso o SOFA apresente uma variação maior ou igual a 2 pontos(90). Se o SOFA não confirmar o resultado, o paciente deve ter sua condição clínica monitorada(90).

Para o tratamento da sepse é necessário devolver o organismo ao seu estado fisiológico, sendo fundamental ventilar, repor a volemia e garantir que os tecidos periféricos recebam aporte de O_2(97,107–110). A suplementação de O_2 é fundamental, pois os tecidos periféricos, além de não conseguirem o aporte suficiente pela circulação sanguínea, apresentam aumento de seu consumo devido ao processo inflamatório(102,111). A forma de ofertar O_2 varia de acordo com o estado clínico do paciente, que pode se dar de maneira passiva, com uso de cateter nasal ou máscara, ou de forma ativa, com ventilação mecânica invasiva ou não invasiva(91,102,106). A reposição volêmica busca restabelecer o sistema circulatório, de modo a recuperar a pressão arterial e o aporte de sangue nos órgãos e tecidos(91,107). Deve ser

realizada por meio de cristaloide com dose aproximada de 30 a 50 mL/kg nas primeiras 6 horas(91,102,107). Caso a pressão arterial não seja reestabelecida, deve-se utilizar droga vasoativa, preferencialmente, noradrenalina para garantir a perfusão sanguínea(99). Essa terapêutica é uma ponte enquanto o estado pró-inflamatório prevalece.

Todavia, para que o processo inflamatório cesse e o organismo restabeleça por si só o estado fisiológico, é fundamental que o patógeno causador seja eliminado. Sendo assim, o início precoce de antimicrobiano adequado é a peça fundamental do tratamento da sepse(112). Segundo Kumar et al., o tempo entre o diagnóstico da sepse e o início da antibioticoterapia é, isoladamente, o principal preditor de mortalidade(112). Para cada hora de atraso no início do antimicrobiano, o risco de óbito aumenta em 7,6%, chegando a mais de 60% após 6 horas(112). Portanto, o início oportuno do antimicrobiano correto, com nível terapêutico adequado é o tratamento real da sepse.

Mesmo com o entendimento da fisiopatologia e da terapêutica que deve ser instituída, a sepse ainda mata 1 em cada 4 pacientes(93,113). Dentre as potenciais causas elencadas para a elevada letalidade está a concentração subterapêutica de antimicrobianos(114). Em 2015, Roberts et al. avaliou a concentração sérica de β-lactâmicos em um estudo multicêntrico em pacientes de 68 UTIs, em 10 países do mundo(114). Concentrações séricas abaixo do esperado foram encontradas em 20% dos pacientes, considerando um alvo terapêutico mais conservador, e em 50%, considerando os alvos sugeridos pelo autor(114).

A otimização terapêutica no uso de antimicrobianos é fundamental não só para maximização do sucesso terapêutico, mas também para evitar o surgimento de resistência antimicrobiana e garantir maior vida útil dos fármacos(115). Embora o processo de otimização terapêutica (dose e espectro) seja desafiador nas UTIs, onde os pacientes apresentam grande variabilidade farmacocinética interindivíduos e intra-indivíduos, o não uso dessa estratégia pode levar a concentrações séricas baixas com falha terapêutica ou a concentrações muito altas com elevada toxicidade(116,117). O monitoramento terapêutico de dose (TDM – therapeutic drug monitoring) faz parte da estratégia de otimização e é recomendado para pacientes críticos(115).

Pacientes críticos são aqueles cujas condições clínicas são potencialmente fatais e necessitam de cuidados especializados em UTI(91). Esses pacientes requerem monitoramento e tratamento devido à disfunção de órgãos vitais(91). Clinicamente, caracterizam-se por mudanças homeostáticas marcantes, impulsionadas pelo processo inflamatório(91,97). Essa condição clínica não é encontrada em pacientes ambulatoriais. Todos esses fatores influenciam significativamente a farmacocinética (PK) antimicrobiana, em especial, o volume de distribuição (Vd) e o clearance das drogas (CL)(115).

TB usualmente afeta os pulmões. Entretanto, ocorre em qualquer órgão ou sistema na forma aguda, podendo mimetizar outras patologias infecciosas ou não(18). As causas mais comuns para internamento são a insuficiência respiratória e a síndrome do desconforto respiratório agudo (SDRA), embora estudos de necropsia mostrem que broncopneumonia por tuberculose pode mimetizar SDRA e disfunção orgânica múltipla(118–120).

Estima-se que de 3% a 16% dos pacientes com TB necessitarão de cuidados em uma UTI devido a insuficiência respiratória aguda, síndrome do desconforto respiratório agudo e/ou falência de múltiplos órgãos(90,119,121). Enquanto a taxa de mortalidade global por TB permanece em aproximadamente 15%, os resultados para pacientes que requerem ventilação mecânica são ruins, com mortalidade hospitalar relatada de 33% a 78%(122–125).

Em um estudo realizado na Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (FMT-HVD), observou-se que 80% das admissões por TB em UTI ocorreram por insuficiência respiratória(125). Dentre esses, 87,3% necessitaram de ventilação mecânica e 71,6% evoluíram com SDRA(125). A proporção de letalidade nesse estudo foi de 78,6%(125). Constatou-se, ainda, que 81,4% dos pacientes admitidos na UTI estavam em tratamento para TB(125). Ao mesmo tempo, nenhum dos medicamentos de primeira linha disponíveis no Brasil para tratamento de TB apresentam formulação parenteral. Dessa forma, comprimidos contendo rifampicina, isoniazida, pirazinamida e etambutol são macerados e misturados em água destilada para a formação de uma suspensão que é administrada ao paciente via sonda nasogástrica ou nasoenteral. Considerando o estado crítico do paciente e a disfunção no trato gastrointestinal que impeça a absorção dos fármacos, é possível questionar

se a alta taxa de letalidade nas unidades de terapia intensiva está associada às concentrações subterapêuticas dos tuberculostáticos.

Alguns estudos demonstraram proporção significativa de pacientes com baixa concentração plasmática de RHZE, o que se associa com falha terapêutica, tanto em pacientes ambulatoriais internados em enfermarias como naqueles admitidos em UTI (68,126).

Diante do exposto, pode-se supor um desfecho desfavorável devido à má absorção da droga pelos pacientes críticos, a partir da hipótese de que a dose de RHZE é insuficiente para o tratamento efetivo de pacientes em UTI.

2. OBJETIVOS

2.1 Gerais

Comparar a farmacocinética do esquema RHZE em indivíduos com tuberculose grave admitidos em uma unidade de terapia intensiva com pacientes não graves em tratamento ambulatorial.

2.2 Específicos

- Desenvolver método de quantificação de RHZE em uma única amostra de plasma.
- Comparar a relação entre farmacocinética (PK) e farmacodinâmica (PD) do esquema RHZE em pacientes internados na UTI e em pacientes ambulatoriais.
- Analisar se as doses atuais de RHZE atingem concentrações terapêuticas.
- Descrever aspectos epidemiológicos, clínicos, microbiológicos e de desfecho dos dois grupos.

3. MÉTODOS

3.1 Modelo de estudo e seleção de pacientes

Este estudo farmacocinético, prospectivo e aberto foi realizado na Fundação de Medicina Tropical Dr. Heitor Vieira Dourado, na cidade de Manaus, Amazonas, Brasil, entre novembro de 2016 e maio de 2018. Foram incluídos indivíduos com mais de 18 anos de idade, com TB ativa pulmonar ou extrapulmonar, com prescrição de comprimidos DFC contendo RHZE.

Foram considerados pacientes com TB ativa, aqueles com a presença de pelo menos dois dos seguintes critérios(54):

1. Baciloscopia positiva para bacilos álcool-ácido resistentes (BAAR) ou GeneXpert MTB/RIF© (Cepheid, Sunnyvale, CA, EUA) detectável no escarro, aspirado traqueal ou qualquer outra amostra clínica;
2. Cultura positiva para *Mycobacterium tuberculosis* no escarro, aspirado traqueal ou qualquer outra amostra clínica;
3. Forte suspeita clínica de TB ativa, que exige pelo menos dois dos quatro sintomas constitucionais associados ao contato conhecido com indivíduo com TB ou história de TB prévia:
    a. perda de peso,
    b. febre aferida (temperatura axilar ≥ 37,8°C) ou referida,
    c. sudorese noturna,
    d. tosse produtiva,
    e. perda de apetite;
4. Forte evidência radiológica de TB ativa

Os pacientes foram recrutados no ambulatório e na UTI da Fundação de Medicina Tropical Dr. Heitor Vieira Dourado. O diagnóstico e o tratamento foram definidos pelo médico assistente dos pacientes e, aqueles que preenchiam os critérios de inclusão foram convidados a participar do estudo. Todos os pacientes realizaram

tratamento diretamente observado, recebendo RHZE em comprimidos DFC, conforme peso (de 20 a 35 kg: 550 mg; de 36 a 50 kg: 825 mg e acima de 50 kg: 1.100 mg), de acordo com protocolo do Ministério da Saúde do Brasil, vigente no momento do estudo(55). Foram excluídas gestantes, indivíduos em hemodiálise, terapia renal substitutiva contínua, diálise peritoneal ou aqueles que as equipes assistentes consideraram inadequados para a inclusão no estudo.

Dados demográficos incluíram gênero, idade, profissão e local de residência. Os dados clínicos compreenderam peso, índice de massa corporal (IMC), comorbidades, escores SOFA e APACHE II, antimicrobianos e medicações em uso durante o estudo e, até 30 dias antes da inclusão, resultados de funções renal e hepática, hemograma, glicemia em jejum, status para HIV, sorologia para hepatite B, C e sífilis. Pacientes HIV positivos tiveram a carga viral e a contagem de linfócitos T $CD4^+$ avaliadas. Os pacientes ambulatoriais não tiveram seu SOFA avaliado por não apresentarem nenhuma disfunção orgânica. Optou-se por realizar a aferição do clearance de creatinina (CrCl) por meio da relação entre creatinina urinária e creatinina sérica, calculado pela seguinte fórmula:

CrCl = (Creatinina Urinária/Creatinina Sérica) x (Volume urinário/Tempo em horas x 60)

Foram coletadas amostras de sangue de cada paciente no tempo zero (imediatamente antes da dose) e aos 30, 60, 120, 240, 360, 480, 720 e 1.440 minutos (imediatamente antes da dose subsequente), no primeiro e terceiro dias após a inclusão no estudo.

As coletas foram realizadas obedecendo aos seguintes critérios:

1. Coleta de 10 mL de sangue em tubo heparinizado.
2. Armazenamento das amostras, imediatamente após a coleta, a 4°C, por no máximo 6 horas até a centrifugação.
3. Centrifugação das amostras a 434 × força gravitacional por 10 minutos.
4. Transferência do plasma (de 1,5 a 1,8 mL) para um criotubo marcado (ver rotulagem)

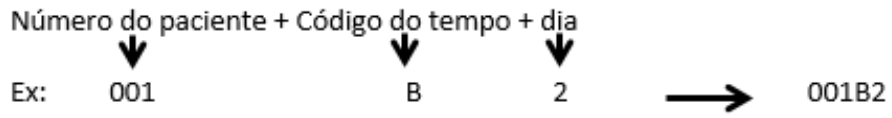
a. Foi garantido que nenhuma célula vermelha fosse transferida.

5. Colocação imediata do criotubo no freezer

a. Inicialmente -20°C (armazenamento de curto prazo).

b. Transferido para -80°C até o envio para análise no Departamento de Farmácia da Universidade Federal do Paraná.

A rotulagem foi realizada da seguinte maneira:



O código do tempo está especificado no Formulário de Coleta de Amostras (Item 9.2)

Todos os pacientes do grupo UTI estavam em ventilação mecânica e receberam os comprimidos DFC por meio de sonda nasogástrica. Antes da administração do medicamento, o enfermeiro do estudo macerava os comprimidos e preparava uma suspensão em 20 mL de água destilada. Em seguida, outros 20 mL de água destilada eram administrados também pela sonda nasogástrica a fim de garantir que a suspensão chegasse ao trato gastrointestinal. Cada paciente da UTI foi avaliado diariamente quanto às suas necessidades individuais de vasopressores e pontuação APACHE II e SOFA. Os pacientes ambulatoriais foram convidados a participar do estudo e a serem admitidos na enfermaria de pesquisa clínica por 72 horas para tratamento diretamente observado com comprimidos DFC e coleta de amostras. Todos os pacientes permaneceram em contato com a equipe do estudo até o final do tratamento.

3.2 Análise das concentrações séricas

As concentrações plasmáticas totais de RHZE foram medidas pelo método de cromatografia líquida de alta pressão com um detector MS/MS em um espectrômetro de massa Waters Xevo G2-S QToF (Waters Corp., Milford, MA, EUA), na faixa de 0,2 a 5 mg/L. O método foi validado pela equipe e publicado (Item 4.1).

Para medir concentrações acima do limite superior de identificação do método (5 mg/mL), as diretrizes de validação de métodos bioanalíticos recomendam a preparação de um controle de qualidade de diluição. Nesse caso, tanto o controle de qualidade quanto a amostra são submetidos a esse processo de diluição. De acordo com a Agência Brasileira de Vigilância Sanitária (Anvisa) e a Food and Drug Administration (FDA) dos Estados Unidos da América, o controle de qualidade da diluição deve ser considerado se a acurácia e a precisão forem 15% da concentração nominal e menor que 15% do desvio padrão relativo. A acurácia foi calculada como o erro relativo e a precisão como o desvio padrão relativo. O erro relativo da acurácia intradiária variou de 0,26% a 13,7%.

3.3 Modelo farmacocinético

Um modelo farmacocinético populacional foi desenvolvido para o etambutol, usando software Pmetrics versão 1.5.0 (Laboratoryof Applied Pharmacokinetics and Bioinformatics, Los Angeles, CA, EUA) em RStudio (versão 0.99.9.3) como um wrapper para R (versão 3.3.1), Xcode (versão 2.6.2) e o Intel Parallel Studio Fortran Compiler XE 2017. Dentro do Pmetrics, foram construídos modelos estruturais de um ou dois compartimentos usando os algoritmos de grade adaptativa não paramétrica (NPAG). O modelo de um compartimento incluiu a eliminação linear de etambutol do compartimento central. O modelo de dois compartimentos testou o uso de constantes de transferência intercompartimental entre os compartimentos central e periférico (Kcp e Kpc), bem como a depuração intercompartimental (Q). Como os pacientes estavam recebendo doses de etambutol a cada 24 horas, a inclusão de ocasião para a primeira e segunda doses foi testada em relação a taxa de absorção, biodisponibilidade, tempo de latência e depuração. A biodisponibilidade absoluta não foi determinada, pois nenhum dos fármacos está disponível no Brasil na forma intravenosa. Modelos de erro aditivo (*lambda*) e multiplicativo (*gama*) foram testados usando uma equação polinomial para desvio padrão em função da concentração observada. Y. (SD = C0 + C1.Y), com ponderação de observação realizada como erro = SD.*gama* ou erro = (SD2 + *lambda2*) 0,5.

A inclusão de covariáveis clínicas biologicamente plausíveis foi avaliada por meio da aplicação de regressão linear entre os parâmetros farmacocinéticos e as

covariáveis categóricas. As covariáveis contínuas foram avaliadas por regressão linear, logarítmica, polinomial e de potência. As covariáveis selecionadas que foram testadas nos parâmetros do modelo estrutural incluem idade, sexo, peso corporal total, índice de massa corporal (IMC), pontuação de SOFA e APACHE II, diabetes, medicação concomitante em uso e antimicrobianos usados nos últimos 30 dias, resultado de funções renal (CrCl) e hepática, contagem de células sanguíneas, status de HIV, sorologias para hepatite B e C e sífilis. O peso e o CrCl foram testados normalizados para valores medianos do paciente e com uma escala alométrica aplicada(127–130).

3.4 Avaliação do modelo

A avaliação do modelo foi realizada por meio de gráficos e exame estatístico para comparação e seleção dos modelos. A triagem inicial foi executada avaliando visualmente, para cada análise, a qualidade do ajuste e o coeficiente de determinação (r2) da regressão linear dos valores observados e previstos dos gráficos (r2 mais próximo de 1, intercept mais próximo de 0). A aceitação do melhor ajuste da estrutura do modelo, do modelo de erro e a inclusão de covariáveis foi identificada por uma mudança na função objetivo (OFV), calculada como uma diminuição no teste da razão de verossimilhança (−2*LL) de −3,84 (correspondente para um $p < 0,05$ com base na distribuição Qui-quadrado e um grau de liberdade) e diminuição no critério de informação de Akaike (AIC). Para a seleção do modelo final foram fatorados viés (erro médio previsto-observado ponderado) e imprecisão (erro médio previsto-observado ponderado ao quadrado). Por fim, para avaliar a consistência interna das predições do modelo com as observações, os erros de distribuição de predição normalizados e a verificação preditiva posterior foram utilizados gráficos de verificação preditiva visual. A proporção de observações entre o percentil 5 e 95 simulados acima de 90% foi considerada adequada.

3.5 Simulações de Dose e Alvo terapêutico

Foram realizadas Simulações de Monte Carlo (n = 1.000) com resultados previstos para intervalos de 24 horas. Os valores das covariáveis de cada um dos pacientes simulados foram fixados na mediana do peso corporal total e do CrCl. Os

regimes de dosagem foram simulados considerando os alvos PK/PD de $AUC_{(0-24)}$/MIC maior que 11,9 mg.h/L e de C_{max}/MIC maior que 0,48 mg/L e, também, 12% de ligação às proteínas plasmáticas(131). Eles também foram simulados em um estado de equilíbrio para depurações de creatinina de 30, 90, 130 e 180 mL/min/1,73 m2 e peso corporal total de 40, 50, 60 e 70 kg com base na dose de FDC. O *probability target attainment* (PTA) para atingir as metas de PK/PD foi avaliada e valores superiores a 95% foram considerados desejáveis. O *fractional target attainment* (FTA) identificou o alcance das exposições alvo aos antibióticos comparando o PTA com a distribuição da MIC para *Mycobacterium tuberculosis*-7H9, do banco de dados do Comitê Europeu de Suscetibilidade e Testes Antimicrobianos (EUCAST) (disponível em www.eucast.org, acessado em 13 de dezembro de 2021). O FTA para terapia empírica foi calculado considerando a distribuição da MIC entre 0,25 e 32 mg/L. As doses foram consideradas aceitáveis se o FTA apresentasse resultado superior a 85%.

3.6 Análise não compartimental

Para rifampicina, isoniazida, pirazinamida e etambutol, foi realizada uma análise não compartimental de farmacocinética. Os parâmetros de PK foram calculados usando Pmetrics v.1.5.0 (Laboratory of Applied Pharmacokinetics and Bioinformatics, Los Angeles, CA, EUA) no RStudio (versão 0.99.9.3) como um wrapper para R (versão 3.3.1), Xcode (versão 2.6.2) e o Intel Parallel Studio Fortran Compiler XE 2017. Foram excluídos da análise PK os pacientes com concentrações plasmáticas do fármaco abaixo do limite inferior de quantificação ou cujas concentrações não decaíram.

3.7 Análise estatística

Foi realizada análise descritiva dos dados com uso da distribuição de frequência e medidas de tendência central. As variáveis categóricas foram expressas em frequência e porcentagem e analisadas pelo teste X^2 de Pearson ou pelo teste exato de Fisher. Para variáveis numéricas, foi utilizado o teste de Mann-Whitney. Para comparar as diferenças entre as ocasiões de dosagem, a taxa de absorção foi constante para a primeira e segunda doses no mesmo grupo, e um teste de

classificação de Wilcoxon foi usado. Todas as análises foram realizadas considerando um nível de significância de 5%, no software R (v.0.99.9.3).

3.8 Aspectos éticos

Este estudo foi conduzido de acordo com as diretrizes de boas práticas clínicas e com a legislação local em vigor.

Este estudo foi aprovado pelo Comitê de Ética da Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (CEP/FMT-HVD CAAE: 60219916.5.0000.0005) (Item 8.1).

Para participação no estudo, os pacientes assinaram o termo de consentimento livre e esclarecido (item 9.3). Esse termo foi lido pelo investigador juntamente com o paciente ou responsável legal. Depois de todas as dúvidas sanadas, o termo foi assinado pelo paciente ou por seu responsável legal de livre e espontânea vontade. Nos casos de paciente analfabeto, o termo foi lido por uma pessoa de confiança do paciente ou que não fizesse parte do grupo de pesquisa.

Os termos de consentimentos assinados ou em branco foram arquivados separadamente para maior sigilo de dados dos pacientes e estão sob a guarda do investigador principal.

A identidade dos pacientes do estudo foi conhecida apenas pelo investigador principal e por um subinvestigador do estudo, que eram responsáveis pela codificação desses pacientes e pela coleta das amostras. Os demais pesquisadores tiveram acesso apenas ao banco de dados codificado, sem acesso a identificação dos pacientes.

4. RESULTADOS

4.1 Artigo publicado descrevendo método de quantificação de rifampicina, isoniazida, pirazinamida e etambutol em uma única amostra de plasma

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RESEARCH ARTICLE

LC-QToF-MS method for quantification of ethambutol, isoniazid, pyrazinamide and rifampicin in human plasma and itsapplication

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Abstract

In this research, we developed and validated a liquid chromatography coupled to mass spectrometry (LC-QToF-MS) method for simultaneous quantification of the anti- tuberculosis drugs ethambutol, isoniazid, pyrazinamide and rifampicin in human plasma. Plasma samples spiked with cimetidine (internal standard) were extracted usingprotein precipitation with acetonitrile containing 1% formic acid. Separation was per-formed using a C_{18} column under flow gradient conditions with water and acetonitrile,both containing 5 mM ammonium formate and 0.1% formic acid. The method was vali- dated according to the ANVISA and US Food and Drug Administration guidelines forbioanalytical method validation. The calibration curve was linear over a concentration

range of 0.2-5 μg ml^{-1} for ethambutol, 0.2-7.5 μg ml^{-1} for isoniazid, 1-40 μg ml^{-1} for pyrazinamide and 0.25-2 μg ml^{-1} for rifampicin, all with adequate precision and accu-racy. The method was reproducible, selective and free of carryover and matrix effects.

The validated LC-QToF-MS method was successfully applied to real samples andshown to be applicable to future therapeutic and pharmacokinetic monitoring studies.

KEYWORDS

anti-tuberculosis drugs, human plasma, LC-QToF-MS, validation

1 | INTRODUCTION

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis*, and is the most lethal infectious disease globally, especially when associated with HIV. According to estimates from the World Health Organization (WHO, 2019), 10 million people developed TB in 2018. However, this scenario can be reversed, since with the correct diagnosis and treatment most patients are cured. Therefore, public health strategies are focused on disease control, seeking to eradicate TB in the near future (Falzon et al., 2017).

Seeking to reduce the number of cases, standardized treatment with adequate pharmacological monitoring and patient support are the best practices for disease control (Brazil, 2012). Thus, the best way to ensure a patient's cure is to use the basic principles for treatment, among which proper drug selection, correct doses and long-term use, while avoiding the development of drug resistance, stand out (Gao et al., 2018).

First-line pharmacological therapy for TB is currently composed of pyrazinamide (PZA), isoniazid (INH), ethambutol (EMB), strepto-mycin (STM) and rifampicin (RIF) (Falzon et al., 2017). For

treatment to be effective, it is often necessary to combine these drugs and adjust their doses according to body weight(McIlleron & Chirehwa, 2019). This leads to frequent problems with treatment adherence, which may still lead to mycobacterial resistance. In addition, these drugs at incorrect doses may cause serious hepatotoxicity as a side effect among other adverse reac- tions, underlining the importance of dose adjustment (Hee, Seo, & Lee, 2015).

In this context, therapeutic drug monitoring of these drugs isan important tool to control dosages in patients under treatment. Some methods are available in the literature for simultaneous anal- ysis of anti-tuberculosis drugs, but use HILIC mode, ion pairs or monolithic columns owing to the polar characteristics of some analytes (Gao et al., 2018; Prahl et al., 2016; Zhou et al., 2013). Therefore, the aim of this study was to develop and validate a rapid and sensitive LC-QToF-MS method using a C_{18} column for simultaneous quantitation of PZA, INH, EMB and RIF in human plasma for application to therapeutic drug monitoring and pharma- cokinetic studies.

2 | METHODS

2.1 | Chemicals and reagents

Isoniazid and pyrazinamide standards (≥98%) were purchased from INCQS/FIOCRUZ (Rio de Janeiro, Brazil). Ethambutol hydrochloride standard was purchased from United States Pharmacopeia (North Bethesda, MD, USA). Rifampicin standard was obtained from European Pharmacopoeia (Strasbourg, France). Cimetidine as the internal standard (IS) (≥ 98%) was purchased from Sigma-Aldrich(St Louis, MO, USA). Acetonitrile and methanol (HPLC grade) were obtained from Tedia (Fairfield, CA, USA). Formic acid (98-100%; LC-MS grade) and ammonium formate (97%; reagent grade) were purchased from Sigma-Aldrich (St Louis, MO, USA). Ultrapure waterwas obtained using a Milli-Q® purification system from Millipore (Milford, MA, USA).

2.2 | LC-QToF-MS development

LC-QToF-MS analysis of anti-tuberculosis drugs was performed on a Waters Acquity ultra-performance liquid chromatograph (UPLC) coupled to a Waters Xevo G2-S QToF mass spectrometer (Waters Corp., Milford, MA, USA) using an electrospray ionization interface. LC was performed on a Agilent InfinityLab Poroshell 120 EC-C_{18} column (150 × 4.6 mm, 2.7 µm) coupled with an Agilent InfinityLab Poroshell 120 EC-C_{18} column (5 × 2.1 mm, 2.7 µm), kept at 40◦C. The mobile phase consisted of water (A) and acetonitrile:water (95:5, v/v) (B), both containing 0.1% formic acid and 5 mM ammonium formate. The elution order gradient was as follows: 0-2.00 min, 10% B; 2.00-5.00 min, 10-90% B; 5.00-6.00 min, maintained at 90% B; 6.00-6.10, 90-10% B; 6.10-12.00 min, maintained at 10%, which resulted in a total runtime of 12 min. The flow rate was 300 µl min^{-1}, and the injection volume was 10 µl. The electrospray ionization source was performed in positive ionization mode. The temperature of the source was set at 150◦C. Nitrogen was used as nebulization gas(25 L h^{-1}) and drying gas (600 L h^{-1} at 400◦C). The capillary voltage was set at 3000 V and the scan range was *m*/*z* 50-1200. All data was collected in centroid mode, acquired using MassLynx™ NT4.1 software, and processed using QuanLynx software (Waters Corp., Milford, MA, USA).

2.3 | Preparation of standards and quality controls

Stock solutions of EMB, INH, PZA, RIF and IS (cimetidine)(1 mg ml^{-1}) were prepared in methanol, stored at −40◦C and protected from light until use. Working solutions (1-200 µg ml^{-1}) were prepared from stock solutions in acetonitrile containing 1%formic acid (v/v). Pooled blank plasma (300 µl) was spiked with a mix of working solution (60 µl) to prepare calibration standards and quality control (QC) samples. Calibration standards were preparedat the following ranges: 0.2-5 µg ml^{-1} for EMB; 0.2-7.5 µg ml^{-1} for INH; 1-40 µg ml^{-1} for PZA; and 0.25-2 µg ml^{-1} for RIF. Quality control (QC) samples were prepared at four concentration levels: 0.2, 0.4, 2 and 4 µg ml^{-1} for EMB; 0.2, 0.5, 3.0 and 5 µg ml^{-1} for INH; 1, 2, 16 and 32 µg ml^{-1} for PZA; and 0.25, 0.5, 1.25 and 1.75 µg ml^{-1} for RIF, representing the lower limit of quantification (LLoQ) and the low, medium and high QCs, respectively. The calibration standards were prepared fresh on the day of analysis, and the QC samples were stored at −40◦C and thawed prior to use.

2.4 | Blood samples

Blood samples were collected in tubes containing sodium heparin 2 h after anti-tuberculosis drug administration in a hospital in Manaus (Brazil). Plasma samples were immediately separated from the blood in a refrigerated centrifuge (1500***g*** for 10 min at 4◦C) and stored at −80◦C until subsequent analysis.

2.4.1 | Sample cleanup

For plasma processing, the frozen plasma samples were thawed at room temperature prior to analysis. An aliquot (300 µl) of the plasma sample was transferred to a polypropylene tube which contained 60 µl of the IS working solution (2.5 µg ml^{-1}). After the tube was vortexed (1 min), 840 µl of acetonitrile containing 1% formic acid (v/v) was added. This mixture was vortexed for 3 min and then centrifuged at 20,817***g*** for 15 min at 4◦C. The supernatant was filtered through a 0.22 µm × 3.0 mm PTFE mem- brane (Millex®, Millipore) into a vial for further LC-QToF-MS analysis.

2.5 | Method validation

LC-QToF-MS was validated with regard to selectivity, linearity, precision, accuracy, carryover, recovery, matrix effect and stability according to US Food and Drug Administration (FDA) and Agência Nacional de Vigilância Sanitária (ANVISA) guidelines (Brazil, 2012; FDA, 2018).

2.5.1 | Selectivity

Selectivity was assessed using six blank plasma samples from different sources (four normal, one lipemic and one hemolyzed plasma) which were compared with a blank plasma sample spiked with analytes at LLoQ concentrations and IS. All samples were checked for any interference of blank and sample responses. LLoQ level samples were determined by the percentage mean accuracy and the percentage CVs (coefficient of variation) were calculated.

2.5.2 | Carryover

Carryover was assessed by injecting a sequence of two blank plasma samples after analysis of the upper limit of quantification. The response of the analytes in blank plasma samples should be <20% ofthe response of analytes at the LLoQ concentration.

2.5.3 | Recovery

Recovery was checked by comparing plasma samples spiked with analytes and IS after sample cleanup (100% recovery) withsamples spiked with analytes and IS prior to sample cleanup (low andhigh QC).

2.5.4 | Matrix effect

Matrix effect, with respect to consistency in signal (suppression/enhancement), was assessed by comparing blank sam- ples spiked with lower, medium and high QC levels with the standardsat the same concentrations (eight replicates). The normalized effect of the matrix was calculated for each level as the response of the anal- yte/IS in the matrix divided by the response of the analyte/IS in the solution. Variations >15% relative to the normalized effect calculated for all samples suggested the presence of the matrix effect.

2.5.5 | Linearity

The calibration curves were constructed by peak area ratios for analyte/IS against the analyte/IS nominal concentration. The curve range was established according to the therapeutic range and plasma concentration of anti-tuberculosis drugs reported in other studies in the literature (Peloquin, 2002; Prahl et al., 2016; Ruslamiet al., 2007; Sturkenboom et al., 2015; Tostmann et al., 2013). Cur- ves were determined in triplicate at six concentration levels. A blank sample (without analyte and IS) and a zero calibrator (blank plus IS) were analyzed along with the curve samples. Variations of up to 15% in the accuracy and precision at each level were allowed, except for the LLoQ, for which maximum variations of 20% were permitted.

The obtained data were then submitted to linear regression analysis for the equation slopes and intercepts. Furthermore, the lin- earity of the method was confirmed by evaluating the analysis of variance (ANOVA) for model significance and lack-of-fit, homoskedasticity of the variances (Brown-Forsythe test), residual normality (Anderson-Darling test) and *t*-test for the slope and inter- cept significances.

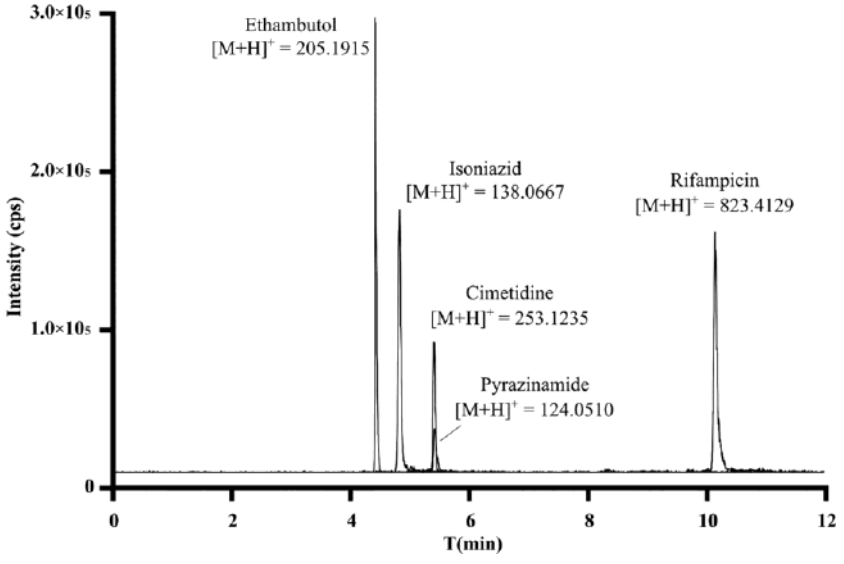

FIGURE 1 Representative chromatogram of plasma spiked with 1.0 μg ml^{-1} of ethambutol, 1.0 μg ml^{-1} of isoniazid, 2.0 μg ml^{-1} of pyrazinamide, 1.0 μg ml^{-1} of rifampicin, and 1.0 μg ml^{-1} of cimetidine (internal standard)

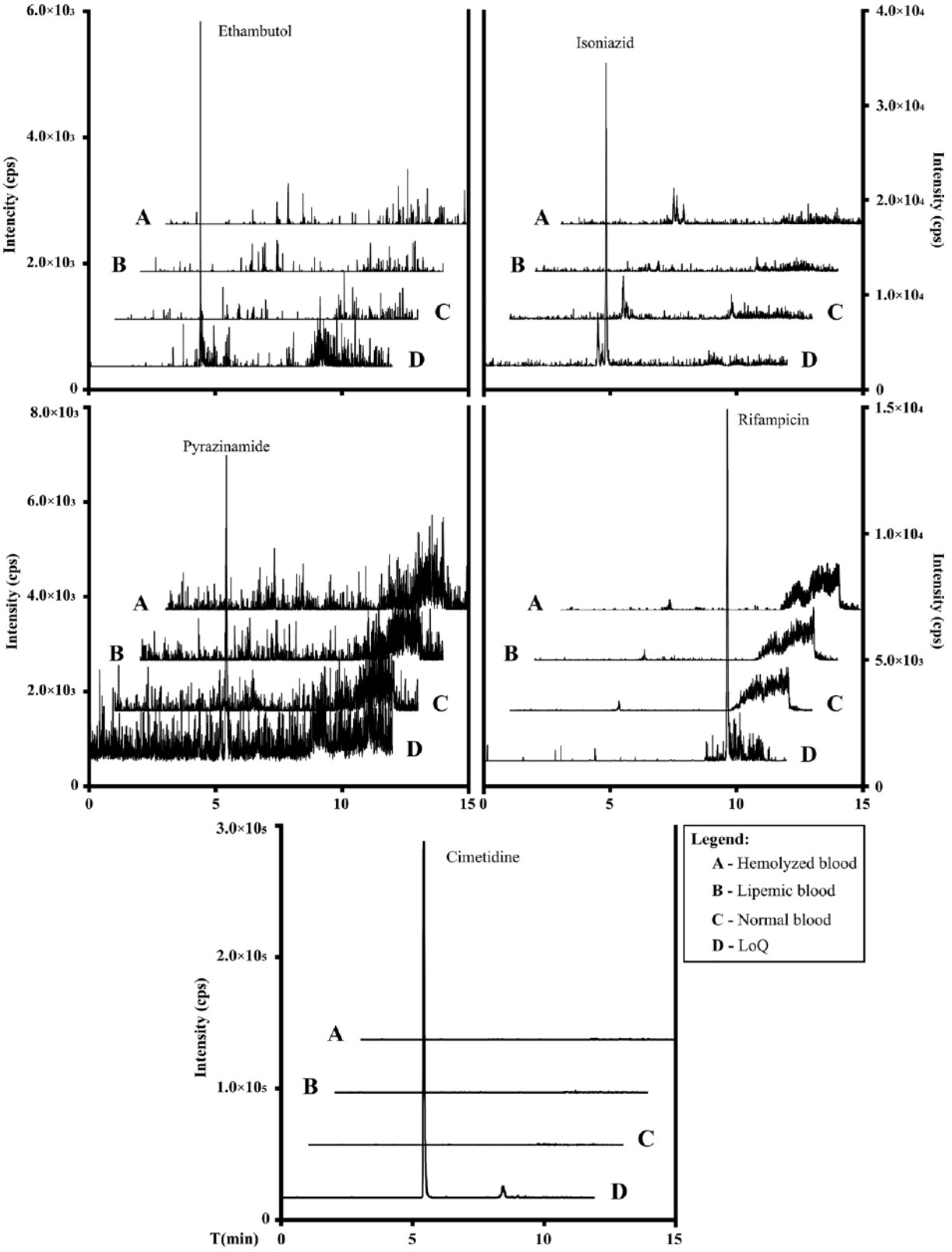

FIGURE 2 Results of selectivity

2.5.6 | Precision and accuracy

The precision and accuracy of the method were determined for a single run and different runs. The precision and accuracy were evaluated by analysis of blank plasma sample spiked with analytesat LLoQ, lower, medium and high QC levels using five replicate determinations. The accuracy was calculated as the relative error (RE), and precision as the relative standard deviation (RSD). In

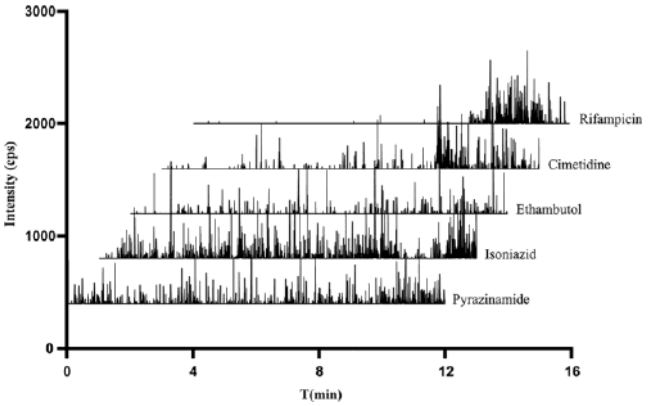

FIGURE 3 Carryover assay of analytes

addition, the intermediate precision was assessed by comparing the precision for different days whilst evaluating the equality of vari- ances using the Bartlett test.

2.5.7 | Dilution integrity

Dilution integrity was evaluated to investigate the influence of sample dilution with a blank matrix. Blank plasma samples (n = 6) were spiked with analytes to obtain concentrations three times higher than medium QC levels. Thereafter, spiked samples were diluted three times with blank matrix, prepared according to Section 2.5, and ana- lyzed. The accuracy was calculated as the RE, precision as the RSD, and the Student's t-test was used to compare means of dilution integ- rity samples and means of medium QC samples.

2.5.8 | Stability

Sample stabilities of EMB, INH, PZA and RIF were evaluated in blank plasma samples spiked with analytes at lower and high QCs levels using five replicate determinations. The spiked plasma samples were submitted to the following conditions: freeze and thaw stability (three cycles of 24 h); short-term stability at room temperature for 8 h; long-term stability at −40°C for 30 days; and post-preparative stability−

TABLE 1 Recovery and matrix effects of ethambutol (EMB), isoniazid (INH), pyrazinamide (PZA) and rifampicin (RIF)

Analyte	Quality control	Concentration (μg ml^{-1})	Recovery (RSD, %)	Mean of matrix factor (RSD, %)
EMB	LQC	0.4	94.99 (11.54)	0.89 (10.67)
	MQC	2	88.00 (7.34)	–
	HQC	4	92.95 (10.96)	0.78 (13.96)
INH	LQC	0.5	89.08 (12.45)	1.27 (12.83)
	MQC	3	85.16 (3.15)	–
	HQC	5	88.25 (8.36)	0.92 (10.32)
PZA	LQC	2	95.51 (13.50)	0.99 (12.20)
	MQC	16	90.13 (8.89)	–
	HQC	32	94.81 (8.48)	0.81 (10.74)
RIF	LQC	0.5	82.66 (10.11)	1.01 (12.75)
	MQC	1.25	88.29 (5.50)	–
	HQC	1.75	93.58 (12.64)	1.10 (11.05)

LQC, low quality control; MQC, medium quality control; HQC, high quality control; RSD, relative standard deviation

TABLE 2 Results obtained from regression analysis

Parameter	EMB	INH	PZA	RIF
Range (μg ml^{-1})	0.2-5	0.2-7.5	1-40	0.25-2
Slope (b)	22.51 ± 1.16	6.80 ± 1.38	0.28 ± 0.03	2.21 ± 0.34
Intercept (a)	−3.50 ± 3.13	−0.67 ± 3.05	0.10 ± 0.17	−0.18 ± 0.43
R^2	0.995	0.992	0.987	0.967
Slope significance	<0.001	<0.001	<0.001	<0.001
Intercept significance	0.267	0.686	0.740	0.678
Regression fit	<0.001	<0.001	<0.001	<0.001
Lack-of-fit	0.840	0.619	0.997	0.996
Homoskedasticity	0.801	0.677	0.758	0.582
Residual normality	0.527	0.055	0.529	0.310

Regression coefficients shown as mean ± SD.

TABLE 3 Intraday and interday accuracy and precision results

Analyte	Quality control	Spiking plasma concentration (μg ml^{-1})	Intraday accuracy (RE, %) Day1	Intraday accuracy (RE, %) Day2	Intraday accuracy (RE, %) Day3	Interday accuracy (RE, %)	Intraday precision (RSD, %) Day 1	Intraday precision (RSD, %) Day 2	Intraday precision (RSD, %) Day 3	Interday accuracy (RSD, %)	Interday (*P*-value)
EMB	LLoQ	0.2	6.10	17.2	11.1	11.46	17.76	17.17	17.02	17.32	0.9841
	LQC	0.4	−11.55	−4.85	−7.4	−7.93	1.93	4.91	6.67	4.50	0.0909
	MQC	2	4.22	−4.84	−2.55	−1.06	2.96	6.14	11.80	6.96	0.0620
	HQC	4	−0.26	8.28	0.72	2.91	4.79	8.40	11.85	8.35	0.2635
INH	LLoQ	0.2	1.70	16.11	13.00	13.93	15.80	4.13	15.83	11.93	0.5561
	LQC	0.5	12.04	−10.08	−7.04	−1.69	6.51	9.40	11.63	9.18	0.7485
	MQC	3	−7.03	−13.59	−13.67	−11.43	6.30	8.23	11.48	8.67	0.5909
	HQC	5	−7.24	7.27	8.54	2.85	4.45	5.24	11.07	6.92	0.1088
PZA	LLoQ	1	9.74	−3.56	−8.16	−0.66	17.49	15.75	16.88	16.71	0.8815
	LQC	2	−0.9	5.23	5.78	3.37	10.21	10.11	13.81	11.38	0.7402
	MQC	16	−8.74	−6.75	−11.34	−8.94	8.10	9.80	7.52	8.47	0.8259
	HQC	32	10.08	−6.31	4.60	2.49	10.97	14.14	11.93	12.35	0.9843
RIF	LLoQ	0.25	−7.12	13.67	4.80	3.78	9.24	1.89	6.42	5.85	0.2129
	LQC	0.5	−16.72	−4.84	−0.6	−7.38	9.40	4.18	6.26	6.62	0.5252
	MQC	1.25	8.67	0.43	−4.54	1.52	2.96	6.21	10.79	6.65	0.3517
	HQC	1.75	−6.63	6.08	−0.76	−0.43	4.79	7.52	11.65	7.99	0.9242
Cimetidine (IS)	–	2	–	–	–	–	7.07	10.93	16.82	11.60	0.5819

RE, Relative error; RSD, relative standard deviation; LLoQ, lower limit of quantification; LQC, low quality control; MQC, medium quality control; HQC, high quality control; DQC, dilution quality control; IS,internal standard. Intraday assays, $n = 5$; interday assays, $n = 15$. *P*-value obtained from Bartlett's test.

TABLE 4 Results from dilution integrity tests

Analyte	Day	MQC	DQC	*P*-value
EMB	Day 1	34.24 ± 4.98	34.74 ± 3.16	0.8541
	Day 2	35.61 ± 3.03	36.52 ± 2.37	0.6113
	Day 3	37.38 ± 6.09	39.09 ± 2.24	0.5726
	Interday	35.95 ± 4.80	37.62 ± 2.23	0.4783
INH	Day 1	21.04 ± 1.06	21.62 ± 3.06	0.7001
	Day 2	18.92 ± 1.84	20.45 ± 2.34	0.2871
	Day 3	19.33 ± 1.87	19.59 ± 2.01	0.8361
	Interday	19.10 ± 1.88	20.41 ± 1.88	0.3268
PZA	Day 1	4.52 ± 0.31	4.63 ± 0.51	0.6784
	Day 2	3.83 ± 0.83	3.96 ± 0.56	0.7831
	Day 3	3.76 ± 0.39	4.10 ± 0.42	0.2305
	Interday	3.95 ± 0.51	4.36 ± 0.61	0.3789
RIF	Day 1	100.64 ± 7.07	93.55 ± 9.93	0.2300
	Day 2	94.56 ± 10.52	84.83 ± 3.90	0.0886
	Day 3	95.58 ± 3.21	96.09 ± 4.26	0.8346
	Interday	96.92 ± 7.51	91.49 ± 7.91	0.0641

Response relative to analyte/internal standard; mean ± SD. *P*-value obtained from Student's *t*-test.

after cleanup procedure (24 h in the sample manager). The stability samples were prepared according to Section 2.5 and analyzed. The mean concentrations of stability samples were compared with those of freshly prepared samples concentrations and results are measured using RSD and Student's *t*-test.

2.5.9 | Clinical application

In order to show the applicability of the method to real samples, the validated method was applied to eight patients diagnosed with TB aged 24–57 years (seven men and one woman). The doses were calculated in accordance with the Brazilian Ministry of Health Guidelines in line with the World Health Organization Guidelines, and administered to the patients in a four-drug fixed-dosed combination regimen as follows: 1100 mg of ethambutol, 300 mg of isoniazid, 1600 mg of pyrazinamide and 600 mg of rifampicin. The Ethics Committee of Fundação de Medicina Tropical Dr Heitor Vieira Dourado approved this study protocol (CAAE 60219916.5.0000.0005). All patients provided written informed consent before enrollment. Plasma was obtained through centrifugation at 1500***g*** for 15 min at 4°C. All samples were stored at −40°C until analysis.

3 | RESULTS AND DISCUSSION

3.1 | LC–QToF–MS development

Initially, both ionization modes were tested; however, because the analyte structures have groups that favor the protonation of these molecules, all presented higher ionization intensities in the positive mode. The composition of the mobile phase was established by the resolution of the peaks and the additive was defined by the high-intensity signal, obtaining better results with the mobile phase comprising water (containing 5 mM ammonium formate and 0.1% formic acid) and acetonitrile. Different chromatographic columns were also tested; however, owing to the polarity of the molecules, the col- umn Agilent InfinityLab Poroshell 120 EC-C_{18} column (150 × 4.6 mm, 2.7 μm) showed better response, peak formation and reproducibility.

Sample preparation is an important step for accurate and reliable LC-MS assays. Protein precipitation was selected for sample preparation, as it is an inexpensive technique and presented satisfactory reproducibility in the recovery of compounds. Different solvents were tested, but acetonitrile containing 1% formic acid obtained the best recovery and reproducibility.

Retention times for EMB, INH, PZA and RIF were 4.23, 5.23, 5.88 and 10.38 min, respectively. The chromatogram shows the fortified plasma with the analytes at a concentration of 1.0 μg ml^{-1} for EMB, INH, RIF and cimetidine, and 2.0 μg ml^{-1} for PZA (Figure 1).

3.2 | Method validation

3.2.1 | Selectivity

Extracts of six blank human plasmas from different sources, including one lipemic and one hemolyzed, were compared with spiked plasma at the LLoQ. No significant interference from endogenous compounds was observed for the retention times of analytes and IS, which indicated that the developed method was selective for the analysis of these four analytes in human plasma. Representative chromatograms of different blank plasmas and plasma spiked with analytes and IS at the LLoQ are shown in Figure 2.

3.2.2 | Carryover

Blank plasma samples injected into the LC-QToF-MS by injection of a plasma sample spiked with analytes of the highest calibration levels presented responses of <20% of the peak area of the analytes and <5% of peak area of the IS. Therefore, no carryover effect was observed between injections (Figure 3).

3.2.3 | Recovery

Recovery was estimated using the mean of the plasma samples extracted against plasma samples spiked post-extraction (considered as 100%). The percentage mean extraction recovery was in the range of 82.66–95.51%, with relative standard deviations <13.50%, ensuring the reproducibility of the extraction process (Table 1).

TABLE 5 Summary of stability of analytes and internal standard in plasma and solution under

Analyte	Control	Spiked concentration (μg ml^{-1})	Plasma						Solution			
			Autosampler stability		Bench-top stability		Freeze-thaw cycles		Long-term stability		Bench-top stability		Long-term stability	
			RSD	*P*-value	RSD	*P*-value	RSD	*P*-value	RSD	*P*-value	RSD	*P*-value	RSD	*P*-value
EMB	LQC	0.4	8.42	0.4952	10.41	0.5492	12.51	0.7623	10.67	0.2267	8.74	0.3954	8.64	0.3173
	HQC	4	11.80	0.1842	9.46	0.1949	12.55	0.8713	12.59	0.1674	9.00	0.6170	5.70	0.3786
INH	LQC	0.5	9.99	0.4525	11.94	0.1516	11.39	0.6717	13.77	0.1076	7.39	0.1291	7.71	0.6093
	HQC	5	10.00	0.6572	10.68	0.0709	9.54	0.1407	8.07	0.3761	6.80	0.7360	7.25	0.7690
PZA	LQC	2	10.50	0.3895	9.15	0.1887	12.09	0.6992	10.47	0.5926	5.19	0.1038	7.85	0.3263
	HQC	32	8.03	0.3509	10.90	0.6101	11.46	0.3377	8.92	0.1281	8.12	0.7423	8.14	0.5206
RIF	LQC	0.5	9.21	0.5056	11.29	0.9322	9.30	0.0654	9.00	0.1000	6.04	0.7886	6.56	0.9950
	[illegible]	1.75	12.68	0.1051	10.97	0.7213	9.88	0.1108	12.56	0.1481	7.48	0.5865	5.70	0.5052

P-value obtained from Student's *t*-test.

3.2.4 | Matrix effect

A matrix effect occurs when there are compounds in the matrix that can compromise analyte ionization, resulting in suppression or enhancement of analyte response. The responses of the plasma sam-ples spiked with all analytes and IS post-precipitation (including lipemic and hemolyzed plasma) showed similar responses to the analytes and IS in solution, as demonstrated by the matrix factor close to 1 and byRSD <15%. Thus, the developed method had no matrix effect. The results for this validation parameter can be found in Table 1.

3.2.5 | Linearity

For all four analytes, the calibration curve was linear when evaluatingthe regression coefficient of determination (R^2) (>0.96) and regression fit (*P*-value <0.05) in the following ranges: 0.2-5 μg ml^{-1} for EMB; 0.2-7.5 μg ml^{-1} for INH; 1-40 μg ml^{-1} for PZA; and 0.25-2 μg ml^{-1} for RIF. Furthermore, the analysis of error by the lack-of-fit parameter (*P*-value >0.05) showed that the model was significant and the error was random. The data showed equality of variances (*P*-value >0.05) and residual normality (*P*-value >0.05). From the *t*-test for slopes and intercepts, all slopes were different from zero (*P*-value <0.05) and intercepts were equal to zero (*P*-value >0.05). The information about ranges, slopes and intercepts for the four analytes is shown in Table 2. Moreover, the RE and RSD were <15% for all concentration levels.

3.2.6 | Accuracy and precision

The RE of intraday accuracy for both analytes ranged from 0.26 to 13.67%, and the RE of the interday accuracy was in the range from 0.76 to 13.93% for the low quality control (LQC), medium quality con-trol (MQC), high quality control (HQC) and dilution quality control (DQC). For the precision parameter, the results obtained for LQC, MQC, HQC and DQC were also within acceptable limits, havingobtained RSD values <17.76% for intraday precision and <17.32% forinterday precision. For the sample at the LLoQ concentration, both parameters (accuracy and precision) showed deviations below the rec- ommended limits (RSD and RE <20%).

According to Bartlett's test to compare the interday precision, all analytes had equal variances at the proposed concentrations. The intra- and interday accuracy and precision for analytes and IS are reported in Table 3.

3.2.7 | Dilution integrity

This parameter was confirmed by the analysis of DQC sample, in which the obtained results presented satisfactory precision and accu- racy (deviations <15%). According to the *t*-test (Table 4), there was no statistical significance between the mean MQC and DQC, ensuring that samples containing the analytes at concentrations above the

upper limit of quantification could be diluted with plasma blank to achieve values within the calibration curve.

3.2.8 | Stability

The stability of analytes and internal standards in plasma under different conditions are summarized in Table 5. The data for autosampler stability (24 h after), bench-top stability (24 h at room temperature), freeze-thaw cycles (three cycles at −40◦C/25◦C) and long-term stability (30 days at −40◦C) indicated that these conditions do not compromise the integrity of the analytes and internal standard. Moreover, ethambutol, isoniazid, pyrazinamide and rifampicin were stable in stock solution (RSD < 10%; Table 5). The results of the Student *t*-test confirmed that there were no significant differences between the values obtained from freshly prepared samples and the tested samples.

3.3 | Clinical application

After validation of the bioanalytical method, samples from eight patients diagnosed with TB were processed to ensure the applicability of the method. The linear equations and correlation coefficients obtained during sample analyses were $y = 22.51x - 3.5$ ($R^2 = 0.995$) for EMB, $6.8x - 0.67$ ($R^2 = 0.992$) for INH, $y = 0.28x + 0.1$($R^2 = 0.987$) for PZA; and $y = 2.21x - 0.18$ ($R^2 = 0.967$) for RIF. The mean (± deviation) and median, respectively, of the measured plasma concentrations were: 10.21 ± 5.05 and 10.03 μg ml^{-1} for EMB;0.88 ± 0.48 and 0.75 μg ml^{-1} for INH; 6.20 ± 2.36 and 5.55 μg ml^{-1} for PZA; and 4.92 ± 2.06 and 4.96 μg ml^{-1} for RIF. The concentrations measured for the analytes are in agreement with previous results reported in other studies (Peloquin, 2002; Tostmann et al., 2013). These results demonstrate the ability of the method to quantify these drugs in patient samples with different metabolic profiles and comorbidities collected at different times.

4 | CONCLUSIONS

An LC-QToF-MS method was developed and validated for the simultaneous quantification in human plasma of drugs used to treat TB: EMB, INH, PZA and RIF. To our knowledge, this is the first publishedassay that simultaneously measures the plasma concentration of all antituberculosis drugs using LC-QToF-MS technique. This method has been shown to be selective, precise, accurate, linear and free of matrix and carryover effects. In addition, protein precipitation sample preparation was a simple, rapid and reproducible preparation technique. The method has been shown to be applicable to future therapeutic monitoring and pharmacokinetic studies.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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4.2 Artigo publicado que descreve modelo farmacocinético do etambutol

antibiotics

MDPI

Article

Is dosing of Ethambutol as Part of a Fixed-Dose Combination Product Optimal for Mechanically Ventilated ICU Patients with Tuberculosis? A Population Pharmacokinetic Study

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Abstract: Background: Tuberculosis (TB) patients admitted to intensive care units (ICU) have high mortality rates. It is uncertain whether the pharmacokinetics of first-line TB drugs in ICU patients are different from outpatients. This study aims to compare the pharmacokinetics of oral ethambutol in TB patients in ICU versus TB outpatients and to determine whether contemporary dosing regimens achieve therapeutic exposures. Methods: A prospective population pharmacokinetic study of ethambutol was performed in Amazonas State, Brazil. Probability of target attainment was determined using AUC/MIC > 11.9 and C_{max}/MIC > 0.48 values. Optimized dosing regimens were simulated at steady state. Results: Ten ICU patients and 20 outpatients were recruited. Ethambutol pharmacokinetics were best described using a two-compartment model with first-order oral absorption. Neither ICU patients nor outpatients consistently achieved optimal ethambutol exposures. The absorption rate for ethambutol was 2-times higher in ICU patients ($p < 0.05$). Mean bioavailability for ICU patients was >5-times higher than outpatients ($p < 0.0001$). Clearance and volume of distribution were 93% ($p < 0.0001$) and 53% ($p = 0.002$) lower in ICU patients, respectively. Conclusions: ICU patients displayed significantly different pharmacokinetics for an oral fixed-dose combination administration of ethambutol compared to outpatients, and neither patient group consistently achieved pre-defined therapeutic exposures.

Keywords: tuberculosis; ethambutol; pharmacokinetics; biological availability; intensive care; Critical Care

1. Introduction

Tuberculosis (TB) is a leading cause of infectious-diseases related deaths worldwide [1]. It is estimated that 3–16% of TB patients will require admission to an intensive care unit (ICU) due to acute respiratory failure, acute respiratory distress syndrome and/or multi-organ failure [2–4]. While global TB mortality remains at approximately 15%, outcomes for patients requiring mechanical ventilation are poor, with in-hospital mortalities reported as being from 33 to 78% [5–8]. TB is a treatable and curable disease, and effective antimicrobial administration is the cornerstone of a proactive approach for the optimal treatment of critically ill patients [5].

Ethambutol displays an initial early bactericidal effect, and its inclusion as part of first-line TB treatment is associated with better clinical outcomes [9,10]. Ethambutol reduces the emergence of resistance to the three other co-administered first-line drugs: rifampin, isoniazid and pyrazinamide [9]. Ethambutol is also used as part of the World Health Organization's (WHO) recommended treatment for multi-drug resistant (MDR)-TB treatment [9,11].

Unfortunately, first-line anti-TB drugs are not available intravenously in many of the high TB burden countries, including Brazil. Oral administration of TB drugs is not recommended for patients in ICU [5,12], so in the absence of other therapeutic options, the crushing and nasogastric tube administration of fixed-dose combination (FDC) tablets (rifampin, isoniazid, pyrazinamide and ethambutol) is used. Despite the successful use of FDC tablets in outpatients [13], it limits the opportunity to tailor doses in special cases, including ICU patients, patients with kidney injury, obese patients or those whose recovery is not progressing as expected. TB treatment using FDC tablets with weight-based regimens for patients in ICU may be suboptimal and lead to poor outcomes [14–16]. Comparing the pharmacokinetics of ethambutol as part of an FDC regimen by studying ICU and outpatients would enable a greater understanding as to whether other approaches to optimize this drug may be required.

The aim of this study was to compare the pharmacokinetics of ethambutol of patients with TB admitted to the ICU to outpatients, where both patient groups are administered their treatment using an FDC tablet. This study also sought to establish whether contemporary dosing regimens using FDC tablets achieved therapeutic exposures. Finally, this study sought to define optimized ethambutol dosing regimens for both ICU and outpatients with TB.

2. Results

2.1. Clinical and Demographic Data

A total of 30 patients were included in the analysis, 10 mechanically ventilated patients admitted to the ICU and 20 outpatients. TB diagnosis was confirmed even by positive culture, sputum smear microscopy for AFB or GeneXpert MTB/RIF© in 50% (5/10) of the ICU group vs. 85% (17/20) of patients in the outpatient group.

As described in Table 1, both patient groups had similar age, weight, gender, creatinine clearance and human immunodeficiency virus (HIV) status. All patients were receiving weight-based FDC treatment for TB. The median time of treatment before sampling was 10 days (IQR = 8.5–13) for ICU and 11 days (IQR = 4.5–14.75) for outpatients. The median time between ICU admission and patient sampling was 4 days (IQR = 2.25 – 8.25).

Table 1. Patients' demographic and clinical characteristics.

Characteristic	ICU; *n* = 10 (IQR)	Outpatients; *n* = 20 (IQR)	*p*-Value
Age (year)	31.0 (29–40)	39.5 (32.7–46.2)	0.13 a
Gender (Male/Female)	8/2	16/4	1.00 b
Weight (kg)	51.2 (46.2–58.6)	58.35 (53.2–67)	0.06 a
SOFA score	10 (6.25–12.0)	-	
APACHE II score	20.5 (17.5–26.5)	6.5 (9–18.3)	<0.0001 a
Vasoactive drugs, *n*(%)	8 (80%)	-	
HIV, *n*(%)	09 (90%)	15 (75%)	0.64 b
Creatinine Clearance (mL/min)	92.3 (36.0–129.1)	113.88 (26.5–157.9)	0.30 a
Albumin (g/dL)	3.1 (2.22–3.6)	3.5 (3.1–4.1)	0.04 a

Data expressed as median; IQR: interquartile range; a: Mann-Whitney test; b: Fisher exact test.

The median C_{max} was 1.11 mg/L (IQR = 0.87–1.50) for outpatients and 2.33 mg/L (IQR = 1.22–3.13) for ICU patients. The therapeutic target C_{max}, 2–6 mg/L, was achieved by 3/20 of outpatients (15%) and by 6/10 of ICU patients (60%). Furthermore, it was observed that most outpatients that reached the target concentrations on one sampling occasion only, whereas 4/6 (67%) of the ICU patients achieved target concentrations on both sampling occasions. Comparative C_{max}, AUC(0–24) and T_{max} for ICU and outpatients are reported in Table 2.

Table 2. Comparative C_{max}, $AUC_{(0–24)}$ and T_{max} of ethambutol for ICU and outpatients.

	ICU (IQR)	Outpatients (IQR)	*p*-Value
C_{max} (mg/L)	2.33 (1.2–3.1)	1.11 (0.9–1.5)	0.04
$AUC_{0–24}$ (mg.h/L)	19.61 (5.4–34)	5.52 (4.7–7.9)	0.06
T_{max} (h)	2.0 (2–4)	2.6 (2–4)	0.58

Data expressed as median and interquartile range (IQR) and Mann-Whitney test.

Of the TB/HIV-coinfected patients requiring intensive care, 1/10 patients had a CD4 count > 100 cells/mm^3, and none of them had an undetectable viral load. Of the outpatients with coinfection, the median CD4 count was 121 cells/mm^3 (IQR = 36–184), and 2/20 had an undetectable viral load.

The 30-day mortality rate among ICU patients was 70% (7/10). Of the remaining three patients, one was discharged from ICU but died in the ward 41 days after commencing treatment, and the remaining two died in the ICU on days 63 and 122 after treatment initiation. Among the outpatient group, a clinical cure was obtained in 94% (16/17) of patients. The patient who failed to achieve clinical cure had resistance to rifampin and isoniazid identified 35 days after diagnosis and treatment initiation. MDR-TB treatment was administered, and the patient presented clinical improvement 21 days after commencing the alternative treatment. Three patients were excluded from the clinical cure analysis, two were lost during follow-up and one abandoned treatment and died. Of the four patients who did not become cured, two were lost during follow-up, and two abandoned treatment. All patients with positive culture had a drug susceptibility test performed.

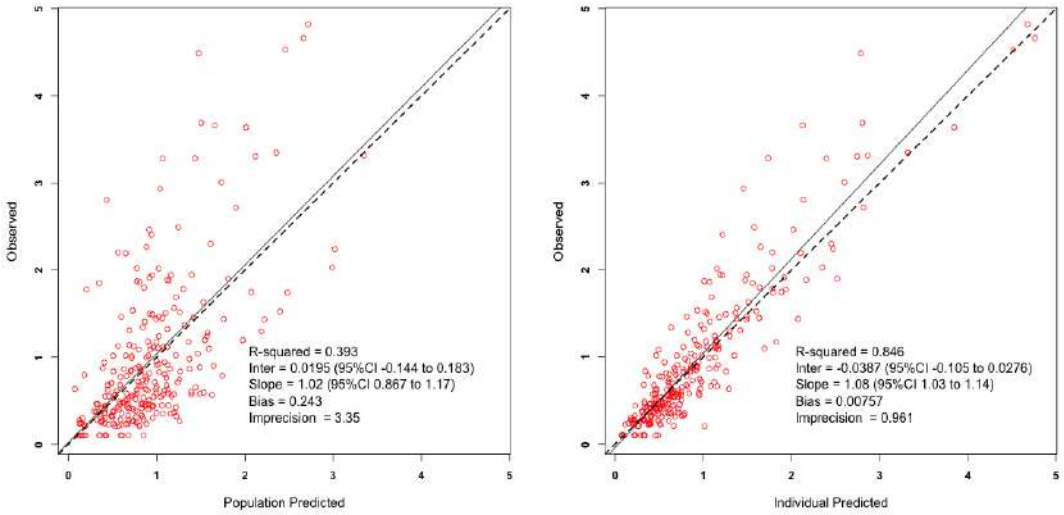
2.2. Pharmacokinetic Model Selection and Evaluation

A total of 352 ethambutol concentrations were included in the model development. All patients had an ethambutol concentration prior to the dosing interval, which was used as the initial condition in the model. All pharmacokinetic parameters for ICU patients were significantly different from outpatients and were estimated separately in the model

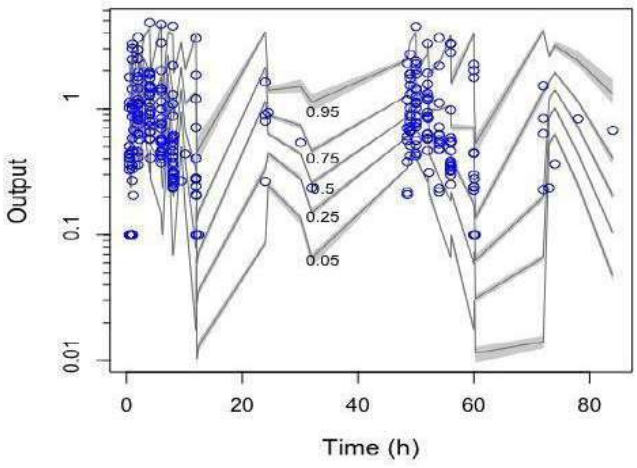
using selective execution statements. Compared to one-compartment, a two-compartment model with first-order absorption, linear elimination from the central compartment, central and peripheral volume of distribution (V) and intercompartmental clearance (Q) with initial conditions (Δ −2*LL: −555.7; ΔAIC: −576.8) best described the data. The inclusion of creatinine clearance

normalized to 101 mL/min on clearance and total body weight normalized to 56 kg as a covariate with an allometric scaler (raised to the 25th power) on central and peripheral volume of distribution resulted in a significant reduction in the log-likelihood ratio (total body weight: Δ −2*LL: −143.1; ΔAIC: −143.1) and an improvement of the model fit as assessed by goodness-of-fit plots. The inclusion of a bioavailability term was tested to support the different routes of administration of the FDC tablet between patient groups. Bioavailability was initially tested in both patient groups broadly across a range of 0 to 1 and then by fixing to 0.65 or 1, and by forcing the range to between 0.65 and 1, in accordance with previous studies. The retention of bioavailability in the model was tested through backwards exclusion for each population group individually and together. In the final model, the bioavailability parameter was accepted based on the population distribution provided by the model. Bioavailability was retained in the model based on an improvement to the goodness of fit plots and a significant decrease in the log-likelihood ratio (−2*LL). HIV status, HIV viral load, CD4 cell count and the antiretroviral therapy used by the subjects were tested as covariates to the pharmacokinetic models, but a linear regression returned a correlation coefficient < 0.2 and did not improve the pharmacokinetic model, and they were therefore not included in the model.

Diagnostic plots are presented in Figure 1, and pharmacokinetic parameter estimates are provided in Table 3.



(**a**)



(**b**)

Figure 1. (**a**) Observed versus population-predicted (left) and individual-predicted (right) ethambutol concentrations diagnostic plots and (**b**) visual predictive check.

Table 3. Estimates of ethambutol pharmacokinetic parameters for the final covariate model.

	ICU (*n* = 10)			Outpatients (*n* = 20)			*p*-Value
Parameter	**Mean (SD)**	**Median**	**%CV**	**Mean (SD)**	**Median**	**%CV**	
Clearance (L/h)	1.2 (1.5)	0.9	120.9	17.5 (13.3)	11.1	75.8	<0.0001
Volume (L)	64.8 (11.7)	61.1	18.1	137.2 (55.1)	170.4	40.1	0.002
Ka1 (h^{-1})	0.72 (0.05)	0.7	7.4	0.35 (0.12)	0.3	35.5	<0.001
Ka2 (h^{-1})	0.75 (0.10)	0.8	13.8	0.39 (0.18)	0.4	44.9	<0.001
F	0.80 (0.06)	0.8	7.9	0.14 (0.13)	0.1	87.1	<0.001
Q (L/h)	7.3 (3.5)	6.6	48.6	2.66 (2.02)	3.8	75.9	<0.001
Vp (L)	348.6 (30.1)	361.6	8.6	343.3 (78.2)	400.0	22.8	0.2

Clearance, relative clearance; Volume, relative volume of distribution of central compartment; Ka, absorption rate constant for the 1st and 2nd dose, respectively; F, bioavailability; Q, Intercompartmental clearance; Vp, Volume of peripheral compartment; SD, standard deviation; CV, coefficient of variation.

The probability target attainment (PTA) for ICU patients and outpatients across a weight range of 40 to 70 kg are presented in Figures 2 and 3, respectively. Finally, the fractional target attainment (FTA) for Mycobacterium tuberculosis-7H9 is presented in Table 4.

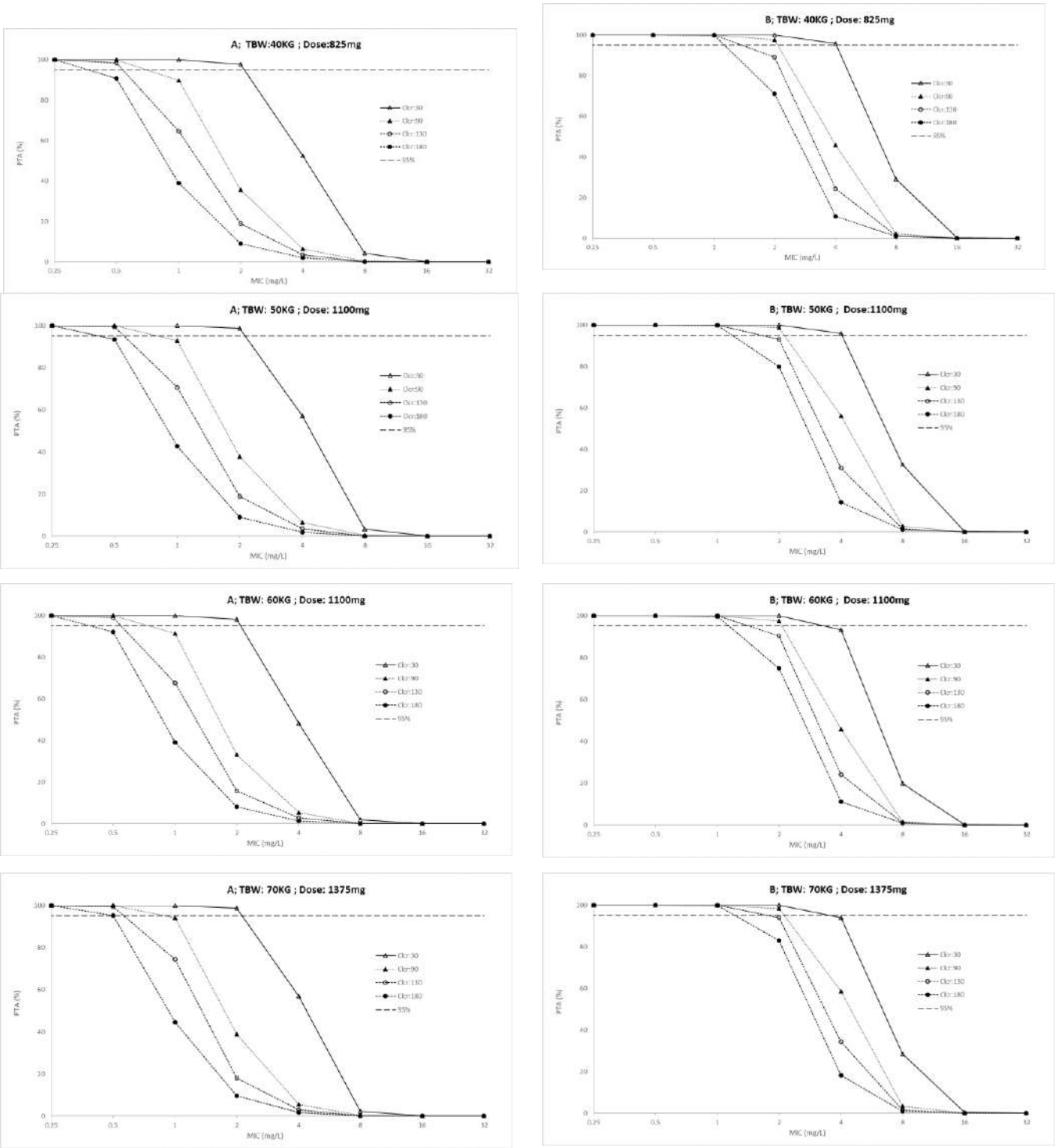


Figure 2. Probability of target attainment (**A**: $AUC_{(0–24)}$/MIC > 11.9 and **B**: C_{max}/MIC > 0.48) in ICU patients for conventional ethambutol dosing regimen according to total body weight (TBW, 40 to 70 Kg) and creatinine clearance (Clcr, 30 to 180 mL/min). PK/PD targets higher than 95% were considered desirable.

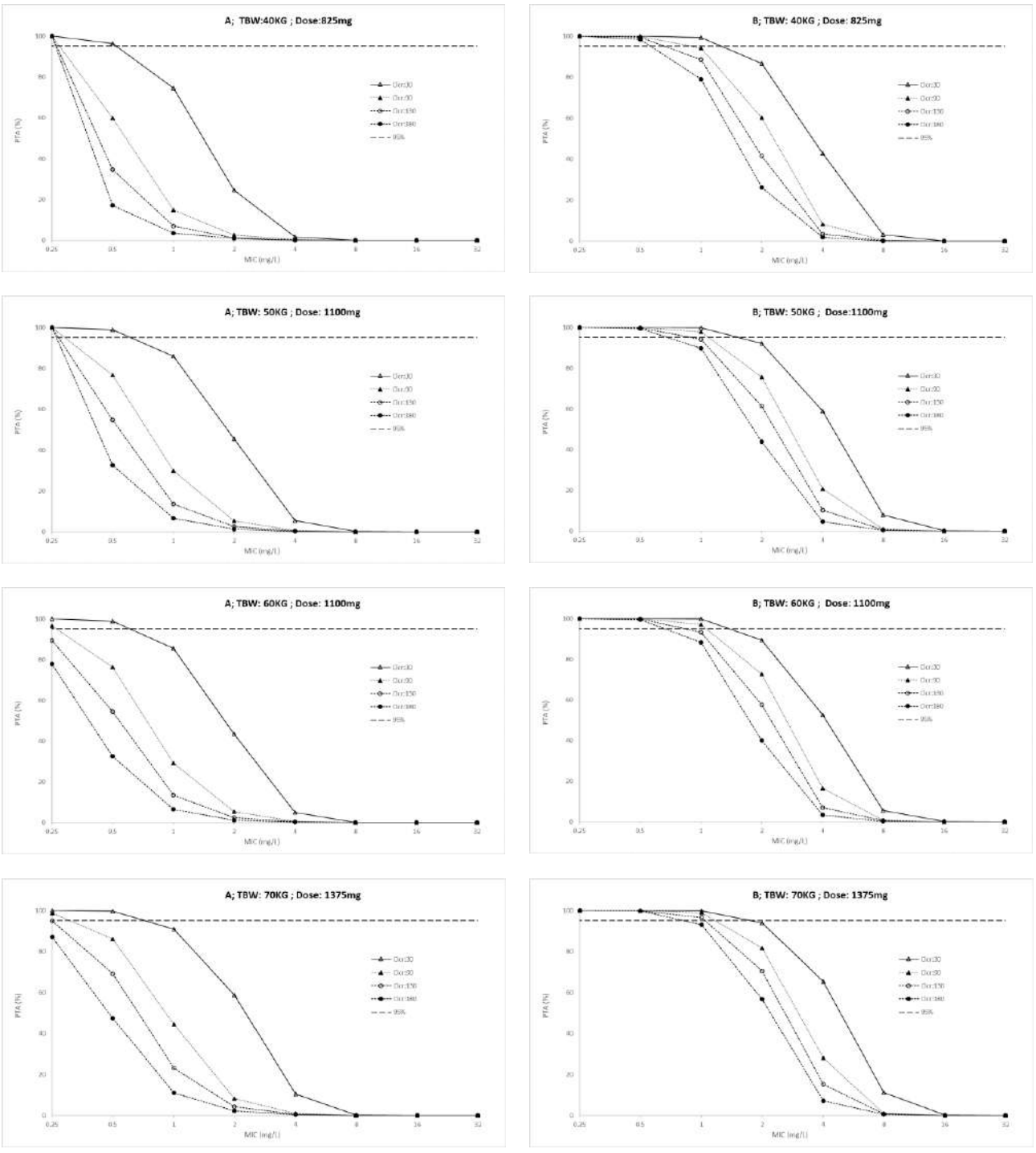


Figure 3. Probability of target attainment (**A:** $AUC_{(0–24)}$/MIC > 11.9 and **B:** C_{max}/MIC > 0.48) in outpatients for conventional ethambutol dosing regimen according to total body weight (TBW, 40 to 70 Kg) and creatinine clearance (Clcr, 30 to 180 mL/min). PK/PD targets higher than 95% were considered desirable.

Table 4. Fractional target attainment for ethambutol in ICU and outpatients according FDC dosing recommendations against the EUCAST MIC distribution for Mycobacterium tuberculosis-7H9 (MIC distribution 0.25 to 32 mg/L).

		Daily Dose (mg)															
		$AUC_{(0-24)}$/MIC								C_{max}/MIC							
		ICU patients				Outpatients				ICU Patients				Outpatients			
TBW kg	Clcr mL/min	550	825	1100	1375	550	825	1100	1375	550	825	1100	1375	550	825	1100	1375
40	30	47.8	52.2	56.6	-	-	-	-	-	63.0	71.9	80.6	-	-	-	-	-
	90	23.3	26.8	29.8	-	22.1	32.7	41.2	-	43.4	50.0	56.4	-	22.1	32.7	41.2	-
	120	15.5	18.5	21.2	-	16.9	27.0	34.9	-	36.2	43.2	48.6	-	16.9	27.0	34.9	-
	180	9.9	11.7	14.0	-	12.7	22.0	29.6	-	29.8	36.5	42.1	-	12.7	22.0	29.6	-
50	30	-	48.8	53.1	57.0	-	44.3	54.1	54.1	-	65.0	73.3	81.9	-	44.3	54.1	54.1
	90	-	24.9	27.8	30.7	-	30.8	39.2	46.0	-	46.5	52.7	58.2	-	30.8	39.2	46.0
	120	-	16.6	19.6	22.3	-	25.2	33.5	40.0	-	39.9	45.5	50.7	-	25.2	33.5	40.0
	180	-	10.7	12.4	14.8	-	20.7	28.0	34.0	-	39.1	39.1	44.2	-	20.7	28.0	34.0
60	30	-	46.3	50.5	81.9	-	41.6	51.2	59.9	-	61.0	67.8	75.6	-	41.6	51.2	59.9
	90	-	23.2	26.2	29.1	-	29.4	37.5	43.9	-	44.1	49.7	55.5	-	29.4	37.5	43.9
	120	-	15.2	18.3	21.0	-	24.0	31.8	38.3	-	37.4	43.3	21.0	-	24.0	31.8	38.3
	180	-	9.6	11.5	13.7	-	19.5	26.6	32.7	-	31.8	37.3	42.2	-	19.5	26.6	32.7
70	30	-	-	48.1	52.7	-	-	49.1	57.1	-	-	64.2	71.3	-	-	49.1	57.1
	90	-	-	25.2	27.9	-	-	35.8	42.3	-	-	47.4	53.3	-	-	35.8	42.3
	120	-	-	17.2	19.9	-	-	30.8	36.8	-	-	41.3	46.4	-	-	30.8	36.8
	180	-	-	10.6	12.8	-	-	25.5	31.7	-	-	35.7	40.5	-	-	25.5	31.7

TBW: total body weight; Clcr: creatinine clearance.

3. Discussion

To the best of our knowledge, this is the first article to compare the pharmacokinetics of FDC administration of ethambutol for the treatment of Mycobacterium tuberculosis in ICU and outpatients.

The WHO recommends the use of a critical breakpoint serum concentration of 5 mg/L to define Mycobacterium tuberculosis susceptibility to ethambutol [17]. However, susceptibility testing of ethambutol is inconsistent, and this may be because the current breakpoint concentration splits the epidemiological cut-off at the upper end of the wild-type MIC distribution, producing results that oscillate between resistant and susceptible [18]. As drug susceptibility testing is a fundamental step in establishing a pharmacokinetic/pharmacodynamic (PK/PD)target, in this study, we included outpatients as a comparative parameter for target attainment. This decision is supported as the treatment success rates for outpatients remain around 85% [1].

A two-compartment model with creatinine clearance and total body weight included as covariates best described the pharmacokinetics of ethambutol for the patients enrolled in the present study, and this is supported by previously published studies [15,19]. The differences between the primary pharmacokinetic parameters of ICU and outpatients were all statistically significant (as reported in Table 3).

The absorption rate was 192% and 205% higher in ICU patients compared to outpatients ($p < 0.05$ on both occasions) for the first and second dose, respectively. The difference in bioavailability of ICU patients was greater than 5-times higher than outpatients ($p < 0.0001$). Unlike the model reported here, most studies do not evaluate the variability of bioavailability and fix it to 1 or 0.65 [11,15,19]. For ICU patients the tablet is crushed and delivered via a nasogastric tube, whereas oral administration was used for outpatients. The difference between ICU patients and outpatients for the parameters of absorption rate and bioavailability may be due to the differences in the administration of ethambutol. The

bioavailability in outpatients was lower but also highly variable compared to the ICU patients. Additionally, a recent study by Sundell et al. identified that mutations in CYP1A2 are associated with a 50% reduction in relative bioavailability in adult patients coinfected with HIV/TB, and this may result in underexposure to ethambutol [11]. A study by Court 2019 has previously demonstrated that tablet crushing did not affect ethambutol C_{max} or $AUC_{(0–10)}$ in patients with multidrug-resistant tuberculosis [20]. Patients receiving whole-tablets displayed an ethambutol C_{max} of 1.9 mg/L [IQR = 1.6–2.3] and patients receiving crushed-tablets a C_{max} 1.8 mg/L [IQR = 1.3–2.9], $p = 0.75$. No difference was seen in $AUC_{(0–10)}$ 11.3 [9.5–12.8] for patients receiving whole tablets and 11 [8.4–15.2] for those receiving crushed-tablets, $p = 0.63$ [20]. However, in this study, there was a significant difference in C_{max} between the ICU patients and the outpatients. The ICU patients had a 210% higher C_{max} ($p = 0.04$).

No patients in the ICU group or the outpatient group achieved the critical breakpoint serum ethambutol concentrations of 5.0 mg/L to inhibit the growth of wild-type strains of Mycobacterium tuberculosis. Furthermore, 85% (17/20) of the outpatients recorded ethambutol concentrations below 2 mg/L for all of the blood samples collected. This result corresponds with others reporting lower-than-expected ethambutol serum concentrations [11,21–23]. ICU patients displayed higher ethambutol serum concentrations compared to outpatients, with a significantly different C_{max} ($p = 0.04$), as shown in Table 3. Only 40% (4/10) of ICU patients failed to achieve a $C_{max} > 2$ mg/L. Although outpatients have a higher rate of treatment success than ICU patients, it is unlikely that the improvement in clinical outcomes was due to the serum concentration of ethambutol.

Relative clearance was significantly lower in ICU patients compared to outpatients ($p < 0.0001$) and lower than that reported for outpatients in other studies, where results range from 2.2 to 77 L/h (bioavailability fixed to 1) [11,15,19,24]. With up to 70% of ethambutol being excreted unchanged in urine [25], the use of creatinine clearance as a covariate on relative clearance is an important inclusion in the final model. A lower clearance for a hydrophilic antimicrobial is not unexpected in critically ill patients [26], and this result is supported by the lower creatinine clearance and sickness severity of the ICU patients enrolled in this study. The APACHE II score was significantly higher in the ICU group. TB patients may develop septic shock, manifest multi-organ failure through cardiovascular dysfunction and acute kidney injury due to a decrease in the effective intravascular volume, requiring fluid resuscitation and vasoactive agents [3,5]. The multi-organ failure expressed by the APACHE II score could explain the lower relative clearance in ICU patients.

The relative volume of distribution from the central compartment was 53% lower in ICU patients compared to outpatients. However, the ICU patients have a higher bioavailability compared to outpatients (mean results 0.8 and 0.14, respectively). The volume of distribution of the central compartment, adjusted for relative bioavailability, is, therefore, higher in ICU patients compared to outpatients. This is not an unexpected result for ICU patients administered a hydrophilic antibiotic [26]. In our study, the volume of the peripheral compartment in ICU patients is similar to the outpatients. There has been a wide range of volumes of the peripheral compartment reported in outpatients (typical values of 16.5 (bioavailability fixed to 1) to 512 (bioavailability fixed to 0.65) [15,19]), and the results of our study fit within these results.

Previous reports had demonstrated that HIV coinfection and antiretroviral therapy interferes with ethambutol oral bioavailability and, an intensified dosing strategy with a supplementary dose of 400 mg of ethambutol is advocated for TB/HIV coinfected patients by Mehta, 2019 [15,27–29]. However, we found no difference in the PK parameters when HIV status, HIV viral load, CD4 cell count and antiretroviral therapy were considered. Brazil and, especially, the Amazonas state, represents a high TB/HIV coinfection burden area [1,30], so for this reason, our sample had 9 (90%) outpatient and 15 (75%) ICU patients coinfected with HIV and did not permit us to find any difference related to the HIV status.

Neither the ICU patient group nor the outpatient group achieved a priori targets of $AUC_{(0–24)}$/MIC > 119 or C_{max}/MIC > 0.48 for the probability of target attainment or fractional target attainment analysis. In vitro studies suggest the use of a PK/PD target of $AUC_{(0–24)}$/MIC > 119. However, applying this target to our data produces a probability of target attainment of 0%. This is unsurprising as the dose of ethambutol in our study produced an $AUC_{(0–24\ h)}$ of 48–144 mg.kg/L [24,31]. This result is similar to that reported by Denti 2015 and McIlleron 2006 in non-ICU outpatients who calculated $AUC_{(0–24)}$ of 23.6 and 59.5 mg.kg/L, respectively [19,29]. Ethambutol accumulates in diseased tissue with a lesion-to-plasma exposure ratio of 10:1 [9,15]. Incorporating this ratio results in a revised PK/PD target of $AUC_{(0–24)}$/MIC > 11.9. On this basis, we incorporated this revised target in the present study [9,15].

In our evaluation of ethambutol efficacy, we need to consider both the PK/PD target and toxicity. Therapeutic drug monitoring previous studies have identified an ethambutol C_{max} of between 2 and 6 mg/L as a therapeutic target [15,16,21,24,32–34]. The median C_{max} of 1.11 mg/L in the outpatients may suggest an ethambutol underdose. In ICU patients, a higher C_{max} may be influenced by a lower clearance.

The low concentration of TB drugs can induce the emergence of drug-resistant TB. However, the prognosis of outpatients with TB is good, while the serum concentration of ethambutol in TB outpatients is low. As the risk of underdosing clearly surpasses the risk of toxicity, doses higher than 1375 mg must be encouraged, as previously suggested by other studies [11,15]. Additionally, therapeutic drug monitoring associated with clinical and bacteriological data plays a main part in patient treatment [23]. While ethambutol lacks sterilizing activity, it is useful in protecting against the emergence of resistance to isoniazid, rifampin and pyrazinamide [9]. Among outpatients achieving clinical cure, there were no reports of the development of resistance. Based on this, ethambutol may not have influenced patient outcomes, but it is likely it is protecting against the emergence of resistance.

The toxicity of ethambutol is not well understood, but one of the main adverse effects, optical neuropathy, appears to be dose and time-related [23,35,36]. Studies report an incidence of 18% in subjects treated for >2 months with >35 mg/kg/day, 5–6% with 25 mg/kg/day, 3% with 20 mg/kg/day and <1% with 15 mg/kg/day [35]. Despite the weak evidence among PK parameters and the recommendation of a C_{max} range of 2 to 6 mg/L for toxicity, ethambutol requires renal elimination, and kidney dysfunction may cause accumulation [23,37]. For these reasons, therapeutic drug monitoring should be encouraged where increased doses are used.

Our research had clear limitations. We have not been able to identify a correlation between antiretroviral therapy and other antimicrobials in use and ethambutol pharmacokinetics [11,24,28]. However, in this study, drug–drug interaction among rifampin, isoniazid, pyrazinamide and ethambutol was not assessed, nor was its joint action against Mycobacterium tuberculosis [19]. It is important to note, however, Chigutsa 2015 evaluated the influence of the four drugs in the outcomes of TB patients using a multivariate adaptive regression splines algorithm and observed that ethambutol C_{max}/MIC ratio was positively correlated with the outcome only when rifampin exposure was low, suggesting that rifampin presents a higher bactericidal effect and also an apparent antagonism of ethambutol [38]. Currently available in vitro kill-curve studies evaluate ethambutol only and do not consider a synergistic effect of the four drugs which are administered in combination [39]. Therefore, kill-curve studies considering drugs synergism should be carried out in order to evaluate the best dose for each drug, considering their joint use.

4. Materials and Methods

This paper was conducted in accordance with the ClinPK checklist report [40].

4.1. Ethics

This study was approved by the Ethics Committee at Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (CEP/FMT-HVD CAAE: 60219916.5.0000.0005). Signed informed consent was obtained from each participant or legal representative for the use of biological materials and publication of data.

4.2. Patients and Study Design

This was a prospective open-label pharmacokinetic study performed in Amazonas, Brazil, from November 2016 to May 2018. We enrolled individuals ≥ 18 years of age with active pulmonary and extrapulmonary TB who were prescribed FDC tablets containing rifampin, isoniazid, pyrazinamide and ethambutol.

Patients were considered to have active TB if at least two of the following criteria were met: (1) smear-positive for acid-fast bacilli (AFB) or GeneXpert MTB/RIF© (Cepheid, Sunnyvale, CA, USA) on sputum, tracheal aspirate or any other clinical specimen; (2) culture-positive for Mycobacterium tuberculosis on sputum, tracheal aspirate or any other clinical specimen; (3) strong clinical suspicion of active TB; or (4) strong radiological evidence for active TB. A strong clinical suspicion of active TB required at least two of four constitutional symptoms (weight loss with accompanying fever, night sweats, productive cough, loss of appetite for 2 weeks) as well as known TB contact or history of previous pulmonary TB [6].

Patients were recruited at the outpatient clinic or at the ICU of Fundação de Medicina Tropical Dr. Heitor Vieira Dourado in Manaus, Amazonas, Brazil, and the diagnosis and the treatment were defined by the patients' assistant physician and, after that, they were invited to the study. Every patient was in directly observed treatment receiving a weight-based dose of ethambutol (20–35 kg: 550 mg; 36–50 kg: 825 mg and > 50 kg: 1100 mg) as FDC tablets in accordance with the Brazilian Ministry of Health Guidelines available at the time of the study [37]. Pregnant women, subjects requiring hemodialysis, continuous renal replacement therapy, peritoneal dialysis or those whose clinician considers the patient unsuitable for enrolment were excluded. Clinical and demographic data include body mass index (BMI), weight, renal and liver function, blood cell count, SOFA and APACHE II score, HIV status, hepatitis B and C, syphilis, diabetes, comorbidities, concomitant medication in use and antimicrobials used in previous 30 days, occupation, age and sex. Outpatients did not have their SOFA assessed since they did not show any organ dysfunction.

Blood samples were collected from each patient on pre-enteral administration and then at 30, 60, 120, 240, 360, 480, 720 and 1440 min (prior to subsequent dose) on the first and third days of enrollment. A measured 8 h creatinine clearance was obtained. All patients in the ICU group were mechanically ventilated and received FDC tablets through a nasogastric tube. Prior to administration, the research nurse crushed FDC tablets and suspended them in 20 mL of distilled water and administered the suspension through the nasogastric tube. After that, another 20 mL of distilled water was flushed through the nasogastric tube to ensure the ethambutol-containing suspension reached the gastrointestinal tract. Each ICU patient was assessed daily for their individual requirements for vasopressors and APACHE II and SOFA score. Outpatients were invited to be admitted to the Clinical Research Ward for 72 h for directly observed treatment with FDC tablets and sample collection. All patients remained in contact with the study staff until the end of treatment.

Blood samples were collected and immediately stored at 4 °C until being centrifuged at 434× *g* for 10 min. Plasma (2 mL) was transferred into a labelled cryotube and stored at −80 °C until analysis.

4.3. Drug Assay

Total ethambutol plasma concentrations were measured according to a previously validated method [41] in high-pressure liquid chromatography with an MS/MS detector on a Waters Xevo G2-S QToF mass spectrometer (Waters Corp., Milford, MA, USA), over the range of 0.2 to 5 mg/L. Bioanalytical method validation guidelines recommend the preparation of a dilution quality control in case of concentrations over the upper limit. Both quality control and sample are submitted to the dilution process. According to the Brazilian Health Surveillance Agency and the United States Food and Drug Administration, dilution quality control should be considered if the accuracy and precision are 15% of the nominal concentration and <15% of the relative standard deviation. This method allows us to measure concentrations over 5 mg/mL. The accuracy was calculated as the relative error and precision as the relative standard deviation. The relative error of intraday accuracy ranged from 0.26 to 13.7%. For the precision parameter, the results obtained for low, medium, high and dilution quality controls were also within acceptable limits, having obtained relative standard deviation values <17.8% for intraday precision.

4.4. Population Pharmacokinetic Modelling

A population pharmacokinetic model was developed using Pmetrics version 1.5.0 (Laboratory of Applied Pharmacokinetics and Bioinformatics, Los Angeles, CA, USA) in Rstudio (version 0.99.9.3) as a wrapper for R (version 3.3.1), Xcode (version 2.6.2) and the Intel Parallel Studio Fortran Compiler XE 2017. One or two-compartment structural models were constructed using the nonparametric adaptive grid (NPAG) algorithms within Pmetrics. The one-compartment model included linear elimination of ethambutol from the central compartment. The two-compartment model tested the use of intercompartmental transfer constants between central and peripheral compartments (KCP and KPC), as well as intercompartmental clearance (Q). As patients were receiving doses of ethambutol every 24 h, the inclusion of occasion for the first and second dose was tested for the rate of absorption, bioavailability, lag time and clearance. Determination of absolute bioavailability was not determined since we do not have intravenous ethambutol available in Brazil. Additive (lambda) and multiplicative (gamma) error models were tested using a polynomial equation for standard deviation as a function of observed concentration, Y. (SD = C0 + C1.Y), with observation weighting performed as error = SD.gamma or error = (SD2 + lambda2) 0.5.

The inclusion of biologically plausible clinical covariates was evaluated by applying stepwise linear regression between the pharmacokinetic parameters and the categorical covariates and evaluated using linear, log, polynomial and power regression for the continuous variables. Selected covariates that were tested on the structural model parameters include creatinine clearance, total body weight, body mass index (BMI), weight, renal and liver function, blood cell count, SOFA and APACHE II score, HIV status, hepatitis B and C, syphilis, diabetes, comorbidities, concomitant medication in use and antimicrobials used in previous 30 days, age and sex. Weight and creatinine clearance were tested normalized to median patient values and with an allometric scalar applied [42–45]. Model retention was governed according to the criteria described below.

4.5. Model Evaluation

Model evaluation was performed using diagnostic plots and statistical examination for comparison and selection of models. Initial screening was conducted by visually assessing, for each run, the goodness of fit and the coefficient of determination (r2) of the linear regression of the observed and predicted plots values (r2 closer to 1, intercept closer to 0). Acceptance of best-fit of the model structure, error model and inclusion of covariates was identified by a change in the objective function (OFV) calculated as a decrease in the log-likelihood ratio test (−2*LL) of −3.84 (corresponding to a $p < 0.05$ based on Chi-square distribution and one degree of freedom) and decrease in the Akaike information criterion

(AIC). We also factored bias (mean weighted predicted-observed error) and imprecision (bias-adjusted, mean weighted squared predicted-observed error) into the selection of the final model. Finally, to evaluate the internal consistency of the model predictions with the observations, normalized prediction distribution errors and the posterior predictive check were assessed graphically using visual predicted check plots and the proportion of observations between 5th and 95th simulated percentiles above 90% were considered adequate.

4.6. Dose Simulations and Target Attainment

Monte Carlo simulations (n = 1000) were performed with predicted outputs at 24 h intervals. Covariate values of each of the simulated patients were fixed on the arithmetic median of total body weight and creatinine clearance. Dosing regimens were simulated considering PK/PD targets of $AUC_{(0–24)}$/MIC > 11.9 and C_{max}/MIC > 0.48 and 12% plasma protein binding[46]. The dosing regimens were simulated at a steady state for creatinine clearances of 30, 90, 130 and 180 //mLmLmin/1.73 m2 and total body weight of 40, 50, 60 and 70 kg based on the FDC dose. PTA for achieving PK/PD targets was assessed, and values higher than 95% were considered desirable. The FTA identified the achievement of target antibiotic exposures by comparing the PTA against the MIC distribution for Mycobacterium tuberculosis-7H9 of the European Committee for Antimicrobial Susceptibility and Testing (EUCAST) database (available at www.eucast.org). FTA for empiric therapy was calculated considering MIC distribution within 0.25 and 32 mg/L. Doses were considered acceptable if the FTA was greater than 85%.

4.7. Statistical Analysis

A descriptive analysis was performed of the data by means of distribution of frequency and measurements of central tendency. The categorical variables were expressed as frequency and percentage and analyzed using Pearson's X^2 test or Fisher exact test. For numerical variables, a Mann–Whitney test was used. To compare differences between dosing occasions, the absorption rate was constant for the first and second dose in the same group, and a Wilcoxon rank test was used. All analyses were performed considering a significance level of 5%, conducted using R software.

5. Conclusions

Understanding the role of the pharmacokinetics of ethambutol in ICU patients remains an important issue, and low serum concentrations can be associated with a worse likelihood of survival [5,23,47]. Based on our dosing simulations, ICU patients do not reach sufficient ethambutol concentrations to achieve the PK/PD targets of $AUC_{(0–24)}$/MIC > 11.9 or C_{max}/MIC > 0.48 using an FDC tablet with a weight-based dosing regimen. Doses higher than 1375 mg of ethambutol must be encouraged for outpatients and ICU patients. Effective treatment of ICU patients for Mycobacterium tuberculosis may require the re-formulation of a combination tablet or the availability of an intravenous combination formulation. Further research to evaluate synergism among rifampin, isoniazid, pyrazinamide and ethambutol is needed.

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Informed Consent Statement: Signed informed consent was obtained from each participant or legal representative for the use of biological materials and publication of data.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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4.3 Comunicação rápida não publicada com análise farmacocinética não compartimental de rifampicna, isoniazida, pirazinamida e etambutol, comparando pacientes em UTI e em tratamento ambulatorial

Is the low serum concentration of rifampicin, isoniazid pyrazinamide, and ethambutol the cause of high mortality in tuberculosis patients admitted to the ICU? A non-compartmental pharmacokinetic analysis

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Running Title: PK for TB treatment ICU vs. outpatients

Title

Is the low serum concentration of rifampicin, isoniazid, pyrazinamide and ethambutol the cause of high mortality in tuberculosis patients admitted to the ICU?

Synopsis

Tuberculosis patients at intensive care units (ICU) have high mortality rates. It is uncertain whether pharmacokinetic parameters of rifampin, isoniazid, pyrazinamide, and ethambutol is the cause of mortality. We compare drugs pharmacokinetics in tuberculosis patients admitted to the ICU to outpatients and conclude ICU patients may present higher drug exposure.

Main article

Introduction

Tuberculosis (TB) is a leading cause of infectious-diseases related deaths worldwide[1]. It is estimated that 3 – 16% of TB patients will require admission to an intensive care unit (ICU), due to acute respiratory failure, acute respiratory distress syndrome and multi-organ failure[2,3]. While global TB mortality remains at approximately 15%, outcomes for patients requiring mechanical ventilation is poor, with in-hospital mortalities reported as being from 33 to 78% [4]. Once sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection, we can assume that a cause for ICU admission would be sepsis due to *Mycobacterium tuberculosis*[3]. Hereafter, delay in initiation of effective antimicrobial therapy could be a critical therapeutic variable associated with mortality[3].

In the absence of other therapeutic options, the crushing and nasogastric tube administration of fixed-dose combination (FDC) tablets containing rifampin, isoniazid, pyrazinamide and ethambutol in a weight-based regimen is used. However, sepsis causes multi-organ failure altering drug absorption and clearance leading to inappropriate pharmacokinetic (PK)/pharmacodynamic (PD) index[4].

The aim of this study was to compare the pharmacokinetics of rifampin, isoniazid, pyrazinamide, and ethambutol of patients with TB admitted to the ICU to outpatients, evaluating drug serum concentrations as a potential cause of mortality.

Methods

Ethics

This study was approved by the Ethics Committee (CEP/FMT-HVD CAAE: 60219916.5.0000.0005).

Patients and study design

This was a prospective open label pharmacokinetic study performed in Amazonas, Brazil from November 2016 to May 2018. We enrolled individuals ≥ 18-years of age, with active pulmonary and extrapulmonary TB who were prescribed FDC tablets containing rifampin, isoniazid, pyrazinamide, and ethambutol.

Patients were considered to have active TB if at least two of the following criteria were met: 1) smear positive for acid-fast bacilli (AFB) or GeneXpert MTB/RIF© (Cepheid, Sunnyvale, CA, USA) on sputum, tracheal aspirate, or any other clinical specimen; 2) culture-positive for *Mycobacterium tuberculosis* on sputum, tracheal aspirate or any other clinical specimen; 3) clinical suspicion of active TB; or 4) strong radiological evidence for active TB. A clinical suspicion of active TB required at least two of four constitutional symptoms (weight loss with accompanying fever, night sweats, productive cough, loss of appetite for 2 weeks) as well as known TB contact or history of previous pulmonary TB[5].

Every patient was in directly observed treatment receiving FDC tablets containing of rifampin 150 mg, isoniazid 75 mg, pyrazinamide 400 mg and ethambutol 275mg in a weight-based strategy, 20-35 kg: 2 tablets, 36-50 kg: 3 tablets and > 50 kg: 4 tablets, in accordance with the Brazilian Ministry of Health Guidelines available at the time of the study[6]. Pregnant women, subjects requiring hemodialysis, continuous renal replacement therapy, peritoneal dialysis were excluded.

Blood samples were collected from each patient on pre-enteral administration, and then at 30, 60, 120, 240, 360, 480, 720, and 1440 minutes (prior to subsequent dose), on the 24h of the first and the third day of enrollment. A measured 8-hour creatinine clearance was obtained. All patients in the ICU group were mechanically ventilated and received FDC tablets through a nasogastric tube. Prior to administration FDC tablets were crushed and suspended it in 20 mL of distilled water and administered through the nasogastric tube. After that, more 20 mL of distilled water was flushed through the nasogastric tube. Each ICU patient was assessed daily for their individual requirements for vasopressors, and APACHE II and SOFA score. Outpatients were invited to be admitted at the Clinical Research Ward for 72 h for directly observed treatment with FDC tablets and sample collection. All patients remained in contact with the study staff until the end of treatment.

Blood samples were collected and immediately stored at 4°C until being centrifuged at 434 g for 10 minutes. Plasma (2 mL) was transferred into a labelled cryotube and stored at -80 °C until analysis.

Patients' outcome was assessed, clinical cure was reached in the absence of constitutional symptoms and weight gain. Microbiological cure was considered negative TB culture and negative sputum smear microscopy at the end of treatment.

Drug Assay

Total plasma concentrations were measured according a previously validated method[7] in a high-pressure liquid chromatography, MS/MS (Waters Xevo G2-S QtoF, Waters Corp., Milford, MA, USA).

Non-compartmental Pharmacokinetics Analysis

Patients with drug plasma concentrations bellow the Lower Limit of Quantification, or whose concentrations did not decay were excluded from PK analysis. PK parameters were calculated using Pmetrics v.1.5.0 (Laboratory of Applied Pharmacokinetics and Bioinformatics, Los Angeles, CA, USA) in RStudio (version 0.99.9.3) as a wrapper for R (version 3.3.1), Xcode (version 2.6.2) and the Intel Parallel Studio Fortran Compiler XE 2017.

Statistical analysis

A descriptive analysis of the data was performed by means of distribution of frequency and measurements of central tendency. The categorical variables were expressed as frequency and percentage and analyzed using Pearson's X^2 test or Fisher exact test. For numerical variables, a Mann-Whitney test was used. All analyses were performed considering a significance level of 5%, conducted using RStudio (v.0.99.9.3).

Results

A total of 33 patients were enrolled, 13 mechanically ventilated in ICU and 20 outpatients. Clinical and demographic characteristics were similar in both groups, mainly composed by men (ICU 10/13 vs non-ICU 16/20; p = 1.00), median age was 38 years-old (ICU 39.5, IQR 32,75 – 46.25; outpatients 32, IQR 30 -52; p=0.53), 81% were HIV co-infected patients (ICU 12/13 vs outpatients 15/20, p=0.42). Median weight was 52kg (46.15 – 60) in ICU group and 58kg.

Kg (53.15 – 67.05) in non-ICU group, p=0.14. Creatinine clearance was significantly lower in ICU group, 45.8 (0 – 97) and 113.9 (86.46 – 157.89) mL/min, p=0,02. Median APACHE-II was significantly higher (P < 0.001) in ICU group, 28 (20 -33) and 5 (3.75 – 7.00) points in outpatients (Table 1).

Clinical and microbiological outcome of patients were also assessed. None of patients in ICU group has reached clinical or microbiological cure. After ICU admission 30-day mortality was 77% (10/13) in ICU group. The other three patients died on days 41, 63 and 122 (in the ward) after ICU admission. On outpatient group, 15/20 patients reached clinical and microbiological cure. From the other 5 patients, one abandoned treatment and developed multi-drug resistant TB, three lost follow-up and one died from accidental trauma.

Data from PK parameters are presented in table 1. For Rifampin, a total of 18 patients were included in the analysis, 5 in ICU and 13 outpatients. The area under the curve (AUC) was almost twice higher in ICU patients. No difference was found on maximum concentration (C_{max}), time to the maximum concentration (T_{max}), constant of absorption, clearance, volume of distribution or half-life ($T_{1/2}$)

For Isoniazid, we analyzed 11 ICU patients and 18 outpatients. No significant difference was seen for AUC, C_{max}, T_{max}, constant of absorption, clearance, volume of distribution and $T_{1/2}$ (Table 2).

For Pyrazinamide, 13 patients in ICU and 20 outpatients were studied. ICU patients presented significantly lower T_{max}, clearance and volume of distribution.

Finally, for ethambutol, a total of 30 patients, 10 in ICU and 20 outpatients have ethambutol PK parameters evaluated. Significantly higher AUC, and $T_{1/2}$ and lower constant of absorption, and clearance were observed.

Discussion

To the best of our knowledge this is the first article to compare the pharmacokinetics of rifampin, isoniazid, pyrazinamide, and ethambutol in a weight-based regimen administered in a FDC tablet through a nasogastric tube after crushing in ICU patients with outpatients receiving the same treatment strategy orally administered for the treatment of *Mycobacterium tuberculosis*.

Although there are not standard PK/PD parameters for first-line TB drugs in the Brazilian population, WHO standard regimen shows 85% of effectiveness in general population[1]. Moreover, in our study, all outpatients who completed treatment has cured TB. Also, susceptibility testing of pyrazinamide and ethambutol can be inconsistent, precluding proper definition of MICs[8]. For these reasons, we can assume outpatients had therapeutic concentrations for rifampin, isoniazid, pyrazinamide, and ethambutol and could be used as a reference standard to know if ICU patients are also achieving those levels.

Rifampin AUC was 85% higher in ICU patients (46.57 mg.h/L) when compared to outpatients (26.07 mg.h/L). The expected AUC after a single dose and after steady state is 72.56 mg.h/L and 38.73 mg.h/L respectively[9]. Outpatients group presented lower-then-expected AUC but, it still above AUC concentration cutoff value of 13 mg.h/L, that predict >91% of long-term clinical outcomes[10]. Despite the target range of C_{max} for rifampicin TDM needs to exceed 8mg/L, expected C_{max} vary form 5.79mg/L to 8.98mg/L depending on treatment duration[9] and, a C_{max} > 6.6 mg/L, reached in both groups studied, predicts microbiological cure in 97% of patients[10].

No difference between both groups were found at the non-compartmental analysis of isoniazid. Considering 90% of early bactericidal activity has been associated with an AUC from time above 10.5 mg.h/L, both groups had reached therapeutic levels for isoniazid[11]. Furthermore, another study suggests AUC would be 17.1 mg.h/L and 9.89 mg.h/L for slow and rapid acetylators respectively[12]. On the other hand, bad clinical outcomes on TB treatment was associated with an isoniazid C_{max} lower than 8.8 mg/L[10].

Pyrazinamide is renally excreted after hepatic metabolization and most of TB patients admitted to the ICU presents renal and hepatic failure[4]. This may be the reason why ICU patients presented lower clearance, T_{max} and volume of distribution when compared to outpatients ($p < 0.05$). There were no difference on C_{max} or AUC in both groups however, both of them were between 6 and 8 times lower than the expected threshold of 32.4 mg/L and 363 mg.h/L[10]. So, pyrazinamide dose was inappropriate for those patients.

ICU patients presented 3-times higher ethambutol AUC and 5-times longer half-life when compared to outpatients. A possible explanation may be that despite a slower absorption, they present also a 5-times lower clearance. In non-ICU outpatients an expected AUC(0-24) is 23.6[12], also it is recommended a C_{max} of 2 to 6 mg/L[13]. This information may suggest our ICU patients would be reaching therapeutic concentrations of ethambutol meanwhile outpatients are not.

Despite no therapeutic level been achieved in both groups, in a non-compartmental analysis, PK parameters were different for rifampin, pyrazinamide and ethambutol and no difference was observed for isoniazid. Rifampin and ethambutol presents higher concentrations in the ICU patients, indeed. For this reason, low serum concentrations may not be associated with worse likelihood of survival of individuals requiring intensive care. Research addressing patients clinical condition and MIC breakpoints must be carried to enlighten the reason of high mortality of TB patients in ICU.

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Transparency Declarations

None to declare

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Tables

Characteristics	ICU (n=13)	Outpatients (n=20)	P-value
Age (yr)	32.0 (30.0-52.0)	39.5 (32.7-46.2)	0.53
Gender (Male/Female)	10 (77%) / 3 (33%)	16 (80%) / 4 (20%)	1.00
Weight (kg)	52.46 (46.1-60,0)	58.35 (53.1-67.0)	0.14
SOFA score	10 (6.25-12.0)	-	
APACHE II score	**28 (20.0-33.0)**	**5 (3.7-7.0)**	**<0.01**
HIV, n (%)	12 (92.3%)	15 (75%)	0.42
Creatinine Clearance (ml/min)	**45.8 (0.0-97.5)**	**113.88 (86.5 - 157.9)**	**0.02**

Table 1 Clinical and demographic data

	Parameter	ICU (n=5)	Non-ICU (n=13)	P-value
Rifampin	AUC (mg.h/L)	46.57 (40.59 - 77.39)	25.07 (21.19 - 31.48)	**< 0.01**
	C_{max} (mg/L)	7.29 (6.63 - 10.12)	6.66 (4.09 - 8.39)	0.33
	Ka (h^{-1})	0.12 (0.09 - 0.25)	0.35 (0.25 - 0.49)	0.20
	Cl (L/h)	9.35 (6.78 - 13.66)	20.73 (16.42 - 24.73)	0.07
	T_{max} (h)	2 (2 - 4)	2 (2 - 4)	0.80
	V (L)	59.17 (53.13 - 78.19)	83.57 (77.49 - 102.10)	0.33
	$T_{1/2}$ (h)	6 (4.05 - 7.50)	1.96 (1.41 - 2.74)	0.19
	Parameter	**ICU (n=11)**	**Non-ICU (n=18)**	**P-value**
Isoniazid	AUC (mg.h/L)	15.17 (6.78 - 27.75)	14.36 (5.43 - 31-00)	0.95
	C_{max} (mg/L)	0.70 (0.29 - 1.59)	0.80 (0.51 - 1.10)	0.72
	Ka (h^{-1})	0.01 (0.01 - 0.06)	0.35 (0.25 - 0.49)	0.20
	Cl (L/h)	8.60 (2.51 - 14.52)	33.89 (3.36 - 47.79)	0.22
	T_{max} (h)	1 (0.5 - 3)	2 (1 - 2)	0.76
	V (L)	1354.29 (415.57 - 2170.85)	1738.4 (841.47 - 1753.4198)	1.00
	$T_{1/2}$ (h)	125.42 (11.52 - 557.50)	23.69 (22.09 - 52.21)	0.84
	Parameter	**ICU (n=13)**	**Non-ICU (n=20)**	**P-value**
Pyrazinamide	AUC (mg.h/L)	46.19 (12.35 - 143.40)	58.36 (34.96 - 74.61)	0.65
	C_{max} (mg/L)	3.51 (2.75 - 13.67)	7.34 (5.62 - 8.54)	0.37
	Ka (h^{-1})	0.1 (0.08 - 0.1)	0.13 (0.09 - 0.15)	0.25
	Cl (L/h)	8.23 (5.47 - 9.82)	21.20 (16.45 - 31.02)	**< 0.01**
	T_{max} (h)	1 (1 - 2)	2 (2 - 4)	**< 0.01**
	V (L)	94.65 (80.97 - 112.97)	220.98 (162.78 - 264.06)	**< 0.01**
	$T_{1/2}$ (h)	7.01 (6.96 - 8.44)	5.40 (4.64 - 7.31)	0.26
	Parameter	**ICU (n=10)**	**Non-ICU (n=20)**	**P-value**
Ethambutol	AUC (mg.h/L)	21.98 (6.82 - 36.13)	6.46 (4.84 - 8.73)	**0.01**
	C_{max} (mg/L)	2.33 (1.04 - 3.13)	1.29 (1.07 - 1.58)	0.13
	Ka (h^{-1})	0.04 (0.03 - 0.08)	0.22 (0.15 - 0.28)	**< 0.01**
	Cl (L/h)	25.11 (10.36 - 61.83)	131.42 (117.08 - 159.23)	**< 0.01**
	T_{max} (h)	2 (2 - 5.5)	2.54 (2 - 4)	0.96
	V (L)	486.49 (399.10 - 811.35)	810.67 (583.86 - 1048.10)	0.06
	$T_{1/2}$ (h)	16.00 (8.57 - 23.67)	3.16 (2.51 - 4.55)	**< 0.01**

Table 2: Rifampin, isoniazid, pyrazinamide, and ethambutol pharmacokinetic parameters estimated by a non-compartmental analysis. Data expressed as median (IQR), Mann-Whitney test

5. DISCUSSÃO

Este é o primeiro estudo a comparar a farmacocinética de rifampicina, isoniazida, pirazinamida e etambutol, em comprimidos DFC para tratamento de TB, administrados por via oral para pacientes ambulatoriais e por sonda nasogástrica para pacientes internados em UTI. Na análise não compartimental, foram observadas diferenças entre os dois grupos em todos os fármacos avaliados, exceto na isoniazida.

Foi verificado que nenhum dos fármacos analisados atinge as concentrações terapêuticas, nem no grupo de pacientes internados em UTI, nem no grupo de pacientes ambulatoriais. Mesmo sem atingir o alvo terapêutico, surpreendentemente, os pacientes em terapia intensiva apresentam AUC/MIC para rifampicina e etambutol superiores às de pacientes ambulatoriais.

Uma provável explicação para esse resultado é que os pacientes com sepse apresentam íleo paralítico, o que pode ter aumentado o tempo de permanência do fármaco no trato gastrointestinal, permitindo maior capacidade de absorção(91,95,97). Isso fica perceptível no etambutol, em que a constante de absorção (Ka) foi maior nos pacientes em UTI. Também, a pirazinamida apresenta um menor tempo para atingir a concentração máxima do fármaco (T_{max}) no grupo UTI. Além disso, os pacientes críticos estudados apresentavam disfunção renal e hepática, o que causa menor taxa de eliminação dos fármacos. Fato comprovado pelo menor CL de etambutol ($P < 0,01$) e por forte tendência de menor CL de rifampicina ($P = 0,07$)

A OMS recomenda o uso de uma concentração sérica crítica de 1 µg/mL para definir a suscetibilidade do *Mycobacterium tuberculosis* à rifampicina, 0,1 µg/mL para isoniazida, 100 µg/mL para pirazinamida e 5 µg/mL para etambutol(77). No entanto, o teste de suscetibilidade para as drogas é inconsistente(77). Rifampicina e isoniazida podem apresentar testes resistentes sem que ocorra necessariamente, falha clínica(73,77). No caso do etambutol e da pirazinamida, o ponto de corte epidemiológico da MIC parece ser superior à MIC efetiva, fazendo com que o teste produza resultados que oscilam entre resistente e suscetível(77). Por esse motivo, a própria OMS sugere que os testes de identificação dos genes indutores de resistência (*rpoB*, *inhA*, *katG* e *pncA*) sejam considerados mais confiáveis(77). Para além disso, há variações na literatura quanto à MIC efetiva que deve ser utilizado para cada

fármaco anti-TB, em que sugere-se, por exemplo, a modificação da MIC de rifampicina de 1,0 µg/mL para 0,0625 µg/mL, de isoniazida de 0,1 µg/mL para 0,0312 µg/mL e de pirazinamida para 50 ou 25 µg/mL(76). Soma-se a isso, a falta de dados com relação às MIC das cepas circulantes no Brasil.

Dessa forma, o uso de MIC para a determinação de alvos farmacocinéticos torna-se inviável. Porém, considerando que, em média, o sucesso terapêutico dos pacientes tratados ambulatorialmente é de 85%, pode-se assumir que estes apresentam concentrações séricas adequadas e seus parâmetros farmacocinéticos podem ser considerados alvos terapêuticos. Neste estudo, dos pacientes avaliados que estavam em tratamento ambulatorial, 94% evoluíram com cura clínica e microbiológica. Portanto, pressupõe-se que as $AUC_{(0-24)}$ e C_{max} encontradas foram suficientes para eliminar o bacilo, podendo ser considerados alvos farmacocinéticos adequados para essa população.

Sendo assim, uma vez que as $AUC_{(0-24)}$ e C_{max}, para rifampicina, isoniazida e etambutol, dos pacientes em terapia intensiva foram iguais ou superiores aos pacientes ambulatoriais, não é possível afirmar que a mortalidade de 70% no grupo UTI está associada a baixas concentrações séricas de RHZE.

Sendo a sepse uma disfunção orgânica causada por uma resposta desregulada do hospedeiro a um processo infecioso e que os pacientes analisados apresentam disfunção orgânica com um SOFA escore médio de 10 pontos, pode-se assumir que o diagnóstico desses pacientes era de sepse por Mtb(90). Portanto, o status inflamatório e o número de disfunções orgânicas podem ser a causa da elevada mortalidade nesse grupo(90,91).

Nagai e colaboradores concluíram, pelo escore A-DROP, que fatores como idade, desidratação, insuficiência respiratória, rebaixamento do nível de consciência e pressão arterial estão diretamente relacionados ao risco de óbito na UTI(132). Embora escores de gravidade e de predição de letalidade, como APACHE II e SAPS III, sejam consagrados em unidades de terapia intensiva, frequentemente, subestimam a mortalidade e a gravidade de pacientes com tuberculose nesse cenário(133,134). Além da ventilação mecânica, a literatura descreve diferentes preditores de mortalidade, como choque séptico, necessidade de vasopressores,

contagem de CD4 e albumina sérica baixa(113,122,125,134,135). Não só fatores do quadro agudo da doença, mas também a falta de diagnóstico precoce e consequente atraso na instituição de terapêutica, bem como a baixa adesão ao tratamento associado a outras comorbidades como tabagismo, abuso de álcool e diabetes mellitus ou questões sociais relacionadas a tuberculose, certamente, facilitam o agravamento da doença(1,7,29,119). Todo esse elenco de fatores reforça a complexidade do paciente crítico com tuberculose.

No estudo, o contraste na condição clínica entre os dois grupos fica bem expresso pelo escore APACHE II. Enquanto aqueles que estão em tratamento ambulatorial têm APACHE II médio de 5 pontos, predizendo 8% de mortalidade, os pacientes críticos apresentam APACHE II médio de 28 pontos, com mortalidade estimada de 55%. Logo, a condição clínica dos pacientes em UTI é muito grave, e seu risco de óbito vai além da terapia antimicrobiana utilizada(104).

Não se pode, todavia, furtar-se ao fato de que os pacientes em ambos os grupos apresentaram concentrações séricas abaixo do esperado para todas as drogas. Cabe salientar que o tratamento da tuberculose não depende apenas de uma droga e, também, que a interação entre essas drogas, bem como sua ação conjunta, não foi abordada neste estudo(136).

Stott e colaboradores, ao estudar a rifampicina, demonstraram que para a redução de 1 log de unidades formadoras de colônia (UFC) *in vivo*, é necessária uma AUC/MIC de 271 mg.h/L(137). Ao mesmo tempo, Aarnoutse e colaboradores observaram que ao dobrar a dose de rifampicina de 600 para 1.200 mg ao dia, a $AUC_{(0-24)}$ aumenta em três vezes e que isso não resultou em redução do tempo de negativação de culturas, portanto, sem gerar impacto no desfecho clínico(138). Por outro lado, Gumbo demonstrou que ao final de sete dias, a maior capacidade bactericida da rifampicina era atingida com $AUC_{(0-24)}$ de 24,14 mg.h/L(66,139). Dessa maneira, pode-se presumir que não é a concentração sérica do fármaco, mas sim sua capacidade de eliminar a bactéria um fator que possa aumentar a letalidade da sepse por tuberculose. Portanto, a busca por medicamentos com melhores *time-kill curves* para pacientes críticos pode ser uma estratégia melhor do que o simples aumento da dose em busca de alvos terapêuticos controversos.

Os conceitos de AUC e AUC/MIC foram definidos pelos estudos de Schentag et al. e Forrest et al., em 1990 e 1993, ou seja, tratam-se de conceitos ainda novos(140,141). Além disso, o alvo terapêutico para TDM da rifampicina, por exemplo, definido por uma C_{max} de 8 a 24 mg/L, foi sugerido na década de 1990 juntamente com o surgimento daqueles conceitos(137,142). Rockwood e colaboradores demonstraram que, embora muitos pacientes apresentassem concentrações séricas abaixo do recomendado, eles obtinham cultura negativa ao final de dois meses de tratamento(143). Além disso, como a rifampicina induz o metabolismo hepático, sabe-se que há uma diferença entre a dose no início do tratamento e após 40 dias(137). Segundo Stott, no padrão de dose recomendada pela OMS, há uma redução significativa de C_{max} e da AUC da rifampicina após 40 dias, quando é atingido seu estado de equilíbrio(137). O C_{max} cai de 8,98 para 5,79 mg/L e a $AUC_{(0-24)}$ diminui de 72,56 para 38,73 mg.h/L(137). Todos esses fatores tornam difíceis a determinação de alvos terapêuticos.

Um C_{max} maior do que 3 mg/mL é a dose terapêutica sugerida para isoniazida(143,144). Segundo Rockwood, concentrações séricas abaixo do alvo para rifampicina, isoniazida e pirazinamida, isoladamente, não interferem na negativação das culturas para Mtb ao final de dois meses(143). Porém, C_{max} de isoniazida abaixo de 4,6 mg/L associado a um C_{max} de rifampicina menor que 28 mg/L apresentam efeito antagônico para a negativação de culturas de Mtb(143). Além disso, Pasipandoya demonstrou que pacientes em uso de isoniazida com desfecho clínico favorável 12 meses após o tratamento apresentavam $AUC_{(0-24)}$ entre 31,26 e 36,37 mg.h/L(126,145). Os pacientes ambulatoriais deste estudo apresentaram concentrações de isoniazida abaixo do alvo em associação com doses de rifampicina menores que as sugeridas e, apesar disso, evoluíram com cura clínica e microbiológica. Dois fatores podem estar associados a isso. O primeiro é o tempo de tratamento de cada indivíduo, que pode influenciar C_{max} e $AUC_{(0-24)}$ de rifampicina, conforme descrito anteriormente. Uma investigação por meio da construção de um modelo farmacocinético para a rifampicina pode ser elucidativa. O outro fator é que, assim como sugerido por Zuur e colaboradores, o MIC para isoniazida seja menor que 1,0 µg/mL (0,125 µg/mL)(76). Considerando a análise não compartimental do presente estudo, um MIC menor que 0,5 µg/mL levaria a uma AUC/MIC entre 31,26 e 36,37 mg.h/L, conforme sugerido por Pasipandoya.

No caso da pirazinamida, Pasipanodya aponta que C_{max} menores que 58,3 mg/L e $AUC_{(0-24)}$ menores que 363 mg.h/L foram preditores de resultados desfavoráveis clínica e microbiologicamente(145). Já Chideya associou desfechos desfavoráveis a C_{max} menor que 35 mg/L. Os pacientes ambulatoriais estudados atingiram C_{max} de 7,34 mg/L e $AUC_{(0-24)}$ de 58,36 mg.h/L, concentrações de 6 a 8 vezes menores que o recomendado(146). No grupo pacientes em UTI, os resultados são mais baixos com C_{max} 3,51 mg/L e $AUC_{(0-24)}$ 46,19 mg.h/L. McIlleron já havia associado baixas concentrações séricas de pirazinamida a pacientes com baixo peso recebendo tratamento com comprimidos DFC(147). Um modelo farmacocinético pode ser capaz de indicar quais variáveis estão associadas a baixa concentração de pirazinamida. Chirehwa sugere uma adição de 400 mg/dia de pirazinamida para que um C_{max} maior que 35 mg/L seja atingido, sem a ocorrência de maior número de eventos adversos associados ao fármaco(148). Além disso, assim como outros tuberculostáticos, o MIC de pirazinamida é questionável, podendo variar de 25 a 100 µg/mL(77,143,148).

Na análise do etambutol, nenhum dos grupos atingiu os alvos definidos a priori de AUC/MIC maior que 119 mg.h/L ou de C_{max}/MIC maior que 0,48 mg/L, fazendo com que a PTA fosse de 0%. Isso não surpreende, uma vez que os pacientes estudados atingiram um $AUC_{(0–24h)}$ de 48 a 144 mg.h/L. Esse resultado é similar ao encontrado por Denti (2015) e McIlleron (2006) em pacientes fora das unidades de terapia intensiva, que resultou em $AUC_{(0–24)}$ de 23,6 e 59,5 mg.h/L, respectivamente(147,149). TDM em estudos prévios de etambutol identificaram um alvo terapêutico de C_{max} entre 2 e 6 mg/L. No presente estudo, a mediana de C_{max} foi 1,11 mg/L nos pacientes ambulatoriais. Isso sugere uma subdose desse fármaco. Considerando que sua toxicidade aumenta com C_{max} maior que 6 mg/L(150,151), o modelo farmacocinético desenvolvido neste estudo sugere que, para a população de pacientes ambulatoriais, a dose de etambutol deve ser maior que 1.375 mg, sem oferecer riscos.

6. LIMITAÇÕES DO ESTUDO

O estudo foi realizado em um único centro, a FMT-HVD, referência em atendimento de pacientes coinfectados TB/HIV para o estado do Amazonas. Portanto, a análise farmacocinética deste estudo pode não ser válida para outras populações. A escolha do centro contribuiu para que 82% dos participantes do estudo fossem pessoas vivendo com HIV, o que potencialmente influenciou variáveis como status HIV, carga viral para HIV, contagem de células $CD4^+$ e uso de antirretroviral a não interferirem no modelo farmacocinético.

O monitoramento terapêutico de fármacos melhora o desfecho clínico dos pacientes, garantindo doses terapêuticas e evitando doses tóxicas de medicamentos. Considerando a facilidade de coleta de amostras de sangue na prática clínica, optamos por realizar a dosagem sérica dos fármacos. Todavia, não avaliamos a concentração do fármaco nos pulmões ou em outros tecidos passíveis de serem acometidos pela tuberculose.

Por tratar-se de um ensaio farmacocinético, direcionado para pacientes de UTI, variáveis prévias ao internamento, como o tempo entre o início dos sintomas e o diagnóstico, bem como o tempo entre o diagnóstico e o início do tratamento, não foram avaliadas. Esses fatores podem ter contribuído para a piora clínica dos pacientes do grupo UTI. Estudos epidemiológicos com maior número de indivíduos devem ser realizados com o objetivo de identificar fatores pré-hospitalares que possam gerar internamento e influenciar no desfecho em óbito de pacientes com TB.

O método do estudo não previu que todos os pacientes obtivessem diagnóstico de tuberculose a partir de cultura e, por isso, os MIC para as cepas de *Mycobaterium tuberculosis* não foram estabelecidos para cada indivíduo. A relação PK/PD foi estabelecida a partir de dados da literatura. Além disso, *time-kill curves* capazes de estabelecer a melhor atividade bactericida de cada fármaco, bem como da combinação entre rifampicina, isoniazida, pirazinamida e etambutol, para as cepas em questão não foram estudados. Ademais, não foram avaliados sinergismo e antagonismo entre os quatro medicamentos estudados.

O modelo de estudo não prevê análise farmacotécnica dos comprimidos DFC, entretanto, é sabido que características físico-químicas dos comprimidos podem influenciar no perfil farmacocinético.

7. CONCLUSÕES

A maioria dos pacientes críticos em tratamento de tuberculose são homens, com mediana de idade de 39 anos, coinfectados pelo HIV. Apresentam disfunção renal, com taxa de filtração glomerular média de 45,8 mL/min e mediana de peso de 52,46 kg. O estado inflamatório dos pacientes em sepse por tuberculose é de forte intensidade, com um SOFA escore médio de 10 pontos e um APACHE II médio de 28 pontos, estimando-se uma letalidade de 55% versus uma letalidade observada de 77%, em 30 dias após a admissão na UTI. Por isso, pode-se inferir que o agravamento da doença causado pelo atraso no diagnóstico, a demora em instituir a terapêutica, a condição clínica prévia do paciente e a adesão ao tratamento são fatores substanciais na elevada taxa de óbitos pela sepse por tuberculose.

Em uma análise não compartimental, observa-se diferença na farmacocinética de rifampicina, pirazinamida e etambutol entre pacientes admitidos em unidades de terapia intensiva e pacientes ambulatoriais. Entretanto, não foram observadas diferenças no perfil farmacocinético da isoniazida. Muito embora essas diferenças garantam maior concentração de rifampicina e etambutol nos pacientes em UTI, as doses avaliadas conforme faixa de peso, neste estudo, não atingiram concentrações terapêuticas para nenhum dos fármacos utilizados.

Todavia, considerando o sucesso terapêutico dos pacientes ambulatoriais, presume-se que não é a concentração sérica do fármaco, mas sim sua capacidade em eliminar a bactéria rapidamente em um paciente séptico um fator que possa aumentar a letalidade pela sepse por tuberculose. Portanto, a busca por medicamentos com melhores *time-kill curves* para pacientes críticos parece ser uma estratégia melhor do que o simples aumento da dose em busca de alvos terapêuticos controversos. Estudos que permitam definir o alvo terapêutico de cada fármaco e de sua combinação, bem como uma concentração inibitória mínima mais precisa, são peças-chave para um tratamento mais assertivo para os pacientes com tuberculose.

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8. ANEXOS

8.1 Aprovação no Comitê de Ética em Pesquisa da Fundação de Medicina Tropical Dr. Heitor Vieira Dourado

FUNDAÇÃO DE MEDICINA TROPICAL DR. HEITOR VIEIRA DOURADO ((FMT-HVD))

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA
Título da Pesquisa: RIPE VERSUS RIPE E N-ACETILCISTEÍNA EM INDIVÍDUOS COINFECTADOS TB/HIV: ENSAIO CLÍNICO RANDOMIZADO, FASE II
Pesquisador: Marcelo Cordeiro dos Santos
Área Temática:
Versão: 2
CAAE: 60219916.5.0000.0005
Instituição Proponente: Fundação de Medicina Tropical do Amazonas - FMT/IMT/AM
Patrocinador Principal: Ministério da Saúde

DADOS DO PARECER

Número do Parecer: 1.774.751

Apresentação do Projeto:

No documento intitulado " PB-INFORMAÇÕES BÁSICAS DO PROJETO -797384.pdf postado em 21.09.2016, item resumo, lê-se "Embora a tuberculose seja uma doença tratável, atualmente trata-se dadoença infecciosa com maior mortalidade no mundo. Os pacientes acometidos podem ser admitidos em unidades de terapia intensiva por diversos motivos, com taxas de mortalidade chegando a 67% em alguns estudos. O Ministério da Saúde, assim como a OMS, recomenda que os pacientes inicialmente devam ser tratados por via oral com rifampicina (R),isoniazida (I), pirazinamida (P) e etambutol(E).A N-acetilcisteína (NAC) teve seu primeiro benefício reportado durante os anos 1960, quando mostrou ser um agente mucolítico eficaz em indivíduos comfibrose cística, exercendo um efeito antioxidante indireto. Recentemente,o estudo PANTHEON propôs a NAC como composto adjunto no tratamento da DPOC, a fim de evitarexacerbações no quadro clínico do paciente, sendo seu uso preconizado a longo prazo (por um ano), com doses diárias de 1.200 mg do composto. A TB e a Aids também são doenças que cursam com um estímulo inflamatório crônico, com

constante formação de radicais livres que em excesso, podem gerar o estresse oxidativo celular

e sistêmico.

Os efeitos da NAC em ambas as populações são estudados. Alguns autores mostraram que

indivíduos com TB possuem subníveis de glutationa.O mesmo ocorreu em um modelo de animais infectados com o Mtb,sugerindo que o estresse oxidativo foi devido, em parte, a defesa antioxidante pobre do hospedeiro. A suplementação de NAC diminui a carga bacteriana no baço e a gravidade da necrose no pulmão.Benefícios adicionais da NAC em indivíduos com TB estão relacionados ao possível efeito protetor hepático às drogas tuberculostáticas e ao efeito antimicrobiano direto demonstrado in vitro. Estudos observaram que o tratamento com NAC em culturas de Mtb reduzia a atividade metabólica bacteriana após 5 dias de
tratamento, não sendo constatado alteração (aumento ou redução) na carga bacteriana durante o períododo experimento. Estes achados sugerem que a NAC tenha ação bacteriostática direta no crescimento do bacilo. O presente estudo visa avaliar os efeitos da NAC como terapia coadjuvante no tratamento da TB. Trata-se de um ensaio Clínico randomizada, fase II onde será avaliada a segurança, a tolerabilidade e a efetividade da NAC como terapia coadjuvante no tratamento da TB. Serão randomizados 50 pacientes em dois grupos. O primeiro grupo receberá o tratamento padrão de tuberculose conforme preconizado pelo Ministério da Saúde; o segundo receberá além deste tratamento 1200mg de NAC por dia durante dois meses. Dessa forma poderá ser avaliada a taxa de conversão da baciloscopia e da cultura para micobactérias, os níveis de glutationa e de biomarcadores de ativação imunológica e inflamação em indivíduos com TB em uso ou não de NAC e o perfil farmacocinético das drogas envolvidas
Trata-se da pesquisa intitulada "Ripe versus Ripe e N-acetilcisteina em individuos coninfectados TB/HIV:ensaio clinico randomizado, fase II", tendo como pesquisador principal Marcelo Cordeiro dos Santos,instituição proponente Fundação de Medicina Tropícal Dr Heitor Vieira Dourado e instituição patrocinadora Ministério da Saúde.

Objetivo da Pesquisa:

OBJETIVO PRIMÁRIO:

1-Avaliar os efeitos da NAC como terapia coadjuvante no tratamento da TB em indivíduos acompanhados na Fundação de Medicina Tropical Dr.Heitor Vieira Dourado (FMT-HVD),

Manaus, Brasil.OBJETIVOS SECUNDÁRIOS:
Avaliar a segurança, a tolerabilidade e a efetividade da NAC como terapia coadjuvante no tratamento da TB;
Observar a taxa de conversão da baciloscopia e da cultura para micobactérias em meio líquido (MGIT®)nas primeiras oito semanas de uso da NAC em indivíduos com TB pulmonar;
Dosar os níveis de glutationa e de biomarcadores de ativação imunológica e inflamação em indivíduos com TB, em uso ou não de NAC.
Descrever a farmacocinética e farmacodinâmica (PK/PD) do esquema RIPE;
Comparar o PK/PD do esquema RIPE em indivíduos internados, dentro ou fora da UTI;
Descrever os aspectos clínicos, epidemiológicos e microbiológicos dos indivíduos com TB acompanhados na FMT-HVD durante o estudo.

Avaliação dos Riscos e Benefícios:

Riscos: A coleta de sangue pode causar uma pequena dor ou hematoma. O exame de escarro, urina e a radiografia de tórax são procedimentos que não causam maiores riscos. A NAC é um medicamento aprovado pelo United States Food and Drug (FDA) e pela ANVISA, categoria B na gestação,quando administrada pode causar náuseas, vômitos e pirose , mas os efeitos colaterais são incomuns. A administração oral de NAC em doses de até 8000 mg/dia não cursou com reações adversas clinicamente significativas.
Benefícios:
A associação de n-acetilcisteína ao tratamento de tuberculose pode acelerar o processo de cura microbiológica, melhorando a qualidade de vida do paciente e reduzindo o tempo de transmissão da doença trazendo melhoria para o indivíduo e para a sociedade. Além disso, o conhecimento sobre a farmacocinéticapermite a individualização da dose trazendo melhor efeito terapêutico com menores eventos adversos.

Comentários e Considerações sobre a Pesquisa:

O presente estudo consiste no recrutamento e acompanhamento Via laboratório de tuberculose de 50 participantes: O laboratório de tuberculose da FMT-HVD será orientado a informar diariamente a equipe doestudo a positividade de algum exame para TB, independente do material biológico analisado.
Via visitas àsunidades de internação e pronto-atendimento: A equipe do estudo realizará visitas diárias a esses setores identificando, junto à equipe assistencial, possíveis indivíduos elegíveis.Sendo então convidado a participar do estudo RIPENACTB através do termo de consentimento livre e esclarecido (TCLE),

aplicado pelo pesquisador. Na impossibilidade de entendimento, o TCLE será oferecido ao familiar mais próximo. Tendo o aceite, será realizada a coleta de exames do protocolo RIPENACTB, conforme a necessidade. Haverá a tentativa de coincidir com a coleta do estudo com a coleta da equipe assistencial, no intuito de diminuir o número de punções. Na presença de acesso venoso central, a amostra sanguínea será aspirada deste dispositivo. Os exames do protocolo RIPENACTB necessários no D0, caso já tenham sido realizados,serão válidos se obedecerem intervalos de tolerância pré-estabelecidos: As consultas do estudo serão programadas nos períodos abaixo e terão os seguintes objetivos: -D0: elegibilidade/TCLE, consulta médica,coleta de exame e liberação de medicação (RIPE/RIPE+NAC);-D1 e D2: coleta de exame;-D7 (semana 1):coleta de exame;-D14 (semana 2): consulta médica, coleta de exame, introdução da TARV (paraaqueles virgens de TARV),seguindo as normas do Ministério da Saúde e liberação de medicação (RIPE/RIPE+NAC); -D28 (semana 4/mês 1): consulta médica, coleta de exame e liberação de medicação (RIPE/RIPE+NAC);- D42 (semana 6): consulta médica, coleta de exame e liberação de medicação (RIPE/RIPE+NAC);-D56(semana 8/mês 2): consulta médica, coleta de exame e término da fase intensiva e da NAC e liberação demedicação (R+I);-Mês 4: consulta de enfermagem, coleta de exame e liberação de medicação (R+I);-Mês6:consulta médica, coleta de exame, avaliação de cura da TB e término da participação no estudo. Após o término do tratamento da TB e dado o individuo como curado, o mesmo será encaminhado para o ambulatório de HIV da FMT-HVD para dar continuidade ao seu acompanhamento.Intervenções: Grupo A - Tratamento com RIPE conforme protocolo do Ministério da Saúde;
Grupo B - Tratamento com RIPE conforme protocolo do Ministério da Saúde + NAC 1200mg/dia durante 2 meses.A NAC é um medicamento aprovado pelo United States Food and Drug (FDA) e pela ANVISA,categoria B na gestação. Análise imunológica: Amostras de plasma EDTA (2 tubos de 4mL), soro (1tubo de 8mL), e células mononucleares do sangue periférico (1 tubo de heparina de 10mL) serão coletadas por punção venosa à vácuo nos tempos D0, D14, D28, D56, D112 e D168 para realização de ensaios imunológicos. As amostras de plasma e soro serão utilizadas para mensuração de um painel de citocinas,quimiocinas, fatores de crescimento, proteínas de fase aguda, marcadores de estresse oxidativo e de remodelamento tecidual utilizando ensaios de luminex. As amostras de células serão analisadas em ensaios de citometria de fluxo para avaliar ativação de células T específicas contra o Mtb.Após a realização

da análise as amostras serão destruídas.

Análise PK/PD- As coletas destinadas a análise de PK/PD serão feitas em intervalos pré-determinados da seguinte maneira: zero minutos (imediatamente antes da dose); 30 minutos após a dose; 60 minutos; 120 minutos; 240 minutos; 360 minutos; 480 minutos; 720 minutos; 1.440 minutos (imediatamente antes da próxima dose).A análise das amostras destinadas a PK/PD será realizada no laboratório da Universidade Federal do Rio de Janeiro (UFRJ) e controle de qualidade será realizado pelaUniversidade de Queensland, em Brisbane, Queensland, Austrália pelo Dr. Steve Wallis. Após a realização do controle de qualidade as amostras serão destruidas.

O RIPENACTB é um ensaio clinico unicêntrico, de grupos paralelos, randomizado, controlado, aberto, de fase II, a pesquisa busca testar a seguinte hipótese: O uso da n-acetilcisteína acelera o processo de cura microbiológica de pacientes com tuberculose.

Critérios de inclusão:

1-Serão elegíveis pacientes que preencham os seguintes critérios:

- Idade maior ou igual 18 anos;
- Aceitação ao exame de HIV;
- Previsão de permanência hospitalar superior a vinte e quatro horas;
- Indicação clínica ou laboratorial de RIPE, conforme decisão da equipe assistente;2-Condições de realizar a punção de acesso venoso ou arterial;
- Em uso de sonda vesical ou condições de coletar a urina em oito horas seguidas;

3-Tratamento diretamente observado de RIPE nos dias em que serão coletadas amostras sanguíneas paraanálise de PK/PD;

- Termo de consentimento assinado e datado antes da avaliação inicial.Critérios de Exclusão:

Serão excluídos da avaliação pacientes que apresentem pelo menos um dos seguintes critérios:

Para o uso de NAC

- Indígenas;
- Recusa em realizar o exame de HIV;
- Gestantes, lactantes ou planos de engravidar durante o período do estudo;
- TB extra pulmonar, sem acometimento pulmonar;
- Não ter condições de realizar a coleta de escarro ou aspirado traqueal para confirmação microbiológica, mesmo que de forma induzida;

- Nenhuma cultura MGIT® positiva para Mtb;
- Mono, poli ou multirresistência ao Mtb, detectada no perfil de sensibilidade;
- Indivíduos em tratamento para broncoespasmos secundários a asma brônquica, conforme a decisão da equipe assistente ou pesquisador do estudo;
- Suspeita clínica de úlcera gástrica ou duodenal, conforme decisão da equipe assistente ou pesquisador doestudo; ou evidência por endoscopia digestiva alta;
- Alanina aminotransferase (ALT) maior que três vezes a normalidade;
- Necessidade de suspensão do esquema RIPE, conforme decisão da equipe assistente ou do médicopesquisador;
- Falta de adesão ao tratamento proposto por mais de sete dias consecutivos;
- Impossibilidade de acompanhamento;
- Retirada de cnsentimento.

Para a realização de PK/PD:

- Indígenas;
- Recusa em realizar o exame de HIV;
- Gestantes, lactantes ou planos de engravidar durante o período do estudo;
- Hemodiálise, diálise peritoneal ou terapia de substituição renal continua;
- Uso de inibidor de protease durante a coleta de amostras sanguíneas para análise de PK/PD;
- Necessidade de suspensão do esquema RIPE nos dias de coleta de amostras sanguíneas para análise dePK/PD, conforme decisão da equipe assistente;
- Impossibilidade de acompanhamento;
- Retirada de consentimento.

O indivíduo poderá retirar sua participação no estudo a qualquer momento e não será obrigado a declarar a razão de retirada. Se isso ocorrer, o indivíduo será remanejado aos ambulatórios especializados da FMTHVD para dar continuidade ao tratamento da TB e do HIV.

Metodologia de Análise de Dados:

Os dados coletados serão digitados em planilha Excel 8.0 e a análise estatistica será realizada no programaestatistico Stata 11.0.

Para a análise descritiva:

1) serão calculadas médias e desvio padrão para variáveis contínuas com distribuição normal;
2) medianas e intervalos interquartis (IIQ,percentil 25-75%) para variáveis contínuas com

distribuição assimétrica;

1) proporções para variáveis categóricas.

Na análise exploratória bivariada será aplicado o teste de hipótese Qui-quadrado ou teste exato de Fisher para analisar variáveis categóricas e nas variáveis contínuas normalmente distribuídas serão analisadascom Teste-t de Student na comparação de médias, com ou sem ajuste de variâncias desiguais. As variáveis contínuas com distribuição assimétrica ou variáveis discretas serão analisadas por meio do teste nãoparamétrico
de Mann-Whitney ou Kruskal-Wallis para comparação das medianas.

A medida utilizada para avaliar a associação entre a variável dependente (o desfecho) e as variáveis explicativas (covariáveis de interesse) será a razão entre a incidência acumulada (risco) dos desfechos nos expostos e nos não expostos (risco relativo - RR). Serão realizadas análises multivariadas por modelo de regressão logística, com derivação de razão de chances (RC ou OR, odds ratio). O confundimento será analisado por meio da exclusão não-automática de variáveis de trás para frentee uma a uma (backwards stepwise). Será adotada uma análise por protocolo (PP) e uma análise por intenção de tratamento (ITT). Será considerado o intervalo de confiança de 95% (IC95%) e os valores de p bicaudais, significativo quando menor que 0,05. Será realizada uma análise preliminar quando se atingir o seguimento da metade dos pacientes a serem randomizados. Modelos farmacocinéticos populacionais serão realizados utilizando avaliação não linear de modelos (NONMEM).

Parâmetros farmacocinéticos (volume de distribuição, clearance de creatinina e clearance nos tecidos) serão estimados. Uma avaliação será feita para estimar as diferenças nos parâmetros farmacocinéticos decada indivíduo com características clínicas e demográficas. As estratégias de dose serão avaliadas a partirde simulação de Monte Carlo.

A pesquisa é factivel e de grande apelo social, pois no final, poderá oferecer dados importantes para a compreensão de uma moléstia que persiste em populações carentes, como comparar o

tempo de conversão da baciloscopia e da cultura MGIT em oito semanas e ainda avaliar a segurança, atolerabilidade e eficácia da NAC.
.Monitorar a hepatotoxicidade medicamentosa aos tuberculostáticos.
.Avaliar o efeito da NAC na carga viral e na contagem de células CD4+.
.Avaliar a concentração sérica do esquema RIPE nos pacientes internados.

Considerações sobre os Termos de apresentação obrigatória:

A maioria dos documentos apensados ao protocolo encontram-se em ordem e devidamente aptos ao julgamento. Entretanto, alguns necessitam ser melhorados e adequados, tanto no aspecto formal, quanto noplano ético.

Conclusões ou Pendências e Lista de Inadequações:

Após análise das respostas postadas em 10/10/2016 na Plataforma Brasil(PB),as conclusões são as seguintes.
PENDÊNCIAS
1. Quanto ao TCLE
- O TCLE, apesar de bem redigido, apresenta as seguintes inadequações:
Uso de termos de dificil compreensão para o participante da pesquisa como RIPENACTB, RIPE e outros. 1.1-Solicita-se que seja adicionado o significado de cada termo técnico empregado no texto (itens II.23 e IV.1, b, da Resolução CNS nº.466 de 2012).

RESPOSTA A PENDÊNCIA 1

O TCLE que se encontra em anexo na PB, foi devidamente melhorado, os termos técnicos foram substituidos para favorecer o esclarecimento ao participante.Possivel verificar o TCLE, intitulado com a seguinte extensão: TCLEcorrigido.pdf-postado em 10/10/2016.

CONCLUSÃO:PENDÊNCIA 1- ATENDIDA

2- Quanto a Anuência da DAM.pdf. Verificou-se que não consta no texto, menção sobre o laboratório de Análises clinicas, que fará os exames de sangue citados no Projeto pag..18. Pede-se para revisar e adequaro texto, e que este documento seja assinado e carimbado pelo gestor

responsável e anexado na PB.

RESPOSTA A PENDÊNCIA 2
Encontra-se em anexo ao protocolo a carta de anuência do Diretor de Assistência Médica, com todas as correções solicitadas, intitulada com a seguinte extensão:AnuênciaDAMcorrigida.pdf postada em 10/10/2016.

CONCLUSÃO:PENDENCIA 2-ATENDIDA.

Diante do exposto, após todas as pendências solucionadas, o voto desta relatoria é pela APROVAÇÃO deste protocolo, por entender que o mesmo se assenta às normas estabelecidas pela Resolução CNS n. 466/2012 e suas complementares.

S.M.J é o parecer

Considerações Finais a critério do CEP:
O presente projeto está APROVADO e os interessados ficam informados de apresentar a este CEP os relatórios parciais e final do estudo, conforme prevê a Resolução CNS nº 466/2012, utilizando o formulário de Roteiro para Relatório Parcial/Final de estudos clínicos Unicêntricos e Multicêntricos, proposto pela CONEP em nossa home page.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_797384.pdf	10/10/2016 11:29:43		Aceito
Outros	AnuenciaDAMcorrigida.pdf	10/10/2016 11:28:59	Francisco Beraldi de Magalhães	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLEcorrigido.pdf	10/10/2016 11:28:08	Francisco Beraldi de Magalhães	Aceito
Folha de Rosto	folhaDeRosto.pdf	21/09/2016 21:24:39	Marcelo Cordeiro dos Santos	Aceito

FUNDAÇÃO DE MEDICINA TROPICAL DR. HEITOR VIEIRA DOURADO ((FMT-HVD))

Continuação do Parecer: 1.774.751

Outros	AnuenciaUFRJ.pdf	21/09/2016 18:18:05	Marcelo Cordeiro dos Santos	Aceito
Declaração do Patrocinador	Apoio.pdf	21/09/2016 18:08:58	Marcelo Cordeiro dos Santos	Aceito
Outros	AnuenciaBA.pdf	21/09/2016 18:08:08	Marcelo Cordeiro dos Santos	Aceito
Declaração de Pesquisadores	compromisso.pdf	21/09/2016 18:06:02	Marcelo Cordeiro dos Santos	Aceito
Projeto Detalhado / Brochura Investigador	projeto.pdf	21/09/2016 18:03:55	Marcelo Cordeiro dos Santos	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

MANAUS, 14 de Outubro de 2016

Assinado por: Marilaine Martins(Coordenador)

9. APÊNDICES

9.1 Apêndice A. Formulário de pesquisa clínica

Número de Inclusão:

Informação Pessoal:

Nome:

Data de Nascimento:

Endereço:

Telefone:

Profissão:

Etnia:

Informação Microbiológica:

BAAR ( ) Positivo ( ) Negativo Data da coleta: Data do resultado:

Cultura( ) Positivo ( ) Negativo Data da coleta: Data do resultado:

Cultura de Urina( ) Positivo( ) Negativo Data da coleta: Data do resultado:

GeneXPERT( ) Positivo ( ) Negativo Data da coleta: Data do resultado:

RIF sensível ( ) Sim ( ) Não

informação clínica:

Data de admissão hospitalar:

Data de admissão na UTI:

Peso (kg):

Primeiro tratamento de TB: ( ) Sim ( ) Não Quando foi realizado?

Suspeita de Tuberculose Meníngea: ( ) Sim ( ) Não

HIV ( ) Positivo ( ) Negativo Data:

Antirretroviral: ( ) Sim ( ) Não Qual esquema?

Data de início:

Última Carga Viral: Data:

Último $CD4^+$: Data:

Anti-HCV ( ) Positivo ( ) Negativo Data:

Anti-HbsAg ( ) Positivo ( ) Negativo Data:

HbsAg ( ) Positivo ( ) Negativo Data:

Sífilis ( ) Positivo ( ) Negativo Data:

Medicação:

Esquema RIPE? ( ) Sim ( ) Não Data de início:

Dose ( ) 1 comprimido ( ) 2 comprimidos
( ) 3 comprimidos ( ) 4 comprimidos

Administração: ( ) Oral ( ) Sonda nasogástrica

Antibióticos nos últimos 30 dias (pré-admissão): ( ) Sim ( ) Não

Antibiótico 1: Data de início: Data de fim:

Antibiótico 2: Data de início: Data de fim:

Antibiótico 3: Data de início: Data de fim:

Antibióticos no hospital: ( ) Sim ( ) Não

Antibiótico 1: Data de início: Data de fim:

Antibiótico 2: Data de início: Data de fim:

Antibiótico 3: Data de início: Data de fim:

Antibiótico 4: Data de início: Data de fim:

Antibiótico 5: Data de início: Data de fim:

Vasopressores: ( ) Sim ( ) Não

( ) Dopamina ( ) Dobutamina ( ) Norepinefrina

Informação de Cuidados Intensivos:

Ventilação mecânica: ( ) Sim ( ) Não

( ) Invasiva ( ) Não invasiva

Sonda nasogástrica: ( ) Sim ( ) Não

Cateter venoso central: ( ) Sim ( ) Não

Cateter arterial: ( ) Sim ( ) Não

Sonda vesical urinária: ( ) Sim ( ) Não

	Admissão	Inclusão	D1	D2	D3	D4
Creatinina Sérica						
Ureia Sérica						
Creatinina Urinária						
Volume Urinário (8h)						
CrCl						

	Admissão	Inclusão	D1	D2	D3	D4
Escala de Coma de Glasgow						
Freq. Cardíaca						
Pressão Arterial Média						
Freq. Respiratória						
PaO2/FiO2						
pH Arterial						
$HCO3^-$ Arterial						
Hematócrito						
Hemoglobina						
Leucócitos						
Plaquetas						
Bilirrubina						
AST						
ALT						
Albumina						
Proteína Total						
APACHE II						
SOFA						

Responsável:

9.2 Apêndice B. Formulário de coleta de amostras

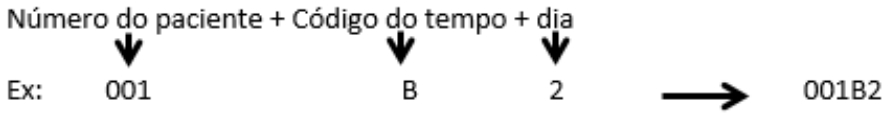
Código do paciente:

Nome do paciente:

Informe a data e o horário exato da coleta

Código		Hora	Dia 1	Dia 2	Dia 3	Dia 4
	Data					
A	0 minuto (pré-dose)	08:00				
B	30 minutos	08:30				
C	60 minutos	09:00				
D	120 minutos	10:00				
E	240 minutos	12:00				
F	360 minutos	14:00				
G	480 minutos	16:00				
H	720 minutos	20:00				

O código da amostra deve ser preenchido conforme o exemplo abaixo:



Responsável:

9.3 Termo de Consentimento livre e esclarecido (TCLE)

RIPE versus RIPE e N-acetilcisteína em indivíduos coinfectados TB/HIV: Ensaio clínico randomizado, fase II

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE)

Você está sendo convidado a participar, como voluntário (a), em um estudo sobre tuberculose chamado "**RIPE versus RIPE e N-acetilcisteína em indivíduos coinfectados TB/HIV: Ensaio clínico randomizado, fase II".** Após conhecer (a) mais sobre esse estudo, você pode dizer se quer ou não participar. Se decidir participar, assine as folhas desse documento que está em duas vias. Uma é sua e a outra é do estudo. Caso não queira, não tem problema. Você receberá o seu tratamento normalmente sem ser prejudicado.

Por que você está sendo convidado (a)? A tuberculose é um problema de saúde pública no Brasil. Um grande número de pessoas, principalmente no estado do Amazonas, tem essa doença. Ela é causada por uma bactéria que tem cura, mas que também pode ter complicações se não tratada corretamente. Por isso, temos interesse em estudar o assunto. Você está iniciando o tratamento com quatro remédios. São eles: rifampicina, isoniazida, pirazinamida e etambutol. Para facilitar, chamamos esse esquema de RIPE usando as letras iniciais de cada um dos quatro remédios utilizados. Você tomará o esquema RIPE por dois meses, uma vez ao dia em jejum ou duas horas após o seu café da manhã. O número de comprimidos dependerá do seu peso, sendo no máximo quatro comprimidos por dia. Depois, na mudança do segundo para o terceiro mês, haverá uma troca e você seguirá o tratamento apenas com dois remédios, rifampicina e isoniazida que,

para facilitar, chamamos de RI que são as letras iniciais de cada uma das medicações. Esses medicamentos também serão tomados uma vez ao dia, em jejum ou duas horas após o seu café da manhã, por no mínimo mais quatro meses. O número de comprimidos dependerá do seu peso, sendo no máximo quatro comprimidos. Essas medicações (RIPE + RI) e o tempo de tratamento (no mínimo seis meses) são os mesmos que recomenda o Ministério da Saúde. Ou seja, participando ou não desse estudo, o tratamento da tuberculose será feito da mesma forma como ocorre em todo o Brasil.

Então o que muda? Nós queremos estudar se outra medicação chamada N-acetilcisteína, que já é usada e recomendada para outras doenças no pulmão, é segura e pode ajudar no tratamento da tuberculose, matando a bactéria mais rápido e protegendo o seu fígado. Para isso, o estudo terá dois grupos. Aqueles que receberão o tratamento recomendado pelo Ministério da Saúde e aqueles que receberão o tratamento recomendado pelo Ministério da Saúde mais a N-acetilcisteína, que serão dois pacotes com pó para diluir em água, e tomar à noite, por dois meses.

A sua participação nesse estudo: Caso aceite participar, você será acompanhado (a) durante todo o tratamento da tuberculose na enfermaria de Pesquisa Clínica (PESCLIN) da Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (FMT-HVD) pelo médico do estudo. O estudo terá um grupo A e um grupo B. Você poderá receber o esquema RIPE (rifampicina + isoniazida + pirazinamida + etambutol) (grupo A) ou o esquema RIPE (rifampicina + isoniazida + pirazinamida + etambutol) + N-acetilcisteína (grupo B). Se você for escolhido para o grupo B, você usará a medicação N-acetilcisteína somente nos dois primeiros meses de tratamento da tuberculose. Quem escolhe se você participa do grupo A ou grupo B é o estudo. Essa escolha não depende de você e nem do seu médico.

Para que o tratamento funcione direito, além de tomar os remédios, você precisará ir às consultas marcadas e fazer os exames que forem pedidos pelo seu médico. Nas consultas, você será examinado e suas queixas e seus exames serão registrados no prontuário do hospital, acessado somente por profissionais autorizados.

Riscos: A coleta de sangue pode causar uma pequena dor ou vermelhidão no local pelo uso da agulha. O exame do catarro, do xixi e a radiografia de tórax ("chapa do pulmão") são procedimentos que não causam maiores riscos. Se você não entender o que está sendo explicado ou ficar com vergonha de coletar o catarro, você poderá falar com o médico, que vai tirar suas dúvidas e ajudar você a encontrar um local mais confortável para a coleta. O material coletado (sangue, xixi e catarro) será utilizado exclusivamente para essa pesquisa e, caso sobre, será devidamente eliminado ao final desse estudo.

Benefícios: Os benefícios esperados com esse estudo são que a medicação N-acetilcisteína diminua o tempo em que o paciente transmite a bactéria da tuberculose para as outras pessoas e o risco do fígado rejeitar as medicações para tratamento da tuberculose.

Custos da participação: Você não precisará pagar nada por sua participação. Se você estiver internado, as refeições serão dadas pelo hospital. Você terá a garantia de que quaisquer problemas devido ao estudo serão tratados e acompanhados por um dos médicos pesquisadores durante todo o tempo que for necessário sem qualquer gasto. Da mesma forma, você terá direito à indenização garantida caso ocorra algum dano permanente devido à participação nesse estudo.

Uso das informações (confidencialidade): Todas as informações particulares serão mantidas em segredo. Em nenhum momento serão publicadas informações que possam causar constrangimento pessoal ou público.

Esclarecimentos e direitos: Sua participação é voluntária. Você poderá participar ou não desse estudo. Caso aceite, você poderá mudar de ideia e desistir de participar a qualquer momento, sem que isso cause prejuízo ao seu tratamento. Você não precisará explicar o motivo, mas deverá apenas comunicar a equipe do estudo. Se não quiser mais participar, não haverá nenhum tipo de punição e você será encaminhado para continuar o tratamento de tuberculose no ambulatório desse hospital ou em postos de saúde. Caso ocorram modificações no estudo, você será esclarecido (a) novamente e deverá assinar um documento como esse, caso esteja de acordo. Em qualquer momento você poderá pedir informações sobre o estudo, seja através do médico ou do comitê de ética desse hospital.

Marcelo Cordeiro dos Santos, Infectologista, CRM AM 3243, RQE 1227.

Fundação de Medicina Tropical Dr. Heitor Vieira Dourado.

Enfermaria Pesclin (2o andar).

Avenida Pedro Teixeira, número 25, bairro Dom Pedro.

Telefone: (92) 99119-9199 / 2127-3481

Horário: Segunda à sexta-feira, 08:00h - 12:00h.

Comitê de Ética em Pesquisa (CEP)

Av. Pedro Teixeira, número 25, bairro Dom Pedro.

Telefone: (92) 2127-3572

E-mail: cep@fmt.am.gov.br

Horário de Funcionamento: Segunda à sexta-feira, 08:00h às 14:00h.

Entendimento pós – informação: Eu,_____ (prontuário:_____), abaixo assinado, li esse documento, entendi o objetivo do estudo e concordo em participar. Fui devidamente informado (a) e esclarecido (a). Foi-me garantido que posso retirar minha participação a qualquer momento, sem que isto leve a qualquer penalidade ou interrupção de meu acompanhamento e tratamento.

Nome do sujeito da pesquisa:

Assinatura do sujeito da pesquisa:

Nome e grau de parentesco do familiar presente (se necessário):

Assinatura do familiar presente (se necessário):

Impressão dactiloscópica (para analfabeto):

Nome da testemunha (para casos de voluntários analfabetos):

Assinatura da testemunha:

Nome do profissional que aplicou o termo:

Assinatura do profissional que aplicou o termo:

Manaus,_____/_____/ 20__

Hora:_____:____

9.4 Apêndice C. Equipe

Nome	Formação	Função	Instituição
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Leandro Sousa Garcia	Enfermeiro	Pesquisador	FMT-HVD
Brenda Karoline Souza Carvalho	Biomédica	Pesquisador	FMT-HVD
Amanda Araújo de Sousa	Biomédica	Pesquisador	FMT-HVD
Mariana Millan Fachi	Farmacêutica	Pesquisador	UFPR
Marcus Vinicius de Liz	Químico	Pesquisador	UTFPR
Roberto Pontarolo	Farmacêutico	Pesquisador	UFPR
Cristina Sanches	Farmacêutica	Pesquisadora	UFSJ
Suzanne Parker	Química	Pesquisadora	UQ
Steven Wallis	Químico	Pesquisador	UQ
Jeffrey Lipmann	Médico Intensivista	Pesquisador	UQ
Jason Roberts	Farmacêutico	Pesquisador	UQ
Marcelo Cordeiro dos Santos	Médico Infectologista	Pesquisador	FMT-HVD/UEA

9.5 Apêndice D. Produção científica durante o programa de doutorado

9.5.1 Artigo 1

G Model
RIAM-446; No. of Pages 2
ARTICLE IN PRESS

Rev Iberoam Micol. 2017;xxx(xx):xxx–xxx

Revista Iberoamericana de Micología

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Letter to the Editor

Long-term cost-effectiveness of lipid formulations of amphotericin B in the empiricaltherapy of invasive mycosis in a developing country

Rentabilidad a largo plazo de las formulaciones lipídicas de anfotericina B en la terapia empírica de micosis invasivas en unpaís en desarrollo

Dear Editor,

Clinical use of lipid formulations of amphotericin B is markedly limited by acquisition costs in developing countries.[6,7] The aim of this study is to assemble a model of cost-effectiveness of ampho- tericin B lipid complex (ABLC) in patients with invasive mycoses in a public Brazilian hospital. This is a pharmacoeconomic analysis from the payers' perspective based on a retrospective observational study published previously about the incidence of acute kidney injury (AKI) in patients using deoxycholate amphotericin B (d-AMB).[8] One hundred and six adult patients were included. The incidence of AKI with ABLC was estimated through another cohort,[1] and in-hospital mortality rate was used as primary endpoint. In the model, the outcome of patients who received ABLC was considered similar to that of patients who received d-AMB, according to a previous publication[5] (Fig. 1). The probability of evolving to chronic hemodialysis (HD) after developing AKI that required acute HD was estimated according to a previous study of Duran et al.[2] Finally, in order to predict the 10 years outcome of every patient under chronic HD after discharge we used reported data from the publication of Gomez et al.[3] In Brazil, patients under chronic HD

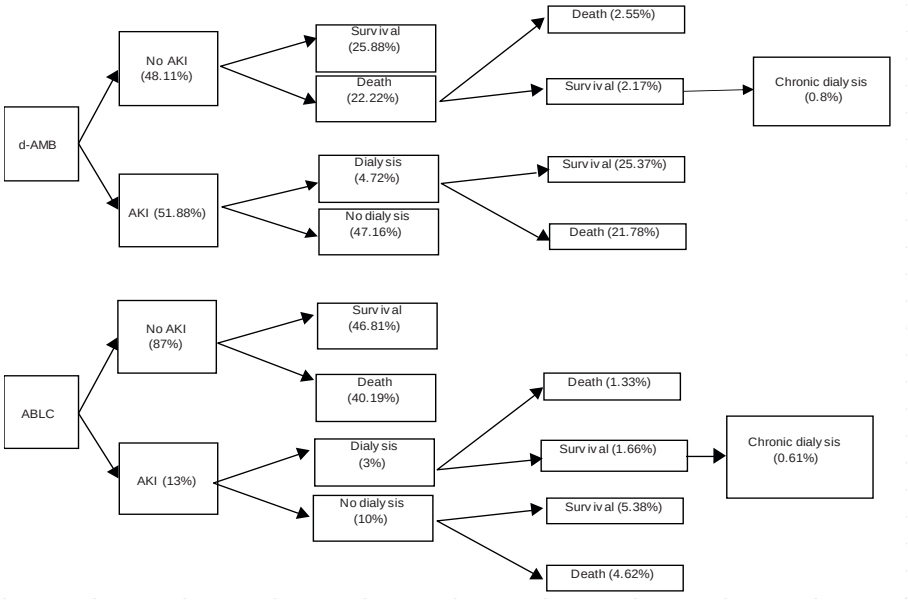

Fig. 1. Tree decision of ABLC to be cost-effective in comparison with d-AMB.

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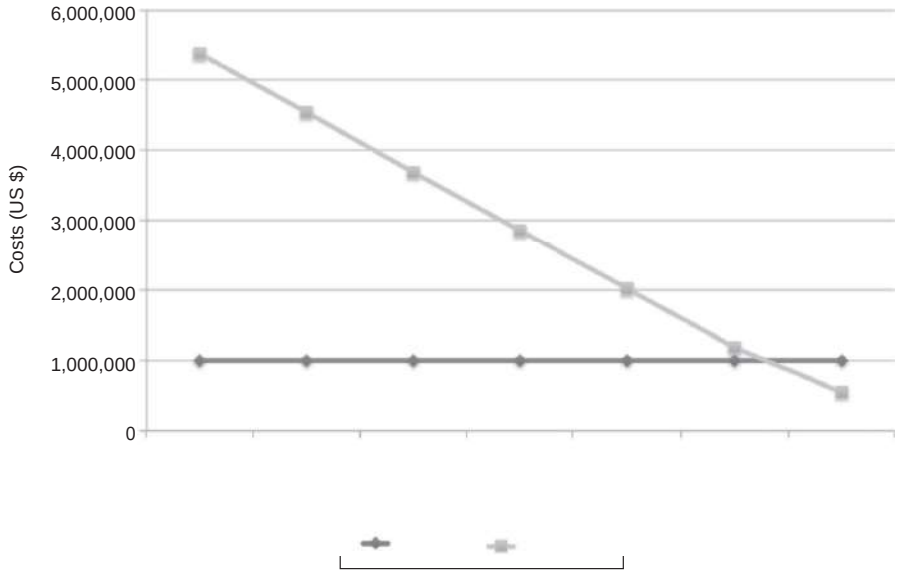

Fig. 2. Break-even value of ABLC to be cost-effective in comparison with d-AMB.

are retired, and the retirement pension fee is the 80% of the mean salary of the last five years (US$ 302.34; minimum salary of the Parana State in Brazil).

Sensitivity analysis was performed considering ±25% of used costs.[4] Only the direct cost of amphotericin B (updated to December 2016) was included, (ABLC = US$ 360.53/50 mg d-AMB = US$ 36.61/50 mg). Costs of chronic HD values were considered those paid by Brazilian public health system to public hemodialysis clinics per HD session (US$ 74.76).

From 106 patients, five were submitted to acute HD due to acute renal failure attributed to treatment with d-AMB (4.72%). Global in-hospital mortality rate was 46%. It may be inferred that 2.17% of the patients were discharged from hospital after acute HD. Considering the previous study of Duran et al.,[2] we estimated that 0.8% of these patients treated with d-AMB would be under chronic HD. Considering 106 patients, the total cost of d-AMB, chronic HD and retirement would be US$ 54,343.66, US$ 27,000.35, and US$ 98,331.33, respectively. On the other hand, if the same group would have received ABLC, total cost of ABLC, chronic HD and retirement would be US$ 1,605,228.17, US$ 10,125.13, and US$ 36,874.25, respectively. The break-even value of ABLC to be cost-effective in comparison with d-AMB is US$ 67.61, a value very different from the current value of US$ 360.56 per vial (Fig. 2).

Despite the high cost of chronic HD and retirement, direct cost of lipid formulations in Brazil is too high to be considered cost-effective. However, a subset of patients with early renal dysfunction should be re-analyzed in the future because ABLC induces less renal injury and consequently fewer patients would be on chronic HD. This aspect is important for a future re-evaluation of the cost of these patients not to be included in the model as well as costs beyond 10 years of life.

Conflicts of interest

This study was supported by TEVA, that had no influence at all on the content of the paper. Felipe Tuon receives grants from TEVA and is a CNPQ researcher.

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9.5.2 Artigo 2

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ORIGINAL ARTICLE

Predictors of mortality among intensive care unit patients coinfected with tuberculosis and HIV

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Study carried out at the Fundação de Medicina Tropical Dr. Heitor Vieira Dourado, Manaus (AM) Brasil.

ABSTRACT

Objective: To identify factors predictive of mortality in patients admitted to the ICU with tuberculosis (TB)/HIV coinfection in the Manaus, Amazon Region. **Methods:** This was a retrospective cohort study of TB/HIV coinfected patients over 18 years of age who were admitted to an ICU in the city of Manaus, Brazil, between January of 2011 and December of 2014. Sociodemographic, clinical, and laboratory variables were assessed. To identify factors predictive of mortality, we employed a Cox proportional hazards model. **Results:** During the study period, 120 patients with TB/HIV coinfection were admitted to the ICU. The mean age was 37.0 ± 11.7 years. Of the 120 patients evaluated, 94 (78.3%) died and 62 (66.0%) of those deaths having occurred within the first week after admission. Data on invasive mechanical ventilation (IMV) and ARDS were available for 86 and 67 patients, respectively Of those 86, 75 (87.2%) underwent IMV, and, of those 67, 48 (71.6%) presented with ARDS. The factors found to be independently associated with mortality were IMV (p = 0.002), hypoalbuminemia (p = 0.013), and CD4 count < 200 cells/mm³ (p = 0.002). **Conclusions:** A high early mortality rate was observed among TB/HIV coinfected ICU patients. The factors predictive of mortality in this population were IMV, hypoalbuminemia, and severe immunosuppression.

Keywords: Mycobacterium tuberculosis; Critical care; Respiration, artificial; Acquired immunodeficiency syndrome.

INTRODUCTION

Among communicable diseases, tuberculosis (TB) is the leading cause of death worldwide. In 2015, there were an estimated 10.4 million new TB cases and 1.8 million deaths worldwide, 400,000 of which occurred among HIV-infected individuals.[1] The reported incidence of TB in Brazil was 32.4 cases per 100,000 population in 2016, with 2.2 TB-related deaths per 100,000 population in 2015. Of the 66,796 new TB cases in Brazil in 2015, 6.8% were cases of TB/HIV coinfection. In 2016, the incidence of TB in Brazil was highest in the state of Amazonas, with 67.2 cases per 100,000 population and a mortality rate of 3.2 per 100,000 population. In the city of Manaus, which is the capital of the state of Amazonas and where 50% of the state population is concentrated, there were 93.2 cases per 100,000 population and 3.5 deaths per 100,000 population in 2016.[2]

Previous studies have shown that people living with HIV are 30 times more likely to develop infection with TB and progress to active disease than are individuals who do not have HIV, which increases the risk of latent TB reactivation up to 20-fold.[3] In TB/HIV coinfected individuals, the virus weakens the host immune response to *Mycobacterium tuberculosis* (*Mtb*), resulting in a more dramatic progression.[4]

Because of immunosuppression, TB is frequently paucibacillary in HIV-infected individuals, meaning that diagnosis and treatment are often delayed.[5] Admission to the ICU is required in 1-3% of cases, invasive mechanical ventilation (IMV) being required in 1.5%. [6] Patients coinfected with TB and HIV usually develop pulmonary lesions accompanied by intrapulmonary shunt and hypoxemic respiratory failure.[7]

Case-fatality rates are notoriously high in TB/HIV coinfected patients, ranging from 22.4% to 67%. [6,8-21] In patients coinfected with TB and HIV, death has been associated with the following: IMV; miliary (i.e., disseminated) TB; renal replacement therapy; use of vasoactive drugs; low Glasgow Coma Scale scores; high Simplified Acute Physiology Score II; high Acute

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Physiology And Chronic Health Evaluation II (APACHE II) scores; high Sequential Organ Failure Assessment scores; lymphopenia; concomitant nontuberculous mycobacterial infection; organ failure; sepsis; and hypoalbuminemia.[8,11-13,16,18-21]

Only a few studies have assessed case-fatality rates in patients with severe TB,[6,10,12,15,17-21] and most had a small sample (of < 100 patients) and were retrospective in design. Prospective studies have focused on investigating ICU patients with TB (n = 83, 44 of whom were coinfected with HIV)[12] or predicting survival among HIV-infected patients (n = 125, 58 of whom were coinfected with TB).[19] There is a lack of studies investigating ICU patients with severe TB/HIV coinfection. In a retrospective study involving a small sample (of 12 patients), the reported mortality was 58.3%.[15]

Since 2004, strategies to minimize the impact of TB/HIV coinfection and improve the treatment of TB/HIV coinfected patients have been adopted, including improved integration between TB and HIV programs and early antiretroviral therapy (ART) to reduce the viral load in patients with a presumptive diagnosis of TB.[22] In the present study, we sought to describe the clinical features of a large cohort of severe TB/HIV coinfected patients admitted to the ICU of a referral hospital in the city of Manaus, Brazil, as well as to identify factors predictive of mortality in that population.

METHODS

This was a retrospective cohort study of TB/HIV coinfected patients admitted to the ICU of a referral hospital for infectious diseases in the city of Manaus, Brazil, between January of 2011 and December of 2014. The study was approved by the local research ethics committee in August of 2014 (Protocol no. CAAE 34073314.3.0000.0005).

HIV-infected patients who were 18 years of age or more and who were diagnosed with TB were included in the study. For a diagnosis of active TB, at least two of the following criteria had to be met[11]: a) two AFB-positive sputum smears; b) one positive *Mtb* culture; c) chest X-ray findings suggestive of TB; and d) postmortem histopathological findings of TB granuloma, caseous necrosis, or AFB. ARDS was defined as low PaO_2/FiO_2, recent appearance of bilateral pulmonary infiltrates, and no clinical evidence of left atrial hypertension.[23]

All of the HIV-infected patients who were included in the present study had serologically confirmed HIV infection, in accordance with the criteria established by the Brazilian National Ministry of Health.[24] The microbiology laboratory in which the tests were performed is quality-controlled within the World Health Organization (WHO) scheme for external quality assurance.

Patients who habitually smoked cigarettes were classified as smokers regardless of the number of cigarettes smoked per day. Alcoholism was defined as consumption of ≥ 60 g of pure alcohol on at least one single occasion at least monthly, in accordance with the WHO criteria.[25] Drug use was defined as use of ecstasy, cocaine, heroin, cannabis, or any combination of the four in the last 12 months.

Sociodemographic and clinical data were collected from the electronic medical records of the participating patients. Laboratory data regarding Xpert MTB/RIF test results, smear microscopy results, *Mtb* culture results, and autopsy findings were collected from the laboratory database. All chest X-rays were assessed with IMPAX digital imaging software, version 1.0 build 1.0389 (Agfa HealthCare, Mortsel, Belgium) and reviewed by the same radiologist, who was unaware of the clinical outcomes.

Age, gender, smoking status, alcohol use, illicit drug use, fever, cough, weight loss, diarrhea, dyspnea, opportunistic infections, and comorbidities were analyzed. Time to anti-TB treatment initiation, therapeutic regimen, ART, time to ICU discharge, and ICU clinical outcome (discharge to the ward or death) were also analyzed.

Glasgow Coma Scale and APACHE II scores were used in order to assess the level of consciousness and prognosis in the ICU. Laboratory parameters included hemoglobin levels, leukocyte count, lymphocyte count, platelet count, albumin levels, and CD4 count.

Patients were treated in accordance with the WHO guidelines recommending at least 6 months of rifampin/isoniazid/pyrazinamide/ethambutol for all clinical forms of TB if the patient has never undergone treatment or has undergone up to 30 days of treatment. In people living with HIV/AIDS with active TB, ART should be started 2-8 weeks after initiation of anti-TB treatment.[26,27]

Data on the study variables were imported into a spreadsheet and analyzed with the Stata statistical software package, version 9.0 (StataCorp LP, College Station, TX, USA) and the IBM SPSS Statistics software package, version 21.0 (IBM Corporation, Armonk, NY, USA). Data were expressed as mean ± standard deviation or median (interquartile range). Normality was assessed by the Kolmogorov-Smirnov test. Patient survival was analyzed by the Kaplan-Meier method and the log-rank test. Variables with values of $p \leq 0.20$ in the univariate analysis were included in a Cox proportional hazards model adjusted for age and gender for survival analysis. The confidence interval was 95%, and values of $p < 0.05$ were considered significant.

RESULTS

Between January of 2011 and December of 2014, 858 patients were admitted to the ICU. Of those, 141 (16.4%) were diagnosed with TB, 131 (92.9%) being coinfected with HIV. A total of 120 patients were included in the study and underwent further analysis.

The mean age of the patients was 37.0 ± 11.7 years, and 70.0% were male. Alcohol consumption, smoking, and illicit drug use were identified in 48.9%, 36.7%, and 25.4%, respectively. As can be seen in Table 1,

the most commonly reported signs and symptoms were weight loss (94.1%), dyspnea (86.4%), and cough (82.9%).

Pulmonary TB was found in 47.0%, and disseminated TB was found in 39.0%. The primary reason for ICU admission was acute respiratory failure (in 80.0%). Data on IMV and ARDS were available for 86 and 67 patients respectively. Of those 86, 75 (87.2%) underwent IMV, and, of those 67, 48 (71.6%) presented with ARDS. The median APACHE II score was 18 (interquartile range, 5-35). Comorbidities were found in 83 (69.2%) of the 120 patients evaluated: neurotoxoplasmosis, in 21.7%; pneumocystis pneumonia, in 15.8%; acute kidney injury, in 13.3%; pneumonia, in 10.8%; and histoplasmosis, in 7.5%.

A total of 80 patients underwent bacteriological screening for TB. Of those, 16 (13.3%) had positive smear results/positive culture results and 8 (6.6%) had negative smear results/positive culture results. Of the 99 patients who underwent chest X-rays or CT scans, 26 had findings suggestive of TB. Autopsy findings were consistent with TB in 5 of the 10 patients in whom an autopsy was performed.

The median length of ICU stay was 5 days (interquartile range, 3-10.5 days). Information on TB treatment initiation was available for 107 patients. Of those, 90 (84.1%) had been receiving anti-TB treatment before ICU admission (for at least 1 month in 33.6%). Of the 120 patients evaluated, 94 (78.3%) died. Of those 94 deaths, 62 (66.0%) occurred within the first week after admission.

In the univariate analysis, mortality was found to be associated with illicit drug use, diarrhea, low CD4 count, hypoalbuminemia, and IMV (Table 1). As can be seen in Figure 1, the Kaplan-Meier method and the log-rank test showed that mortality was associated with low CD4 count ($p = 0.008$), hypoalbuminemia ($p = 0.001$), and IMV ($p < 0.001$).

All of the variables showing $p \leq 0.20$ in the univariate analysis were included in a Cox proportional hazards model adjusted for age and gender. The factors found to be independently associated with mortality were IMV (hazard ratio [HR] = 0.10; 95% CI: 0.02-0.45; $p = 0.002$), hypoalbuminemia (HR = 0.47; 95% CI: 0.26-0.85; $p = 0.013$), and low CD4 count (< 200 cells/mm^3; HR = 0.26; 95% CI: 0.08-0.87; $p = 0.02$; Table 1).

DISCUSSION

The objective of the present study was to describe the clinical features of a large cohort of severe TB/HIV coinfected individuals admitted to the ICU of a referral hospital in the Brazilian Amazon, as well as to identify factors predictive of mortality in that population. We found a mortality rate of 78.3% in the study population, most of the deaths having occurred within the first week after admission. The factors found to be independently associated with mortality were IMV, hypoalbuminemia, and low CD4 count.

The case-fatality rate observed in our cohort was higher than those reported by Balkema et al. (57%)[12] and Silva et al. (65%)[6] in South Africa and Brazil, respectively, as well as being higher than those reported by Zahar et al. (26.7%),[18] Lanoix et al. (28%),[17] and Valade et al. (42%)[10] in France. However, none of these cohorts were designed for studying TB/HIV coinfected patients in the ICU; such patients were primarily evaluated in a subanalysis of larger studies. In addition, as previously mentioned, TB is usually paucibacillary in HIV-infected individuals, and diagnosis remains a challenge. Of the 120 patients in our sample, only 24 (20.0%) had a microbiological diagnosis of TB. Therefore, the case-fatality rate found in the present study can be attributed, at least in part, to histoplasmosis and other fungal diseases (which are generally underdiagnosed), as well as to noninfectious diseases that mimic TB. It is also of note that 89.0% of those patients had been receiving treatment. It is possible that some patients were diagnosed late, meaning that treatment was also delayed. Given the severity of the clinical conditions, it is possible that the doses of the anti-TB drugs used were lower than required, that adherence was suboptimal, or both. Therefore, the directly observed treatment strategy should be revised.

In Brazil, 25% of patients have a low CD4 count at diagnosis of HIV infection.[28] In the state of Amazonas, as many as 30% have a mean count of 282 cells/mm^3 at diagnosis.[28] Most (79.0%) of the deaths among the patients included in the present study occurred in those with a CD4 count of < 200 cells/mm^3 at ICU admission, a finding that is consistent with those of other studies.[12,29] This is probably due to delayed HIV diagnosis and advanced AIDS. In highly immunosuppressed individuals requiring critical care, it is best to "hit hard and hit early" with active bactericidal agents in order to stop TB progression and save time in the ICU. Another issue that merits further investigation is whether there is a need to wait 2 weeks before initiating ART or whether ART should be initiated earlier. In HIV-infected patients, a low CD4 count is known to be associated with early ICU admission and increased case-fatality rates.[30,31]

Belperio & Rhew reported the prevalence and outcomes of anemia in HIV-infected individuals,[32] in whom anemia is commonly caused by disseminated TB.[33] Although low hemoglobin levels are common among HIV-infected patients and have previously been described as constituting an important predictor of mortality in such patients,[32] we found no association between anemia and mortality in our cohort. However, anemia is a common sign of TB and HIV infection, being present not only in critically ill patients in the ICU but also in recently diagnosed patients in an outpatient setting. Therefore, it might have no impact on ICU prognosis.[33]

In the present study, acute respiratory failure was the main reason for ICU admission (in 80.0% of the patients) and a variable that was associated with high mortality rates among our patients. These results

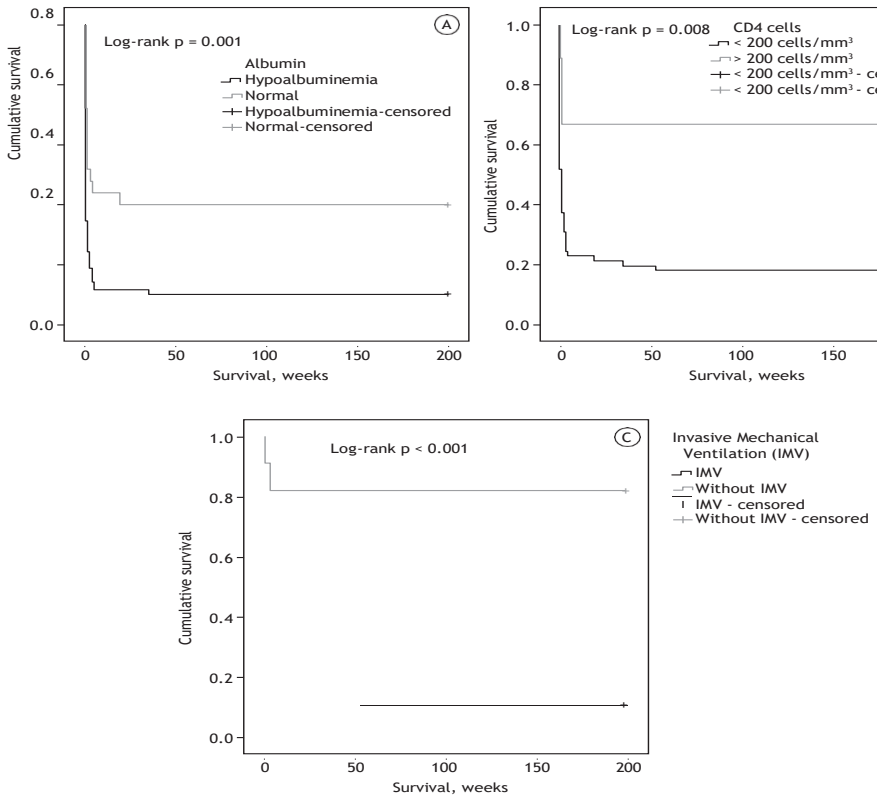

Figure 1. Kaplan-Meier curves for survival among TB/HIV coinfected patients in the ICU. In A, albumin levels; in B, CD4 cell count; and in C, invasive mechanical ventilation.

are similar to those of studies analyzing patients undergoing IMV.[6,12,13]

Potential factors responsible for low rates of bacteriological confirmation include the lack of quality assurance schemes[34] and the empirical approach to TB treatment in the ICU. Suboptimal diagnostic quality can hinder differential diagnosis as well. In the present study, the rate of bacteriological confirmation among patients facing high case-fatality rates was found to be low (i.e., 27.5%). Despite evidence of increased mortality among patients without microbiological confirmation because of HIV-related immunosuppression,[35] we found no significant differences in mortality rates between TB cases with and without microbiological confirmation.

Few studies have examined treatment adequacy and patient adherence.[5] It is of note that although 75.0% of our patients were started on TB treatment before ICU admission, the mean time from admission to treatment in most studies is 1.6-5 days.[9,10,12] The high prevalence of TB/HIV coinfection in Brazil pushes health professionals to the edge. There are currently few ART regimens that can be prescribed in combination with anti-TB drugs; new regimens based on different drugs might make it easier to combine the two in the future.

ICU patients with severe TB pose a major challenge in TB diagnosis (microbiological confirmation of TB) and treatment (poor absorption of anti-TB drugs; organ dysfunction; and apparent deterioration of TB during appropriate treatment, i.e., paradoxical reactions).[7] The potential role of malabsorption of anti-TB drugs in severe cases and the potential utility of therapeutic drug monitoring have been poorly studied and deserve more attention.[36,37] To our knowledge, this is the first

Table 1. Demographic, clinical, and laboratory characteristics of TB/HIV coinfected ICU patients who either survived or died in the 2011-2014 period.[a]

Characteristic	Total sample (N =120)	Patients who survived (n = 26)	Patients who died (n = 94)				
Age, years		34.3 ± 12.0	37.7 ± 11.54	-	0.393	-	-
Male gender	84 (70.0)	18 (21.4)	66 (78.6)	1.04 (0.40-2.68)	0.884	-	-
Alcoholism	44/90 (48.9)	13 (29.6)	31 (70.5)	0.42 (0.15-1.20)	0.167	1.32 (0.81-2.16)	0.264
Smoking	33/90 (36.7)	10 (30.6)	23 (69.7)	0.48 (0.17-1.34)	0.254	-	-
Drug use	17/67 (25.4)	8 (47.1)	9 (52.9)	0.18 (0.05-0.63)	0.012	0.50 (0.23-1.06)	0.074
Comorbidities	83 (69.2)	19 (22.9)	64 (77.1)	0.78 (0.29-2.07)	0.804	-	-
Cough	73/88 (82.9)	16 (21.9)	57 (78.1)	0.25 (0.03-2.08)	0.284	-	-
Fever	31/109 (28.4)	6 (19.4)	25 (80.1)	1.07 (0.37-3.06)	0.897	-	-
Weight loss	80/85 (94.1)	13 (16.3)	67 (83.8)	1.28 (0.13-12.4)	1.000	-	-
Diarrhea	37/84 (44.0)	2 (5.4)	35 (94.6)	5.34 (1.10-25.8)	0.034	1.34 (0.83-2.17)	0.220
Dyspnea	89/103 (86.4)	15 (16.9)	74 (83.3)	2.74 (0.80-9.33)	0.140	1.57 (0.78-3.16)	0.199
Clinical form of TB	120			-	0.938	-	-
Pulmonary	57 (47.5)	13 (22.8)	44 (77.2)	0.88 (0.36-2.09)	0.946	-	-
Disseminated	47 (39.2)	10 (21.3)	37 (78.7)	1.03 (0.42-2.53)	0.885	-	-
Extrapulmonary	16 (13.3)	3 (18.8)	13 (81.3)	1.23 (0.32-4.69)	1.000	-	-
TB treatment initiation[b]	107			-	0.232	-	-
< 30 days before ICU admission	54 (50.5)	11 (20.3)	43 (79.6)	-	-	-	-
≥ 30 days before ICU admission	36 (33.6)	10 (27.8)	26 (72.2)	-	-	-	-
After ICU admission	17 (15.9)	4 (23.5)	13 (76.5)	-	-	-	-
CD4 count	71			7.53 (1.65-34.28)	< 0.009	0.29 (0.09-0.94)	0.04
< 200 cells/mm^3	62 (87.3)	13 (21)	49 (79)	-	-	-	-
≥ 200 cells/mm^3	9 (12.7)	6 (66.7)	3 (33.3)	-	-	-	-
Hemoglobin level	83	18 (21.6)	65 (78)	0.70 (0.22-2.21)	0.747	-	-
Males (8-13 g/dL)	55 (66.3)	13 (23.6)	42 (76.4)	-	-	-	-
Females (7-12 g/dL)	28 (33.7)	5 (17.9)	23 (82.1)	-	-	-	-
Lymphocyte count	114			-	0.081	0.73 (0.46-1.14)	0.175
Lymphocytosis	4 (3.5)	0 (0.0)	4 (100.0)	-	-	-	-
Lymphocytopenia	84 (73.7)	14 (16.7)	70 (83.3)	-	-	-	-
Normal	26 (22.8)	9 (34.6)	17 (65.4)	-	-	-	-
Albumin level	94			5.99 (2.03-17.64)	0.001	0.48 (0.26-0.88)	0.018
Hypoalbuminemia	69 (73.4)	8 (11.6)	61 (88.4)	-	-	-	-
Normal	25 (26.6)	11 (44.0)	14 (56.0)	-	-	-	-
APACHE II score	96			-	0.362	-	-
1-15	37 (38.4)	6 (16.2)	31 (83.8)	-	-	-	-
16-30	51 (53.1)	14 (27.5)	37 (72.5)	-	-	-	-
31-45	8 (8.3)	1 (12.5)	7 (87.5)	-	-	-	-
IMV	75/86 (87.2)	10 (13.3)	65 (86.7)	29.25 (5.50-155.4)	0.000	0.12 (0.03-0.51)	0.004
ARDS	67			0.80 (0.22-2.88)	1.00	-	-
Yes	48 (71.6)	12 (25.0)	36 (75.0)	-	-		
No	19 (28.4)	4 (21.1)	15 (79.0)	-	-		

TB: tuberculosis; HR: hazard ratio; APACHE II: Acute Physiology and Chronic Health Evaluation II; and IMV: invasive mechanical ventilation. [a]Values expressed as n, n (%), or mean ± SD, except where otherwise indicated. [b]Information on TB treatment initiation was unavailable for 13 patients.

study to evaluate TB/HIV coinfected ICU patients in theAmazon region, being the largest of its kind. Information on how to improve TB/HIV coinfection managementin the ICU is still anecdotal, and important issues (such as uncertainty regarding severity classification, mortality scores, vulnerable populations, and effective treatment) have yet to be resolved.

Our study has several limitations. First, all data wereobtained retrospectively by reviewing patient medical records and were probably not as complete or accurate as are data that are collected prospectively. Second,although our cohort is the largest available sampleof TB/HIV coinfected patients in the ICU, its power was too low to allow subanalyses to be undertaken.Despite these limitations, our results provide importantimplications for similar demographic areas and clinicalsettings. In addition, our study poses questions onhow to approach TB/HIV coinfected patients andhow to predict their prognosis while providing timelyinterventions.

The high mortality rate found in the present study underlines how difficult it is to manage TB in the ICU.Pre-ICU interventions (including early diagnosis and effective treatment) can have a major impact on TB/HIV mortality in the ICU, as well as improving thequality of TB control.

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9.5.3 Artigo 3

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Short Communication

Examination of respiratory specimens improves microbiological diagnosis of patients with presumptive extrapulmonary tuberculosis

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ABSTRACT

Objectives: Bacteriological confirmation of extrapulmonary tuberculosis (EPTB) is challenging for several reasons: the paucibacillary nature of the sample; scarce resources, mainly in middle and low-income countries; the need for hospitalization; and unfavorable outcomes. We evaluated the diagnostic role of respiratory specimen examination prospectively in a cohort of patients with presumptive EPTB.

Methods: From July 2018 to January 2019, in a tuberculosis (TB)/HIV reference hospital, a cohort of 157patients with presumed EPTB was evaluated. Xpert[1] MTB/RIF Ultra or a culture-positive result was considered for bacteriologically confirmed TB.

Results: Out of 157 patients with presumptive EPTB, 97 (62%) provided extrapulmonary and respiratoryspecimens and 60 (38%) extrapulmonary specimens only. Of the 60 patients with extrapulmonary samples, 5 (8%) were positive. Of those with respiratory and extrapulmonary samples, 27 (28%) were positive: 10 in both the respiratory and extrapulmonary samples, 6 in the extrapulmonary sample only,and 11 in the respiratory sample only. A respiratory specimen examination increased by 6-fold the chanceof bacteriological confirmation of TB (odds ratio = 5.97 [1.11–47.17]).

Conclusion: We conclude that respiratory samples should be examined in patients with presumptive EPTB.

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Introduction

Of the 7 million new tuberculosis (TB) cases reported worldwide in 2018, 15% were extrapulmonary tuberculosis (EPTB) (World Health Organization, 2019). EPTB is usually paucibacillary, and presentation may be atypical; therefore, it is more challenging to diagnose. Invasive procedures may be required, resulting in a delayed diagnosis, need for hospitalization, unfavorable outcomes and higher costs (Norbis et al., 2014).

Extrapulmonary presentations such as lymph node and pleural TB occur with concomitant pulmonary TB (Shaw et al., 2019; Züker et al., 2019). However, most current TB guidelines do not include respiratory specimen examination in the evaluation of

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presumptive EPTB. The new Xpert® MTB/RIF Ultra increased the yield of paucibacillary sample examination compared to smear microscopy or the previous generation of the molecular test (Zhang et al., 2020). We evaluated whether the examination of respiratory samples for bacteriological confirmation of TB could improve patients' diagnosis with presumptive EPTB.

Methods

The study is a *post hoc* analysis of a prospective observational cohort study conducted at *Fundação de Medicina Tropical* Doutor Heitor Vieira Dourado (FMT-HVD), an academic reference center for TB/HIV co-infection, located in the Brazilian Amazonian region. Between July 2018 and January 2019, 157 out- and in-patients aged ≥18 years with presumptive EPTB were tested for TB in FMT-HVD's mycobacteriology laboratory and included in the study cohort. The laboratory had received 100% proficiency on the biannual INSTAND test (INSTAND, 2021), which assesses the quality of sample processing, for the previous 4 years. The clinical team made the decision on which specimen should be obtained without the researchers' intervention.

We considered the World Health Organization's definition of a bacteriologically confirmed case: a positive result of smear, culture or WHO-approved rapid diagnostics such as the Xpert RIF/MTB Ultra in any sample (World Health Organization, 2020). Clinically diagnosed TB is defined in the presence of symptoms and radiological images compatible with TB without a positive result of any of the tests above (missing or negative). If there was evidence of concomitant involvement of extrapulmonary sites and lung parenchyma or the tracheobronchialtree, thepatientwasclassifiedashavingpulmonary TB (PTB). At FMT-HVD, all biological samples from patients with presumptive TB are submitted to liquid culture and molecular test (Xpert® MTB/RIF Ultra, Cepheid, Sunnyvale, CA, USA).

Clinical and demographic data (age, sex, HIV status, CD4 count and viral load) were gathered from patients' electronic medical records. Data on respiratory symptoms (dyspnea, chest pain or cough) and X-ray abnormalities were collected from patients with bacteriologically confirmed TB in any anatomic site. The sensitivity and specificity of Xpert Ultra was calculated using culture as the reference standard.

Written informed consent was obtained from participants. Clinical investigations were conducted following the Declaration of Helsinki. All the information handled by the research team was de-identified.

Descriptive data are expressed as proportions with their 95% confidence interval (95% CI) levels or median values with their interquartile range (IQR). We used a multivariate regression model to evaluate the association of having a respiratory sample examined with the diagnosis confirmation using the odds ratios (OR) and 95% CI levels, adjusted for age (in years), sex and CD4 count.

Results

Among 157 patients with presumptive EPTB, the median (IQR) age was 37 (30–43) years, 60% were male, and 75% were people living with HIV. Of these, 62% had a CD4+ T-cell count below 200 cells/mm³, and 64% had a detectable viral load. Extrapulmonary specimens examined were cerebrospinal fluid (n = 56), urine (n = 37), skin (n = 29), lymph node (n = 21), pleural fluid (n = 12) and ascitic fluid (n = 2). In total, there were 32 positives results from respiratory or extrapulmonary samples. Sixty patients (38%) provided extrapulmonary specimens only; 5 (8%) were positive. Extrapulmonary and respiratory specimens were provided by 97 (62%) patients; 27 (28%) were positive, 10 in both the respiratory and extrapulmonary specimens, 6 in the extrapulmonary specimen only, and 11 in the respiratory specimen only. The results for patients with respiratory specimen only corresponded to 34% of all positive results and represented an 11% overall added value. Of 21 patients with a positive respiratory sample, 4 (19%) patients had no respiratory symptoms. Adjusted for age, sex and CD4 cell count <200 (Figure 1), having a respiratory specimen examined was associated with bacteriological confirmation of EPTB (OR = 5.97 [1.11–47.17]). A summary of all rapid molecular test results is displayed in Supplementary Table S1.

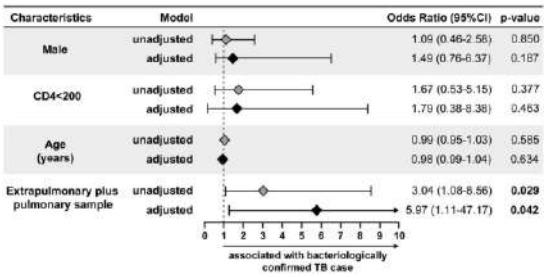

Figure 1. Factors analyzed for the association with bacteriological confirmation of extrapulmonary tuberculosis.

The overall sensitivity and specificity (95% CI) of Xpert MTB/RIF Ultra compared to *M. tuberculosis* culture were, respectively, 100% (95% CI 73.5–100) and 94.3% (95% CI 88.6–97.7). In one sputum sample, the DNA of a rifampicin-resistant strain was identified using Xpert Ultra and confirmed in the drug susceptibility test against first-line drugs. Information on antiretroviral treatment and respiratory symptoms was only available for 32 patients and was not included in the multivariate analysis.

Discussion

In this cohort of patients with presumptive EPTB, the chance to bacteriologically confirm the diagnosis of TB increased by 6-fold when a respiratory sample was obtained. In poor resource settings, adding a respiratory sample examination to the EPTB workup may increase the chances of rapidly confirming TB and initiating therapy and be more cost-effective than hospitalization for invasive procedures (this analysis was beyond the scope of the current study). Although most patients with a positive respiratory sample did have respiratory symptoms, 1/5 of them did not, suggesting that this test could be useful regardless of symptoms.

Our findings have public health implications as they corroborate previous concerns about EPTB patients' contagiousness and imply the need for contact surveillance and infection control actions, usually neglected in EPTB (Hernández-Garduño et al., 2004; Schirmer et al., 2010).

Finally, in settings where high-quality free-of-charge chest X-rays are unavailable, as in many low- and medium-income countries, Xpert Ultra is now provided by the public health system and more accessible for disadvantage populations. In our study, the sensitivity and specificity of Ultra were high, confirming previous findings (Horne et al., 2019). Relying exclusively on chest X-rays may have adverse consequences regarding transmission, as up to 10% of people living with HIV may have normal radiographs (Pepper et al., 2008; Palmieri et al., 2002).

Our study has limitations. Firstly, despite being a prospective study, the analysis was performed *post hoc*. The clinical team managed patients and there were no clear guidelines for respiratory sample collection in patients with presumptive EPTB in Brazil (Ministério da Saúde, 2018). Patients with respiratory

symptoms had more respiratory sample examination than those without respiratory symptoms, leading to a bias in selection; there was also an absence of a TB history that could have affected the analysis. Furthermore, antiretroviral treatment could not be analyzed as the information was missing for most patients. Thus, robust conclusions on the yield of respiratory specimen examination in all EPTB patients are not possible. Despite these limitations, we believe that systematically screening patients with presumptive EPTB using respiratory specimens should be considered in clinical settings while awaiting results from further studies.

Ethical approval statement

The study was approved by the Fundação de Medicina Tropical Doutor Heitor Vieira Dourado Ethics Committee (#2.525.182).

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Conflict of interest statement

None of the authors has any conflict of interest (financial or personal) in this study.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2021.03.022.

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9.5.4 Artigo 4

PLOS ONE

RESEARCH ARTICLE

Safety and efficacy of N-acetylcysteine in hospitalized patients with HIV-associated tuberculosis: An open-label, randomized, phase II trial (RIPENACTB Study)

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Abstract

Despite the availability of effective antimicrobials, tuberculosis (TB) is still a serious health threat. Mortality is even higher in people living with HIV who are diagnosed with TB. New therapies are needed to shorten the time required to cure TB and decrease fatality rates in this population. N-acetylcysteine (NAC) is a glutathione precursor and has shown recently in experimental setting to present *in vitro* and *in vivo* anti-mycobacterial activity. We test the hypothesis that NAC is safe, well tolerated and secondarily efficacious as adjunctive anti-TB therapy in hospitalized individuals with HIV-associated TB. Patients were enrolled sequentially in a tertiary care center, in the Brazilian Amazon. We performed a randomized, parallel group, single-center, open study trial of two arms, in hospitalized patients over 18 years of age, with microbiologically confirmed pulmonary TB in HIV: one with rifampicin, isoniazid, pyrazinamide and ethambutol at standard doses (Control Group), and a second in which NAC 600 mg bid for eight weeks was added (NAC Group). A total of 21 and 18 patients were enrolled to the Control Group and NAC Group, respectively. Adverse event rates were similar in the two arms. Our findings suggest that in the more critical population of hospitalized patients with HIV-associated TB, the use of NAC was not unsafe, despite the low

Data Availability Statement: All relevant data arewithin the manuscript and its Supporting Information files.

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Competing interests: The authors have declaredthat no competing interests exist.

sample size, and a potential impact on faster negative cultures needs to be further exploredin larger studies.

Introduction

Worldwide, tuberculosis (TB) is one of the top 10 causes of death. Despite significant efforts to control the disease, World Health Organization (WHO) estimates that 250,000 people living with HIV died in 2018 due to TB [1]. Treatment scheme of TB in people with HIV is the same as in HIV-negative patients. The recommended regimen for drug-susceptible disease is a com-bination of Rifampicin, Isoniazid, Pyrazinamide, Ethambutol (RIPE) for 2 months, followed by at least 4 months of rifampicin and isoniazid [2]. Although it is a curable and treatable dis- ease, TB is the leading cause of death (40%), admission to hospital (18%), and in-hospital death (25%) in people living with HIV (PLWH) [3,4].

Glutathione (GSH) is the main nonprotein thiol responsible for cellular homeostasis and maintenance of the cellular redox balance [5]. HIV infection is associated with increased oxi- dative stress (OS). Intracellular GSH levels in macrophages of HIV individuals are compro- mised, contributing to the loss of innate immune function observed in these patients and anincrease in the growth of *Mycobacterium tuberculosis* (Mtb) [6]. Agents that assist in the resto-ration of GSH levels in macrophages isolated from individuals with HIV infection promote better control of Mtb [7]. N-acetylcysteine (NAC), a GSH precursor, is an agent that restoresGSH levels. T lymphocytes derived from HIV infected individuals are deficient in GSH, and this deficiency correlates with decreased levels of Th1 cytokines and enhanced growth of Mtb inside human macrophages [8]. NAC was shown to tailor macrophages to induce enhanced Th1 response that may be helpful to control TB [9].

NAC is included in the list of essential medicines of WHO [10]. It is widely used in patients with a wide range of respiratory diseases due to its mucolytic and anti-oxidant activities, mak- ing it attractive as a potential chronic obstructive pulmonary disease therapy [11]. NAC poten-tially protects against anti-TB drug-induced hepatotoxicity in individuals with TB without HIV [12]. NAC treatment in Mtb-infected human macrophages resulted in a decrease of oxi- dative stress and enhanced anti-mycobacterial activity [13]. In a model of Mtb infection of mice, NAC treatment resulted in diminished mycobacterial loads in lungs [13], highlighting the therapeutic potential of this drug.

NAC as an adjuvant appears to be an effective agent in terms of early bacteriological and radiological improvement in treatment of pulmonary TB [14]. However, to our knowledge, no evidence exists for patients with HIV-associated TB, and especially in those more complicated cases requiring hospitalization. Such clinical trial play innovative and strategic role in WHO New Global Elimination Tuberculosis Strategy (Pillar III research strategy) [15].

Methods

Ethics

The study was approved by the Ethics Review Committee of *Fundação de Medicina TropicalDr Heitor Vieira Dourado* (CAAE 60219916.5.0000.0005). Written informed consent was obtained from all participants (or relatives in case of unconscious patients), after detailed information about the study protocol was given.

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Study design

RIPENACTB Study was an open-label, single center, randomized, phase II trial to test whetherNAC-containing treatment regimen was as safe as the standard regimen for TB treatment in hospitalized patients with HIV, besides exploring efficacy upon respiratory sample culture conversion. The study was conducted at *Fundação de Medicina Tropical Dr Heitor Vieira Dourado* (FMT-HVD), a tertiary care reference institution for coinfection TB/HIV in Manaus,Western Brazilian Amazon, from December 2016 to April 2018. This is a reference public institution for infectious diseases in the Amazonas State, with ~150 beds available for hospitali-zation and 7 intensive care unit (ICU) beds, where all cases of TB/HIV coinfection are referredto.

Study participants

Either gender 18 years or older patients with pulmonary TB diagnosed through positive Xpert-MTB/RIF and hospitalized (at clinician's discretion) for more than 24 hours, were eligible to be included in the study. Patients without HIV, with extrapulmonary TB only, unable to collect respiratory sample, pregnant and lactating women, exposed to quinolones in the last 7 days, and in use of anti-TB drugs for more than 72 hours or in use of anti-TB drugs as second line drugs were not included in the study. Enrolled patients were subsequently excluded if their baseline culture failed to grow Mtb or grew a strain of Mtb that was resistant to any anti-TB drug.

For the sample size calculation, a percentage of 37.5% of hepatotoxicity among the RIPE group and no episodes for RIPENAC was considered [12]. A 1:1 ratio with a power of 80%and a significance level of 95% was used. A total sample size of 36 was estimated.

Randomization and study treatments

Patients were randomized into **Control Group** or **NAC Group** in a 1:1 ratio using a com- puter-generated randomization table. The groups received standard anti-TB treatment with RIPE (150 mg, 75 mg, 400 mg, 275 mg), fixed dose tablets combined according to weight, for eight weeks. RIPE was supplied by Farmanguinhos [1] , Rio de Janeiro, Brazil. In addition, NAC group received two effervescent tablets containing N-acetylcysteine (Fluimucil[1]) 600 mg bid, for eight weeks, following the same dose used in a preliminary study on the effect of NAC onTB [14]. Tablets were dissolved in water before oral ingestion or administration though the nasoenteral tube.

Patients and involved infectious disease physicians were aware of the treatments, except laboratory team, to whom the study was blinded. While hospitalized, all the medication was administered in a supervised way by the nursing team. After discharge, patients were asked to take anti-TB drugs accordingly, and NAC only until eight weeks was completed. During everyvisit to the clinics, patients were requested to bring medication packages for tablet counting, asa proxy of adherence. Adherence was considered low when patients did not take the medica- tion for more than seven consecutive days. The study was registered at Clinicaltrials.gov. (https://clinicaltrials.gov/ct2/show/NCT03281226)

Study procedures

All participants underwent a baseline clinical evaluation, which included physical examina-tion, sputum (spontaneous or induced whenever sputum production was considered insuffi-cient) or tracheal aspirate in unconscious patients, $CD4^+$ lymphocyte count, viral load, aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubins, screening of

concomitant drug exposures and chest radiograph. Safety assessments were performed at base-line and weeks 1, 2, 4, 6 and 8. Additional exams were solicited whenever needed.

Respiratory samples were submitted to smear Ziehl–Nielsen staining technique, Xpert-MTB/RIF for Mtb, and sown in liquid culture BACTEC MGIT™ 960 and solid culture Löwen- stein-Jensen. Xpert-MTB/RIF for Mtb, even being more expensive, was used as inclusion crite-rion because of its higher sensitivity [16].

Study outcomes

The primary endpoint was clinical and laboratorial safety, and tolerability. Radiology alter-ations recovery, respiratory specimen culture conversion rate on liquid and solid media at theend of eight weeks of treatment were secondary endpoints.

Definitions

Culture conversion and rate of culture conversion. We defined culture conversion as the first negative respiratory sample (sputum or tracheal aspirate) cultures on liquid or solid media, without an intervening positive culture. Negative cultures followed by contaminated cultures were also regarded as culture conversion. Culture conversion was also defined as a case where the participant could not expectorate after one negative sputum culture. The rate ofculture conversion was defined as the time elapsed from day 1 to the first negative culture [17].

Radiology assessment. Chest radiography was performed at baseline and week 8. Com-parative assessment was performed by a single specialist in radiology, blinded to the group ofenrollment, which evaluated both exams and classified them as: (1) improvement or no change, or (2) worsening.

Hepatotoxicity. Hepatotoxicity was defined as ALT and/or AST increased more than 3times the upper limit of normal range with the presence of hepatitis symptoms, or increasedup to 5 times the upper limit of normal range in the absence of symptoms or total levels of bilirubinemia greater than twice the upper normal limit, as described elsewhere [12]. Refer- ence values adopted were 38 UI/mL (AST), 44 UI/mL (ALT) and 1.3 mg/dL (bilirubins). Allpatients were tested for HBsAg and anti-HCV.

Adverse events. Adverse events were graded according to the modified toxicity events cri-teria of the *National Institute of Allergy and Infectious Diseases*, Division of AIDS (DAIDS) Table for Grading the Severity of Adult and Pediatric Adverse Event (Corrected Version 2.1, July 2017) [18].

Statistical analysis

All analyses were performed according to the intention-to-treat principle. Differences in cate-gorical variables were tested using Fisher's exact test. Univariate log-binomial generalized lin- ear regression with respective 95% confidence intervals (CI) was used to estimate relative risks (RR) in order to assess associations with the major outcomes of the study. P-values $< .05$ were considered statistically significant. The statistical analyses were performed using Stata 13.0.

Results

Between December 2016 and April 2018, 162 participants were assessed for eligibility, and 50 underwent randomization (Fig 1). Out of those, 21 were included in the Control Group and18 in the NAC Group. Demographic and clinical characteristics of participants were similar between the study arms, except that more males were included in the control arm (Table 1). Overall, most of the included patients had CD4+ lymphocyte counts under 200 cells/mm^3.

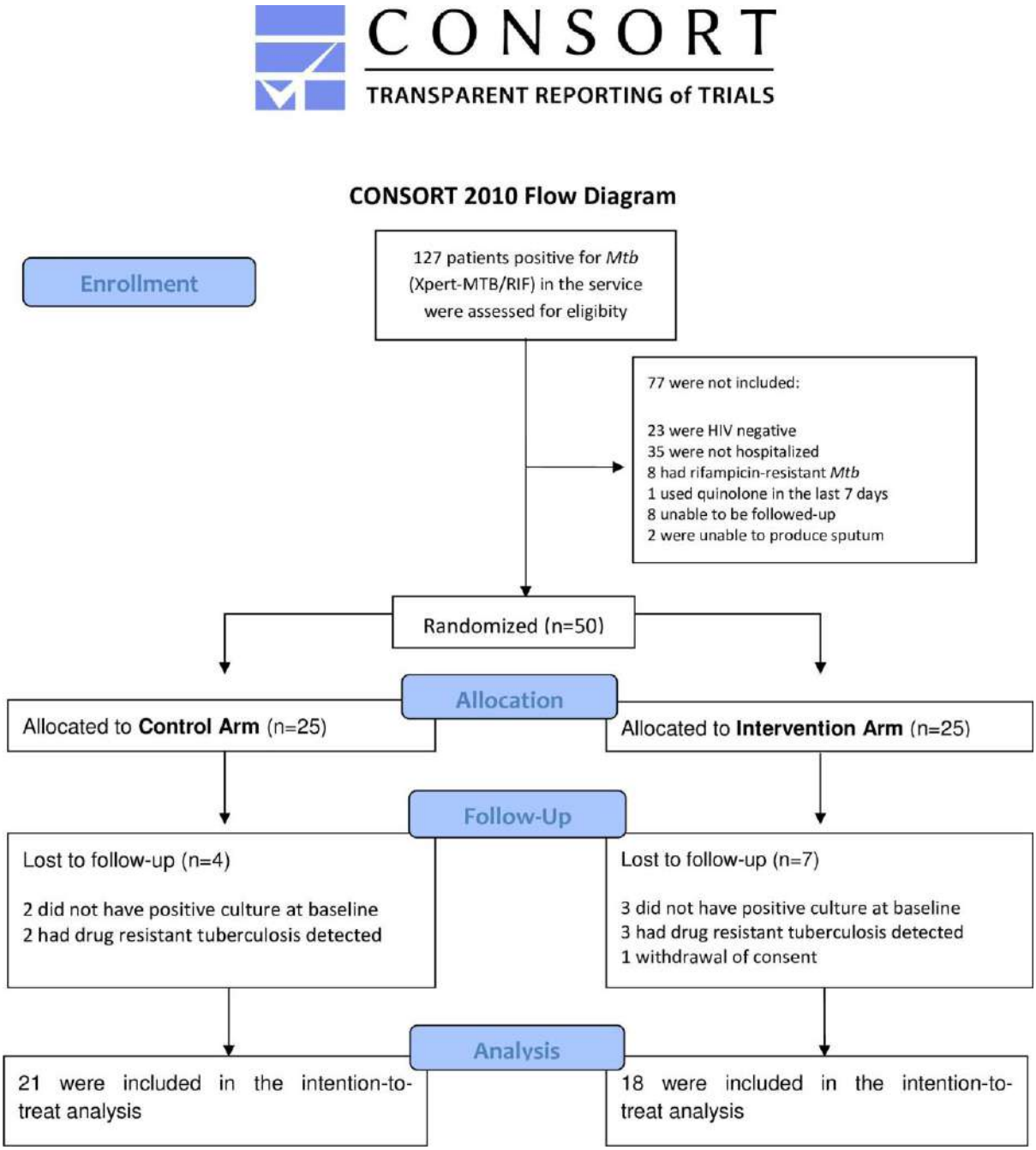

Fig 1. Flowchart of eligible, randomized and enrolled patients in the study.

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Table 1. Baseline characteristics of patients enrolled in both groups.

Characteristic	Control Group (n = 21)	NAC Group (n = 18)	P-value
	n (%)	n (%)	
Male sex	19 (90.5)	10 (55.6)	**.025**
Age group, in years			.972
<25	4 (19.1)	4 (22.2)	
25–34	6 (28.6)	4 (22.2)	
35–45	8 (38.1)	7 (38.9)	
>45	3 (14.3)	3 (16.7)	
CD4⁺ lymphocyte count			.336
<50 cells/mm³	6 (28.6)	9 (50)	
50–199 cells/mm³	12 (57.1)	8 (44.4)	
≥200 cells/mm³	3 (14.3)	1 (5.6)	
Viral load			.873
≥ 400 copies/mL	5 (23.8)	4 (22.2)	
401–3,000 copies/mL	1 (4.8)	1 (5.6)	
3,001–10,000 copies/mL	1 (4.8)	0 (0.0)	
10,001–100,000 copies/mL	3 (14.3)	4 (22.2)	
>100,000 copies/mL	11 (52.4)	9 (50)	
Hemoglobin <8g/dL	4 (19.1)	5 (27.8)	.706
Albumin ≥2.7mg/dL	4 (19.1)	8 (44.5)	.163
Time between TB diagnosis and ART in naïve patients			.549
< 2 weeks	5 (55.6)	4 (50)	
2 weeks—8 weeks	3 (33.3)	4 (50)	
> 8 weeks	1 (11.1)	0 (0.0)	
Concurrent opportunistic infection			.196
No	16 (76.2)	10 (55.6)	
Yes	5 (23.8)	8 (44.4)	
Concurrent extrapulmonary TB	14 (66.7)	11 (61.1)	.750
Disseminated TB (≥2 sites)	3 (14.3)	4 (22.2)	.683
Years since HIV diagnosis			.471
<1 year	15 (83.4)	13 (62)	
>1 year	8 (38.1)	3 (16.7)	
ICU hospitalization at enrollment	4 (19.1)	0 (0.0)	. . .

ART: antiretroviral therapy

https://doi.org/10.1371/journal.pone.0235381.t001

Overall, TB resistance was seen in 5 out of 50 enrolled patients (10%). Geometric means ofviral load in the control group was 4.133 copies/mL and in the NAC group 4.569 copies/mL.

As per protocol, no patient in the NAC Group had low adherence to NAC. Table 2 shows similar types of adverse events seen in both groups, and Table 3 shows the similar grading. Nopatient was positive for HBV or HCV.

Fig 2 shows the major outcomes related to the efficacy of the NAC arm. No differenceswere seen. Fig 3 details the quantification of ALT over the weeks, reinforcing that no changewas seen between the groups.

Discussion

This trial conducted with TB/HIV coinfected hospitalized patients aimed to estimate whetherthe use of NAC together with RIPE was not unsafe.

Table 2. Major adverse events seen in both groups.

Adverse event	Control Group (n = 21)	NAC Group (n = 18)	
Gastrointestinal disorders			
Gastric fullness	0	1 (5.5)	. . .
Dysphagia	0	2 (11.1)	. . .
Nausea	1 (4.7)	3 (16.6)	0.345
Vomiting	2 (9.5)	4 (22.2)	0.414
Hepatotoxicity	7 (33.3)	10 (55.5)	0.562
Respiratory disorders			
Dyspnea	0	1 (5.5)	. . .
Other disorders			
Pyrosis	0	1 (5.5)	. . .
Pruritus	0	1 (5.55)	. . .
Rash	1 (4.7)	0	. . .

NS: Non-Significant

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Hospitalized patients, used here as a proxy of clinical severity, were the targeted population because of their increased likelihood of evolving to death, and therefore, more prone to adhereto adjunctive therapy. Non-severe HIV/TB patients are already in use of many drugs simulta- neously, and any adjunctive therapy would compromise adherence if a major benefit is not clearly seen by the patient. Therefore, we believe that adjunctive therapy in HIV/TB coinfec-tion must be designed to give priority to more complicated patients. That requests that safety and efficacy studies are performed in this population since the very beginning of the evidence generation process.

For non-severe TB patients, NAC has been pursued as an adjunctive drug to decrease hepa-totoxicity, a problem that still persists in ~25% of patients, impacting adherence to RIPE and TB cure, ultimately [19]. The only clinical trial in which NAC was concurrently used in pulmo-nary TB, NAC was significantly associated to faster sputum negativity, improved radiological response, weight, serum glutathione peroxidase level, and amelioration of the deregulated immune response [20].

In PLWH, up to 30% of the patients experience hepatotoxicity, HIV infection apparentlybeing one predisposing factor [21]. However, not only hepatotoxicity is an expected effect of NAC adjunctive therapy in this population, but also culture conversion, used routinely as a

Table 3. Grading of adverse events seen in both groups.

	Control Group (n = 21)		NAC Group (n = 18)		
Adverse event	**Number of events**	**Number of participants (%)**	**Number of events**	**Number of participants (%)**	**p-value**
No events	. . .	6 (28.6)	. . .	2 (11.1)	0.427
Any event, except death	33	13 (61.9)	41	14 (77.8)	0.322
Grade 1	15	10 (47.6)	18	12 (55.6)	0.201
Grade 2	10	7 (33.3)	12	7 (38.9)	0.750
Grade 3	4	3 (14.3)	10	5 (27.8)	0.682
Grade 4	4	2 (9.5)	1	1 (5.6)	1
Death	. . .	4 (19.1)	. . .	5 (27.8)	0.706

NS: Non-Significant

https://doi.org/10.1371/journal.pone.0235381.t003

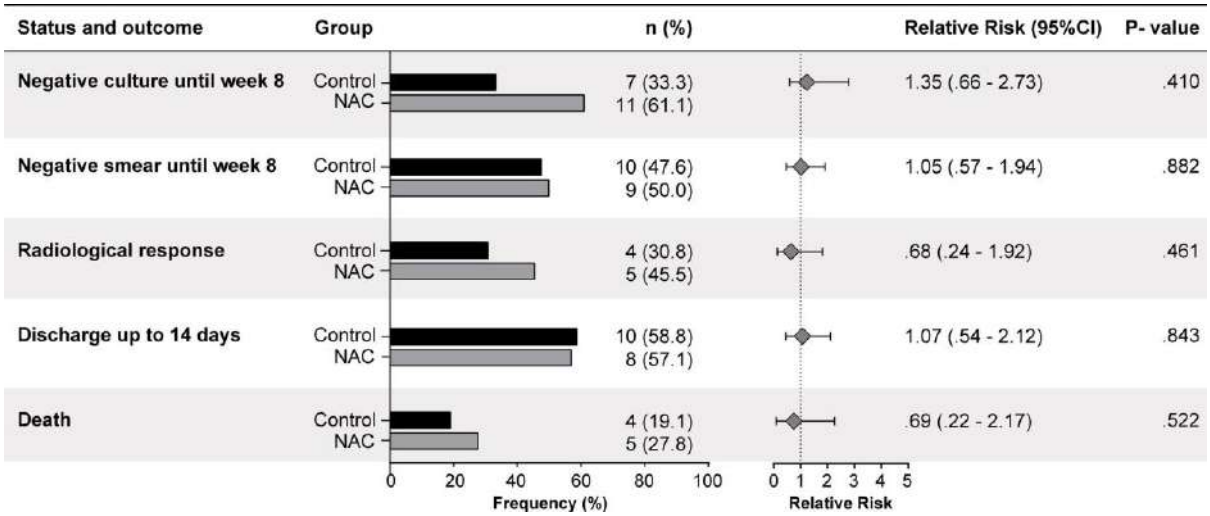

Fig 2. Major outcomes and respective 95% confidence intervals. P-values refer to RR estimates.

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marker of TB clinical recovery. This is critical in patients with immune deficiency, and increased risk of death. To date, however, no proof of concept study has been performed tar- geting this high-risk population of PLWH and TB coinfection. The selected dose of NAC waschosen based on the lack of immunological effect in PLWH using 600 mg qd [22], and the promising dose of 600 mg bid in TB patients [20].

Safety was the major outcome of interest in our study, considering that TB/HIV hospital- ized patients are a special group of subjects under enormous stress, with very low $CD4^+$ lym- phocyte counts, and in use of many drugs with potential interactions. During the eight weeks in which NAC was used in one of the arms, adverse events were seen in similar proportions inboth arms, gastrointestinal events being the most frequent (Table 2). When the total number of events and grading were assessed, no significant findings were seen, evidencing that NACadjunctive to RIPE is not unsafe as compared to RIPE by itself in coinfected patients. No trends in decrease of hepatotoxicity in patients using NAC was seen, suggesting that oxidativestress only partially explains liver damage in these patients in use of RIPE.

TB resistance, even not being a major focus of this work, was found in 10% of the enrolledpatients, which is pretty much the same percentage as seen in other similar settings in Brazil [23].

Major limitation of the trial was the reduced sample size, which did not allow for a more robust statistical analysis. However, the universe of eligible patients seen in such a reference unit is not much bigger, and the whole study recruited patients over 16 months. A multicentricapproach will certainly be needed in further phase III studies. It is also known that more males present HIV/TB coinfection in Brazil [24], but in this randomized trial, Table 1 shows that more males were enrolled in the Control Group, what may be explained by chance and the small sample size. No clear bias was considered. Noteworthy to say that results found here maynot be extrapolated to non-severe patients seen in the outpatient clinics. Likewise, results are not applicable to multidrug-resistant Mtb [25], a condition in which adjunctive therapies arealso needed. Some patients were already hospitalized in the ICU during enrollment, and there-fore, ICU hospitalization as an endpoint had limitation in the analyses. Data from our group

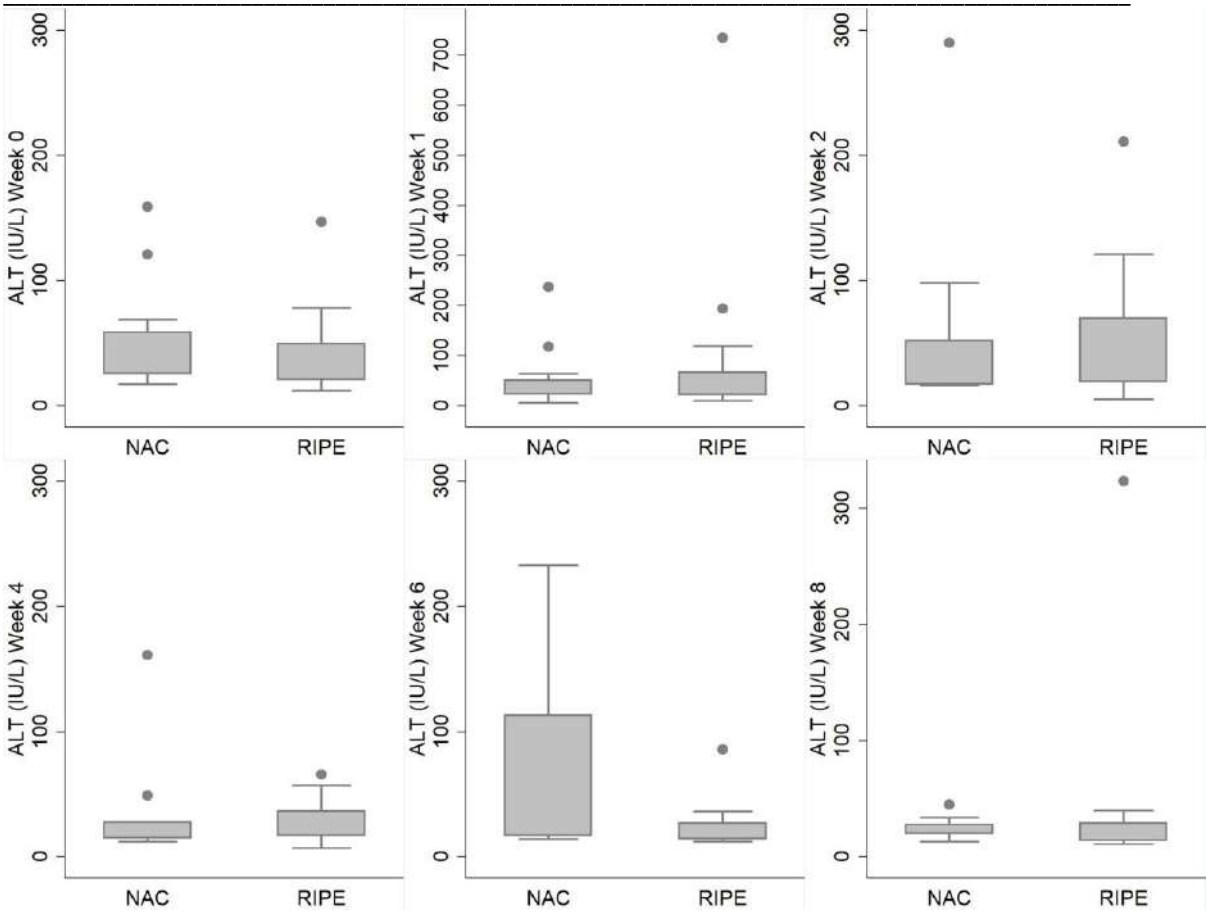

Fig 3. ALT levels between control and NAC groups over the weeks of the follow-up.

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show high early mortality rate amongst TB/HIV coinfected ICU patients. The factors predictive of mortality in this population were invasive mechanical ventilation, hypoalbuminemia, and severe immunosuppression [26]. Indigenous population was also excluded from the analy-sis, but represents a major burden of the disease in the Brazilian Amazon [27,28].

As the use of NAC in the HIV/TB population seems promising in terms of safety, our results indicate that RIPE plus NAC regimen is suitable for a larger phase III trial. It is worth mentioning that NAC has a well-known safety profile safety, even in much higher doses [29], is tolerable in pregnant women, is quite economically affordable, and requests no major medical supervision during the administration of oral presentations, mostly flavored. During oral administration, deacetylation reaction of NAC happens while passing along the small intestine as well as liver, thus its bioavailability is only 4–10% decreased [30]. The ongoing TB-SEQUEL cohort study (ClinicalTrials.gov Identifier: NCT03702738) aims to evaluate similar endpoints, using a higher dose of 1,200 mg bid of NAC in patients with TB, with and without HIV coinfection. Therefore, in the near future, more evidence will be generated to support the use of

this safe drug in coinfected patients, still a major contributor to mortality in developingcountries.

Supporting information

S1 Checklist. CONSORT 2010 checklist of information to include when reporting a rando-mised trial[ω].
(DOC)
S1 Dataset.
(XLSX)
S1 File.
(PDF)

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9.5.5 Artigo 5

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Early death by tuberculosis as the underlying cause in a state of Southern Brazil: Profile, comorbidities and associated vulnerabilities

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ABSTRACT

Aim: To know the profile of adults who died by tuberculosis as the main cause, the time interval between the diagnosis and death, associated comorbidities and vulnerabilities.

Method: Observational study of secondary data regarding deaths by tuberculosis that occurred in the State of Paraná, Brazil, from 2008 to 2015. A linkage between the databases of mortality and tuberculosis notification system was conducted for data enrichment. Frequency tables, Exact Fisher test and Z test have identified statistical associations.

Results: Linkage points out 12.1% (115/944) of under-reporting in the 944 deaths identified. Early deaths accounted for 74.6% (705/944). The male sex (75.8%) was associated with the early death group. Almost half of the deaths reported in notification system (414/829) had one or more vulnerabilities. Early death were associated with respiratory system diseases and symptoms (p = 0.0001) and mental and behavioral disorders (p = 0.0001).

Conclusion: High number of early deaths due TB indicate the need to seek out the respiratory symptomatic and use faster diagnostic methods. Strategies for treatment adherence, adequate monitoring of comorbidities and multisectorial support may prevent early and late death. The presence of vulnerabilities indicates that efforts beyond the health sector are needed in order to eliminate tuberculosis as public health problem.

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Introduction

Tuberculosis (TB) continues to make new cases in Brazil and worldwide. A significant number of deaths are attributed to this disease until the present day. In Brazil, approximately 4,400 deaths due TB occurred in 2014 (Brasil, 2016). The World Health Organization (WHO) estimates that in 2017, the disease has killed 1.6 million people worldwide. Among them, 300,000 were positive for Human Immunodeficiency Virus (HIV) (WHO, 2018). The high lethality of TB has caught the attention of government authorities and justifies efforts to achieve global agreements goals in order to eliminate TB as a public health problem (Zumla and Petersen, 2018).

Although the incidence of TB declines over the years (Brasil, 2018; WHO, 2018), it has been observed that patients have presented the occurrence of diseases and vulnerabilities associated with tuberculosis and this fact has negatively impacted the treatment outcome (Reis-santos et al., 2013; Reis-Santos et al., 2013; Rocha et al., 2015). A review regarding the risk factors associated with TB death, such as HIV positivity, high age, comorbidities, use of alcohol and drugs, points out differences between results for regions with low and high incidence of tuberculosis (Waitt and Squire 2011).

The incidence rate of TB per 100 thousand inhabitants was 34.1 in Brazil in 2014 (Brasil, 2016). In the state of Paraná, southern Brazil, the incidence was 19.7 in the same year (Brasil, 2016). The

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mortality rate for TB in HIV-negative individuals, on the other hand, was 2.2 and 1.0 in Brazil and Paraná, respectively (Brasil, 2016). Study from 1998 to 2012, also in Paraná, had already indicated an increase in mortality due TB since 2010 (Cecilio et al. 2018).

A systematic review regarding tuberculosis mortality indicated few studies on early deaths, i.e., the deaths that occurred in the first two months of treatment. A limitation in the studies would be the focus on hospitalized patients and the fact that death due to TB would be related to non-infectious comorbidities, organ failure and malnutrition (Waitt and Squire 2011; Shuldiner et al., 2014). The state of Paraná is one of the five states of Brazil with the best Human Development Index (0.749) (IPARDES, 2019) and adhered to the United Nations Sustainable Development Goals (Paraná, 2016). Although it is not in TB epidemic, the occurrence of deaths due to this disease still occurring in the present day points out to the need for local researches to guide feasible TB control actions and thus eliminate TB as a public health problem (Lönnroth and Raviglione, 2016; Kyu et al., 2018).

Researches using linkage between databases of TB reporting and mortality has shown results to identify under-reporting, correct divergences, and enrichment of the records (de Oliveira et al., 2012; Pinheiro et al. 2012; Rocha et al., 2015). In view of these facts, the aim of the present study was identify the profile of the people who died and the presence of comorbidities and vulnerabilities associated with early death with TB as the underlying cause in the state of Paraná.

Methods

This was a cross-sectional, observational and secondary data study regarding deaths due to TB in the state of Paraná, Brazil from 2008 to 2015. We identified all cases of death with TB as the underlying cause, available in the database of the Mortality Information System (SIM). In this system, when TB and HIV infection are present in the death certificate, the underlying cause of death is automatically defined as AIDS (Rocha et al., 2015). Considering that there are already studies that discuss the lethality of HIV/TB co-infection (De Melo et al., 2017), in the present study we opted to analyze the deaths by TB as underlying cause, i.e., HIV-negative individuals.

Another information system used was the SINAN (Notifiable Diseases Information System). This system can be considered the main source of information on cases reported with TB in the Ministry of Health from Brazil (Brasil, 2011). The data were extracted from SIM and SINAN in May 2017 and updated in March 2018.

Using common variables present in the analyzed databases, we estimated the probabilities of certain records in the two databases belong to the same individuals. The comparison was performed using the software OpenReclink, which uses the method of probabilistic record linkage (Camargo and Coeli, 2015). The reference variables for the linkage were name, date of birth and mother's name. From this data, a relationship file was generated aggregating the information present in the two systems referring to each case of death.

The "diagnosis date" variables of the notification form (SINAN) and "death date" in the death certificate (SIM) were related and the time interval in days was calculated. There were considered as early deaths the cases under-reported, with a notification date after or on the same date of death, and those with an interval of 1 to 60 days between diagnosis and death. The 60-day period was adopted because it corresponds to the attack phase of the basic treatment regimen in Brazil (Brasil, 2011). The deaths that occurred after 61 days of diagnosis were considered as late deaths.

Associated causes described in death certificate (SIM) were added to the information present in the variables "diseases and associated injuries" in the SINAN notification form. The information were grouped according to the chapters of the International Statistical Classification of Diseases and Related Health Problems, 10th revision (ICD-10) (Selig et al. 2010; Rocha et al., 2015). When more than one cause was reported in the same ICD chapter, it was quantified only once. The percentage of each chapter or specified cause was calculated by the total number of deaths in the early or late group.

The SINAN notification form was altered and the variables about deprivation of liberty, homeless population, beneficiary of government program, have been grouped. In this sense, the use of alcohol, tobacco and other psychoactive substances were also evaluated as vulnerabilities (Brasil 2018; Carter et al., 2018).

The data obtained were entered in a spreadsheet of the Software Microsoft Excel 2010[1] and analyzed statistically using the softwares Statistica Single User version 13.2 and R 3.5.1. The following descriptive measures were calculated for the quantitative variables: Mean, standard deviation, maximum, minimum. Qualitative variables were organized into simple frequency and double input tables. Fisher Odds Ratio (OR) was calculated to verify possible risk or protection factors, and to compare the proportions assessed in early and late deaths. The Z test was used to compare pairs of proportions. The level of significance used in the tests was 5%, that is, the comparisons were considered significant when $p < 0.05$.

The Standing Committee on Ethics in Research at the Hospital do Trabalhador, Curitiba, Paraná, appreciated and approved the research project with Registration No. 1.632.073. This research project was developed according to the guidelines disciplined by Resolution 196/96 of the National Health Council (1996).

Results

There were identified 952 deaths due to TB (ICD A15 to A19) in Paraná from 2008 to 2015 in individuals over 15 years of age without any duplicity. Among these deaths, eight did not present identification of name, age or date of birth and/or mother's name. Seven people were male and five had as their underlying cause of death pulmonary TB without bacteriological or histological confirmation (ICD A162). Two individuals died due to unspecified respiratory TB (ICD A169) and another patient, due to unspecified miliar TB (ICD A199). These eight individuals were excluded of the analysis, since the lack of identification data made it impossible to perform the linkage of the records, an essential step to achieve the objectives of this work.

The 944 TB deaths analyzed occurred in all 22 regional health in the state of Paraná, distributed among 222 of the 399 municipalities. The three regional health with the highest number of TB deaths were the Curitiba Metropolitan Area, Paranaguá and Londrina respectively, and together they were responsible for almost half of the deaths (48.9%) in Paraná. Curitiba, capital of the state, was the place where 13.6% of deaths occurred.

The deaths was compared to the spreadsheet extracted from SINAN with all cases of TB diagnosed in Paraná until 2015 (N = 44,884). Following the linkage blocking steps, the question- able pairs were manually analyzed, excluding duplicities, and records that referred to the same treatment were linked. Nevertheless, in 115 deaths (12.1%) no reports regarding individu- als who died due to TB were found at SINAN.

Among these 829 individuals who died due to TB and were found at SINAN, in 10.6% (88/829) there were more than one notification referring to treatments of the same individual (2 to 6 notifications per individual). Other 741 cases of death (78.5%) had only one notification form. For analysis of the time interval

between diagnosis and death, for cases in which there was more than one notification form, the date of diagnosis of the most recent form before death was used.

Deaths were categorized as early or late according to the number of days between diagnosis and death. Early deaths accounted for 74.7% of the total, according to the following categories: under-reported 12.1% (115/944), with diagnosis dates after death 6.1% (58/944) and between one and 60 days after diagnosis 56.3% (532/944). Late deaths, which occurred after 61 days of diagnosis, totaled 25.3% (239/944).

The proportion of early cases in 2008 (14.2%) was statistically lower (p = 0.0268) than the late cases (19.7%). Only in 2014 the late deaths was significant evidenced. Female sex has a greater proportion of late deaths occurred (p = 0.0124), whereas in men the proportion of early deaths was higher than the late ones

Table 1
Distribution of year of death, age, gender, race, marital status, schooling, occupation and underlying cause of early and late deaths by tuberculosis as the main cause in the death certificate from 2008 to 2015, Paraná, Brazil.

	Group				*OR*	*OR (IC 95%)*	*p*
	Early		Late				
	74.7% (n = 705)		25.3% (n = 239)				
	n	%	n	%			
Year of death							
2008	100	14.2	47	19.7	Reference		
2009	81	11.5	31	13	0.81	(0.45;1.44)	0.4958
2010	81	11.5	27	11.3	0.71	(0.38;1.28)	0.2645
2011	91	12.9	30	12.6	0.70	(0.39;1.24)	0.2231
2012	71	10.1	23	0.1	0.69	(0.37;1.28)	0.2454
2013	106	15	28	11.7	0.56	(0.31;0.99)	0.0529
2014	86	12.2	19	7.9	0.47	(0.24;0.89)	0.0139*
2015	89	12.6	34	14.2	0.81	(0.46;1.42)	0.5053
Age group							
15–30 years old	52	7.4	22	9.2	Reference		
31–45 years old	157	22.3	52	21.8	0.78	(0.42;1.49)	0.4430
46–60 years old	263	37.3	74	31	0.66	(0.37;1.23)	0.1722
61–75 years old	155	22	66	27.6	1.01	(0.55;1.89)	0.9998
76 years old or more	78	11.1	25	10.5	0.76	(0.37;1.57)	0.4907
Gender							
Female	156	22.1	71	29.7	0.67	(0.48;0.95)	
Male	549	77.9	168	70.3	Reference		
Race							
White	484	68.7	158	66.1	Reference		
Black	48	6.8	22	9.2	1.40	(0.78;2.46)	0.2461
Yellow	6	0.9	1	0.4	0.51	(0.01;4.26)	0.9998
Brown	150	21.3	56	23.4	1.14	(0.79;1.65)	0.4620
Indigenous	2	0.3	0	0	0.00	(0.00;16.40)	0.9998
Ignored or blank	15	2.1	2	0.8	0.41	(0.04;1.79)	0.3877
Marital status							
Not married	272	38.6	85	35.6	Reference		
Married/Stable union	254	36	95	39.7	1.19	(0.84;1.70)	0.3017
Separated/divorced	59	8.4	26	10.9	1.41	(0.80;2.43)	0.2112
Widow/Widower	76	10.8	21	8.8	0.88	(0.49;1.55)	0.6875
Ignored	44	6.2	12	5	0.87	(0.40;1.78)	0.8654
Schooling							
No schooling	2	0.3	0	0.0	0.00	(0.00;14.60)	0.9998
Elementary School	310	44	114	47.7	Reference		
High School	227	32.2	74	31.0	0.88	(0.62;1.26)	0.4930
(In)complete College	90	12.8	25	10.5	0.75	(0.44;1.26)	0.2818
Ignored	76	10.8	26	10.9	0.93	(0.54;1.55)	0.8044
Occupation							
Occupied	406	57.6	134	56.1	1.01	(0.66;1.55)	0.9998
Student, housewife and retired	174	24.7	64	26.8	1.12	(0.70;1.82)	0.6462
Ignored or unemployed	125	17.7	41	17.2	Reference		
Underlying Cause							
Respiratory tuberculosis, with histological bacteriological confirmation (ICD A15)	175	24.8	71	29.7	1.26	(0.89;1.78)	0.192
Tuberculosis of the respiratory tract, without bacteriological or histological confirmation (ICD A16)	446	63.3	143	59.8	Reference		
Tuberculosis of the nervous system (ICD A17)	16	2.3	4	1.7	0.78	(0.19;2.47)	0.7951
Tuberculosis of other organs (ICD A18)	23	3.3	10	4.2	1.36	(0.56;3.05)	0.4127
Miliar tuberculosis (ICD A19)	45	6.4	11	4.6	0.76	(0.35;1.55)	0.5137

* p significant value by Exact Fisher test considering significance level of 5%.

(p = 0.0124). By the way, the OR of the variables in Table 1 was not significant.

Pulmonary TB, a transmissible form of the disease, was responsible for almost 80% (755/944) of deaths. Still according to the underlying cause, among these deaths record at SIM, only 32.5% presented bacteriological or histological confirmation (246/755). The clinical form of the disease described in the underlying cause of death was not statistically associated with the early or late group.

The mean age of patients who died due to TB was 54.0 16.3. There was no significant difference between the group of women (54.9 18.0) and men (53.7 15.7). People over 65 years old represented 27.7% of all deaths (262/944) (Table 2).

The mean time between diagnosis and death was 150.5 479.1 days. In women, this time was 173.1 503.1 and in men it was slightly lower, being 172.4 864.8 (in this group the variation was higher than the variation in women).

In the middle of the causes associated with early death due to TB, we can mention other respiratory diseases (p = 0.0001), mental and behavioral disorders (p = 0.0001) and other infectious diseases (p = 0.0001), which occurred more frequently. Late death due to TB occurred proportionally in individuals with diabetes (p = 0.0077), illicit drugs users (p = 0.0467), presence of diseases in the circulatory system (p = 0.0010) and diseases in the (p = 0.0467) genitourinary tract (Table 3).

Among the deaths due to pulmonary TB without bacteriological or histological confirmation (ICD A16.1 to A16.9), more than 55% (320/580) of patients had a positive examination record for tuberculosis on baciloscopy, molecular rapid test, culture or histopathological in the SINAN notification form. Of these, 225 died within 60 days of diagnosis, i.e., they belonged to the group of deaths considered early (Table 4).

Early death due to TB had a statistical association to patients relapse (p = 0.0001), return after abandonment (p = 0.0048) and transference (p = 0.0066) than the new cases. Regarding the closure situations, it was also observed a significant association of late death with cure, abandonment of treatment, transference and drug-resistant TB. The contacts indicated by the individual were examined more frequently among those patients who had late death, i.e, they had been in treatment for longer period.

Almost half of the deaths reported in SINAN (414/829) had one or more vulnerabilities. Early death due to TB was also associated with situations of vulnerability such as the population deprived of liberty (Table 5).

Individuals who had more than one notification record in SINAN, i.e., who were attendend in more than one health service or began TB treatment for more than once were associated with late death (p < 0.0001). On the other hand, the population deprived of liberty were associated with early death. The population with one or more types of vulnerability were also more associated with early TB death.

Discussion

Tuberculosis is still present in Brazil and was responsible for the deaths of more than 100 adults per year on average in the State of Paraná, from 2008 to 2015. Considering that tuberculosis is a curable disease, these deaths should not happen. An important finding in the present study was that the vast majority (74.7%) of these patients deaths occurred within 60 days of diagnosis release, i.e, at an early stage. It was considered as early death the cases in which the patients probably did not receive treatment (under-reported or diagnosed after death) or were in the first phase of treatment yet (Brasil, 2011).

A study regarding the tendency of TB mortality in Paraná from 1998 to 2012 demonstrated that there was initially a decrease, and an increase tendency in the years 2010 to 2012 (Cecilio et al. 2018), which was not confirmed by the present study.

Tuberculosis is a compulsory notifiable disease in Brazil (Brasil, 2011), cases that were either under-reported or diagnosed after death indicate that they were probably not diagnosed in health services before death and did not receive adequate treatment. The absence of notification in SINAN of these 115 deaths that occurred in the state represents 12.1% of the deaths in the period studied. The comparison with other studies shows that there has been improvement in the qualification of information systems over the years in Paraná. A linkage study between SIM and SINAN carried out with TB deaths in 2006 demonstrated 23.4% of under-reported cases in Paraná and 39.4% in Brazil (de Oliveira et al., 2012). A survey performed in Rio de Janeiro demonstrated even higher under-reporting rates, exceeding 40% (De Melo et al., 2017).

The diagnoses performed after death evidence the importance of epidemiological surveillance in monitoring these situations, and the adoption of research protocols may contribute to the quality of this information (Saúde, 2017). Early deaths may indicate late diagnosis and this fact points out the need for faster examinations in order to detect the disease among the respiratory symptomatics (Brasil, 2011). In this sense, the incorporation of the rapid molecular test for TB using the GeneXpert MTB/RIF system in the Brazilian Unified Health System in the year 2013 can contribute to a faster diagnosis and a consequent change in this scenario (Pinto et al. 2017).

Individuals with more than one notification form (88/829) have attended more than one health service or have initiated treatment for TB repeatedly. These cases have an increased risk of death (Waitt and Squire 2011) and points out the importance of the contact between the individual and health professionals for treatment adherence. Directly observed treatment is an important

Table 2
Distribution of the gender and age of patients who died due to tuberculosis as the underlying cause in the death certificate according to the number of days between diagnosis and death, Paraná, Brazil, from 2008 to 2015.

Variables	n	Mean Standard deviation	Minimum	Maximum
Number of days between diagnosis and death (n = 829)*				
All patients	829	150.5 479.1	−351	4530
Female	189	173.1 503.1	−351	4324
Male	640	172.4 864.8	−193	18482
Age (n = 944)				
All patients	944	54 16.3	15	95
Female	227	54.9 18	15	94
Male	717	53.7 15.7	17	95

* 115 cases that were not reported in Notifiable Diseases Information System (SINAN) were excluded.

Table 3
Distribution of early and late deaths due to tuberculosis by mention of associated causes in the notification form and/or in the death certificate according to the chapters of the International Statistical Classification of Diseases and Related Health Problems, 10th revision (ICD-10), from 2008 to 2015, Paraná, Brazil.

Associated causes	Group				*p*
	Early		Late		
	74.7% (n = 705)		25.3% (n = 239)		
	n	%	n	%	
Some infectious and parasitic diseases (A00-B99)	222	31.5	68	28.5	0.0001*
Neoplasms (tumors) (C00-D48)	24	3.4	5	2.1	0.0866
Blood and hematopoietic organ diseases and some immune disorders (D50-D89)	17	2.4	2	0.8	0.0277*
Endocrine, nutritional and metabolic diseases (E00-E90)	120	17	39	16.3	0.0001*
Diabetes mellitus (E10-E14)	55	7.8	23	9.6	0.0077*
Mental and behavioral disorders (F00-F99)	279	39.6	94	39.3	0.0001*
Mental and behavioral disorders due to the use of alcohol (F10)	212	30.1	75	31.4	0.0001*
Mental and behavioral disorders due to the use of psychoactive substances (F11-14, F16, F18-19)	38	5.4	15	6.3	0.0467*
Mental and behavioral disorders due to tobacco use (F17)	98	13.9	28	11.7	0.0001*
Diseases of the nervous system (G00-G99)	16	2.3	7	2.9	0.3078
Diseases of the circulatory system (I00-I99)	99	14	40	16.7	0.0010*
Diseases of the respiratory tract (J00-J99)	471	66.8	146	61.1	0.0001*
Diseases of the digestive system (K00-K93)	42	6	22	9.2	0.0721
Skin and subcutaneous tissue diseases (L00-L99)	2	0.3	2	0.8	0.9999
Osteomuscular system and connective tissue diseases (M00-M99)	10	1.4	0	0	0.1208
Diseases of the genitourinary system (N00-N99)	33	4.7	17	7.1	0.0467*
Congenital malformations, deformities and chromosomal abnormalities (Q00-Q99)	2	0.3	1	0.4	0.9999
Symptoms, signs, and abnormal findings in clinical and laboratory tests, not elsewhere classified (R00-R99)	198	28.1	74	31	0.0001*
Injuries, poisonings and some other consequences of external causes (S00-T98)	2	0.3	2	0.8	0.9999
External causes of morbidity and mortality (V01-Y98)	10	1.4	9	3.8	0.9999

Note: Chapters of the ICD not mentioned were excluded.
* p significant by the Z test for comparison of proportions considering level of significance of 5%.

strategy to favor this contact between health professional and patient, which is especially performed by primary health care teams (Maciel et al., 2018).

The association between late death and cure can be explained by situations when the pacient died after the treatment and was not notified again (Rocha et al., 2015). In the same way, the individuos who descontinuoed the treatment and died have to be reported at the SINAN again. The TB-drug resistant has a longer treatment and could explain the association with late death too. Studies about the delay to diagnose TB and TB drug resistant like studies about health care cascade could contribute do explain and to avoid the TB deaths (Cazabon et al. 2017).

There was no significant difference between mean age and mean number of days between diagnosis and death of men and women. However, the men group was related to early death. Men may delay to seek healthservices, have advanced TB at the time of diagnosis and die early. However, a systematic review regarding the delay in the diagnosis of TB has not reached a conclusion on this aspect (Storla et al. 2008). Further studies are needed to understand whether the differences of the results between men and women occur due to biological or sociocultural factors (Waitt and Squire 2011).

Early death was mostly related to the presence of other diseases and symptoms described in Chapter J of the ICD. Symptom such as coughing may be trivialized by patients who are slow to seek health services and also may be confused with other diseases like a pneumonia. This fact was observed in other studies that report the difficulty and the delay in the diagnosis of TB in these situations (Waitt and Squire, 2011; Rocha et al., 2015).

The present study identified the presence of diabetes in 8.2% (73/944) of the cases; it was significantly associated with late death due to TB (p = 0.0077). Endocrine, nutritional and metabolic disorders were mentioned in 159 of the 944 deaths, i.e., 16.8%. Considering the projected increases in these diseases worldwide, health policies to prevent multi-morbidities must be extended (Goldhaber-fiebert et al., 2011).

Aging may lead to immunosuppression, but this may also occur with other conditions, such as use of corticosteroids or other immunosuppressants, renal insufficiency or transplantation, diabetes, and neoplasms. The advancing age is related to the occurrence of concomitant diseases since individuals over 60 years of age are 44 times more likely to have TB associated with two or more chronic conditions (Reis-Santos et al., 2013). In the present study, 34.3% of deaths occurred in people in this age group, a quite different result from that found in Israel, in which 70% of TB deaths were related with age over 65 years (Shuldiner et al., 2014). Elderly people present the classic symptoms of TB less frequently, hindering and delaying the diagnosis, and thus decrease the effectiveness of anti-TB treatment (Silva et al., 2010). However, in the present study, we did not identify association (p = 0.6596) in early or late deaths in people over 75 years.

Vulnerabilities were reported in approximately 50% of the deaths that were recorded in SINAN. The use of alcohol, tobacco and other psychoactive substances were mentioned in 30.4%, 13.3% and 5.6% of the deaths, respectively. These are individual behavioral factors that cause vulnerability, as they make it difficult to adhere to the treatment and, consequently, cure of the patients. Studies indicate the need for actions beyond the health sector in order to reach the social determinants of the disease, and thus obtain better results (Furlan et al., 2012; Maciel et al., 2018).

The eight deaths excluded at the beginning of the study due to the lack of identification data can be attributed to people living in the street, a population at increased risk of developing the disease due to the vulnerability situation and consequently death due to TB (Waitt and Squire, 2011; Maciel et al., 2018). Fighting TB, and

Table 4
Distribution of early and late tuberculosis deaths according to clinical form, type of treatment, chest X-ray, baciloscopy or positive molecular rapid test, culture of sputum or other positive material, histopathological test, contact examination, outcome situation and laboratory confirmation registered in the Notifiable Diseases Information System (SINAN)**, from 2008 to 2015, Paraná, Brazil.

Variables	Group				*OR*	*OR (IC 95%)*	*p*
	Early		Late				
	74.70%		25.30%				
	(n = 590)		(n = 239)				
	n	%	n	%			
Clinical form							
Pulmonary	491	83.2	198	82.8	Reference		
Pulmonary + extrapulmonary	29	4.9	8	3.3	0.68	(0.27;1.57)	0.4545
Extrapulmonary	70	11.9	33	13.8	1.16	(0.72;1.85)	0.4876
Type of treatment							
New case	519	88	174	72.8	Reference		
Not known	2	0.3	1	0.4	1.49	(0.03;28.80)	0.9998
Post-death	8	1.4	0	0	0.01	(0.00;1.77)	0.2108
Relapse	35	5.9	37	15.5	3.15	(1.86;5.32)	0.0001*
Return after abandonment	15	2.5	15	6.3	2.97	(1.33;6.69)	0.0048*
Transference	11	1.9	12	5	3.25	(1.29;8.29)	0.0066*
Chest X-ray							
Suspicious	513	86.9	203	84.9	Reference		
Normal	21	3.6	13	5.4	1.56	(0.70; 3.34)	0.2446
Other pathology	7	1.2	1	0.4	0.36	(0.01;2.84)	0.4527
Ignored	3	0.5	1	0.4	0.84	(0.01;10.56)	0.9998
Not performed	46	7.8	21	8.8	1.15	(0.64;2.03)	0.6716
Bacilloscopy or positive molecular rapid test							
Yes	363	61.5	142	59.4	Reference		
Not	227	38.5	97	40.6	1.09	(0.79;1.50)	0.5830
Positive culture							
Yes	81	13.7	42	17.6	Reference		
Not	509	86.3	197	82.4	0.75	(0.49;1.15)	0.1621
Histopathological							
Positive bar	58	9.8	17	7.1	Reference		
Suggestive of tuberculosis	26	4.4	18	7.5	2.34	(0.97;5.72)	0.0597
Not suggestive of tuberculosis	9	1.5	2	0.8	0.76	(0.07;4.21)	0.9998
Ignored	497	84.2	202	84.5	1.38	(0.77;2.60)	0.2827
Contacts examination							
Yes	280	47.5	139	58.2	Reference		
Not	310	52.5	100	41.8	0.65	(0.47;0.89)	0.0058*
Outcome situation							
Cure	1	0.2	43	18	153.50	(25.74;6038.89)	0.0001*
Abandonment of treatment	4	0.7	11	4.6	9.90	(2.87;43.20)	0.0001*
Death due to tuberculosis	505	85.6	140	58.6	Reference		
Death due to other causes	69	11.7	31	13	1.62	(0.98;2.62)	0.0543
Transference	5	0.8	8	3.3	5.75	(1.63;22.70)	0.0025*
Change of diagnosis	4	0.7	1	0.4	0.91	(0.02;9.21)	0.9998
Change of treatment	0	0	1	0.4	Not calculated	(0.09-infinito)	0.2183
Drug-resistant tuberculosis	2	0.3	4	1.7	7.19	(1.02;80.03)	0.0238*
Laboratory confirmation							
Yes	373	63.2	152	63.6	Reference		
Not	217	36.8	87	36.4	0.94	(0.71;1.36)	0.9367

* p significant value by Exact Fisher test considering significance level of 5%.
** 115 cases that were not reported in SINAN were excluded.

especially cases with comorbidities and vulnerabilities, requires a multisectoral approach that includes health and social protection organizations (Reis-santos et al., 2013). In order to promote the results of the TB control programs, it is necessary to think about strategies beyond the health sphere, i.e., the other Sustainable Development Goals must be achieved (Carter et al., 2018; Kyu et al., 2018; Zumla and Petersen, 2018).

Among the 829 individuals who died and had notification forms, 13 (1.56%) had diagnostic dates after death (ranging from 1to 351 days). This fact can be attributed to typing error or to examinations and investigation with results obtained in dates after the death of the individual. Other 45 cases had the same date for diagnosis and death.

As the present study was limited to analyzing variables present in the epidemiological record in SINAN and in the death certificate of the SIM, it was not possible to measure the severity of the diseases and injuries reported by the individuals to the professionals who filled the form. The coding according to the ICD in the death certificate may present errors because it depends on the knowledge of the

Table 5
Distribution of vulnerability situations described in the Notifiable Diseases Information System (SINAN)** for patients early and late deaths by tuberculosis as the underlying cause in the death certificate from 2008 to 2015, Paraná, Brazil.

Variables	Group				p
	Early 74,70% (n = 590)		Late 25,30% (n = 239)		
	n	%	n	%	
Case type					
Case with one record	548	92.9	193	80.8	0.0001*
Last record (multiple records)	42	7.1	46	19.2	0.0001*
Drugs					
Yes	36	6.1	15	6.3	0.9136
Not or ignored	554	93.9	224	93.7	0.9136
Smoking					
Yes	94	15.9	28	11.7	0.1218
Not or ignored	496	84.1	211	88.3	0.1218
Alcoholism					
Yes	202	34.2	75	31.4	0.4387
Not or ignored	388	65.8	164	68.6	0.4387
Homeless population					
Yes	7	1.2	3	1.3	0.9058
Not or ignored	583	98.8	236	98.7	0.9058
Beneficiary of government programs					
Yes	4	0.7	2	0.8	0.8781
Not or ignored	586	99.3	237	99.2	0.8781
Population deprived of liberty					
Yes	44	7.5	6	2.5	0.0063*
Not or ignored	546	92.5	233	97.5	0.0063*
Other institutionalized population					
Yes	41	6.9	10	4.2	0.1419
Not or ignored	549	93.1	229	95.8	0.1419
Vulnerable					
Yes	311	52.7	103	43.1	0.0123*
Not	279	47.3	136	56.9	0.0123*

* Significant Z test considering level of significance of 5%.
** 115 cases that were not reported in SINAN were excluded.

professional who filled the document and the person who did the coding. In addition, the two databases, SIM and SINAN, may contain typing errors.

Conclusions

Tuberculosis is still the underlying cause of a significant number of deaths in the state of Paraná. The finding of under- reported cases reinforces the need to improve epidemiological surveillance systems. The high percentage of early deaths suggests that the diagnoses may be occurring late, which can be improved with the strengthening and expansion of primary health care coverage for the search of respiratory symptomatic. It is also highlighted the implementation of diagnostic tests with fastresults, which may already be changing the TB scenario in the stateof Paraná.

Communication between health services at the different levels such as primary, secondary, laboratory and hospital is fundamental to promote integrated care. The presence of comorbidities and individual behaviors that cause vulnerabilities associated with early and late death reinforces the need for more frequent contact between patients and health professionals, individualized thera- peutic regimens and multisectorial support to ensure better adherence to treatment and thus prevent death caused by TB.

Conflict of interest

The authors have no conflict of interest.

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Ethical approval

The Standing Committee on Ethics in Research at the Hospital do Trabalhador, Curitiba, Paraná, appreciated and approved the research project with Registration No. 1.632.073. This research project was developed according to the guidelines disciplined by Resolution 196/96 of the National Health Council (1996).

Contributions

SPO, RRO, LA and RFC made the study design. SPO was responsible for data collection and analysis. SPO, JTPS and FBM wrote the initial version. All the authors revised and approved the last version.

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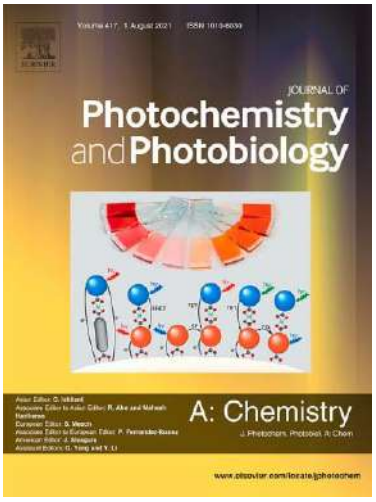
9.5.6 Artigo 6

Journal Pre-proofs



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Study of Isoniazid degradation by Fenton and photo-Fenton processes, by-products analysis and toxicity evaluation

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ABSTRACT

This paper presents the degradation of the most widely used antibiotics for the treatmentof tuberculosis, isoniazid (INH), by Fenton, photo-Fenton on bench-scale, and solar photo-Fenton on a pilot scale. The INH dosage in tuberculosis treatment varies accordingto disease stage, between 300–400 mg d^{-1}patient^{-1}, and its excretion range via urine is 50–70% within 24 h. The photodegradation processes were performed with 25 mg L^{-1} of INH,10 mg of Fe^{2+}, and 125 mg L^{-1} of H_2O_2 for 120 min. The degradation of INH was monitored by HPLC-DAD and dissolved organic carbon (DOC). The degradation by- products were analyzed by UPLC QToF-MS and the toxicity was evaluated by *Daphniamagna* (acute toxicity) and *Lactuca sativa* (phytotoxicity). INH removal was higher than99% at 60 min for Fenton, 10 min for photo-Fenton, and 15 min for solar photo-Fenton. The DOC results indicated that under light irradiation (photo-Fenton and solar photo-Fenton), the mineralization rate was > 70%. The nitrite ion analysis indicated the complete oxidation of nitrogen. Besides the main INH degradation by-products, isonicotinic acid, isonicotinamide, and *N*'-(pyridyl-4-carbonyl)-hydrazide, we detected four unknown by-products by mass spectrometry. The Fenton treatment decreased the toxicity of *D. magna* by 75% in 15 minutes, achieving 87% in 60 minutes of treatment, while the photo-Fenton process conducted with artificial radiation reduced 87% of the toxicity of *D. magna* and resulted in no significant effect in *L. sativa* seeds in 15 minutes(Q_{UV-A} 1.29 kJ L^{-1}). However, solar photo-Fenton (Q_{UV-A} 3.38 kJ L^{-1}) did not reduce toxicity.

Keywords: AOP, solar photo-Fenton, pilot-scale, *Daphnia magna*, *Lactuca sativa*, mass spectrometry

1. Introduction

Pharmaceutical active compounds, such as antibiotics, affect physiological function and are usually designed to be persistent in the target organism [1,2]. Since conventional wastewater treatment plants were not designed to remove these contaminants, they can be found in wastewater discharge at a concentration range from ng L^{-1} to µg L^{-1}, reported as contaminants of emerging concern (CECs) [3–5].

Antibiotics play an essential role in developing antibiotic-resistant bacteria strainsin different matrices [6,7]. The upward trend of tuberculosis cases observed in Brazil during 2016–2018 instigated a particular worry in the Region of the Americas. The isoniazid (INH) molecule, chemically expressed as pyridine-4-carbohydrazide ($C_6H_7N_3O$; molecular weight: 137.14), is the first-line antibiotic used in tuberculosis treatment, especially in developing countries, such as Brazil [8]. Following the Brazilian Ministry of Health guideline, the INH dosage in tuberculosis varies with treatment stageand body weight, from 300–400 mg d^{-1} $patient^{-1}$, as recommended by the World Health Organization [9]. According to Douglas and McLeod [10], when considering adults withnormal renal function who take 5 mg kg^{-1} per 24 hours of INH, the excretion of this drugvia urine in its unchanged form or as metabolites varies from 50–70%. Therefore, in the absence of proper treatment, these compounds can be discharged into water bodies.

Over the recent years, different advanced treatment techniques have been appliedfor CEC removal. Among them, advanced oxidation processes (AOPs) are used to treat highly non-biodegradable substances. AOPs mainly generate strongly oxidizing species, such as free hydroxyl radicals ($HO^{\bullet}$) *in situ* [11], which can degrade macromolecules into short-chain organic acids, inorganic ions and CO_2 as final products.

Since INH is not readily biodegradable according to guideline test results [12], few AOP studies have evaluated its degradation. These AOPs include photocatalysts [13– 15], electrochemical processes [16], and UV light processes [17], which require an extended time for treatment or high accumulated energy to degrade it. On the other hand,Fenton (F) and photo-Fenton (PF) in the homogeneous and heterogeneous processes haveshown to be excellent alternatives to the degradation of several classes of organic water pollutants in short times of reaction [18]. For example, Stets et al. [19] reported the INHdegradation of 70% and 7% for the homogeneous and heterogeneous F processes at 60 minutes, respectively.

The advance of liquid chromatography technology as Liquid Chromatography Mass Spectrometry (LC-MS) promotes the analysis of specific compounds through mass fragmentation. Guevara-Almaraz et al. [17] reported that INH degradation byphotocatalysts under UV light leads to the following by-products: isonicotinamide,isonicotinic acid, and pyridine. However, this was not found in the literature on INH by-products by F and PF treatments, to the best of our knowledge.

Studies have showed that by-products formed as a result of AOPs may result in compounds more toxic than their parent molecules[20,21]. To date, data regarding INH by-products toxicity is still scarce [22], whereas studies have focused on monitoring methods, environmental risks and removal efficiency for this compound [17,19,23,24]. In general, there is a lack of knowledge on the toxicity effects of CEC degradation and how its presence in the environment can negatively impact the ecosystem and human health [11]. In this perspective, monitoring toxicity changes by bioassays are crucial for evaluating INH degradation by AOPs. Phytotoxicity evaluation by *Lactuca sativa* is an alternative in the search for the possible effects of fertigation with water treated by AOPs

[25]. Toxic substances can interfere in germination and typical root development in the first days of the seedling. Also, although *L. sativa* is not an aquatic ecosystem species, data generated from this bioassay provides information regarding the possible effects on plant communities near the margins of wastewater discharge [26]. In addition, acute toxicity of *Daphnia magna* can indicate a potential toxicological impact on the aquatic ecosystem when these treated matrices are discharged into surface water bodies [27].

In this study, we evaluated INH degradation by the AOPs (Fenton [F], photo- Fenton [PF] and solar photo-Fenton [solar-PF]on a pilot scale) using liquid chromatography to analyze the efficiency of the processes. Mass spectrometry verified its by-products during treatment. In addition, bioassays with *L. sativa* and *D. magna* tookplace to monitor acute ecotoxicity before and after the degradation processes.

2. Materials and Methods

2.1 Chemicals and solutions

Isoniazid (99% purity; Sigma-Aldrich) was used as the analytical standard in the photodegradation processes. Acetonitrile (ACN), phosphoric acid (H_3PO_4), and methanol (MeOH) were (HPLC) grade (JT Baker). Acetone (HPLC grade; Honeywell), sulfuric acid (H_2SO_4, 97%; Fluka), oxalic acid ($C_2H_2O_4.2H_2O$, >99.5% purity; Fisher Chemical),and Nitrite TraceCERT® (Supelco) were used for ionic chromatography. Iron (II) sulfateheptahydrate, sodium bisulfite, and potassium chloride were purchased from Vetec (99%purity). Hydrogen peroxide (30% w/v) and Allper® reagent were obtained from Peróxidos do Brasil Ltda. Hydroquinone and 1,10-phenanthroline (99% purity) were supplied by Sigma-Aldrich. Bovine liver catalase (2000–5000 units per mg of protein, Sigma-Aldrich) was used to remove residual H_2O_2 before bioassays. For other analyses

and to stop the reaction by H_2O_2, $NaHSO_3$ (40% w/v) was used. This work was conductedin partnership with a hospital specializing in tuberculosis treatment in the state of Paraná,Brazil, with a capacity of 40 inpatients, that prescribes INH in the range of 7500–10000 mg d-1.

All chemicals were used without further purification. Stock standard solutions were prepared by dissolving INH in ultrapure water from Millipore (Bedford, MA) at a concentration of 25 mg L^{-1} at a pH 3. The Fe^{2+}/Fe^{T} and residual H_2O_2 concentrations weredetermined by UV-Vis spectrometry (Varian, Cary 50 Bio, Australia). The iron ions were analyzed using 1,10-phenanthroline as a complexing agent (ISO 6332:1998) with limits of quantification (LOQ) of 0.20 mg L^{-1}. The residual H_2O_2 was evaluated by Schick et al.

[28] methodology using Allper® reagent with a limit of quantification (LOQ) of 0.5 mg L^{-1}. A TOC analyzer (HiperTOC®, Thermo Scientific, United Kingdom) determined thedissolved organic carbon with a LOQ of 0.5 mg L^{-1}. The INH (5 mg L^{-1}) stability test wasperformed for seven consecutive days and the solution was kept at 4 °C in the absence oflight and quantified by chromatographic analysis.

2.2 Isoniazid quantification by HPLC-DAD

The detection and quantification of INH were performed by High-Performance Chromatography with diode-array detection (HPLC-DAD) (Agilent Technologies, 1260 Infinity) equipped with a quaternary pump (G1311B) and autosampler (G1329B), coupled with a diode array detector (G4212B). Agilent ChemStation Software® was usedto process the data. Chromatographic separation took place in a Zorbax Eclipse Plus C18analytical column (250 mm x 4.6 mm; particle size 5 μm; Agilent Technologies) using mobile phase acetonitrile and dibasic hydrogen phosphate buffer solution ($6.29x10^{-3}$ $molL^{-1}$, pH adjusted to 6.8 with H_3PO_4) with a gradient program set by USP-NF 37 (injection

of 50 µL and flow rate of 0.8 mL min^{-1}). The percentual RSD, accuracy, and selectivity were evaluated (Table S1; Figures S1 and S2). The limit of detection (LOD) and the LOQwere 1 and 50 µg L^{-1}, respectively.

2.3 Degradation experiments

The F and PF experiments were conducted on a bench scale in a conventional photochemical borosilicate reactor with 600 mL of INH solution, equipped with water recirculation and magnetically stirred for 120 minutes. According to the INH dosage usedin the tuberculosis hospital, the initial concentration of INH was 25 mg L^{-1} for the F and PF processes. The experiments were performed in an aqueous solution at an initial pH adjusted to 3.0, H_2O_2 at 125 mg L^{-1}, and Fe^{2+} at 10 mg L^{-1}. After every 10 minutes of treatment, the H_2O_2 concentration was corrected to the initial condition (125 mg L^{-1}). Forthe PF process, the solution was irradiated with UV-A light (range from 315–800 nm) using a 125 W high-pressure mercury vapor lamp (without its original glass bulb) covered with a Pyrex bulb into the solution. Aliquots (3 mL) were withdrawn at regular intervalsand filtered through a 0.45 µm Millex-HA filter (Millipore). The residual H_2O_2 was removed using $NaHSO_3$ (40% w/v) to determine the INH concentrations by HPLC-DADand DOC analyses. For the bioassays, the pH of the sample was adjusted to 6–7, and residual H_2O_2 was removed by adding bovine catalase solution (1% w/v). Experiments ofF and PF using INH concentration of 75 mg L^{-1} were performed to identify by-products generated during the treatments at the times of 5, 10, 15, 30, and 45 minutes in the sameconditions previously mentioned.

2.3.1 Solar photo-Fenton pilot plant

Solar-PF experiments were performed in Curitiba, Brazil (25°26'37.2" S 49°21'12.6" W), by a pilot compound parabolic collector (CPC) solar plant designed for

solar photocatalytic applications. The reactor is comprised of five Pyrex glass tubes (2 cm internal diameter and 95 cm length) arranged in high reflectivity aluminum solar collectors on a fixed platform tilted (45º), with a total illuminated area of 1.25 m^2 and a total volume of 1.5 L (Figure S3). The pilot-scale experiments were carried out at bench scale experiment conditions (25 mg L^{-1} of INH, 125 mg L^{-1} of H_2O_2, 10 mg $L^{-1}$of Fe^{2+}) ata flow rate of 0.3 L min^{-1}. The hydraulic residence time was five minutes and after this time, the sample was recirculated into the reactor for 120 minutes (24 cycles of 5 minutes). The INH concentrations were measured before and after each five minutes of the treatment cycle.

Considering the differences between the conventional photochemical reactor at the bench scale and the CPC at pilot-scale as the average irradiance, surface area, and total volume of solution, the incident radiation was measured using a global radiometer (Instrutherm, MRUR 202) for UV-A radiation (320–390 nm). The accumulated energy per unit of volume ($Q_{UV\text{-}A}$, kJ L^{-1}) was determined according to Malato et al. [29] (Eq. 1),which was also used by other authors for different reactor features [30–32].

$$Q_{UV,n} = Q_{UV,n-1} + \Delta_t UV_{G,n} \left(\frac{A_r}{V_t}\right) \quad (1)$$

where t_n is the sampling time, V_t is the total reactor volume, A_r is the illuminated collector surface area, and $UVA_{G,n}$, the average solar or artificial UV-A radiation, is measured during the period $\Delta_{tn} = t_n - t_{n-1}$.

2.4 Degradation characterization by ionic chromatography

After INH (75 mg L^{-1}) degradation by F and PF processes, 10 mL of every treatment time was analyzed by ionic chromatography (930 Compact IC Flex, Metrohm) with conductivity detection after sequential suppression with a self-regenerating anion

suppressor, Metrohm Suppressor Module MSM (100 mmol L^{-1} H_2SO_4, 140 mmol L^{-1} oxalic acid, and 680 mmol L^{-1} acetone). An isocratic separation of anions was performedusing an anion-exchange column, Metrosep A Supp 7 (250 x 4.0 mm, 5 µm) and sodiumcarbonate 3.6 mmol L^{-1} as an eluent at 1.0 mL min^{-1}. The column temperature was kept at 58 ºC, and the injection volume was 20 µL. Before analysis, the samples were filteredby a PTFE filter (0.45 µm). The accomplished retention times were 8.88 minutes and
12.36 minutes of nitrite and nitrate, respectively. The detection range was estimated between 0.0025–5.0 mg L^{-1}. LOD (for a signal-to-noise ratio [S/N] of 3) and LOQ (S/N of 10) were also determined (Table S2).

2.5 Isoniazid by-product identification by UPLC-QToF-MS

The INH by-products for F and PF processes were carried out using 75 mg L^{-1} ofINH. The fragmentation assignment spectra were supported by the MS/MS profile of eachcompound. The UPLC system consisted of an Acquity UPLC H-Class system (Waters Corp., Milford, U.S.A.) coupled to a hybrid quadrupole time-of-flight mass spectrometerXevo G2-S (Waters Corp., Milford, U.S.A.). The mass spectrometer was equipped with an electrospray (ESI) ionization operating in the positive and negative modes. Chromatographic separation occurred in an Agilent InfinityLab Poroshell 120 EC-C18 column (150 x 4.6 mm, 2.7 µm) coupled with an Agilent InfinityLab Poroshell 120 EC-C18 column guard column (5 x 2.1 mm, 2.7 µm), and kept at 30 °C. The elution wasisocratic with a mobile phase system consisting of water containing formic acid (0.1%) and a mix of acetonitrile and formic acid (0.1%) in the ratio of 95:5 (v/v), at a flow rate of 0.3 mL min^{-1}. The total run time was 10.0 minutes, and the injection volume was 10 µL. Electrospray parameter conditions were the same as developed by Fachi et al. (2020)[33]. Leucine enkephalin (m/z 556.2771 $[M+H]^+$ and m/z 554.2671 $[M-H]^-$) was

the lockmass for mass shift correction. Data acquisition was ascertained by MassLynx™NT4.1 software (Waters Co., Milford, U.S.A.) in centroid mode. MS data were acquiredover the m/z range of 40–300. Peak identification performance followed the massaccuracy in MS function considering suitable values of mass error of less than 11 ppm, with subsequent fragmentation of positive and negative in the MS/MS mode.

2.6 Ecotoxicity bioassays

For the ecotoxicity evaluation, samples were collected in previously washed glassflasks at initial, intermediate, and final treatment times. When necessary, the pH was adjusted to 6–7, and bovine catalase (1% v/v) was added to remove residual H_2O_2. Samples were kept frozen at −20 °C for a maximum of 20 days until the tests were performed. Two bioassays were selected: *L. sativa* seeds (phytotoxicity, producer) and *D. magna* (acute toxicity, primary consumer).

2.6.1 Phytotoxicity with *Lactuca sativa* seeds

The seed germination and root elongation phytotoxicity test was adapted from themethodology described by Sobrero and Ronco [26] and Young et al. [34], using commercial lettuce seeds (white Boston variety). The tests were carried out in Petri dishes(100x15mm) lined with filter paper (80 g/m^2) and 15 seeds each, containing 4 mL of sample or negative/positive control (ultrapure water and commercial glyphosate solution6%, respectively), within triplicate. The seeds were incubated at 22 ± 2 °C in the dark for120 h. At the end, the germination index (GI) and relative growth index (RGI) were calculated [34,35]. Data were subject to the Kolmogorov-Smirnov normality test, and as it was normally distributed, it proceeded to analysis using the one way analysis of variance (ANOVA) and Dunnett post-test ($\alpha = 0.05$).

2.6.2 Acute ecotoxicity with *Daphnia magna*

The acute ecotoxicity of *D. magna* was assessed according to the methodology described by the Brazilian Guideline [36]. Ten neonates (6–24 h) were used for every three replicates. Samples were diluted in a culture medium at 100 (non-diluted), 50, 25, 12.5, and 6.25% (v/v). The negative control was the culture medium, and the positive control was potassium chloride. All tests were maintained at 20 ^{o}C. After 48 h of exposure, the number of immobile organisms was recorded. The results were expressed in terms of the toxicity factor (TF), which corresponds to the highest sample solution at which no toxic effect is observed [36].

3. Results and Discussion

3.1 Control experiments: radiation, H_2O_2, and Fe^{2+}

The stability tests of INH (Figure S2) in an aqueous solution showed that after seven days of analysis, INH remained stable. The pH is a critical operating variable in thetreatment process that influences degradation. The optimum pH of the classic Fenton reaction is 2.8–3.2 due to the presence of iron species with a more significant light absorption coefficient and quantum yield for $HO^{\bullet}$ production [37]. Razak et al. [38] observed that the acidic medium favors INH stability and does not significantly contributeto the formation of INH by-products [38]. Hence, the pH value measured during the reaction remained constant (pH ~3) until the end of the treatments. In addition, we performed control experiments to determine the effect of radiation, H_2O_2 (125 mg L^{-1}), Fe^{2+} (10 mg L^{-1}), and H_2O_2/radiation (Table S3). The isolated radiation, Fe^{2+}, and H_2O_2 achieved a maximum of 9.5% INH degradation in 120 minutes, which is mostly achievedin the first 15 minutes of reaction. The combined radiation with H_2O_2 removed 96.0% ofINH within a gradual degradation during the reaction time. However, it achieved only

9.6% of mineralization (DOC analysis). These results agree with the control tests of isolated radiation and chemicals in removing CECs [39]. Hence, standalone reactants could not effectively remove INH, making AOPs necessary to remove INH, minimize itstreatment time or achieve mineralization.

3.2 Fenton process

The chromatogram profiles for F treatment (INH, 25 mg L^{-1}; pH, 3.0; Fe^{2+},10 mg L^{-1}; and H_2O_2, 125 mg L^{-1}) are shown in **Figure 1.** The concentration of H_2O_2 as remained constant during 120 min of reaction with H_2O_2 reinjection into the system when

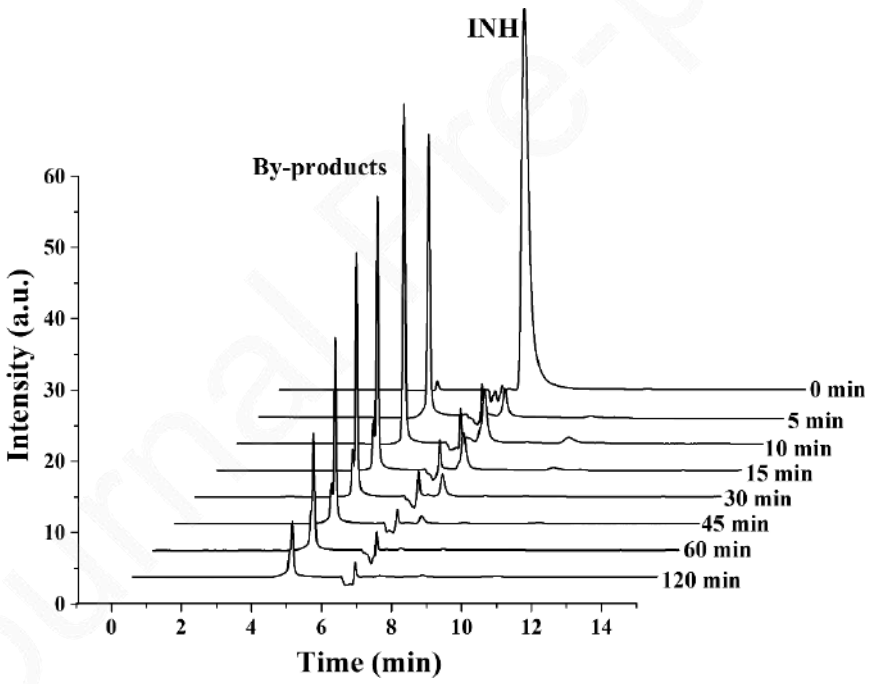

Figure 1. Isoniazid chromatograms before and after the Fenton process (HPLC-λ = 262 nm, 25 mg L^{-1} of INH, 10 mg L^{-1} of Fe^{2+} and 125 mg L^{-1} of H_2O_2) duringtreatment times.

In 60 minutes of treatment, the F process removed the initial concentration of INHto at least 0.001 mg L^{-1} (< LOD), achieving efficiency higher than 99.99%. It was observed that INH degradation was faster in the initial five minutes of treatment, in which

~14% of available Fe^{2+} was converted to Fe^{3+}, resulting in 86% of INH degradation. Also,in 15 minutes, 91% of initial H_2O_2 was consumed. According to the speciation of iron compounds, the main reaction occurred at 45–60 minutes of treatment, in which INH removal efficiency were 94% and >99.99%, respectively (Figure S4a).

For the F process, the HPLC-DAD analysis (Figure 1) showed two leading by- product elution bands in the first 5 minutes of the reaction. The first peak showed a retention time (t_R) of 6.38 minutes (λ = 260 nm) with low intensity, corresponding to isonicotinamide and isonicotinic acid [17,22]. The second peak presented an overlappingat t_R = 4.8 minutes, which can be related to unidentified by-products as a non-specific signal. The intensity of these peaks decreased throughout the reaction. Although they were present at the end of the treatment, this indicates the feasibility to degrade these compounds. Also, another small peak at t_R = 9.4 minutes was generated at 10 minutes ofthe reaction, and it was completely degraded after 15 minutes of F treatment. The DOC decreased only 22% in 120 minutes (Figure S4a), demonstrating drawbacks to themineralization by the F process.

3.3 Photo-Fenton process

The chromatographic results for INH degradation by PF are presented in **Figure**

2.

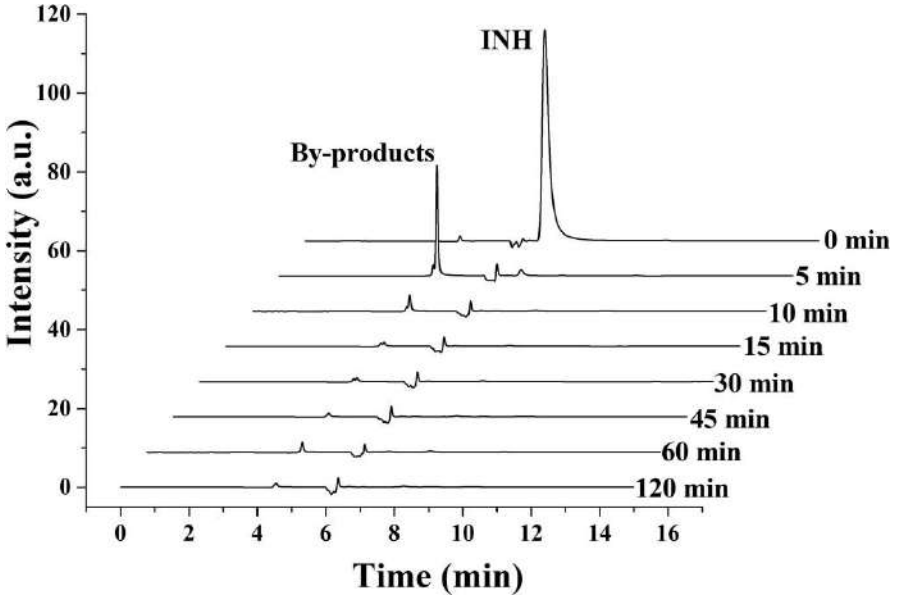

Figure 2. Isoniazid chromatogram before and after the photo-Fenton process (HPLC- DAD; λ = 262 nm, 25 mg L^{-1} of INH, 10 mg L^{-1} of Fe^{2+} and 125 mg L^{-1} of H_2O_2) during treatments.

The INH concentration decreased from 25 mg L^{-1} to 0.51 mg L^{-1}, achieving a degradation efficiency of 91.8% after 5 minutes with PF treatment with a Q_{UV-A} of 0.43 kJ L^{-1}. At 10 minutes of treatment (Q_{UV-A} of 0.86 kJ L^{-1}), INH was no longer detected bythe analytical method (< LOD), resulting in degradation higher than 99.99%. Throughoutthe PF treatment, unknown by-products generated from INH were identified under the same chromatographic conditions as the F process, with similarity in the absorbance spectrum profile and retention times (Figure 1). The light radiation (320 nm > λ < 600 nm) can accelerate the oxidation rate compared to the F process. In addition, UV light irradiation produces extra $HO^{\bullet}$, formed by two additional mechanisms: a) photoreductionof Fe^{3+} to Fe^{2+} ions (Eq. 2–3) and b) H_2O_2 photolysis via shorter wavelengths (Eq. 4) [40].

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^\bullet \quad (2)$$

$$Fe^{3+} + H_2O + h\nu \rightarrow Fe^{2+} + HO^\bullet + H^+ \quad (3)$$

$$H_2O_2 + h\nu^{(\lambda < 300\ nm)} \rightarrow 2HO^\bullet \quad (4)$$

While the INH concentration decreased in the first 5 minutes of treatment, the treatment evaluation presented the same peaks at t_R = 6.38 and t_R = 4.8 min (Figure 2), asobserved in the F process with the same chromatographic conditions (Figure 1). However,the intensity of these peaks decreased faster than with the F process, indicating the feasibility of the PF approach compared to the F process. In first 10 minutes of the reaction, the accumulated energy $Q_{UV\text{-}A}$ was 0.86 kJ L^{-1} and practically all oxidant H_2O_2 available was consumed (residual concentration ≤ 20 mg L^{-1}). Therefore, its reinjection was necessary to achieve the initial H_2O_2 concentration value (125 mg L^{-1}) every 10 minutes until the end of the PF treatment. On the other hand, during the F reaction, Fe^{2+} presented oscillations and converted slightly to Fe^{3+}, with the expressive transformation of Fe^{2+} within 45 minutes of treatment (Figure S4a). The photoirradiation-based process consumed more oxidant H_2O_2 than the Fenton classic reaction. Thus, it increased $HO^\bullet$ in the aqueous solution and consequently, the reaction rate. Nevertheless, the high amount of $HO^\bullet$ generation can produce unwanted side reactions that consume available $HO^\bullet$ and H_2O_2 in the system (Eq. 5–6) [41,42]. In small amounts of Fe, ferrous regeneration is therate-limiting step of the catalytic iron cycle. Nevertheless, PF treatment presented an oscillation of the $Fe^{2+/3+}$ transformation (Figure S4b), which, combined with H_2O_2 consumption, indicated the occurrence of the PF process [43].

$$HO^{\bullet} + H_2O_2 \rightarrow HO^{\bullet}_2 + H_2O \quad (5)$$

$$HO^{\bullet} + HO^{\bullet}_2 \rightarrow H_2O + O_2 \quad (6)$$

The DOC results (Figure S4b) showed only 2.5% of INH mineralization in the first 5 minutes of the PF process (Q_{UV-A} of 0.43 kJ L^{-1}), demonstrating the persistence of intermediate products. However, in 120 min of reaction during the PF process conductedwith artificial radiation, mineralization of the solution achieved 86.6% the accumulated energy Q_{UV-A} of 10.30 kJ L^{-1}. Nitrite and nitrate anions were expected to remain in the samples after degradation based on the chemical structure of INH. Treated solutions of the F and PF processes analyzed by ionic chromatography showed only nitrate ions in allsamples above the LOQ (0.040 mg L^{-1}), as shown in Figure S5. The presence and increase of nitrate ion concentration indicated that the initial N converted into NH_4^+ ion, which was oxidized subsequently until NO_3^-, confirming the observed overall mineralization. Most likely, part of N from INH was also lost as volatile nitrogenated compounds [44].

3.4 Solar photo-Fenton on a pilot-scale

The PF under solar irradiation proved to be efficient for INH degradation in the first 15 minutes, with an accumulated energy Q_{UV-A} of 0.42 kJ L^{-1} achieving removal > 99.99%. This result is consistent with that obtained during the PF process performed on the bench scale, in which a Q_{UV-A} of 0.43 kJ L^{-1} degraded 91.8% of INH and a Q_{UV-A} of
0.86 kJ L^{-1} achieved efficiency > 99.99%. Furthermore, in comparison to PF performed with artificial radiation (~87% of mineralization with Q_{UV-A} of 10.30 kJ L^{-1}), the mineralization of DOC completed ~72% at the end of this process with a Q_{UV-A} of 3.38 kJ L^{-1}. The accumulated energy achieved in this study is in agreement with the values obtained by Micheletto et al. [31] using a CPC reactor in the solar-PF performed in the

municipality of Curitiba, Brazil. A comparison of INH degradation efficiency and DOC mineralization evaluated in this study is summarized in **Table 1**.

Table 1. Comparison of isolated radiation, Fe^{2+}, Fe^{2+} and radiation, H_2O_2, H_2O_2 andradiation, during the Fenton, photo-Fenton performed by artificial radiation, and solar-PF processes in the INH degradation

Process	Radiation	Fe^{2+} (10 mg L^{1})	H_2O_2 (125 mg L^{-1})	Radiation and H_2O_2 (125 mg L^{-1})	Fenton	PF	Solar-PF
Final time (min)	120	120	120	120	120	120	120
Q_{UV-A} (kJ L^{-1})	10.30	-	-	10.30	-	10.30	3.38
INH degradation (%)	3.83	1.96	9.48	96.04	>99.99	>99.99	>99.99
DOC (%)	7.16	-0.43	7.56	9.68	22.52	86.61	71.68

The F, PF, and solar-PF processes promoted INH degradation > 99.99% (INH concentrations < LOD); however, the time to achieve this was 10 minutes for PF (Q_{UV-A} 0.86 kJ L^{-1}) by artificial radiation, 15 minutes for solar-PF (Q_{UV-A} 0.42 kJ L^{-1}), followed by 60 minutes for F. Trovó et al. [45] evaluated the PF process ([Fe^{2+}] = 11.8 mg L^{-1}, [H_2O_2] = 188.1 mg L^{-1} and pH = 2.5–2.8) under artificial radiation (400 W high-pressuremercury vapor lamp) and solar radiation on a CPC reactor to degrade the herbicide paraquat (50 mg L^{-1}), reporting Q_{UV-A} of 1284 and 146.9 kJ L^{-1}, respectively, during 120minutes of treatment. Despite the highest power of artificial radiation, the authorsreported that both experiments obtained the same efficiency with ~89% of mineralizationby artificial radiation and ~83% by sunlight. Therefore, notwithstanding the nominal potency of artificial radiation, according to Malato et al. [29], the CPC reactor being a static reactor that consists of some Pyrex tubes coupled in series and irradiated by

concentrated solar radiation by the parabolic reflective surface, it can achieve high removal efficiencies [46].

These results showed that the light-dependent process, PF under artificial radiation and natural sunlight, achieved more than 70% of mineralization. Therefore, it may be due to Fe^{2+} regeneration and reaction rate acceleration generating additional $HO^{\bullet}$. Although PF with artificial irradiation demonstrated ~15% higher mineralization than solar-PF, the feasibility of solar-powered treatment becomes apparent, even though solar- PF requires a higher accumulated energy. Besides that, solar light is believed to be one ofthe most environmentally friendly and cost-effective processes [41]. Since solar degradation experiments by solar photocatalytics in the treatment of several compounds achieve successful results, economic studies have been conducted to obtain information on possible costs for this technology application [47,48].

3.5 Analysis of intermediate products

The identification of INH degradation by-products from samples treated by F andPF under artificial radiation on a bench scale was performed by mass spectrometry. The analysis was developed by the positive electron spray ionization (ESI) mode for the drugand its by-product identification by single and tandem high-resolution MS/MS data/mode. In order to identify the by-products during INH degradation processes, F andPF experiments were carried out using a higher concentration of INH (75 mg L^{-1}). In theseexperiments, aliquots were withdrawn at 5 and 10 minutes, and the chromatographic profile of PF and F processes after 5 and 10 minutes of treatment were similar. An example of the chromatographic profile of the PF process after 10 minutes of treatment is shown in Figure 3. Detected were six major by-products, besides INH, during the PF process. Furthermore, the exact mass of the compounds at each elution band was

compared to the exact mass of known INH degradation by-products from the literature [16,22,49–52].

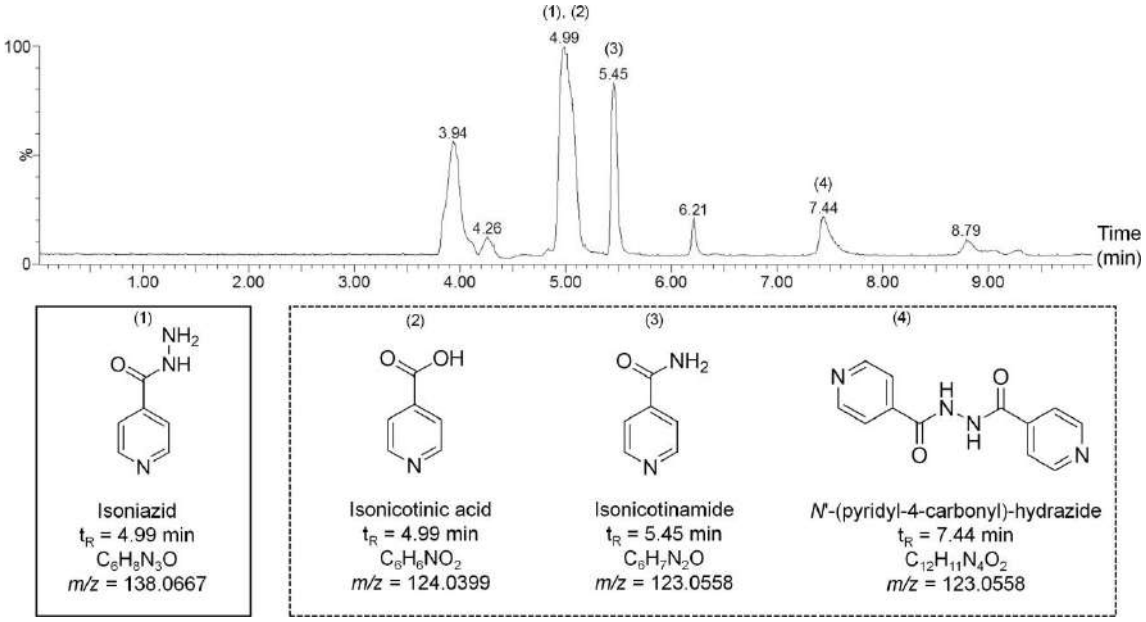

Figure 3. Standard isoniazid chromatogram after the photo-Fenton process performed by artificial radiation on a bench scale (HPLC-TOF-MS, 75 mg L^{-1} of INH, 10 mg L^{-1} of Fe^{2+} and 125 mg L^{-1} of H_2O_2) after 10 minutes of treatment and identified by-products

INH was detected at t_R of 4.99 minutes since the ESI (+)-MS/MS analysis of thiselution band showed the molecular ion for the protonated compound with *m/z* $[M+H]^+$ 138.0682 (calculated exact mass for $C_6H_8N_3O$, 138.0667). Additionally, at t_R of 4.99 minutes, we also identified isonicotinic acid, a well-known by-product of INH degradation by oxidation or photolysis [51], with the molecular ion in its protonated formin the ESI (+)-MS/MS analysis with *m/z* $[M+H]^+$ 124.0428 (calculated exact mass for $C_6H_6NO_2$, 124.0399). The ESI (+)-MS/MS analysis of the elution band at t_R of 5.45 minutes is related to isonicotinamide since the molecular ion of the protonated form has

the *m/z* $[M+H]^+$ 123.0594 (calculated exact mass for $C_6H_7N_2O$, 123.0558). Furthermore,at t_R of 7.44 minutes, there exists *N*'-(pyridyl-4-carbonyl)-hydrazide, which, in the ESI (+)-MS/MS analysis, shows the molecular ion in the protonated form with *m/z* $[M+H]^+$ 243.0896 (calculated exact mass for $C_{12}H_{11}N_4O_2$, 243.0882). It is worth mention that Bhutani et al. [51] proposed *N*'-(pyridyl-4-carbonyl)-hydrazide as a decomposition product of INH exposure to light. Despite the observation of well-known INH degradationby-products by ESI (+)-MS/MS, we were not able to identify the compounds related to the elution bands at t_R of 3.94, 4.26, 6.2, and 8.79 minutes corresponding to molecular ions with *m/z* 158.0043, 226.9532, 202.1819 and 292.1060, respectively.

3.6 Ecotoxicity evaluation

Evaluating by-products generated during treatment is crucial since these by- products could result in either lesser or greater toxicity than the target compound. Thus, acute toxicity in microcrustacean *D. magna* (consumer) and *L. sativa* seeds (lettuce, producer) were assessed before and after treatments. The results are summarized in **Table2**.

Table 2. Acute toxicity of INH before and after degradation by Fenton, photo-Fenton, and solar photo-Fenton processes

Time	Fenton		Photo-Fenton		Solar photo-Fenton	
(min)	*D. magna* (TF)	*L. sativa*	*D. magna* (TF)	*L. sativa*	*D. magna* (TF)	*L. sativa*
0	16	I	16	I	16	I
15	4	I	2	NSE	> 32	I
60	2	I	2	NSE	> 32	I

Note:TF = toxicity factor; I = inhibition of seed growth; NSE = Non-significant effect

Initially, INH (25 mg L^{-1}) solution without treatment was toxic to *D. magna* and *L. sativa*, with TF 16 and root growth inhibition, respectively. The EC_{50} of INH for *D. magna* is ~23 mg L^{-1}, similar to the initial concentration INH (25 mg L^{-1}) in this work [53], explaining the higher TF value before treatment. However, after 15 minutes of treatment, the PF process conducted with artificial radiation reduced the toxicity of *D. magna* by 87% (TF 2) and resulted in no significant effect in *L. sativa* seeds, and these results remained constant after 60 minutes of treatment. It might be related to the greaterdegree of degradation and mineralization of INH (Figure S4b), which may have led to theformation of lesser toxic by-products, such as isonicotinic acid and pyridine, which are intermediates in the photocatalytic degradation of INH [17]. In the case of the F process,the toxicity for *D. magna* decreased by 75% (TF 4) in 15 minutes, achieving 87% (TF 2)in 60 minutes of treatment.

In contrast, solar-PF resulted in a more toxic matrix after treatment, showing TF > 32 in 15 and 60 minutes. These responses were probably due to the different mineralization levels obtained during the processes and the different by-products formedduring treatment. The AOPs first generated isonicotinic acid and isonicotinamide, followed by isonicotinaldehyde and pyridine. Finally, organic acids and other lower molecular weight compounds were present at the end.

Regarding the *L. sativa* seeds, there are no reports in the literature of INH EC_{50} value. However, except for the PF process, all treatment settings and times resulted in theinhibitory growth of the seeds. The results obtained by Liu et al. [54] also demonstrated that the toxicity of pyridine to *Aliivibrio fischeri* is minor (EC_{50} = 4.73 mmol L^{-1}), in comparison to that of isonicotinic acid (EC_{50} = 0.36 mmol L^{-1}). Sax et al. [55] observed

that the pyridine compound was less toxic than INH in rats. Faria et al. [56] and Garcia- Segura and Brillas [57] reported that the organic acids and aldehydes generated at the endof INH degradation are less toxic than other intermediates and INH itself. Coronado- Castañeda et al. [14] studied INH degradation by heterogeneous photocatalysis with β- $Bi2O_3$ and observed that the toxicity decreased after treatment, regardless of the matrix used (deionized water or wastewater). According to Wang and Wang [58], AOPs can result in different responses in organisms: i) toxicity reduction during AOP treatment; ii)toxicity increases in the beginning of the treatment and then a decrease; or iii) toxicity increases during AOP treatment. The toxicity responses after AOPs can be due to many factors, such as the types of reactive species, structure of organic pollutants, the concentration of reactive species, toxicity bioassay used, experimental parameters, residual oxidizers, and heterogeneous catalysts. For instance, Angeli et al. [21] verified the oscillation in TF of *D. magna* during the homogeneous photocatalysis treatment of the commercial pesticide chlorpyrifos. Therefore, toxicity reduction occurred at differenttimes and achieved efficiency following the sequence of 15 minutes for PF conducted with artificial radiation (Q_{UV-A} 1.23 kJ L^{-1}) > 60 minutes for F > toxicity increase for solar-PF (Q_{UV-A} 0.42–1.69 kJ L^{-1}). These observations reveal the overall treatment efficiencies, importance and need for toxicity evaluation according to the possible by- products generated throughout the treatment processes.

4. Conclusion

The degradation of INH by PF treatment was more efficient than the F process, achieving INH degradation > 99.99% in 10 minutes (Q_{UV-A} 0.86 kJ L^{-1}) and 60 minutes, respectively. In addition, PF performed by artificial radiation resulted in a higher

mineralization percentage (87%) compared to the F process (22%). Although INH degradation by solar PF in a CPC reactor resulted in > 99.99% removal of INH in 15 minutes of treatment (Q_{UV-A} 0.42 kJ L^{-1}), the results for solar PF showed mineralizationof 72% in 120 minutes of treatment (3.38 kJ L^{-1}). Therefore, it is noted that, under natural sunlight, treatment requires an extended time, compared to PF under artificial radiation,due to the radiation intensity difference between them. The MS/MS analyses for the Fand PF process showed that the INH molecule degraded to known degradation by-products, such as isonicotinic acid and isonicotinamide, and four unknown by-productsin the first 10 minutes of the treatments. At the end of the process, the transformation ofINH and its by-products to organic acids and other low molecular weight compoundsformed in different proportions, which justifies the responses in the different levels oftoxicity in *D. magna* and *L. sativa.* Toxicity reduction occurred at different times andachieved efficiency following the sequence: 15 minutes for PF conducted with artificialradiation (Q_{UV-A} 1.29 kJ L^{-1}, TF 2 in *D. magna*, and non-significant effect in *L. sativa*), >60 min for F (TF 2 in *D. magna*, and inhibition of seed growth in *L. sativa*) > toxicityincrease for the solar PF process (Q_{UV-A} 1.69–3.38 kJ L^{-1}, TF > 32 in *D. magna*, andinhibition of seed growth in *L. sativa*). These observations reveal the overall treatmentefficiencies, importance and need for toxicity evaluation according to the possible by-products generated throughout the treatment processes. Also, it indicated that, for solarPF, treatment time might be higher than PF under artificial radiation, resulting in non-toxic effects in these organisms due to the accumulated energy obtained in these cases. **Acknowledgments**

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Declaration of competing interest

The authors declare no competing interest.

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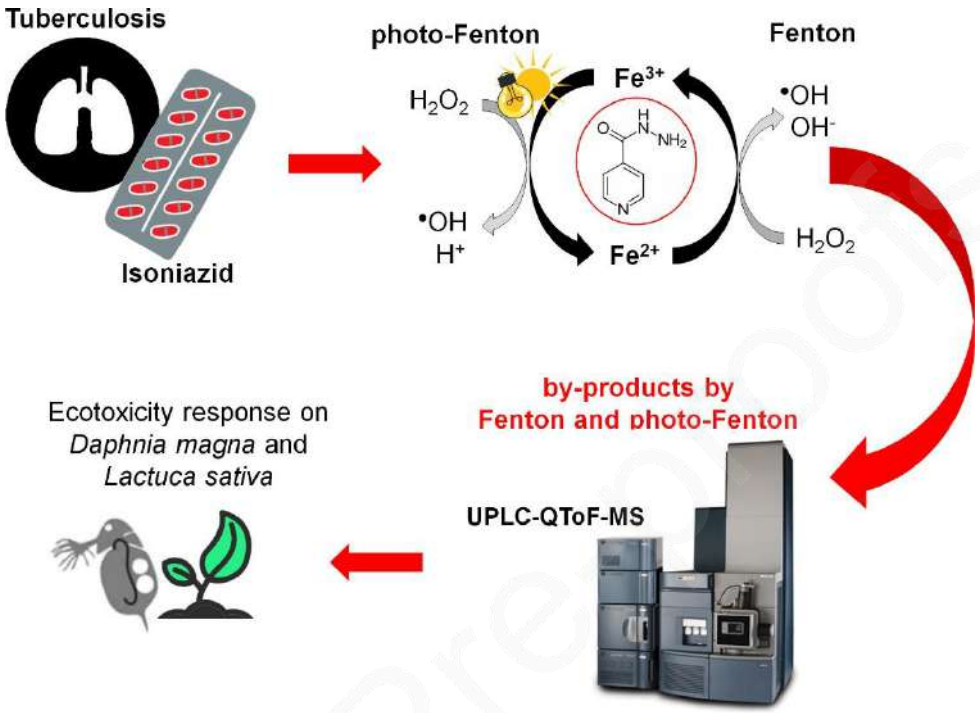
Graphical abstract



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Highlights

- Fenton-based processes can successfully degrade the antibiotic isoniazid used for tuberculosis treatment.
- Photo-Fenton by artificial and solar radiation achieves high mineralization of isoniazid.
- Fenton and photo-Fenton processes generate isonicotinic acid, isonicotinamide, *N*'-(pyridyl-4-carbonyl)-hydrazide and four new by-products in the first 10 minutes of treatment.
- Fenton reaction reduces acute toxicity in *D. magna* yet inhibits seeds growth in *L. sativa*, and photo-Fenton performed by artificial radiation reduces toxicity in both test organisms.
- Besides the Isoniazid degradation, solar photo-Fenton needs prolonged time to generate non-toxic results.

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Fernando R. da Silva: Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Camila R. Lemos:** Investigation, Methodology, Validation, Formal analysis, Writing – original draft. **Andressa A. Naico:** Formal analysis, Writing – original draft. **Mariana M. Fachi**: Investigation, Methodology, Writing – original draft. **Bianca do Amaral:** Validation, Investigation, Writing – original draft. **Vinícius C. S. de Paula:** Formal analysis, Validation, Writing – original draft. **Daniel S. Rampon:** Investigation, Writing – original draft Writing – review & editing**. Francisco Beraldi-Magalhães**: Investigation, Writing – original draft Writing – review & editing. **Liziê D. T. Prola**: Investigation, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Roberto Pontarolo**: Supervision, Project administration, Funding acquisition, Writing – review & editing. **Adriane M. de Freitas:** Conceptualization, Methodology, Supervision, Project administration, Funding acquisition, Writing – review & editing. **Marcus V. de Liz**: Conceptualization, Methodology, Supervision, Project administration, Funding acquisition, Writing – review & editing.

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Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal

relationships that could have appeared to influence the work reported in this

☐The authors declare the following financial interests/personal relationships which may beconsidered as potential competing interests:

9.5.7 Artigo 7

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High-performance immune diagnosis of tuberculosis: Use of phage display and synthetic peptide in an optimized experimental design

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ABSTRACT

Immunoassays are practical and cost-effective approaches suitable for large-scale tuberculosis (TB) screening. This study identified new peptide mimotopes of *Mycobacterium tuberculosis* and applied them in the serodiagnosisof TB. Thereby, linear (X_{15}, X_8CX_8) and constrained (LX-4 and LX-8) phage display peptide libraries were screened with purified Immunoglobulin G antibodies from TB-positive patients, and eight mimotopes were selected. The mimotope peptides were screened using the SPOT-synthesis technique followed by immunoblot- ting. Peptides P.Mt.PD.4 and P.Mt.PD.7 demonstrated the highest binding affinity and were chemically syn- thesized and used as antigens for enzyme-linked immunosorbent assay (ELISA) assays. Experimental designs were used to optimize the assays and to assess each variable's influence. Peptide P.Mt.PD.7 was differentiated between positive and negative samples and achieved 100% sensitivity and specificity when tested on a 100-sera panel. Therefore, the selected peptide was applied to the ELISA assay as a screening method for diagnosing TB represents a potential tool for helping to combat the disease.

1. Introduction

Tuberculosis (TB) is an infectious disease caused by bacteria of thegenus *Mycobacterium*, especially those of the *M. tuberculosis* complex (MTC) (Kanabalan et al., 2021). In 2020, data reported by 198 countries and territories accounting for more than 99% of the world population estimated that TB cases in 2019 were around 10 million, in addition to 1.4 million deaths (Koegelenberg et al., 2021). Up to then, TB was the leading cause of death from a single infectious agent. However, in 2020, COVID-19 exceeded TB in the number of deaths and had a negative impact on ongoing actions for TB prevention and control (Koegelenberg et al., 2021). The reallocation of TB resources, such as reagents for diagnosis, human resources, and financial funds to respond to the COVID-19 pandemic, may have led to an increase of 0.2 to 0.4 millionTB deaths in 2020 (World Health Organization, 2020; Togun et al., 2020). The main tests applied for TB diagnosis are sputum smear mi- croscopy, bacilli culture, molecular tests, delayed-type hypersensitivity reaction, X-ray image, and interferon gamma release assay (IGRA), each with its own qualities and limitations. This scenario highlights the need for better diagnostic tools to fill the gaps from existing methods (MacLean et al., 2019). Immunological tests have acquired significance in TB diagnosis, as they can detect anti–*M. tuberculosis* (anti-Mtb) anti-bodies in body fluids, such as blood and serum, and they provide fast results that can be used for large-scale screening in endemic regions (Yong et al., 2019).

The quality of an immunological test depends on the selection of adequate antigens and their ability to bind to specific antibodies against Mtb (Ireton et al., 2010). Antigens include proteins from the pathogen as well as their variations, including protein subunits, fusion proteins, and peptides (Hill et al., 2005; Meier et al., 2018). The use of peptides as antigens is based on the identification of individual-specific epitopes with the goal of avoiding cross-reactions with non-specific antibodies (Kashyap et al., 2013). Peptides can be identified and isolated for im- munoassays using the phage display technique (Anand et al., 2021).

In light of the above, this study was aimed at selecting novel antigens and applying them in the serological diagnosis of TB. Peptides were identified and selected using phage display, SPOT-synthesis, immuno-detection, and in silico analyses. The selected peptides were evaluated in

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an immunosorbent assay (ELISA) to determine their performance (sensitivity and specificity) in the diagnosis of TB.

2. Materials and methods

2.1 Phage display

2.1.1. Library

The linear phage-displayed peptide libraries used in this study were 15-mer (X_{15}) and 17-mer (X_8CX_8). The constrained libraries were 8-mer (LX-4) and 12-mer (LX-8). They showed random peptides at the N-ter- minus of the pVIII protein of phage vector f88.4 (Bonnycastle et al., 1996). The libraries were obtained from J. Scott (Simon Fraser Uni- versity, Canada).

2.1.2 Serum samples

The Regional Center of Specialties-Barão (Curitiba, Brazil) and the São Sebastião Regional Hospital of Lapa (Lapa, Brazil) provided serum samples from 50 positive TB patients. The patients were diagnosed via bacilli cultures via a specialized medical service in the health units, and they were in the first three months of TB treatment. For the negative controls, serum was obtained from 50 negative volunteers who had no histories of TB infection but were vaccinated with bacillus Calmette–Guérin.

2.1.3 Purification of anti-M. tuberculosis Immunoglobulin G (IgG)

The precipitation of IgG from positive serum samples was performed with saturated ammonium sulfate (Kumar et al., 2008). The IgG fraction was separated by affinity chromatography using protein G-agarose resin. Specific anti-Mtb immunoglobulins were purified by eluting the antibodies bound to the Polyvinylidine Fluoride (PVDF) membrane (Millipore) with previously attached soluble *M. tuberculosis* antigens via the Western blot method (Alban et al., 2013). The eluted antibodies were dialyzed and concentrated using a 10 kDa Amicon Ultra-15 column (Millipore). The protein concentration was measured by using the Bradford method. Purity was evaluated by applying 3 μg of isolated antibodies in a 10% SDS-PAGE followed by staining with Coomassie blue. The IgG reactivity was determined in enzyme-linked immunosor- bent assay (ELISA) assays using soluble *M. tuberculosis* as the antigen as well as serial diluted (1:1–64 *v*/v) total IgG or anti-Mtb IgG. The soluble *M. tuberculosis* antigens used for the Western blotting and ELISA methods were previously prepared using an *M. tuberculosis* (ATCC 27294) inactivated biomass. The biomass was resuspended in 2 mM of EDTA buffer containing 100 μg/mL of phenylmethanesulfonyl fluoride proteinase inhibitor, lysed via sonication (four cycles of 15 min each), and isolated via centrifugation (10,000 × g for 20 min at 4 °C) after the supernatant was filtered through a membrane filter (0.22 μm pore size) .

2.1.4. Selection of reagent phages

A Nunc immuno-tube with 5 μg/1.5 mL of anti–*M. tuberculosis* IgG was incubated overnight in 100 mM of $NaHCO_3$ (pH 8.6) at 4 °C. The tube was washed with 0.05% Tris Buffered Saline, with Tween 20 (TBST), filled with blocking solution (0.05% TBST, 3% BSA), and incubated for two hours at 37 °C. After a washing step (0.05% TBST), the tube was incubated with 1.5 × 10^{11} phages from libraries X_{15} and X_8CX_8, as well as 2.5 × 10^{10} phages from libraries LX-4 and LX-8 diluted in 0.05% TBST. After another washing step, the attached phages were eluted with 1.5 mL of 0.1 M glycine (pH 2.2) and 1 mg/mL of bovine serum albumin (BSA). After neutralization with Tris HCl 2 M (pH 9.0), the eluted phages were amplified via the infection of *Escherichia coli* K91 bacteria (NCTC 10650), which J. Sott kindly provided (Simon Fraser University, Burnaby BC, Canada). After incubation in a Luria Bertani (LB) medium supplemented with 20 μg/mL of tetracycline overnight (225 × g at 37 °C), the culture supernatant obtained was precipitated using Polyethylene glycol/NaCl solution (20% PEG 8,000 and 2.5 M NaCl) overnight at 4 °C and resuspended in TBS buffer. Three The most reactive clones were selected for DNA extraction with the subsequent cycles were performed with 2.5 μg/1.5 mL ofanti–*M. tuberculosis* IgGs and 2.0 10^{11} phages from previous rounds (Alban et al., 2014). After the fourth selection cycle, the phage clones were isolated, and affinity to anti–*M. tuberculosis* IgGs was confirmed via ELISA (Zeng et al., 2012).

2.1.5. Immunological screening of phage clones

The phage clones were isolated and evaluated for their ability to bind to anti–*M. tuberculosis* IgGs. For this purpose, ELISA plates were coated with 50 μL of anti-bacteriophage antibody (Sigma-Aldrich, USA) at a dilution of 1:800 (100 mM NaHCO3, pH 8.6) and stored overnight at4 °C. Plates were washed three times with Phosphate buffered salinePBS Tween-20 (PBST-0.05%) and then blocked with 2% skim milk powder in PBST-0.05% for one hour at 37 °C. The supernatant (50 mL) from the culture of each isolated clone was added to a different well of the plate and incubated for 2 h at 37 °C. After washing, the plates were incubated with 100 μg/mL of total IgG from TB patients in blocking solution for 1 h at 37 °C. A further wash was performed, and a peroxidase-conjugated anti-human Fc IgG antibody (Sigma-Aldrich, USA) diluted 1:10,000 in blocking solution was added and incubated for1 h at 37 °C. After a further wash, a reaction was revealed following the addition of substrate solution (0.2 mg/mL *o*-phenylenediamine dihydrochloride - OPD) (Sigma-Aldrich, USA) diluted in citrate buffer pH 5.0 containing 0.2 μL/mL of 30% H_2O_2. The plates were incubated at room temperature in the dark for 15 min, and the reaction was stopped through the addition of 10 μL of 1:20 H_2SO_4. Absorbance readings were taken in a spectrophotometer at a wavelength of 492 nm. The most reactive clones were selected for deoxyribonucleic acid (DNA) sequencing.

2.1.6. Sequencing and translation

QIAprep Spin M13 kit (Qiagen, Hilden, Germany), and sequencing was performed using a reverse primer (5'-TCG GCA AGC TCT TTT AGG-3′) and Sanger sequencing. DNA to amino acid conversion was performed with the "translate" tool from the Expasy website (https://web.expasy.org/translate/).

1.1. SPOT-synthesis and immunodetection

The peptides identified from phage sequences were chemically synthesized via the SPOT-synthesis method anchored to a cellulose membrane (Merrifield, 1963; Frank, 2002). The technique was performed on cellulose membranes containing free amino groups and polyethylene glycol spacers—amino-PEG membranes (Intavis Bioanalytical Instruments AG, Nattermannallee, Köln, Germany)—using a ResPep SL automated peptide synthesizer (Intavis Bioanalytical Instruments AG). The synthesis was carried out according to the manufacturer and usingFmoc amino acids (9-fluorenylmethoxycarbonyl). Briefly, Fmoc amino acids were activated for coupling with N,N′-diisopropylcarbodiimide (DIC) and Oxyma Pure. After coupling, free amino groups were blockedvia acetylation. The Fmoc group was removed through treatment with25% 4-methylpiperidine in *N*,*N*-dimethylformamide (DMF) to prepare the membrane for the next cycle of coupling. A final acetylation step was performed after the last synthesis cycle. The removal of the sidechain protecting groups was performed by treating the membrane with a compound solution of 92.5% trifluoroacetic acid, 2.5% triisopropylsilane, 2.5% β-mercaptoethanol, and 4% water for 4 h, followed bywashes with dichloromethane (DCM), DMF, and ethanol. After drying at room temperature, the membrane was stored at −20 °C. Immunodetection was performed as previously described by exploiting the affinity of immobilized peptides to antibodies from TB-positive serum samples. The membrane with immobilized peptides were blocked using blocking solution (3% casein, 0.5% sucrose, 0.1% Tween 20 in TBS buffer) overnight at 4 °C. This was followed by incubation with diluted TB- positive serum samples (1:400 v/v), anti-human IgG-biotin (1:10,000

v/v), and streptavidin-horseradish peroxidase (HRP) (1:10,000 v/v). Dilutions were performed using incubation buffer (0.25% casein, PBSpH 7.4, 0.05% Tween 20). Affinity was visualized using a chemilumi-nescent substrate (Pierce™ ECL Plus Western Blotting Substrate Thermo Scientific™) and Hyperfilm ECL (GE Healthcare) (Alban et al., 2014).

1.2. Homology analysis

The BLASTp tool (NCBI) was used to evaluate the similarity betweenthe peptide mimotopes and *M. tuberculosis H37Rv* (taxid:83332), as well as *M. bovis* Moreau RDJ (taxid:413996) from a non-redundant protein sequence (nr) database (Altschul, 1997).

2.4. Chemical synthesis

The selected peptides were chemically synthesized using cleavable resin as a solid support, allowing for the production of soluble peptidesin aqueous buffer (Jensen, 2013). The method employed solid-phase peptide synthesis (SPPS) using an automatic synthesizer (Intavis Bio-analytical Instruments, Nattermannallee, Germany) and Fmoc-protected amino acids.
Peptide sequences that were considered to be hydrophobic were modified by adding three amino acids to the N-terminal, such as S-G-S or K-K-G.

2.5. Experimental design (DoE)

A full factorial design (2^5) was created to standardize the ELISA conditions using the Minitab© Statistical Software *v. 18.1* (2017 Minitab, Inc.), including central points (Table 1), by considering five factors: antigen concentration (A), BSA (Sigma Aldrich) concentration in blocking solution (B), serum dilution (C), conjugated antibody anti-Human IgG (Fc specific)-Biotin (Sigma Aldrich Clone Hp-6017) dilu- tion (D), and neutravidin-HRP (Thermo Scientific™) dilution (E). The response variable was defined as the ratio between the positive and negative sample absorbance readouts for each ELISA condition.
The experiment was set to maximize the response variable. New points were added to fit a curvature model in cases where the results did not fit the linear model.

2.6. Method validation

The ELISA was performed in high-binding polystyrene 96-well flat-bottom plates (Costar® 96-Well EIA/RIA Stripwell™ Plate). First, the antigen was diluted in coating buffer (0.16% Na_2CO_3, 0.29% $NaHCO_3$, pH 9.6) and was incubated overnight at 4 °C. Next, the wells were washed with washing solution (0.9% NaCl, 0.05% Tween 20). Blocking was performed with 3–5% BSA in PBS (pH 7.4) and was incubated for 1h at 37 °C. After washing, the plates were incubated with serum diluted in incubation buffer (0.25% casein, PBS pH 7.4, 0.05% Tween 20) for 1 h at 37 °C. Subsequently, anti-human IgG conjugated (Invitrogen™, USA) with biotin was diluted in incubation buffer and then added to the plate, followed by incubation for 1 h at 37 °C. The plates were washed and incubated with neutravidin-HRP (Invitrogen™, USA). The chromogenic substrate was 3,3′,5,5′-Tetramethylbenzidine (TMB) (Invitrogen™, USA), and the reaction was stopped with 20 μL of 5 N HCl after 30 min. Absorbance was read in a spectrophotometer at a wavelength of 450 nm.

Table 1
Actual and coded values of the independent variables from the full factorial experimental design (2^5) to optimize the ELISA test.

Level (ng/well)	Antigen	Blocking solution (% BSA)	Serum (Dilution)	Conjugated antibody (Dilution)	Neutravidin (Dilution)
—1	30	2	1:100	1:10,000	1:6000
0	75	3	1:200	1:12,000	1:8000
1	120	4	1:300	1:14,000	1:10,000

2.7. Ethics committee approval

The study was carried out in accordance with the Brazilian National Research Ethics Committee (CEP-CONEP) and Conselho Nacional de Saúde resolution No. 196/96. The Ethics Committee of the Federal University of Paraná (CAAE 36541020.9.0000.0102) and the Human Research Ethics Committee of the Health Department of the State of Paraná (Process No. 002/2008 and CAAE 36541020.9.3001.5225) granted approval of the study.

2.8. Statistics

The experimental design results were analyzed using the Minitab© Statistical Software *V. 18.1* (2017 Minitab, Inc.). In addition, sensitivity, specificity, Youden index **J**, and receiver operating characteristic (ROC) curve results were determined using MedCalc Statistical Software version 18.2.1 (MedCalc Software bvba, Ostend, Belgium; http://www. medcalc.org; 2018). A cutoff point for optimal sensitivity and specificity for the ELISA tests was determined using the ROC curve analysis via a method of DeLong et al. (1988), considering unknown TB incidence. ROC curve performance was evaluated via Youden index **J** (Youden, 1950).

3. Results

3.1. Purification of anti–M. tuberculosis IgGs

Specifically, anti-Mtb antibodies were necessary for use in a later selection step via the phage display method. Therefore, these antibodies were obtained through the purification of serum samples from TB pa- tients. First, total IgG was purified from the pool of TB patients by using precipitation with saturated ammonium sulfate followed by chroma- tography with G-agarose resin. Then, elution from a Western blotting membrane containing antigens from the *M. tuberculosis* allowed for isolating anti-Mtb IgG antibodies. The purity of isolated antibodies was verified via SDS-PAGE (Fig. 1.A) and immunoaffinity in ELISA assays (Fig. 1.B). SDS-PAGE results demonstrate the high purity of the isolated anti-Mtb, where only two bands, near 25 and 50 kDa, were visible as expected from IgGs. In the ELISA assays, the absorbance of antigen- specific immunoglobulins (anti-Mtb IgG) is higher than in the total IgG in all of the dilutions tested, even considering that the total IgG concentration was five times greater than that of the anti-Mtb IgG. Therefore, the method allows for obtaining concentrated antigen-specific antibodies, which were used for the phage-displayed technique.

3.2. Selection of reagent phages

Mycobacterial antigen mimotopes were isolated from phage- displayed peptide libraries through four biopanning rounds against purified anti–*M. tuberculosis* IgG. The total amount of recovered phages was determined in each cycle (Table 2). Each cycle increased the per- centage of phages bound to purified antibodies, highlighting the enrichment of the phage population.
A total of 110 isolated bacterial colonies were randomly selected after the four rounds of biopanning, and the corresponding phages were evaluated for their binding capacity to total IgG from TB patients via ELISA. Third, three clones were recognized through antibody binding.
Each phage clone, after amplification, was evaluated via ELISA againstsera from TB patients and healthy individuals. The results showed that the reactivity of the phage clones was higher than that of the wild-type phage, with the serum samples being positive against the phage clones. This indicates that the observed reactivity occurred between the serum samples and the phages expressing peptides on their surface. ELISA

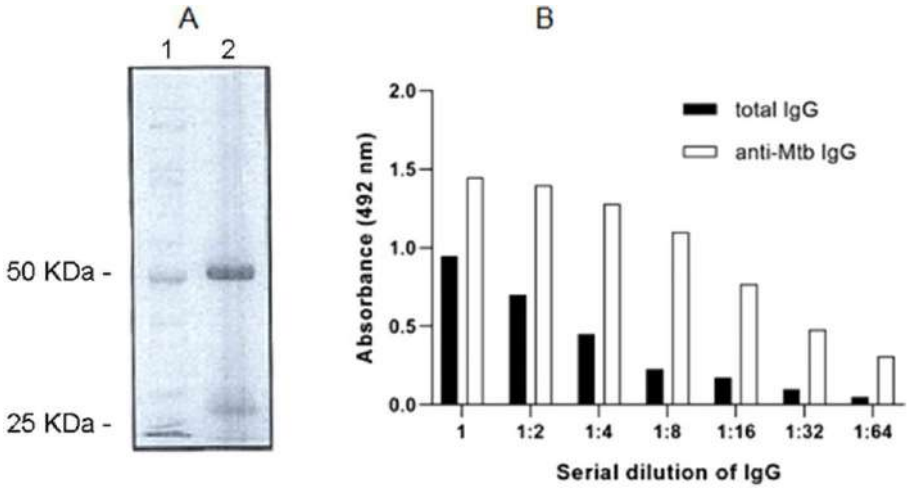

Fig. 1. A - SDS-PAGE under reducing conditions of purified IgGs from TB patients after immunoblotting. IgGs are visible in two bands of approximately 25 and 55 kDa. Column 1: molecular-weight size marker; 2: purified anti-Mtb IgG isolated from TB patients. Three micrograms of protein were loaded on 10% poly-acrylamide gel and stained with Coomassie blue. B- Reactivity of purified IgG via ELISA. An ELISA plate was sensitized with 10μg/mL of soluble Mtb antigen and was incubated with total IgG starting at 25 μg/mL(dark bars) and 5 μg/mL for specific anti-Mtb IgG (light bars). Detection was performed with the Fc-specific anti-human IgG antibody conjugated with horseradish peroxidase (HRP) and OPD as the chro-mogenic substrate. (For interpretation of the refer-ences to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Input and output phages from each biopanning round of phage display with specific anti–*M. tuberculosis* Immunoglobulin G.

Round (cfu)[a]	Input phage	Output phage (cfu)[b]	% bound phages ($x10^{-4}$)	Enrichment (x)
1	7.98×10^{11}	9.9×10^{5}	0.012	
2	2.0×10^{11}	2.9×10^{6}	0.145	12.1
3	2.0×10^{11}	1.05×10^{8}	5.250	437.5
4	2.0×10^{11}	1.05×10^{9}	5.450	454.5

[a] Number of phage colony-forming units (cfu) incubated in each round.
[b] Total number of phages (cfu) contained in the eluate.

plates were sensitized with antiphage antibody (1:800 v/v) and incu- bated with 2 10^{10} phages/mL, and then, patient serum (1:100 v/v) was added. The detection of the reaction was performed with a specific anti-IgG Fc antibody conjugated to peroxidase and OPD as the chromogen.

3.3. Sequencing and translation

The 33 clones were sequenced, and eight DNA sequences were identified. After the DNA–to–amino acid conversion, the peptides weredesignated from P.Mt.PD.2 to P.Mt.PD.9. The length of the sequences varied from 8 to 17 amino acids. Peptide P.Mt.PD.4 belongs to the X_{15} library, whereas peptide P.Mt.PD.7 belongs to the LX-8 library.

3.4. Immunodetection

The eight peptides were synthesized anchored to a cellulose mem- brane via SPOT-synthesis. Immunodetection was performed to confirm peptide reactivity with specific anti–*M. tuberculosis* antibodies and to screen for high-affinity peptides. No reactive spot was observed for any of the peptides when incubation was performed with a negative serum pool (data not shown). However, four peptides were reactive (P.Mt. PD.4, P.Mt.PD.5, P.Mt.PD.7, and P.Mt.PD.9) when incubation was per- formed with a pool of positive sera (Fig. 2). Peptides P.Mt.PD.4 and P. Mt.PD.7 showed intense reactions for the three independent spots. P.Mt. PD.5 showed two positive spots with faint signals, whereas peptide P.Mt. PD.9 showed a strong reaction at just one spot. Because peptides P.Mt. PD.4 and P.Mt.PD.7 demonstrated intense immunoreactivity with sera from patients infected with *M. tuberculosis*, they were selected for in silico analysis.

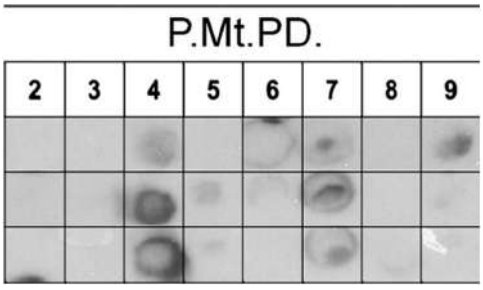

Fig. 2. Immunodetection of peptides P.Mt.PD.2-9 incubated with sera from TB patients (1:400 *v*/v sera, 1:10,000 v/v anti-human IgG-biotin, and 1:10,000 v/v streptavidin-HRP). Each peptide was synthesized 3 times in the same cellulose membrane via SPOT-synthesis.

3.5. Homology analyses

Peptide P.Mt.PD.4 showed up to 80% query coverage with the 1-acyl-sn-glycerol-3-phosphate acyltransferase protein, and its highest identity was 100% with glycosyl hydrolase, both from the *M. tuberculosis* complex (Table 3). Also, the fatty acid desaturase from *M. tuberculosis* H37Rv and the probable conserved membrane protein from *M. bovis* Moreau RDJ showed 53% coverage and 62.50% identity with P.Mt. PD.4. On the other hand, P.Mt.PD.7 reached 100% query coverage with the phenyloxazoline synthase protein from Mtb, whereas *M. bovis* Moreau RDJ achieved the highest coverage of 66% with putativetransposase. Overall, both selected peptides showed similarities to pro- teins from *M. tuberculosis* H37Rv, enhancing the mimotope character of peptides and contributing to the sensitivity of the diagnostic test.

3.6. Peptide synthesis and immunoassay standardization

Peptides P.Mt.PD.4 and P.Mt.PD.7 were chemically synthesized and purified to 80 and 90% purity. Subsequently, they were evaluated via ELISA with 51 parameter combinations determined from the experi- mental design. As a result, it was possible to define the ELISA conditions that provided the greatest difference between positive and negative samples for each selected peptide mimotope (Table 4). In addition, the influences from the independent variables were evaluated (Fig. 3).

3.7. Statistical analysis

Statistical analysis was performed via multifactorial analysis ofvariance (ANOVA) using the Minitab© Statistical Software. The aim was

Table 3
Coverage and identity of peptides P.Mt.PD.4 and P.Mt.PD.7 with sequences from Mtb and *M. bovis* Moreau RDJ. Only the results with the highest e-value and overall similarity are shown.

Peptide	Reference organism	Protein name	*E*-value	Query coverage (%)	Identity (%)
P.Mt.PD.4	*M. tuberculosis* complex	1-Acyl-sn-glycerol-3-phosphate acyltransferase (WP_003411341.1)	1.5	80	55.56
	Glycosyl hydrolase(WP_003900047.1)		1.5	33	100.00
	M. tuberculosis H37Rv	Fatty acid desaturase (WP_003900326.1)	3.0	53	62.50
	M. bovis Moreau RDJ	Probable conserved membrane protein (CCC63992.1)	2.2	53	62.50
P.Mt.PD.7	*M. tuberculosis* H37Rv	PhenyloXazoline synthase (WP_003899299.1)	3.6	100	71.43
	M. bovis Moreau RDJ	3-Oxoacyl-[acyl-carrier protein] synthase 2 kasB (CCC64840.1)	16	50	83.33
		Putative transposase (CCC65482.1)	16	66	50.00

Table 4
Optimized ELISA parameter for peptides P.Mt.PD.4 and P.Mt.PD.7 calculatedusing the Minitab© Statistical Software.

Peptide	Antigen (ng/well)	Blocking solution (% BSA)	Serum (Dilution)	Conjugated antibody (Dilution)	Neutravidin (Dilution)
P.Mt. PD.4P.Mt.	30	4	1:100	1:14,000	1:10,000
PD.7	30	2.63	1:100	1:10,000	1:10,000

to determine the individual and combined effects of the five independent variables on the response variable. The ANOVA (presented in Tables 1S and 2S) corresponds to the data generated for P.Mt.PD.4 and P.Mt.PD.7. For each main effect, interaction, and quadratic effect, the table includes the sum of squares (SS), the degree of freedom (DF), the mean square (MS), an F-ratio calculated using the residual mean square, and the significance level of the *P* value.

For P.Mt.PD.4 (Fig. 1S), the main significant factors were serum and antigen for the dependent variable at the 0.05 level of significance. This implies that different serum and antigen values result in different dependent variable performance values. The Conjugated antibody*Conjugated antibody interaction was significant at the 0.05 levelof significance, whereas the other combinations were not significant. The R-squared value of 88.69% implies that the model explains about 86.05% of the total variability.

The P.Mt.PD.7 (Fig. 2S) data analyzed showed that the main significant factors were serum, antigen, and blocking solution ('S' symbol in the last column) for the dependent variable at the 0.05 level of significance. This indicates that different values of the independent variables (serum, antigen, and blocking solution) result in different performance values of the dependent variable. The Serum*Blocking solution, Serum*Conjugated antibody, and Blocking solution*Blocking solution interactions were also significant at the 0.05 level of significance, whereas the other combinations were not significant. The R- squared value of 98.49% implies that the model explains about 98.22%of the total variability.

After optimization, peptide P.MT.PD.7 proved to best differentiate samples from infected and not infected individuals (Fig. 4). Therefore,this peptide was selected to investigate sensitivity and specificity with a panel of sera.

3.8. Method validation

A total of 100 serum samples (50 positive and 50 negative) were screened to determine the sensitivity and specificity of the ELISA assay using P.Mt.PD.7 as the antigen (Fig. 5.A). The test reached 100% sensitivity and specificity. Therefore, the area under the ROC curve (AUC) was 1.000 (Fig. 5.B).

4. Discussion

TB remains a major public health issue, and a reliable, fast, and ac- curate diagnosis is a key aspect for mitigating the burden of this disease. Immunological tests are suitable for this purpose due to their rapid and

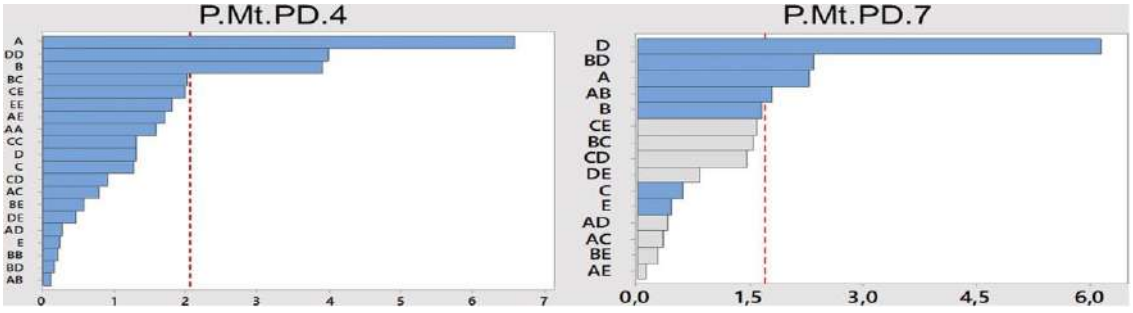

Fig. 3. Pareto chart of the experimental design for the analysis of the independent variables: (A) serum, (B) antigen (C) blocking so lution, (D) conjugated antibody, and (E) Neutravidin. The red line is the effect size at significance level ($\alpha < 0.05$) using ANOVA. The grey bars represent non-significant terms that were removed from the model. Model fit: *P value* < 0.001. Individual *P value* from variables are found in Table 1S (P.Mt.PD.7) and Table 2S (P.Mt.PD.4). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

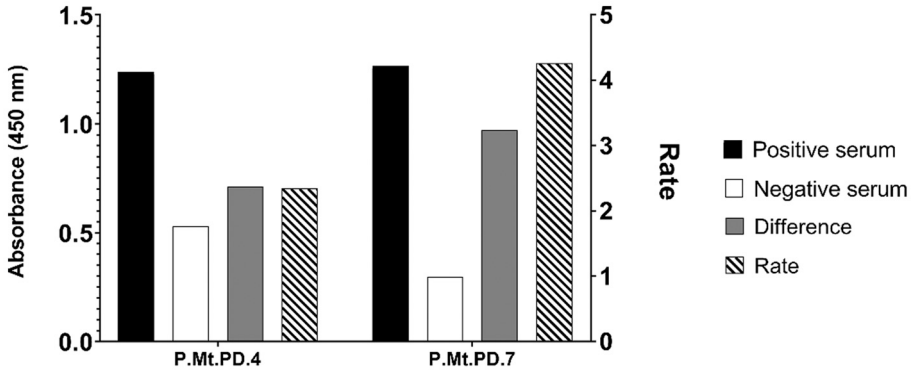

Fig. 4. ELISA reactivity of peptides P.Mt.PD.4 and P.Mt.PD.7. Positive and negative serum pools were tested at the optimized conditions for each peptide. The difference showed is the absorbance of the positive serum minus the absorbance of the negative serum. The rate is the proportion between positive and negative serum absorbance readouts.

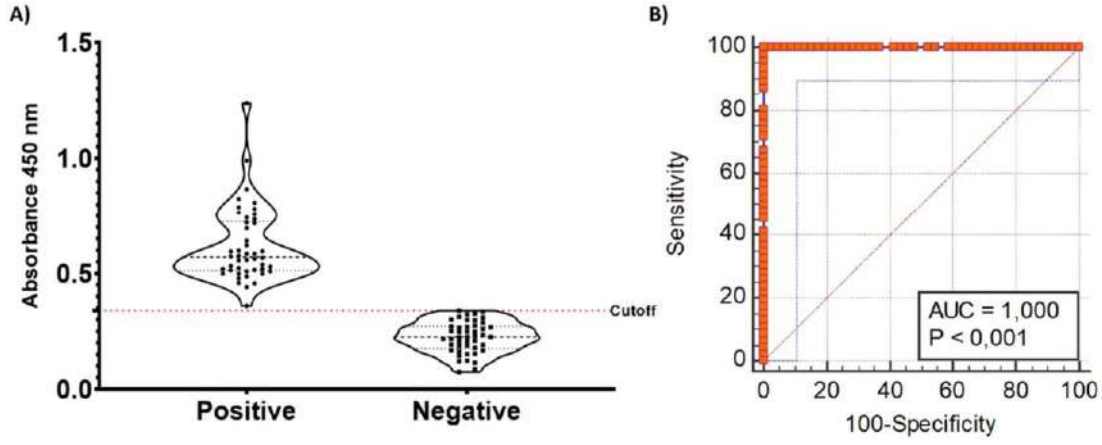

Fig. 5. Reactivity of 50 positive and 50 negative serum samples for TB when ELISA is performed using peptide P.Mt.PD.7 as the antigen. (A) Violin plot of positiveand negative samples created in GraphPad Prism 8. Absorbance is read at 450 nm. The 0.340 cutoff was determined via MedCalc. (B) ROC curve, statistical analysis, and parameters were calculated via MedCalc. Sensitivity: 100%, Specificity: 100%, AUC: 1.000, Yonden index: 1, Cl 95%: >0.335 to >0.34, *P value* < 0.001.

cost-effective performance and applicability in various stages of the disease. Besides, these tests can be used in large-scale testing and can be implemented in adverse conditions, which is often the case in under-developed countries. Identifying new antigens using phage display is an excellent tool that has positively impacted health science by targetingand selecting specific binding peptides or proteins (Anand et al., 2021; Kumar et al., 2019). Phage display has been used to identify antigens for immunodiagnostics of a range of diseases, such as COVID-19 (Guo et al., 2021) and TB (Wang et al., 2016; Zhao et al., 2017; Luo et al., 2019).

SPOT-synthesis, which consists of having synthesized peptides immobilized in cellulose membranes, can be a powerful tool for investigating peptide interaction with different molecules (antibodies, in this case). It can also screen for highly active peptides from hundreds of peptide sequences that can be displayed at the same time (López-Pérez et al., 2017). This analysis is crucial for saving time and reagents whenone is screening various peptides as demonstrated in previous studies (Guedes et al., 2021; Link et al., 2017; Molina-Molina et al., 2018; Ramliet al., 2019; Soares et al., 2021), thus optimizing the process. In this study, SPOT-synthesis and immunodetection were used to assess the immunoreactivity of eight peptides (P.Mt.PD.2 to P.Mt.PD.9) using serum samples from TB patients. Four peptides were reactive (P.Mt. PD.4, 5, 7, and 9), but peptides P.Mt.PD.4 and P.Mt.PD.7 showed an intense reaction in the three replicates, and they were therefore selected for homology analysis and chemical synthesis.

Phage-displayed peptides that bind to serum antibodies can mimic the epitopes of protein antigens (Liu et al., 2013). BLASTp is a bioinformatics tool used to match the mimotopes with the correspond- ing proteins. This approach has been used before to determine the ho- mology of peptides selected via phage display with *Hemiscorpius lepturus* toXins (Jahdasani et al., 2016) and BoHV-1 (Almeida et al., 2015). Here, BLASTp was used to verify the correspondence of P.Mt.PD.4 and P.Mt. PD.7 with proteins from *M. tuberculosis* and *M. bovis* Moreau RDJ. Pep- tide P.Mt.PD.4 showed a query coverage of up to 80% with 1-acyl-sn- glycerol-3-phosphate acyltransferase from the *M. tuberculosis* complex, as well as 53% query coverage with fatty acid desaturase from *M. tuberculosis* H37Rv, and probable conserved membrane protein from *M. bovis* Moreau RDJ. In contrast, peptide P.Mt.PD.7 showed 100% coverage and 71.43% identity with phenyloxazoline synthase from Mtb. The most similar protein from *M. bovis* Moreau RDJ had 50% coverage, showing that both peptides share epitopes with Mtb proteins, favoring their application in diagnostic tests. The presence of similarity with proteins from *M. bovis* Moreau RDJ is not ideal. However, based on the strong reaction in immunodetection assays, we believed that peptides could potentially discriminate between positive and negative sera, and therefore, they were synthesized. Peptide P.Mt.PD.4 showed some reactivity with negative sera, although it was lower compared with positive samples. Nevertheless, peptide P.Mt.PD.7 had a higher resem- blance with Mtb-specific epitopes and little or no reaction with the negative controls.

According to the results from the DoE analysis, the variable with the greatest influence on P.Mt.PD.4 was serum dilution, followed by the dilution of anti-human IgG antibody. For P.Mt.PD.7, serum dilution was

the most influencing variable, followed by the antigen concentration. However, all variables directly influenced the test results. When the test condition was determined via the DoE, P.Mt.PD.7 was the only antigen that showed a high difference between positive and negative serum samples, as well as a rate of 4.27, which is one of the most important characteristics of a diagnostic test (OIE, 2019). Thus, P.Mt.PD.7 coulddiscriminate between TB-positive and healthy individuals at a higher rate compared with the other peptides described here. For this reason, P. Mt.PD.7 was chosen to be further evaluated with a larger number ofsamples.

The diagnostic performance of peptide P.Mt.PD.7 was evaluated with 50 sera from TB-positive individuals and 50 sera from healthy in-dividuals. Sensitivity is defined as the ability of the test to identify infected individuals as positive, whereas specificity is the ability of healthy individuals' ability to be interpreted as negative (OIE, 2019). Of the 100 samples tested, the ELISA described in this study resulted in 100% sensitivity and specificity. Other currently researched serological tests have shown sensitivity and specificity values of 77.1 and 81.1%, respectively, using the Rv0674 protein as the antigen (Xiao et al., 2019). The ribokinase protein was recently evaluated for its potential as an antigen and showed 90% sensitivity and 86% specificity (Luo et al., 2019). In another study, a multi-antigen ELISA of six Mtb epitopes (PstS1, ESAT6, CFP10, Ag85B, Ag85A, and PPE54) showed 90% sensi-tivity and 93.3% specificity (Gao and Zhao, 2019). Another similar study with mixed antigens achieved 69.9% sensitivity and 77% specificity (Yan et al., 2018). Although some of them showed satisfactory results, recombinant proteins require a number of laborious steps, such as het- erologous protein production (i.e., *E. coli*), protein extraction, purifica-tion, and refolding, demanding a long time for antigen production. However, synthetic peptides, such as the one used in this study, can overcome the previously mentioned limitations due to their faster pro- duction (Mustafa, 2013; Vordermeier et al., 2001). Other studies based

on peptides for diagnosing TB have shown 78–92% sensitivity and 76–100% specificity, with a Youden Index of 0.7 (López-Ramos et al., 2018). In another study, two 12-mer peptides showed 89.41 and 85.88% sensitivity and 90.63 and 87.50% specificity (Wang et al., 2016). In contrast, our P.Mt.PD.7 achieved 100% for both sensitivity and speci-ficity. Current tests available in the market, such as T-SPOT.TB and IGRA-ELISA, have shown 82.9% and 81.7% sensitivity and 78.6 and 75.2% specificity, respectively, when evaluated in a large number of samples (Wang et al., 2018).

In summary, our study selected and evaluated a novel antigen for the serological diagnosis of TB with ideal sensitivity and specificity. Also, it has the potential to be marketed and widely applied in diagnosis, especially in low-income countries. For further analysis, a larger number of more diverse samples would be required to support the results pre- sented here. Also, serum samples from latent *M. tuberculosis* infection (LTBI) would be needed to investigate if the P.Mt.PD.7 antigen coulddiscriminate between active and latent TB infection.

5. Conclusion

The peptide P.Mt.PD.7 presented in this study was selected via phage display and in silico and in vitro tools. The ELISA assay with this antigen showed 100% sensitivity and specificity for the diagnosis of TB in a 100- sample panel, which is higher than previously reported similar tests. Therefore, this novel ELISA assay for TB has high diagnostic accuracyand can be used to screen endemic regions and help to combat the spread of TB.

Authors' contributions

V.T.S. and C.R.S. conceived the original idea; drafting the work revising it critically and supervised the project. S.M.A. performed the phage-display and chemical synthesis. N.N.S. performed the ELISA im- munoassays. M.H.S. and R.A.B. conceived the SPOT-synthesis and immunodetection assays. M.H.S. planed the DoE. V.D.P. performed the immunoassay tests and wrote the paper. J.M.D.V.C. performed the homology analysis and wrote the paper. F.B.M. and C.W. obtained the biological samples. All authors revised and approved the final version of the manuscript.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2022.113242.

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