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Renata Streck Fernandes

POTENCIAL PREVENTIVO DA ALAMANDINA NA FIBROSE PULMONAR EXPERIMENTAL

Porto Alegre

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Tese submetida ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Doutora.

Orientadora: Prof.ª Dr.ª Katya Vianna Rigatto

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C E R T I F I C A D O

Certificamos que **Renata Streck Fernandes** orientada pela professora Katya Vianna Rigatto, apresentou a Tese de Doutorado intitulada "Avaliação do potencial Preventivo da alamandina na fibrose pulmonar experimental" no dia 21/02/2022, junto ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal de Ciências da Saúde de Porto Alegre, sendo considerada **aprovada**. Após a homologação da Tese de Doutorado receberá o título de Doutora em Ciências da Saúde: Fisiologia e Patogênese.

Porto Alegre, 21 de fevereiro de 2022.

Giovana Maria Roth Lopes
Secretária Executiva
PPG Ciências da Saúde - UFCSPA

“Todas as verdades são fáceis de perceber depois de terem sido descobertas,
o problema é descobri-las.”

Galileo Galilei

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SUMÁRIO

LISTA DE ABREVIATURAS

ALA: Alamandina
Ang A: Angiotensina A
Ang I: Angiotensina I
Ang II: Angiotensina II
Ang III: Angiotensina III
Ang IV: Angiotensina IV
Ang-(1-7): Angiotensina 1-7
Ang-(1-9): Angiotensina 1-9
AT1: Receptor de Angiotensina tipo 1
AT2: Receptor de Angiotensina tipo 2
ATS: American Thoracic Society
BLM: Bleomicina
CVF: Capacidade vital forçada
DIP: Doença intersticial pulmonar
ECA: Enzima conversora de Angiotensina
ECA2: Enzima conversora de Angiotensina 2
ERNs: Espécies reativas ao nitrogênio (ERNs)
EROs: Espécies reativas ao oxigênio
ERS: European Respiratory Society
FP: Fibrose pulmonar
FPI: Fibrose pulmonar idiopática
MEC: Matriz extracelular
NEP: Endopeptidase Neutra
PA: Pressão arterial
PI: Pneumócitos tipo I
PII: Pneumócitos tipo II
PIU: Pneumonia intersticial usual
RL: Radicais livres
SARS-CoV-2: Síndrome Respiratória Aguda Grave 2
SRA: Sistema Renina Angiotensina
SUS: Sistema Único de Saúde
TCAR: Tomografia computadorizada de alta resolução
TGF-β: Fator de Crescimento Transformador – β
UFCSPA: Universidade Federal de Ciências da Saúde de Porto Alegre
VEF1: Volume expiratório forçado no primeiro segundo

RESUMO

A doença intersticial pulmonar (DIP) engloba um grupo heterogêneo de doenças fibrosantes que causam cicatrizes pulmonares após lesões de causas conhecidas ou desconhecidas. A fibrose pulmonar idiopática (FPI) é a variante mais comum de DIP e, devido ao caráter progressivo e crônico, possui alta morbidade e mortalidade em todo o mundo. As limitações terapêuticas agravam o prognóstico do paciente, o qual possui uma sobrevida média de apenas 3 anos após o diagnóstico. Apesar do mecanismo patogênico da FPI ainda não estar totalmente elucidado, o significativo declínio na função pulmonar é a principal característica responsável pelo prejuízo funcional e impacta diretamente na qualidade de vida do paciente. Atualmente, os dois únicos medicamentos aprovados mundialmente para controlar os sintomas da FPI não são distribuídos no sistema único de saúde (SUS) devido à ausência de evidências robustas que justifiquem o alto investimento. Dessa forma, compreender a fisiopatologia da FPI pode auxiliar a identificação de novas opções terapêuticas. Nesse contexto, por ser um modulador essencial da homeostase tecidual, o sistema renina angiotensina (SRA) deve ser explorado como um sistema ativo também nestadoença. Evidências consistentes mostram o papel do eixo clássico do SRA napatogênese da doença. Entretanto, nosso grupo mostrou, pela primeira vez, que a alamandina (ALA), um importante peptídeo contrarregulatório do SRA está reduzido 365% no plasma de pacientes com FPI, enquanto os peptídeos do eixo clássico estavam similares aos controles. Esse achado demonstrou a importância do equilíbrioentre os eixos do SRA na FPI e permitiu a hipótese de que a administração exógena de ALA pode, ao menos em parte, proteger da fibrose pulmonar (FP) e representar umapromissora alternativa terapêutica futura. Portanto, o objetivo desse estudo foi avaliaro potencial preventivo da ALA no desenvolvimento da FP experimental induzida por bleomicina (BLM). Para isso, 40 ratos Wistar foram divididos aleatoriamente em 4 grupos: CO (saudáveis), ALA (saudáveis e tratados com alamandina), BLM (fibróticos) e BLM+ALA (fibróticos e tratados com alamandina). Nos animais que receberam BLM,houve uma correlação positiva entre a pontuação de Ashcroft, que indica o grau de fibrose pulmonar, e a elastância do sistema respiratório. A administração de ALA atenuou o desenvolvimento da fibrose, reduziu a elastância pulmonar e preservou o ganho de peso em animais fibróticos sem afetar o controle autonômico da pressão arterial e frequência cardíaca. Nossos dados representam um importante avanço nasterapias antifibróticas e, provavelmente, também para as sequelas fibróticas daCOVID-19.

Palavras-chave:
Fibrose pulmonar; alamandina; bleomicina; sistema renina angiotensina; modelo animal.

ABSTRACT

Interstitial lung disease (ILD) comprises a heterogeneous group of fibrous diseases that cause lung scarring after lesions of known or unknown causes. Idiopathic pulmonary fibrosis (IPF) is the most common variant of ILD and, due to its progressive and chronic character, has high morbidity and mortality worldwide. Therapeutic limitations worsen the prognosis of the patient, who has an average survival of only 3 years after diagnosis. Although the pathogenic mechanism of IPF is not yet fully elucidated, the significant decline in lung function is the main characteristic responsible for functional damage and impacts directly on the quality of life of the patient. Currently, the only two drugs approved worldwide to control IPF symptoms are not distributed in the Brazil's Unified Health System due to the absence of robust evidence to justify the high investment. Thus, understanding the pathophysiology of IPF can help to identify new therapeutic options. In this context, as it is an essential modulator of tissue homeostasis, the renin angiotensin system (RAS) should be explored as an active system also in this dis Consistent evidence shows the role of the classical axis of RAS in the pathogenesis of this disease. However, our group showed for the first time that alamandine (ALA), an important counter-regulatory peptide of RAS, is reduced by 365% in plasma of patients with IPF, while the classical axis peptides were similar to controls. This finding demonstrated the importance of balance between the axes of RAS in FPI and allowed one to hypothesize that exogenous administration of ALA may, at least in part, protect from pulmonary fibrosis (PF) and represent a promising future therapeutic alternative. Therefore, the objective of this study was to evaluate the preventive potential of ALA in the development of bleomycin (BLM)-induced experimental FP. For this, 40 Wistar rats were randomly divided into 4 groups: CO (healthy), ALA (healthy and treated with alamandine), BLM (fibrotic) and BLM+ALA (fibrotic and treated with alamandine). In the animals that received BLM, there was a positive correlation between Ashcroft score, which indicates the degree of PF, and the elastance of the respiratory system. ALA administration attenuated the development of fibrosis, reduced pulmonary elastance, and preserved weight gain in fibrotic animals without affecting autonomic blood pressure and heart rate control. Our data represent an important advance in antifibrotic therapies for several causes, including for the sequelae of COVID-19.

Keywords: Pulmonary fibrosis; alamandine; bleomycin; angiotensin renin system; animal model.

INTRODUÇÃO

1. REPARO TECIDUAL E O PROCESSO FIBROGÊNICO

1.1 Processo de cicatrização

Uma vez que os seres humanos estão em constante exposição a lesões, sejam elas acidentais, por doença ou intervenção cirúrgica, o reparo tecidual é um processo crucial para a sobrevivência.[1] Nesse contexto, para compreender os mecanismos de resposta aos danos celulares e desenvolver abordagens terapêuticas, é fundamental entender como o tecido responde fisiologicamente aos insultos.

A cicatrização é um fenômeno complexo, com alta demanda metabólica e intensa atividade celular. Ele é caracterizado por processos controlados que resultam na substituição do tecido lesado por células regeneradas e/ou cicatriciais. Portanto, o processo inflamatório pode ser resolutivo, com a proliferação de células do mesmo tipo, ou, como na maioria dos casos, envolver algum grau de comprometimento funcional pela deposição de tecido conjuntivo em substituição ao parenquimatoso, fase conhecida como fibroplasia ou fibrose.[2,3]

Conforme bem descrito na revisão de Wang *et al*. (2018)[4], a cicatrização pode ser dividida, didaticamente, em 3 fases sobrepostas: hemostasia/inflamação, proliferação e remodelação. Resumidamente, após as alterações vasculares, visando a redução do extravasamento sanguíneo, e processos inflamatórios para evitar infecção, a fase proliferativa é a responsável efetivamente pelo início da formação do novo tecido.

Quando o tecido formado é o substitutivo, ou seja, tecido conjuntivo, ele é composto por colágeno e outros componentes da matriz extracelular (MEC), possuindo alta capacidade de contração e sendo responsável, portanto, pelo fechamento da ferida. Muitas citocinas e fatores de crescimento participam dessa fase, como, por exemplo, a família do fator de crescimento transformador-β (TGF-β), a interleucina 6 e a interleucina 8.

Em resposta ao TGF-β, fibroblastos são ativados, ocorrendo uma intensa proliferação e diferenciação em miofibroblastos que, por definição, são células que expressam proteínas do músculo liso, como α-actina, e são responsáveis pela retração do tecido. Essa fase de estabelecimento da fibrose pode ser observada em torno do 7° dia após a injúria tecidual.[4]

A última etapa da cicatrização é caracterizada pelo equilíbrio entre a apoptose das células miofibroblásticas e a produção de novas células para formar propriamente a cicatriz. Nessa fase, também ocorre a degradação gradual da MEC e a formação de colágeno tipo I maduro, passos críticos para o sucesso do reparo. Portanto, qualquer anormalidade nessa etapa pode resultar em cicatrização excessiva e crônica.[4] Além disso, distúrbios na deposição de MEC também podem estar associados à presença persistente de um insulto, sustentando a produção de citocinas e fatores de crescimento.[3]

1.2 Mecanismo fibrogênico

Conforme descrito anteriormente, embora benéfica ao organismo, a fibrose pode se tornar patogênica quando estimulada de forma inadequada. Essa remodelação permanente e progressiva da arquitetura tecidual resulta em disfunção e/ou falência do órgão. Na fibrose patológica, os miofibroblastos permanecem no tecido e são responsáveis pelo aumento da síntese da MEC e pelo aumento da elasticidade do tecido.[5]

Dados da literatura demonstram que, com a idade, aumenta a possibilidade de uma cicatrização anormal e pode ocorrer, com maior prevalência em tecidos expostos constantemente a agentes químicos, como medicamentos, e/ou agentes biológicos, como os vírus.[6,7] Dessa forma, os pulmões, pele e trato digestivo são considerados os órgãos mais suscetíveis.[4,8]

Assim como na cicatrização, os processos moleculares que conduzem a fibrose envolvem muitos tipos celulares e mediadores inflamatórios. Rockey *et al.* (2015)[8] propuseram quatro fases fibrogênicas principais: lesão primária, ativação de células efetoras, deposição excessiva da MEC e falência do órgão (figura 1). Para os autores, o insulto celular inicial e posterior ativação de células inflamatórias resulta em um mecanismo autócrino, com feedback positivo para produção de proteínas da MEC e recrutamento de mais células efetoras como fibroblastos, fibrócitos e miofibroblastos.

A distorção da arquitetura e a contração do tecido promovem a patogênese da doença fibrótica e, como consequência, a falência do órgão. Além disso, Chappell e Zayadneh (2017)[9] apontam que, somando à estimulação das vias fibróticas, a perda das vias contrarregulatórias pode contribuir para o agravamento do processo.

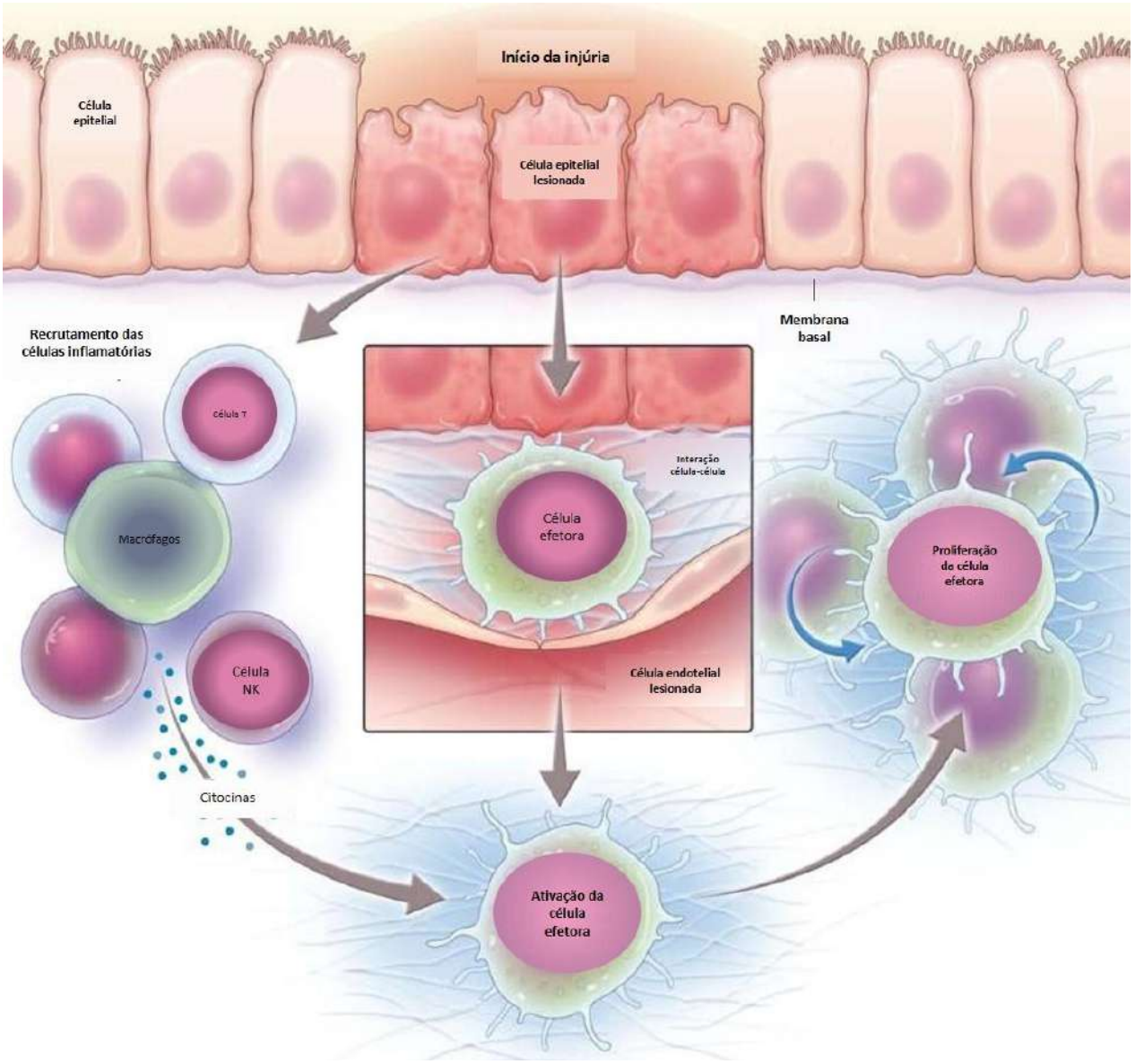


Figura 1: Injúria celular e fibrogênese. (Traduzida de Rockey *et al.*, 2015)[8]

Dentre os mediadores inflamatórios envolvidos na fibrogênese, o TGF-β desempenha um papel amplo e fundamental. Os processos celulares regulados por essa molécula estão implicados no remodelamento do tecido e envolvem a super-expressão de células epiteliais e ativação e diferenciação de fibroblastos. Todas essas ações ocorrem pela sinalização clássica das proteínas SMADs e modulação da expressão de diferentes genes-alvo.[10] Portanto o TGF-β, sintetizado pelas células inflamatórias e efetoras, pode ser considerado um mediador chave do estabelecimento da fibrose, tanto por vias autócrinas quanto parácrinas.[8]

O declínio funcional dos órgãos acometidos faz com que as doenças fibróticas

tenham altas taxas de morbidade e mortalidade[8]. Mesmo com os avanços e inúmeros estudos pré-clínicos, não se tem disponível nenhuma terapia eficaz que atendam às necessidades clínicas e, portanto, a fibrose impacta substancialmente a qualidade de vida do paciente e os sistemas de saúde.[3] Nesse contexto, investigar alternativas terapêuticas que proporcionem um ambiente ideal para que o reparo ocorra de forma controlada e sem estímulos excessivos pode ser promissor.

2. DOENÇA INTERSTICIAL PULMONAR

O tecido pulmonar é composto por células epiteliais chamadas pneumócitos, vasos sanguíneos e espaço intersticial também conhecido MEC. Os alvéolos podem ser revestidos por pneumócitos tipo I (PI), células pavimentosas que formam a interface adequada para as trocas gasosas e pneumócitos tipo II (PII), células cúbicas responsáveis pela produção do surfactante. Além dos pneumócitos, a presença de macrófagos e fibroblastos no tecido pulmonar é bem descrita na literatura.[11] Outra importante função dos PII está associada a alta capacidade proliferativa dessas células, sendo, portanto, a fonte celular para o reparo de lesões, como as observadas na doença intersticial pulmonar (DIP).[12]

DIP é a abreviatura de um termo abrangente utilizado para descrever um grupo heterogêneo de mais de 200 doenças que causam inflamação e/ou fibrose nos pulmões e possuem achados radiológicos, patológicos e clínicos comuns. A etiologia da DIP está associada ao processo anormal de reparação após uma lesão nos pulmões. Apesar de existirem variações entre essas doenças, o acúmulo de tecido cicatricial colagenoso, comum em todas elas, causa espessamento do interstício e rigidez pulmonar (figura 2).[13–15] Essa cicatriz afeta a capacidade respiratória dos pacientes e dificulta a obtenção de oxigênio suficiente para a perfusão tecidual e, por isso, ainda é um grande desafio para os pneumologistas.[15]

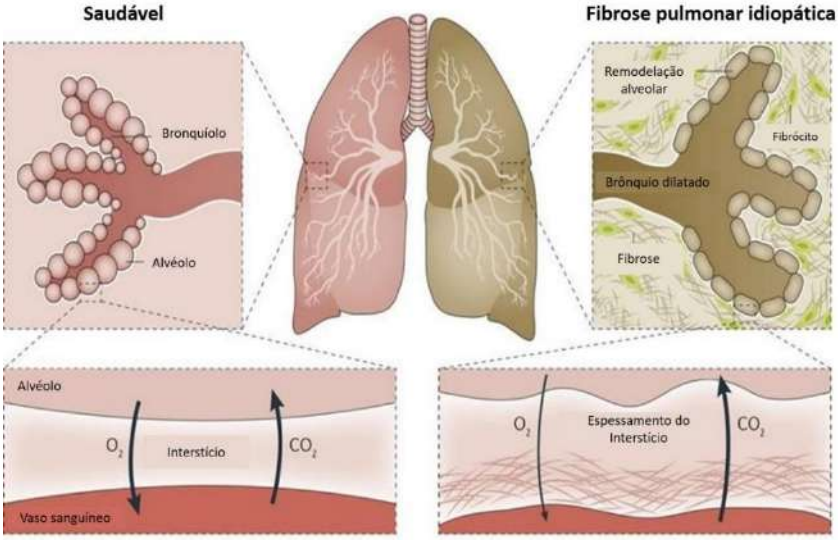


Figura 2: Representação do comprometimento respiratório na DIP (Traduzida de Martinez *et al*., 2017)[16]

Os primeiros sinais clínicos da DIP são inespecíficos, como por exemplo, tosse, dispneia, fadiga e perda de peso, prejudicando o diagnóstico precoce. Desses, a dispneia é o mais comum e, inicialmente, manifesta-se apenas diante de esforços físicos e, com a progressão da doença, ocorre até mesmo em repouso. Com o avanço da doença, os pacientes também podem apresentar taquipneia e taquicardia.[14,17] Geralmente, quando os sintomas são perceptíveis, os danos pulmonares já estão em um estado avançando, necessitando acompanhamento médico imediato.

Apesar de desafiador, o diagnóstico é fundamental para o adequado manejo dos pacientes. Para isso, uma anamnese detalhada, biópsia e exames de imagem são essenciais. Dentre os exames, destaca-se a importância da tomografia computadorizada de alta resolução (TCAR) devido a visualização clara das anormalidades estruturais, permitindo o diagnóstico antes mesmo do aparecimento das alterações funcionais.[13,17]

Em termos gerais, as DIPs podem ser (a) pneumonias intersticiais idiopáticas, ou seja, de causa não identificada ou (b) secundárias a uma causa conhecida.[18] Dessa maneira, a classificação correta da DIP requer uma abordagem precisa e multidisciplinar para a exclusão de todas as possíveis causas.[19] Entre elas, encontram-se exposições ocupacionais ou ambientais, como asbestose[20], doenças sistêmicas, como esclerose[21] e COVID-19[23,24], ou resultantes da toxicidade de medicamentos, como a bleomicina[22]. Embora algumas causas possam ser identificadas, a variante mais comum de DIP é a idiopática[18], sendo a fibrose pulmonar idiopática (FPI) a sua principal forma.[16]

3. FIBROSE PULMONAR IDIOPÁTICA

3.1 Conceito e patogênese

A FPI é caracterizada por diferentes graus de ativação de fibroblastos, deposição excessiva de colágeno na MEC, espessamento das paredes alveolares e distorção da arquitetura pulmonar e alveolar. Esse conjunto de alterações teciduais implica diretamente na função respiratória, comprometendo a capacidade funcional e exercendo um considerável efeito negativo na qualidade de vida dos pacientes.[16]

Assim como em outros órgãos, a FPI se desenvolve em resposta a lesão persistente. Porém, diferentemente do observado nas demais DIPs, a biópsia de pacientes com FPI mostra uma inflamação discreta, sugerindo que ela não tenha participação direta no processo.[25]

Inicialmente, acreditava-se que a FPI era resultante de um processo inflamatório exacerbado e reparo ineficiente. Entretanto, durante as últimas décadas, essa teoria tornou-se menos aceita. Um dos motivos é a ausência de benefícios clínicos com o uso de anti-inflamatórios, tão pouco a melhora no prognóstico dos pacientes.[26,27]

Os eventos iniciais da FPI ainda são mal compreendidos, mas a hipótese mais aceita atualmente é a exposição de indivíduos geneticamente suscetíveis à doença.[15] Sendo assim, a resposta epigenética envolvida no desenvolvimento da FPI é uma hipótese cada vez mais aceita. Além disso, fatores como tabagismo e alguns agentes infecciosos, como vírus da hepatite C, também estão sendo associados ao padrão histológico e clínico da FPI, porém ainda sem evidência comprovada de relação causal.[28]

Resumidamente, a FPI pode ser considerada uma doença multifatorial na qual estímulos ambientais ativam fatores endógenos, resultando em um processo de reparo celular desordenado.[16] A proliferação de fibroblastos e miofibroblastos, em última instância, leva a insuficiência respiratória.[30] Nesse contexto, alguns autores sustentam a ideia de que a denominação “idiopática” está cada dia menos adequada.[27,29] Didaticamente, é possível explicar a patogênese da FPI em fases, conforme visualizado na figura 3.

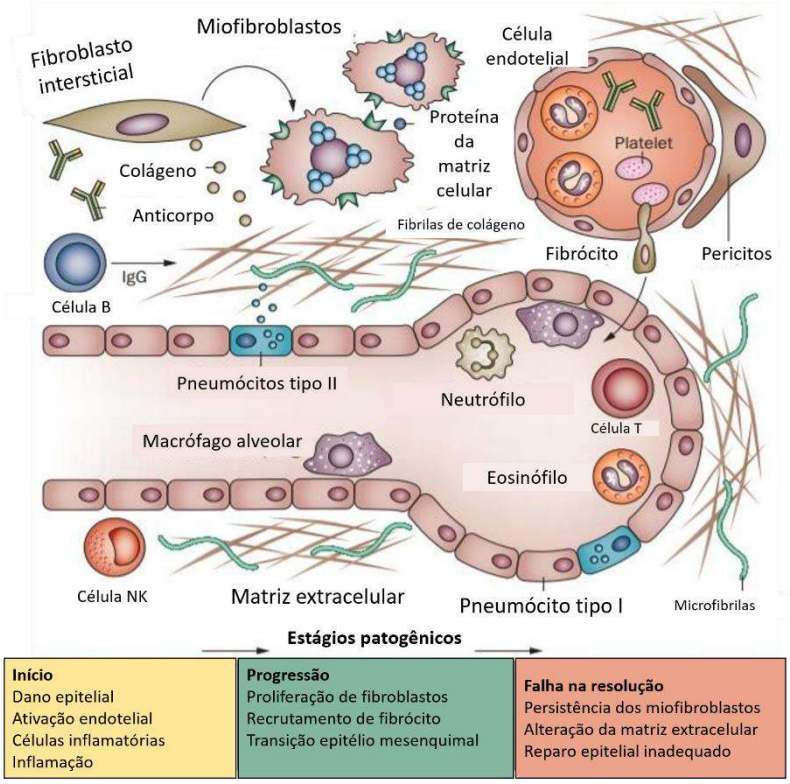


Figura 3: Estabelecimento da FPI.

Uma vez iniciada o estabelecimento da fibrose, até o momento, não existe alternativa de reversão do processo, tendo a doença um curso gradual ou rápido, mas sempre com declínio inevitável na função do órgão que, muitas vezes, leva ao óbito.[26] Estudos mostram que o TGF-β1, expresso pelos macrófagos e pelos PII, está diretamente envolvido na progressão da FPI e, portanto, parece ser um importante alvo terapêutico. [31]

À medida que o mecanismo de reparo avança, a liberação de citocinas e fatores de crescimento intensifica a deposição de MEC, demonstrando a importância das moléculas presentes no tecido para o desenvolvimento da FPI. Além disso, a literatura indica que a presença de estresse oxidativo também deve ser considerado como um importante mecanismos para o estabelecimento da FP.[1–3]

O estresse oxidativo é uma condição na qual há um desequilíbrio entre a produção de espécies reativas ao oxigênio (EROs) e/ou espécies reativas ao nitrogênio (ERNs) e as defesas antioxidantes do organismo. EROs são radicais livres (RL), ou compostos que produzem RL, que interagem com componentes da célula como lipídeos, proteínas e DNA, causando lesões altamente prejudiciais ao funcionamento celular e, muitas vezes, irreversíveis.[4,5] Conforme muito bem destacado na revisão de Birben *et al.* (2012), seres aeróbicos possuem componentes antioxidantes integrados, com ações enzimáticas e não

enzimáticas, potencialmente eficazes em condições normais. No entanto, durante o estabelecimento de doenças, como a FPI, esses sistemas de defesa estão sobrecarregados, contribuindo com o mecanismo patogênico.

Além da depleção das defesas antioxidantes, as toxinas ambientais, as mitocôndrias/NADPH oxidase das células inflamatórias e pulmonares são importantes origens do estresse oxidativo na FPI.[5] Kinnula e Myllärniemi (2008)[6] destacam que tanto em humanos quanto em modelos experimentais de FP o estado redox exerce um papel modulador na síntese da MEC nos pulmões, a qual está intimamente ligada ao processo de estabelecimento e agravamento da FP. Para os autores, além da ativação de citocinas reguladores do crescimento, a sinalização desencadeada pelo estresse oxidativo resulta na fibro-proliferação, constituindo mais uma importante via de estabelecimento da doença. Além disso, diversos estudos indicam que o estresse oxidativo está intimamente associado também a progressão e piora no prognóstico da FPI.[1,6–9]

No entanto, é improvável que um desequilíbrio único seja responsável pelas múltiplas vias afetadas durante a FPI e, por esse motivo, estudos que investiguem potenciais mecanismos associados são de extrema necessidade e importância. Nesse contexto, componentes responsáveis pela homeostase tecidual podem ser considerados potenciais alvos terapêuticos na FPI. Por esse motivo, investigar a participação dos peptídeos do sistema renina angiotensina (SRA) na fisiopatologia dessa doença é de grande relevância para avanços na ciência e na clínica, podendo representar uma importante via de tratamento futuramente.

3.2 Epidemiologia

A FPI é considerada a DIP com o pior prognóstico e com uma mortalidade precoce.[15,32] Conforme consta em Mikolasch, Garthwaite e Porter (2017)[15], a FPI é a pneumonia intersticial idiopática mais comum, afetando principalmente homens e com sobrevida média de apenas 3 anos após o diagnóstico. Ainda segundo a revisão, a FPI é um problema crescente e pode ser explicado, ao menos em parte, pelo envelhecimento da população.

Uma vez que o conceito de doença rara é definido pela União Europeia como a que afeta 5 em 100.000, uma única doença pode afetar até 250.000 mil pessoas. Países como Brasil, Rússia, Índia e China, com 2,9 bilhões de habitantes ao todo, podem ter uma doença rara em 1 milhão de pessoas, representando desafios médicos

para o sistema de saúde.[33]

Embora a FPI seja considerada uma doença rara, a mortalidade vem aumentando mundialmente no século XXI, alcançando entre 4-10 óbitos/100.000 habitantes em países da Europa, América do Norte, Ásia e Oceania.[34] Ainda segundo o estudo de Hutchinson *et al.* (2014)[34], associações positivas com o sexo masculino e aumento da idade foram identificadas.

As taxas epidemiológicas da FPI no Brasil são difíceis de determinar e os dados sobre a doença são raros. Uma das razões deve-se à dificuldade de diagnóstico que tem sido por exclusão. Dessa maneira, as estimativas podem variar de acordo com os critérios utilizados, podendo ser amplos ou restritos. Nesse sentido, na análise temporal da mortalidade no Brasil, entre 1979-2014, os coeficientes de mortalidade estão menores aos de outros países, podendo indicar a subnotificação.[35] Ainda assim, os dados de Algranti *et al*.(2017)[35] mostram que o país acompanha a tendência mundial de sexo e idade (figura 4).

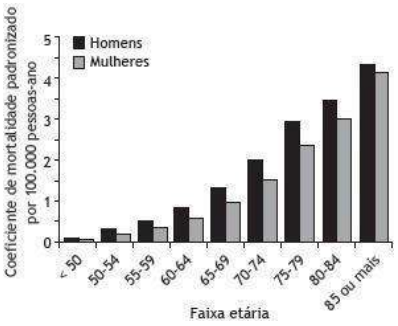


Figura 4: Taxas de mortalidade padronizadas por 100.000 pessoas-ano, por gênero e faixas etárias, 1979-2014, Brasil. (Algranti *et al*.,2017)[35]

Em relação a incidência, um estudo de estimativa supôs que a incidência anual de casos de FPI no Brasil está entre 6.841 e 9.997 casos/100.000 habitantes,enquanto sua prevalência varia entre 13.945 e 18.305 casos/100.000 habitantes.[36] Damesma forma que a mortalidade, a incidência também vem aumentando ao longo dosanos. Entretanto, para Baddini-Martinez e Pereira (2015)[36] não é possível saber se esse aumento é decorrente do maior reconhecimento da doença, ao aumento da sobrevida da população ou a fatores ambientais.

Com a incidência crescente, há um potencial para que centros especializados fiquem sobrecarregados e os custos tornem-se extremamente onerosos para o sistema de saúde. Portanto, estudos que visem elucidar os mecanismos envolvidos na FPI, bem como a identificação de vias de tratamento são de extrema relevância

não apenas para o paciente e seus familiares como para a saúde pública e ainda para o setor econômico.

3.3 Características histológicas, radiológicas e funcionais

Assim como para a classificação das DIPs, os exames de imagem são de extrema importância para a confirmação do diagnóstico e determinação da gravidade da FPI. A TCAR de tórax é o exame radiológico padrão para a FPI e costuma ser a primeira investigação em pacientes com DIP.[15] Os critérios diagnósticos observados na TCAR envolvem o predomínio de lesões basais e/ou subpleurais, anormalidades reticulares e faveolamento (figura 5).[32,37] A importância da TCAR é tamanha que pode, inclusive, eliminar a necessidade de procedimentos mais invasivos.[15]

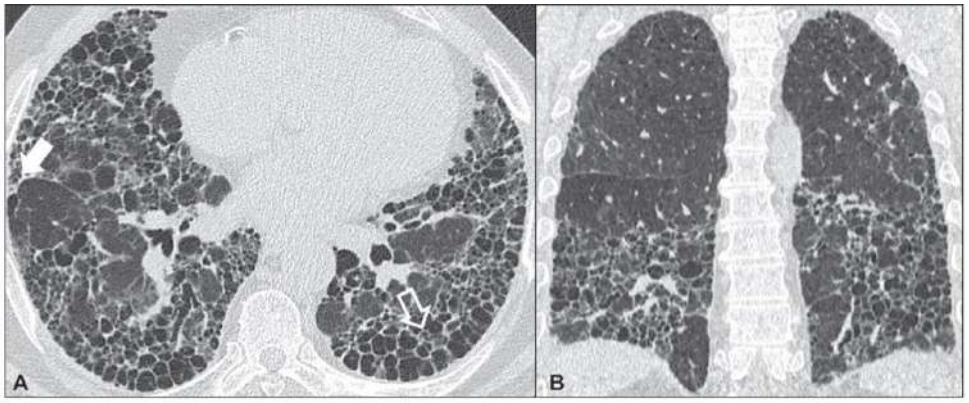


Figura 5: Imagem axial de TCAR do tórax. (A) opacidades reticulares (seta cheia) e cistos de faveolamento (seta vazada). (B) padrão de distribuição principalmente periférico e basal. (Oliveira *et al.*, 2018)[32]

Em alguns casos, a biópsia pulmonar é necessária para a confirmação da DIP clínica, mas não classificável. A diretriz publicada em 2011 pelas American Thoracic Society e European Respiratory Society (ATS/ERS)[37] fornece critérios bem estabelecidos para o padrão histológico conhecido como pneumonia intersticial usual (PIU). Conforme pode ser visualizado na figura 6, o padrão PIU é caracterizado por acúmulo de colágeno, presença de focos fibróticos, alterações subpleurais e/ou basilares e faveolamento com ou sem bronquiectasia de tração. Uma vez que as aparências típicas estão presentes em dois terços dos pacientes[15], a realização da biópsia nem sempre é necessária e, portanto, a decisão de realizar ou não deve ser individualizada.

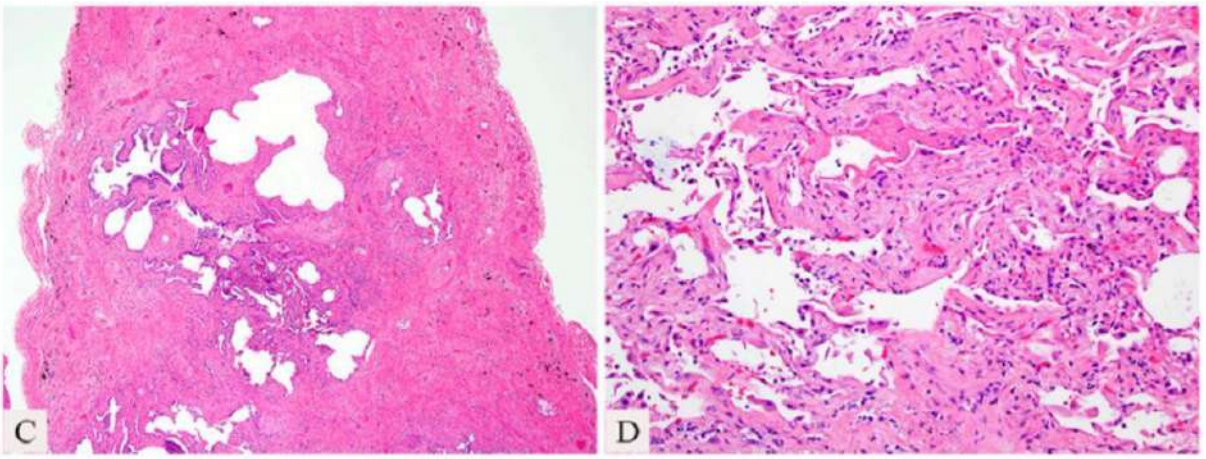


Figura 6: Características histológicas de padrão PIU. (C) Fibrose com faveolamento e focos fibróticos. (D) Dano alveolar difuso e espessamento das paredes alveolares. (Travis *et al.,* 2013)[18]

Esse conjunto de alterações histológicas, inevitavelmente, alteram a função do órgão. Nesse sentido, a FPI é descrita como uma doença respiratória restritiva, na qual há redução dos volumes pulmonares e prejuízo na oxigenação do paciente, fatores diretamente associados aos sintomas clássicos da doença.[38]

A dificuldade dos pulmões fibróticos de se expandirem durante a inspiração é explicada, não só pelo acúmulo de colágeno no parênquima pulmonar, mas também pela diminuição da produção do surfactante pelos PII lesados. Em conjunto, essas anormalidades funcionais representam o aumento da elastância e, consequentemente, redução da complacência pulmonar.[39]

Segundo Cottin *et al.* (2019)[14], esses prejuízos funcionais podem ser analisados através do parâmetro denominado capacidade vital forçada (CVF). Esse parâmetro é avaliado pela mudança na linha de base em mL ou também considerando a percentagem do valor previsto.

O grau de comprometimento da função respiratória reflete diretamente a progressão da doença e, nesse contexto, o declínio da CVF é um preditor bem estabelecido de mortalidade. Além disso, devido ao aumento da força elástica dos pulmões, a relação entre o volume expiratório forçado no primeiro segundo (VEF1) e a CVF pode apresentar-se normal ou elevada.[40,41]

3.4 Terapêutica da fibrose pulmonar idiopática

Apesar da crescente evolução na compreensão das vias envolvidas no desenvolvimento da FPI, as opções terapêuticas ainda são muito limitadas e o óbito é um desfecho clínico comum em pouco tempo após o diagnóstico.[15] Ainda assim, conforme Cottin *et al.* (2019)[14], a FPI é a única DIP com terapia já estabelecida.

Os primeiros tratamentos para FPI incluíam protocolos com terapia combinada de prednisona, N-acetilcisteína e Azatioprina. Porém, além de não atingir resultados eficientes, esses imunossupressores mostraram piorar o prognóstico, com aumento do risco de hospitalizações e mortalidade, fornecendo evidências contra o uso desse protocolo.[42]

Atualmente, a terapia básica é apenas de suporte e não há medicamento que interrompa a progressão, nem reverta a fibrose existente. De todas as substâncias investigadas até o momento, apenas o nintedanibe e a pirfenidona foram recomendadas para o tratamento da FPI.[43] Embora sejam eficazes para retardar a progressão da doença e reduzir a taxa de declínio da função pulmonar, elas não modificam a alta taxa de mortalidade e possuem efeitos colaterais debilitantes, como vômito e diarreia.[16,43–45]

Essas substâncias têm como alvo receptores de fatores de crescimento essenciais na patogênese da FPI. Dessa forma, experimentos demonstraram a eficácia em diversos processos como proliferação, migração e diferenciação de fibroblastos e deposição de MEC.[43,46,47] Entretanto, apesar de serem considerados seguros e com recomendação na diretriz de prática clínica internacional para FPI[43], no Brasil ele não está disponível no Sistema Único de Saúde (SUS). Conforme os relatórios do SUS[44,45], os benefícios de ambas as drogas não justificam a distribuição pelo sistema público.

Em casos mais avançados, o transplante pulmonar é a única opção para possibilitar um aumento considerável na sobrevida de pacientes. Entretanto, no Brasil, essa opção é muito limitada, devido aos poucos centros médicos aptos a realização de um procedimento tão complexo e ao baixo número de doadores.[48]

Devido ao caráter crônico da FPI, a terapia precoce pode estar diretamente correlacionada com a eficácia terapêutica, uma vez que a arquitetura pulmonar não sofreu modificações significativas. Portanto, estudos de caráter preventivo, avaliando o papel de reguladores endógenos da homeostase tecidual, como o SRA, parece ser

uma estratégia fundamental para a investigação de possíveis terapias para essa doença limitante.

4. SISTEMA RENINA ANGIOTENSINA: ALÉM DO CONTROLE CARDIOVASULAR

O organismo humano é composto por diferentes e complexos sistemas que interagem para um adequado funcionamento. Entre eles, o SRA é vital para a sobrevivência. O SRA pode ser definido como uma cascata de reações, envolvendo hormônios vasoativos, enzimas conversoras e receptores com funções essenciais na homeostase. Classicamente, o SRA foi reconhecido como parte do sistema cardiovascular, sendo fundamental para regular o tônus vascular e o balanço hidroeletrolítico.[49] Portanto, já está bem descrito que perturbações no SRA podem causar alterações pressóricas e a morte.[50–52]

Porém, desde a sua caracterização, ele vem sendo extensivamente estudado, proporcionando a identificação de novos componentes, vias e locais de ativação. Atualmente, a presença do SRA é descrita em tecidos como cérebro, coração, músculo esquelético e tecido adiposo com importantes funções fisiológicas (figura 7).[53–55] Dessa forma, é de se esperar que o desequilíbrio desse sistema esteja envolvido na patogênese de diferentes doenças como fibrose pulmonar[56,57], esclerose[58], sepse[59] e COVID-19[60].

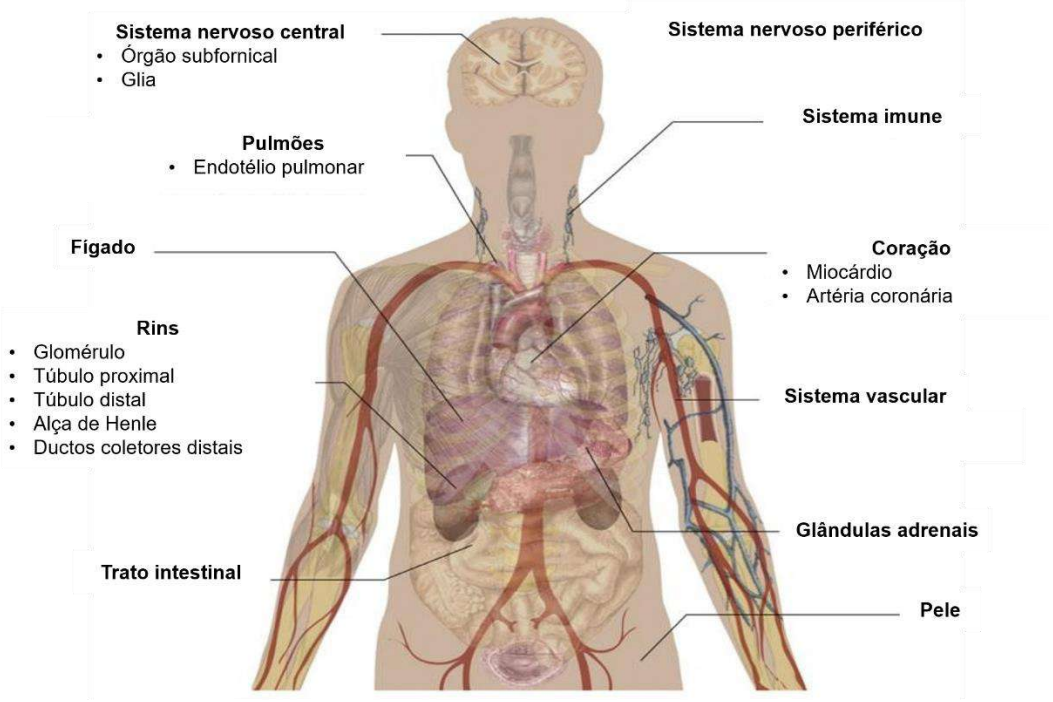


Figura 7: Localização do SRA tecidual. (Traduzida de Bitker e Burrell, 2019)[53]

Com o avanço das pesquisas e a descoberta dos novos componentes, o SRA passou a ser dividido, didaticamente, em dois eixos, para facilitar a compreensão da cascata enzimática, bem como os efeitos nos órgãos alvo.

4.1 Eixo clássico

A via canônica do SRA inicia com a secreção da enzima renina, uma aspartil protease, pelas células justaglomerulares localizadas na arteríola aferente renal. A secreção dessa enzima pode ser estimulada por diversos fatores como a diminuição do fluxo sanguíneo renal e/ou de sódio no túbulo renal distal, fortes indicativos de redução da pressão arterial.[61,62] Ao ser liberada na corrente sanguínea, a renina realiza a conversão do peptídeo angiotensinogênio, proveniente principalmente do fígado, no decapeptídeo angiotensina I (Ang I).[53]

A Ang I é considerada um peptídeo biologicamente inativo por possuir baixos efeitos vasoativos, sendo então substrato para a enzima conversora de angiotensina (ECA), presente na superfície luminal das células endoteliais. A ação da ECA é baseada na clivagem dos dois últimos aminoácidos da Ang I, formando o octapeptídeo angiotensina II (Ang II), que possui importantes funções no sistema cardiovascular.[63] Essa conversão ocorre grande parte nos pequenos vasos dos pulmões.[64]

Apesar de rapidamente degradada em angiotensina III (Ang III), IV (Ang IV) ou 1-7 [Ang-(1-7)], a Ang II é o peptídeo central desse eixo. As suas ações podem ser através da ligação com os receptores de angiotensina tipo 1 (AT1) ou tipo 2 (AT2), pertencentes à família de receptores acoplados à proteína G.[64]

As clássicas funções cardiovasculares da Ang II, como vasoconstrição, aumento do débito cardíaco e promoção da síntese de aldosterona são desempenhadas pela ligação do peptídeo ao receptor AT1. Por outro lado, os efeitos da Ang II via receptor AT2 são menos conhecidos. Apesar disso, todas as ações já observadas são antagônicas às produzidas via AT1, como, por exemplo, vasodilatação, antifibrótica e anti-inflamatória. Além disso, sabe-se também que a expressão tecidual do receptor AT2 está reduzida em comparação a do AT1.[64] A figura 8 representa um esquema simplificado desse eixo.

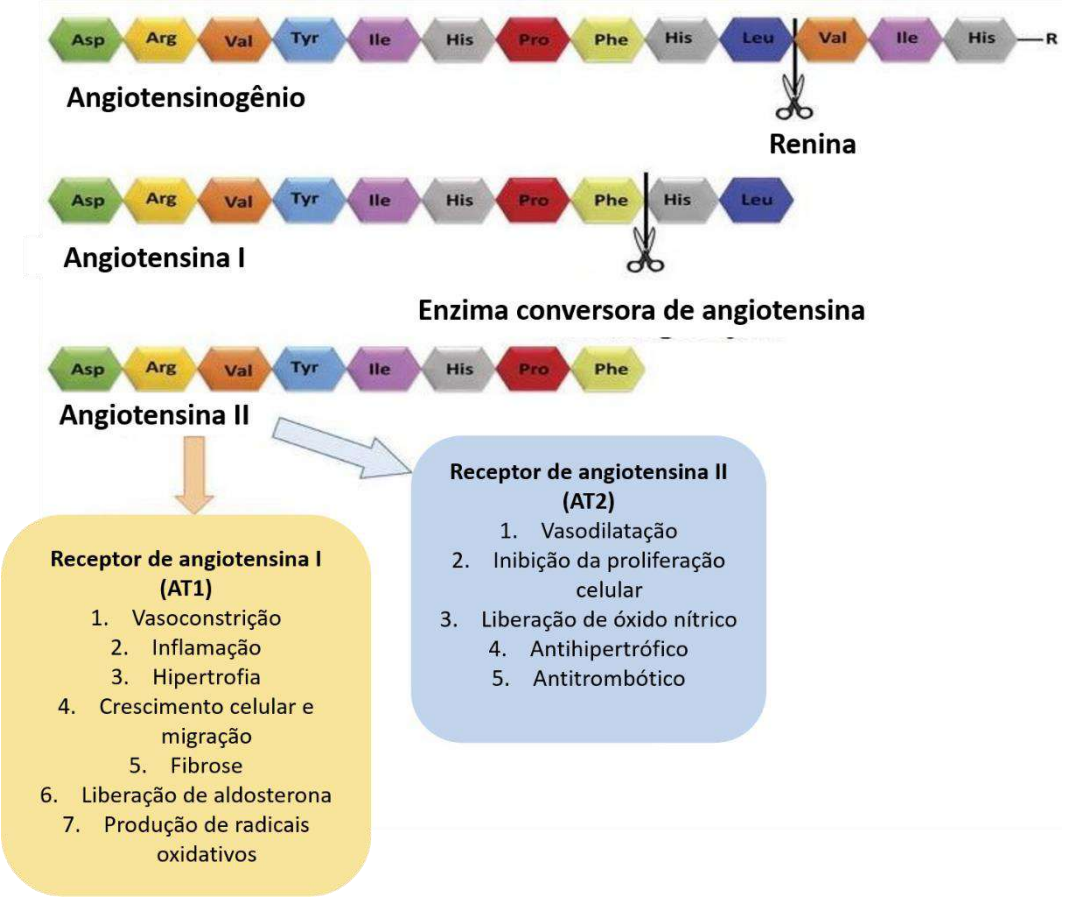


Figura 8: Visão clássica do sistema renina angiotensina. (Traduzida de Hussain e Awan, 2018)[64]

A Ang III também é um potente vasoconstritor desse eixo, estimulador do apetite pelo sal e indutor da aldosterona, assim como a Ang II. Enquanto que a Ang IV tem efeitos pressóricos opostos, aumentando o fluxo sanguíneo renal e reduzindo a reabsorção de sódio e água.[53]

Apesar das funções regulatórias bem conhecidas e importantes da Ang II, a maior expressão tecidual de AT1 ou o aumento plasmático do peptídeo podem resultar no desequilíbrio do sistema. Essa modulação do SRA causa efeitos deletérios como vasoconstrição, estimulação do sistema nervoso simpático, inflamação, apoptose, aumento do estresse oxidativo e proliferação dos fibroblastos.[65] Dessa forma, inúmeras evidências apontam o envolvimento do SRA em condições patológicas além do sistema cardiovascular como nos pulmões[57], fígado[66] e sistema nervoso[58].

Outro importante componente da via clássica é a Angiotensina A (Ang A), um octapeptídeo também com afinidade pelos receptores AT1 e AT2. Isso se deve a semelhança estrutural com o seu precursor Ang II, diferindo apenas no aminoácido alanina na posição N-terminal e não aspartato. Apesar de ter maior afinidade pelo

receptor AT2, as ações vasoconstritoras e vasopressoras da Ang A são comparáveis às da Ang II.[67]

4.2 Visão atual do SRA

Por décadas, os pesquisadores estudaram o SRA, não apenas para compreender os mecanismos fisiológicos, mas também para identificar alvos terapêuticos. Essas investigações levaram a descoberta de vias alternativas que despertaram o papel protetor do SRA em diversos tecidos.[53,65,69,70]

O SRA contrarregulador é composto de vários peptídeos, receptores e enzimas. O primeiro componente descrito foi o heptapeptídeo Ang-(1-7), sintetizado em diferentes reações. A primeira via observada foi a partir da Ang I, independentemente da ECA.[71] Uma década após a descoberta da Ang-(1-7), aidentificação da enzima conversora de angiotensina 2 (ECA2), revelou a rota de formação do peptídeo. Essa enzima tem a capacidade de clivar a Ang I, formando Ang-(1-9) que, posteriormente, poderá formar Ang-(1-7) pela ação catalítica da ECA[72]ou pela endopeptidase neutra (NEP)[73]. Porém, apesar da Ang I representar um precursor importante, a via mais relevante ocorre através da ação direta da ECA2 naAng II (figura 9).[68]

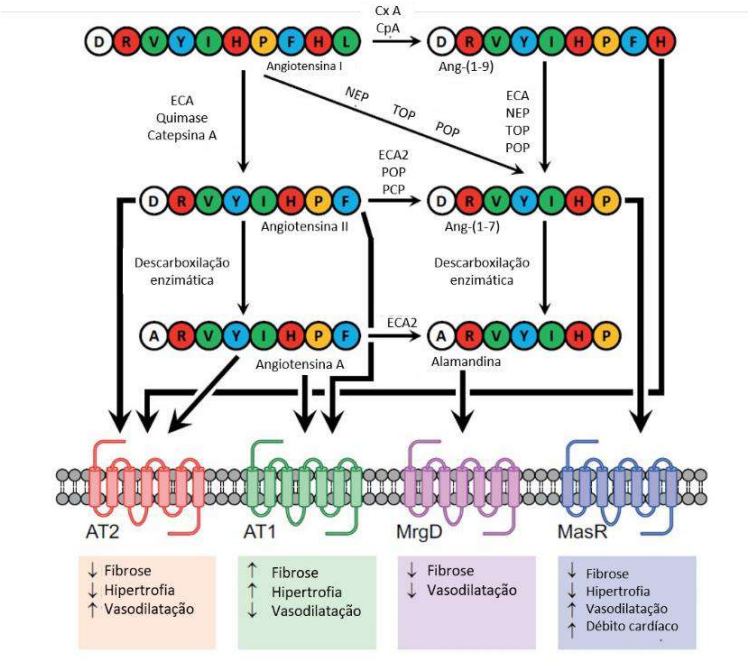


Figura 9: Vias clássicas e vias protetoras do sistema renina angiotensina.

ECA, enzima conversora de angiotensina; ECA2, enzima conversora de angiotensina 2; AT1, receptor de angiotensina tipo 1; AT2, receptor de angiotensina tipo 2; MrgD, receptor acoplado a proteína G relacionado

ao Mas tipo D; MasR, receptor Mas; Cxa, carboxipeptidase; CpA, catepsina; NEP, endopeptidase neutral; PCP, prolil carbopeptidase; POP, prolil endopeptidase; TOP, thimet oligopeptidase.
(Traduzida de Torres *et al.*,2015)[68]

Apesar de muito semelhante do ponto de vista estrutural, a ECA2 e a ECA se contrapõem do ponto de vista funcional. Os peptídeos resultantes da ECA2 ativam potentes receptores vasodilatadores e anti-inflamatórios e, portanto, foram considerados contra regulatórios do SRA clássico (figura 11).[68]

De todos já identificados, a Ang-(1-7) e a alamandina (ALA) são os peptídeos com as ações mais investigadas. Os efeitos da Ang-(1-7) são mediados pela ligação com o receptor Mas e já foram demonstrados em diversos tecidos.[69,74–77] Em muitas condições fisiológicas, como, por exemplo, na gestação, a expressão de Ang-(1-7) é essencial para neutralizar a elevação progressiva da Ang II circulante e manter os níveis pressóricos normais.[76] Além disso, Barroso *et al*. (2017)[75] demonstraram que a Ang-(1-7) é um importante mediador de resolução da inflamação por inibir o fator de transcrição NF-κB.

A descoberta, em 2013, de outros dois componentes do eixo não clássico amplia as possibilidades terapêuticas do SRA. Pesquisadores brasileiros identificaram e caracterizaram o peptídeo ALA e seu receptor *Mas-related G-protein coupled receptor type D* (MrgD), com ações vasodilatadoras e protetoras semelhantes à já conhecida Ang-(1-7).[78]

A ALA é um heptapeptídeo análogo a Ang-(1-7), possuindo apenas uma diferença estrutural na posição 1, com a substituição do aminoácido aspartato pela alanina. A ALA pode derivar da descarboxilação da própria Ang-(1-7) ou a partir da Ang A, pela ação da ECA2[79] e, apesar de recentemente descoberta, já possui efeitos comprovados em diversas condições.[80–82]

Segundo experimentos *in vivo*, a administração de ALA pode ter diferentes desfechos em relação a regulação da pressão arterial, dependendo da via de administração e condição fisiopatológica do corpo. Em ratos hipertensos, a infusão subcutânea de ALA por 6 semanas foi capaz de reduzir a pressão arterial (PA) e a hipertrofia cardíaca.[83] Em contraponto, Wang *et al*. (2019)[84] mostraram que a ALA intraperitoneal é capaz de atenuar a fibrose cardíaca de forma independente da PA. Essa característica pode representar uma vantagem em futuras intervenções terapêuticas em doenças não associadas à hipertensão, como a FPI.

Além do efeito pressórico, a capacidade antioxidante, anti-inflamatória e antifibrótica já foram descritas para a ALA.[82,85,86] Efeitos esses altamente desejáveis no manejo de pacientes com doenças fibrosantes. Em modelo experimental, a ALA reduziu a síntese de colágeno e a remodelações tecidual, mostrando ter um potente efeito antiproliferativo no sistema cardiovascular.[80]

Além disso, a capacidade antifibrótica foi comprovada, recentemente, também no fígado.[82] Huang *et* al. (2020)[82] demonstraram que a administração de ALA mudou o equilíbrio do SRA a favor do eixo da ECA2, reduzindo o estresse oxidativo e a produção de colágeno.

4.3 SRA e fibrose pulmonar

Recentemente, os efeitos protetores do eixo da ECA2 no sistema respiratório ficaram mais evidente com a pandemia COVID-19.[60] Entretanto, as implicações do desequilíbrio entre os eixos do SRA nos pulmões já foram bem descritas.[53,65,87–89]

Considerando que os pulmões são um importante sítio de ativação do SRA, é evidente a participação desse sistema na fisiopatologia de doenças pulmonares, como a FPI.[65] De fato, nosso grupo já demonstrou que pacientes com FPI apresentam desequilíbrio nos componentes desse sistema.[56,57] Segundo Uhal *et al*. (2012)[90], o aumento da produção de angiotensinogênio, com o objetivo de estimular a fibrogênese, pode contribuir para a maior geração de Ang II pelas células epiteliais alveolares lesadas e miofibroblastos e estar envolvida com o estabelecimento da FP.

No estudo de Raupp *et al.* (2020)[57], em comparação ao grupo controle, a expressão do receptor AT1 estava significativamente maior no tecido pulmonar de pacientes com FPI, enquanto a do receptor da Ang-(1-7) estava menor. Além disso, os autores mostraram uma correlação negativa entre a expressão do receptor AT1 e os importantes parâmetros respiratórios VEF1 e CVF.

Esse desequilíbrio no SRA também foi demonstrado, no plasma, por Sipriani *et al*. (2019)[56]. Os autores analisaram a concentração plasmática de Ang I, Ang II, Ang-(1-7) e ALA em pacientes com FPI e verificaram que apenas esse último peptídeo estava 365% menor. Em conjunto, os dados do nosso grupo mostram que o desequilíbrio do SRA a favor do eixo fibrosante pode estar envolvido diretamente no desenvolvimento e progressão da FPI.

Além disso, em modelo animal de FP, foi demonstrado que a Ang II formada no tecido pulmonar é fundamental para estimular a expressão de TGF-β, impulsionandoa proliferação de fibroblastos e a diferenciação em miofibroblastos.[87,88] Wang *et al.* (2015)[91] posteriormente comprovaram que, além do papel crítico da via Ang II/AT1 para os eventos moleculares fibrogênicos, esse eixo também é responsável pela redução da complacência respiratória em camundongos.

Embora pouco se saiba sobre os efeitos do SRA na FPI em humanos, o dano epitelial e endotelial parece criar um desequilíbrio que favorece a ação deletéria do eixo clássico sobre o não clássico. Esses efeitos incluem o aumento da secreção de mediadores pró inflamatórios e a produção local de espécies reativas ao oxigênio, todos diretamente implicados na patogênese da doença.[14,30]

A inibição do eixo Ang II/AT1 já foi investigada em ensaios clínicos como alternativa terapêutica para FPI e não obtiveram resultados satisfatórios.[92] No entanto, como é possível perceber, nosso conhecimento sobre o SRA está crescendo e expondo, cada vez mais, sua complexidade. Novos capítulos, direcionados para um novo conceito na utilização desse sistema, precisam ser escritos.

O potencial do eixo contrarregulatório já foi demonstrado em modelos experimentais de FP induzidos por bleomicina.[93–96] Segundo os achados, a modulação do SRA pode ser um importante aliado no tratamento da fibrose. Para Prata *et al.* (2017)[96], a ativação do eixo da ECA2, associado ao exercício, pode representar melhoras significativas na redução da FP. Além disso, restabelecer o equilíbrio do SRA pode ser mais importante do que a inibição do eixo fibrosante, conforme sugerido por Wang *et al*. (2015).[94]

Considerando o desequilíbrio do SRA observado nos pacientes com FPI[56,57] e as ações antifibróticas da ALA[79,82,97], é possível acreditar que a administração exógena desse peptídeo possa atenuar o desenvolvimento da fibrose e auxiliar na redução dos sintomas, melhorando a qualidade de vida desses pacientes. Apesar da importância do eixo envolvendo a ECA2 estar bem fundamentada para a homeostase tecidual, nenhum estudo foi encontrado na literatura avaliando o potencial terapêutico da ALA em doenças respiratórias.

5. MODELO EXPERIMENTAL DE FIBROSE PULMONAR

O reparo tecidual é um mecanismo universal nos seres multicelulares e,

portanto, modelos experimentais são considerados seguros para estudos fisiopatológicos e investigação de potenciais terapias. Diversos modelos de administração de drogas, exposição a alérgenos, irradiação e infecção já foram descritos na literatura.[98] Segundo as recomendações da ATS, a indução por bleomicina (BLM) intratraqueal em ratos é o que melhor mimetiza a doença nos humanos sendo o mais indicado para os testes pré-clínicos.[99]

A BLM é um antibiótico isolado do fungo *Streptomyces verticillus,* descoberto em 1966, com propriedades quimioterápicas.[100] Esse medicamento tem sido utilizado como tratamento de diferentes tipos de tumores malignos, como linfomas, entretanto, a eficácia terapêutica é limitada pelo desenvolvimento de FP.[7] A toxicidade para o tecido pulmonar está associada a baixa atividade local da enzima bleomicina hidrolase, protease capaz de inativar a BLM.[101]

O mecanismo antitumoral da BLM envolve a produção de radicais livres que, através da clivagem do DNA, induzem a morte celular. Além disso, o estresse oxidativo leva a uma resposta inflamatória e ativação dos fibroblastos. A patogênese induzida pela BLM nos pulmões parece estar associada a danos no endotélio e epitélioalveolar, liberando citocinas e fatores de crescimento que estimulam a FP.[7,102]

Desde a descoberta das rápidas reações inflamatórias e fibróticas em ratos[103], o modelo tem sido amplamente utilizado.[104–106] Ainda que muitos pesquisadores utilizem uma única administração intratraqueal ou injeções peritoneais repetitivas, a via orofaríngea leva à FP com menor sedação do animal e sem procedimento cirúrgico, permitindo uma recuperação mais rápida e segura.[107]

A evolução da FP após a instilação da BLM pode ser dividida em três fases: lesão pulmonar aguda (dias 0-7), fibroproliferação (dias 3-14) e fibrose estabelecida (dias 14-28)[108] (figura 10). Portanto, com esse modelo, é possível investigar o efeito preventivo e terapêutico de compostos.[109]

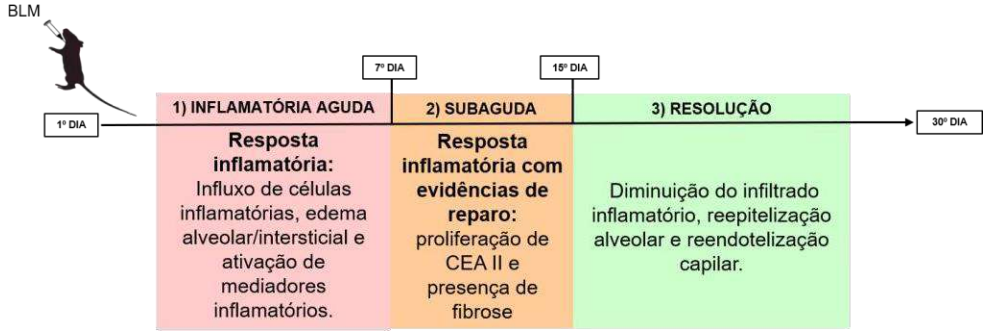


Figura 10: Evolução temporal da FP após instilação de BLM. (Autoria própria,2020)

Para que esses estudos sejam conduzidos de forma adequada, Moeller *et al*. (2008)[102] reconhecem como tratamento preventivo aqueles iniciados antes do 7º dia de protocolo, enquanto o terapêutico deve ser administrado apenas após a fase de inflamação aguda. Nesse contexto, o melhor momento para a avaliação do efeito preventivo da ALA é o 14º dia após a instilação. Nessa fase, os animais apresentam fibrose estabelecida, porém, sem níveis extensivos a ponto de causar mortalidade.

Além de mimetizar as alterações da FPI, o modelo de BLM é de fácil execução e boa reprodutibilidade, critérios importantes para um bom modelo animal.[102] Por esses motivos, foi o método de avaliação pré-clínica do nintedanibe[110] e de elucidação da participação do TGF-β como um dos fatores chave no desenvolvimento da fibrose.[111]

De acordo com as diretrizes oficiais, a análise precisa da FP envolve vários desfechos. Entre eles, a avaliação histológica e a função pulmonar são consideradas importantes parâmetros pré-clínicos.[99]

O grau de fibrose é mensurado pela escala de Ashcroft, sendo classificado de 0 (pulmão saudável) a 8 (obliteração fibrosa total).[112] As alterações fibróticas observadas podem ser confirmadas através da deposição de colágeno, pela coloração tricrômica de Masson.[99]

Essas modificações teciduais refletem na função pulmonar que, apesar de ter a avaliação desafiadora, é muito útil para examinar a resposta terapêutica. A análise dos parâmetros funcionais na FP experimental é realizada através de métodos invasivos, Buxco-force ou FlexiVent, que realizam manobras de ventilação forçada em animais anestesiados e traqueostomizados.[99]

6. DIRETRIZES FUTURAS: ALAMANDINA E COVID-19

O primeiro caso da COVID-19, doença causada pelo novo coronavírus da síndrome respiratória aguda grave (SARS-CoV-2), teve seu primeiro caso relatado em Wuhan, na China, em dezembro de 2019 e foi reconhecida como pandemia pela Organização Mundial da Saúde (OMS) em março de 2020.[113] Devido a acelerada disseminação, a COVID-19 despertou intenso interesse de grupos de pesquisa em todo o mundo. O árduo esforço da pesquisa mundial permitiu a rápida identificação da estreita relação do vírus com o SRA[70,114] e do potencial terapêutico desse sistema para

a doença.[115,116]

Os estudos demonstraram que a infecção pelo SARS-CoV-2 é dependente, ao menos em parte, da ligação com a ECA2, levando a redução da atividade da enzima.[70,114] O desequilíbrio do SRA resultante tem como consequência o prejuízo dos efeitos anti-inflamatórios, antifibróticos e antitrombóticos, favorecendo os distúrbios da COVID-19.[117] Além disso, uma vez que os pulmões são a fonte principalda ECA2, a interação do vírus com o tecido pulmonar explica as graves consequênciasrespiratórias, além do amplo espectro clínico e patológico da doença.[118]

Apesar do reconhecimento aos esforços centrados na resolução do quadro agudo da doença e no desenvolvimento de vacinas, evidências emergentes apontam para a necessidade do acompanhamento a longo prazo dos pacientes recuperados da infecção, devido a possível sequela fibrótica.[24,119–124] Embora os pacientes se recuperem dos sintomas, o desenvolvimento da FP pós COVID-19 pode trazer sérias complicações aos pacientes e aos cofres públicos, por se somar às causas já conhecidas de FP.

Segundo os estudos de Pan *et al.* (2020)[125], Zhou *et al*. (2020)[126] e Shi *et al*. (2020)[127]*,* as alterações fibróticas no tecido pulmonar já podem ser observadas na fase inicial, inclusive assintomática[127], da doença. Ainda que seja cedo para determinar se a fibrose irá progredir em todos os pacientes, alguns dados deixam claro a importância clínica da FP no mau prognóstico.[6,128] Sendo assim, e, considerando os desafios relacionados ao tratamento da FP, esforços devem ser direcionados para reduzir o risco entre os pacientes com COVID-19.

Embora, neste momento, ainda não seja possível prever quantas pessoas desenvolverão sequelas permanentes devido a pandemia, as altas taxas de contaminação demandam profunda atenção. Diante disso, estudos que identifiquem alvos terapêuticos para, futuramente, atenuar o desenvolvimento da fibrose representa um grande avanço na pesquisa básica.

Nesse sentido, a literatura sugere possíveis efeitos benéficos do uso da Ang-(1-7) e da ALA para o tratamento da COVID-19.[115,116,129–131] As inúmeras evidências do papel anti-inflamatório e antifibrótico desses peptídeos, já descritas nesse referencial, estimulam as investigações para a COVID-19 e/ou suas sequelas, uma vez que são efeitos altamente desejáveis.

Nossos dados até o momento corroboraram com essa hipótese e, por isso, mais estudos estão sendo realizados para demonstrar o potencial antifibrótico da

alamandina, pela primeira vez, nos pulmões. Uma vez que o mecanismo fibrótico da FPI e da COVID-19 são semelhantes, acreditamos que os achados descritos na presente tese poderão, futuramente, se confirmar em modelos in vitro e *in vivo* de infecção por SARS-CoV-2. Importante ressaltar que, devido aos relevantes achados e a possibilidade da aplicação para a atual pandemia, as informações contidas no artigo anexado na tese foram classificadas pela OMS como de interesse em saúde pública para possívelmente tratar sequelas da COVID-19. Além disso, foi solicitada a *PCT International Application No. PCT/US2021/029559 Filed: April 28, 2021* (ANEXO 2).

HIPÓTESE

A hipótese do estudo foi de que a alamandina seria protetora aos danos inflamatórios, oxidativos e funcionais em um modelo de fibrose pulmonar experimental. Além disso, é plausível acreditar que, futuramente, ela possa ser considerada como uma importante alternativa adjuvante no tratamento de pacientes com fibrose pulmonar por múltiplas causas.

OBJETIVOS

Objetivo geral

Investigar o potencial preventivo da alamandina no desenvolvimento da fibrose pulmonar experimental.

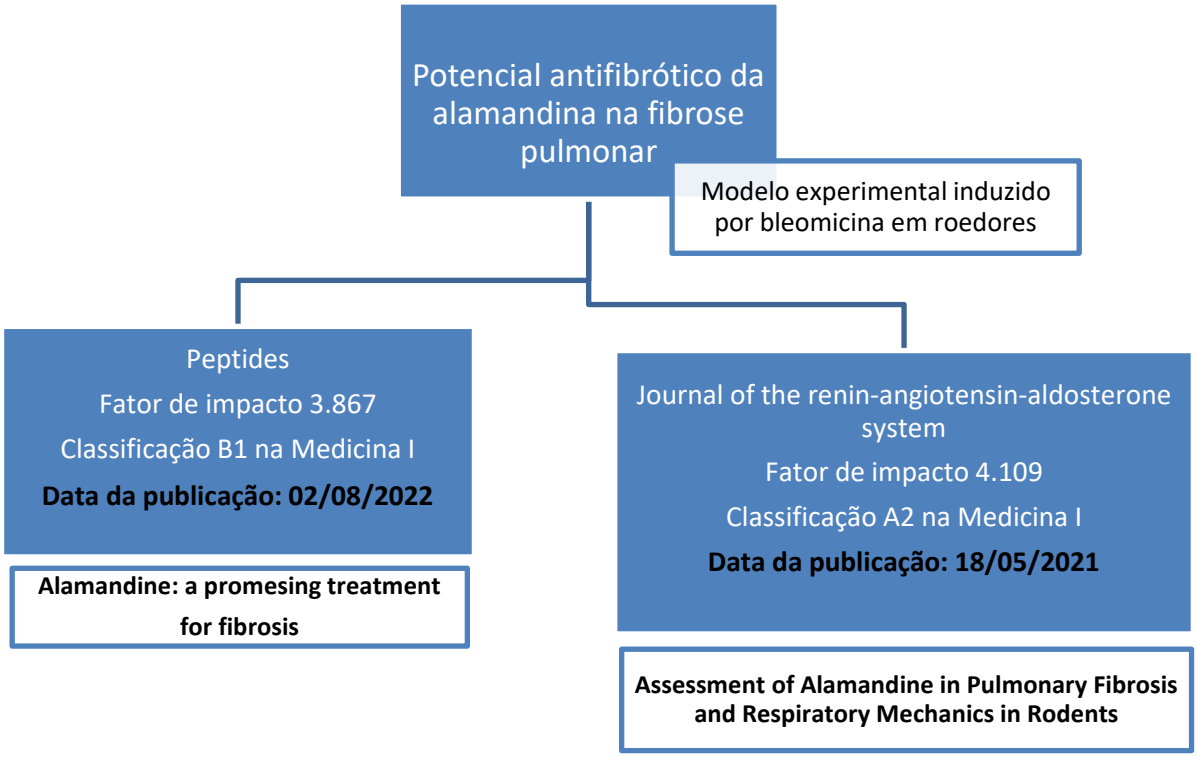
Objetivos específicos

Analisar o efeito da administração de alamandina em ratos sobre a(o):

- Mecânica pulmonar;
- Pressão arterial sistêmica;
- Balanço simpatovagal para o coração;
- Histopatologia e deposição de colágeno na matriz extracelular;

RESULTADOS

Os resultados obtidos durante o estudo foram publicados em 2 artigos internacionais. A ordem de apresentação segue o diagrama abaixo e tem como objetivo a melhor compreensão dos objetivos propostos no estudo.



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Review

Alamandine: A promising treatment for fibrosis

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ABSTRACT

Angiotensin (Ang) II, the main active member of the renin angiotensin system (RAS), is essential for the maintenance of cardiovascular homeostasis. However, hyperactivation of the RAS causes fibrotic diseases. Ang II has pro-inflammatory actions, and moreover activates interstitial fibroblasts and/or dysregulates extracellular matrix degradation. The discovery of new RAS pathways has revealed the complexity of this system. Among the RAS peptides, alamandine (ALA, Ala[1] Ang 1–7) has been identified in humans, rats, and mice, with protective actions in different pathological conditions. ALA has similar effects to its well-known congener, Ang-(1–7), as a vasodilator, anti-inflammatory, and antifibrotic. Its protective role against cardiovascular diseases is well-reviewed in the literature. However, the protective actions of ALA in fibrotic conditions have been little explored. Therefore, in this article, we review the ability of ALA to modulate the inflammatory process and collagen deposition, to serve as an antioxidant, and to mediate protection against functional disorders. In this scenario, we also explore ALA as a promising therapy for pulmonary fibrosis after COVID-19 infection.

1. Introduction

In 1898, the physiologist Professor Robert Tigerstedt and Pen Gunnar Bergman, one of his fellows, showed the pressor function of a substance obtained from the kidney. The substance, which they named renin, was isolated from the renal cortex [1] and was the beginning of an impactful and broad area of research in the biological field. Since the discovery of renin, several related substances have been studied, presently known as the renin-angiotensin system (RAS).

Currently, it is well-established that dehydration and hyponatremia stimulate renin release from the kidneys, more specifically the juxtaglomerular apparatus. This is a response to decreased renal perfusion pressure and/or sodium (Na) concentration in the distal tubules. Renin release is also stimulated by activation of the sympathetic nervous system. In the systemic circulation, renin cleaves angiotensinogen, a glycoprotein produced by the liver, to form angiotensin I (Ang I; Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu). Ang I is cleaved by angiotensin converting enzyme (ACE) into angiotensin II (Ang II; Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), mainly in the vasculature of the lungs. The classic actions of Ang II are associated with hydromineral balance and blood pressure (BP) control by different mechanisms, such as renal Na excretion, vasoconstriction, and sympathetic nervous system stimulation through AT1 receptors [2–4] (Fig. 1).

For many years, the RAS was mainly considered in terms of the hemodynamic functions of Ang II. However, investigations have shown that sustained stimulation of the Ang II/AT1 axis is implicated in several pathological conditions, including cell proliferation, inflammatory responses, and coagulation disorders [5–8].

Since the discovery of renin over 100 years ago, much progress has been made towards a better understanding of the RAS in homeostasis and in disease. Several new RAS components have been discovered in the blood and tissues: angiotensin (1–7) (1–7) [Ang-(1–7); Asp-Arg-Val-Tyr-Ile-His-Pro] [9], the Mas receptor [10], and ACE subtype 2, (ACE2) [11–13]. These findings have led to a new interpretation of the original cascade of RAS, with the ACE2/Ang-(1–7)/Mas axis being antagonistic to the Ang II/AT1 axis.

When discovered in 1983, Tonnaer et al. [13] demonstrated that Ang-(1–7) had no biological activity in the brain of rabbits. These authors hypothesized that Ang-(1–7) was an inactive product from Ang II degradation. Subsequently, Santos et al. [14] showed the exogenous

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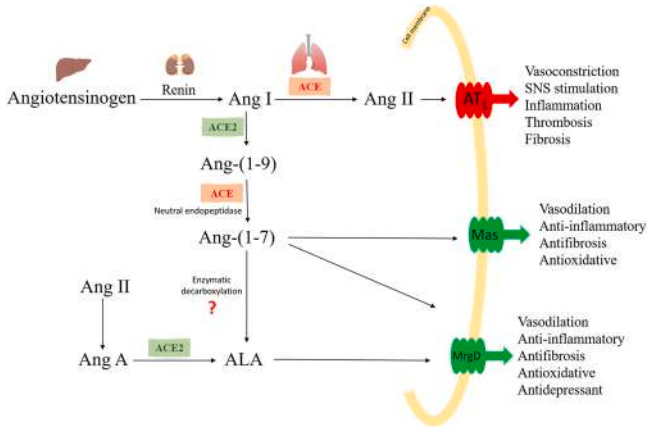

Fig. 1. Classical and non-classical actions of the renin-angiotensin system components. Ang I = Angiotensin I; Ang II = Angiotensin II; Ang-(1–9) = Angiotensin 1–9; Ang-(1–7) = Angiotensin (1–7); ALA = Alamandine; Ang A = Angiotensin A; ACE = Angiotensin-converting enzyme; ACE2 = Angiotensin-converting enzyme type 2; AT1 = Angiotensin receptor type 1; Mas = Ang-(1–7) receptor; MrdG = Mas-related G-protein coupled receptor type D.

conversion of Ang I into Ang-(1–7) in the brainstem and spinal cord regions of dogs, even in the presence of an ACE inhibitor. Similarly, Chappell et al. [9] demonstrated in rats that plasma and tissue concentrations of Ang-(1–7) suggest that the endogenous generation of this peptide is from Ang I or Ang II.

Surely, the characterization of this non-classical RAS began in 1988 with Santos et al. [15], who showed Ang-(1–7) formation from Ang I in an ACE-independent pathway. From that time, its cardiovascular and cerebral effects were discovered reveling new RAS interactions [16,17], including a specific receptor for Ang-(1–7) [18,19].

After the discovery of ACE2 in 2000, the unprecedented enzymatic pathway to form Ang-(1–7) was unveiled [11,12]. In 2003, the hypotensive effects of Ang-(1–7) were associated with bradykinin [20], opposing the vasoconstrictor and proliferative effects of the Ang II/AT1 axis [21]. Finally, still in 2003, Santos et al. demonstrated that the functional activities of Ang-(1–7) occur through the Mas receptor [10].

Currently, there are abundant data for the ACE2-independent generation of Ang-(1–7) [22–28]. Studies show that vascular neprilysin (NEP) is responsible for the extracellular conversion of Ang I to Ang-(1–7), particularly in the presence of ACE inhibitors [26,27]. Additionally, Domenig et al. [29] identified NEP as the main source of renal Ang-(1–7) in mice and humans.

With the advancement of research, the ACE2/Ang-(1–7)/Mas axis has been increasingly recognized for its counterregulatory and protective actions in different physiological and pathological conditions. Numerous studies have shown that Ang-(1–7) has opposite effects to those of Ang II, particularly as an antihypertensive peptide [30], inducing vasodilation [31], anti-inflammatory [32], and antifibrotic effects [33], while also reducing oxidative stress [34] under various conditions (Fig. 1). Thus, in view of these new findings, a new interpretation of the therapeutic perspective of RAS is necessary.

Since the discovery of this protective axis, several studies have been focused not only physiological function of RAS [29–32], but also in its involvement in pathological mechanisms provoked by the RAS imbalance that favor Ang II actions [33–36]. These studies contributed to the discovery of another component that also counterbalances Ang II actions: alamandine (ALA) and its receptor Mas-related G- coupled receptor D (MrgD) [37]. The literature extensively reports on the deleterious role of upregulating the Ang II/AT1 axis. Currently, there is increasing awareness that Ang-(1–7) and ALA formation could constitute a counterregulatory axis that needs to be better explored.

Studies have already demonstrated that ALA has important therapeutic roles in different tissues and pathophysiological contexts, as in sepsis [38], in the cardiovascular system [39], and in pulmonary [40] and hepatic fibrosis [41]. In addition, the idea is growing that the balance between the two axes is more important than the participation of individual mediators. Therefore, this article reviews the newest findings about ALA, focusing on its potential antifibrotic role, a subject that is still little discussed in the literature. For this, we will address (i) the synthesis and biological diversity of ALA; (ii) the pathophysiological mechanisms involved in tissue repair; (iii) the potential therapeutic targets for ALA in pathological fibrosis; (iv) the protective effects of ALA in cardiovascular, hepatic, and pulmonary fibrosis (PF) and, finally, (v) the potential use of ALA in the adjuvant treatment of COVID-19. Currently, little is known about the actions of ALA on different tissues and systems. Thus, this review tries to provide an overview of the mechanisms already described in the literature.

2. Biosynthesis pathways of alamandine

In 2013, two possible ALA biosynthesis pathways were demonstrates [37]. Lautner et al. showed that, in addition to Ang II, Angiotensin A (Ang A; Ala-Arg-Val-Tyr-Ile-His-Pro-Phe) can also be a substrate for ACE2 and could participate in ALA formation [37]. These researchers described ALA (Ala-Arg-Val-Tyr-Ile-His-Pro) formation from Ang A hydrolysis by ACE2 [37], possibly due to the high structural similarity between Ang A and Ang II [42].

Lautner et al. [37] also reported that ALA can be derived directly from the decarboxylation of the N-terminal aspartate amino acid residue of Ang-(1–7). Intriguingly, the enzyme responsible for this decarboxylation is still unknown and constitutes a new challenge in the field. In 2020, Jha et al. hypothesized that a bacterial aspartate decarboxylase, present in the gastrointestinal tract, heart, or the systemic circulation, could metabolize Ang-(1–7) to ALA in mammals. According to the authors, this enzyme probably converts aspartate to alanine and, therefore, transforms Ang-(1–7) into ALA by β-decarboxylation. Moreover, Jha et al. [43] believe that this β-decarboxylation could also be responsible for the conversion of Ang II into Ang A. In both situations, from Ang A and/or from Ang-(1–7), the reaction leads to ALA formation (Fig. 1). Studies should be carried out to unravel alternative candidates that provoke ALA formation.

Although it is known that ALA is synthesized from Ang A or Ang-(1–7), little is known about its degradation. Due to the similarity of ALA to Ang-(1–7), it is possible that ALA can be cleaved by NEP into several smaller peptides. Using mass spectrometry, Brar et al. [44] showed that, in addition to generating Ang-(1–7), NEP is able to cleave this peptide at three different sites, corresponding to six smaller peptide products. However, studies should be conducted to investigate the fate of ALA.

3. The diversity of alamandine's biological actions

Although ALA differs from Ang-(1–7) by the replacement of the aspartate for an alanine residue at the amino terminus, they present several functional similarities [45]. In addition, it was very well-demonstrated that the biological effects of ALA occur through its interaction with MrgD receptor [37,39,46,47]. Expression of this receptor has been detected in the membrane, cytoplasm, perinuclear and nuclear regions of the cells of the brain [48,49], cardiomyocytes [50], the aorta [5], endothelial cells [48,51,52], and the retina [53]. Moreover, MrgD can also be expressed in macrophages [54] and fibroblasts [48], which are important cells in the fibrotic process.

Recently, Valenzuela et al. [55] found a new Mas-related G- coupled receptor E (MrgE) in dopaminergic neuronal mitochondria that is capable of recognizing Ang-(1–7) and ALA. The authors showed that intracellular ACE2 and its products Ang-(1–7) and ALA are highly concentrated in the mitochondria and bind to MrgE to produce nitric oxide, which counterbalances the increase in reactive oxygen species (ROS) at the cell and mitochondrial levels. Knowing the importance of

the counter-regulatory peptides to maintain tissue homeostasis, discovery of the ACE2/Ang-(1–7)-ALA/MrgE axis reinforces the need for a new look at RAS in inflammatory, oxidative, and fibrotic diseases.

Important physiological effects have already been attributed to ALA, such as vasodilation [37,51,56], improvement in cardiovascular function [39,51,52,57,58], and anti-inflammatory [47] and antifibrotic [37, 39–41,46,58,59] actions. Collectively, these findings indicate the versatility and pathophysiological impact of ALA on homeostasis.

Interestingly, although ALA administration frequently causes hypotension, studies have shown that its actions are dependent on the route of administration and on the pathophysiological situation [5,37,40,46, 50,56,59–62]. In this sense, Hekmat et al. [56] suggested that, under normal conditions, ALA could also act via the AT1 receptor. On the other hand, under pathological situations, its effect on MrgD probably masks the effect on the AT1 receptor. In hypertensive rats, a subcutaneous infusion of ALA reduced blood pressure (BP) and cardiac hypertrophy [50]. In contrast, Wang et al. [46] showed that an intraperitoneal infusion of ALA attenuates cardiac fibrosis independently of BP. ALA probably reduces BP only in hypertensive disease and could be an advantage in therapeutic interventions when the disease is not associated with hypertension, such as idiopathic PF (IPF).

In early 2021, Almeida-Santos et al. demonstrated an unprecedented role of ALA [49]. Considering that MrgD is expressed in the brain, the authors evaluated the potential of ALA to decrease the symptoms of depression in transgenic rats. They reported that ALA attenuated the depression-like behavior observed in transgenic rats with low brain angiotensinogen. This study extends the importance of the counter-regulatory arms of the RAS to include neuropsychiatric diseases.

Briefly, all the biological actions of ALA point to a promising future for this peptide as an antifibrotic therapeutic compound. However, despite the strong evidence of ALA involvement with the pathophysiological mechanisms of fibrosis, as well as the functional impact of the imbalance between the two axes of the RAS in patients with fibrosis, this field has been little explored.

4. Tissue repair and the fibrogenic process

4.1. The healing process

Healing is a complex phenomenon with high metabolic demand, intense cellular activity, and oxidative involvement [33]. It is characterized by processes that result in tissue replacement by regenerated and/or scar cells. Therefore, the initial inflammatory process can be solved or, as in most cases, involve some degree of functional impairment. In this last case, myofibroblasts remain in the tissue and increase extracellular matrix (ECM) proteins and tissue elasticity [63]. This deposition of connective tissue to replace the parenchyma is known as fibroplasia or fibrosis [64,65].

Many cytokines and growth factors participate in this fibrogenic mechanism, such as the transforming growth factor-β (TGF-β) family, synthesized by inflammatory and effector cells, and responsible for fibroblast activation. There is intense proliferation and cell differentiation into myofibroblasts that express smooth muscle proteins, such as α-actin, responsible for tissue retraction. The last stage of healing is characterized by the balance between myofibroblast apoptosis and the production of new cells to form the scar. In this phase, there is also a gradual degradation of the ECM and mature collagen type I formation for successful repair. However, abnormalities in any of these phases can result in excessive and chronic scarring and serious functional damage to the organ [66].

4.2. Pathological fibrosis

In pathological fibrosis, the persistence of the initial cellular insult, and subsequent activation of inflammatory cells, results in a feedback mechanism. This positive feedback increases ECM protein production and/or the recruitment of more effector cells such as fibroblasts, fibrocytes, and myofibroblasts [67]. The permanent and progressive remodeling of tissue architecture can result in total organ failure [63, 68], emphasizing the importance of counter-regulatory pathways that can initiate anti-fibrotic effects [33].

Therefore, considering the functional impairment and the poor quality of life of patients with fibrotic disease, it is crucial to avoid the pathological tissue transformation that is responsible for the high morbidity and mortality rates among these patients [67]. Unfortunately, regardless of advances in research and in preclinical studies, there is still no effective therapy for pathological fibrosis that reduces mortality and the high cost to the health system [65]. Thus, investigating therapeutic alternatives to provide an ideal environment for tissue repair may be promising.

5. Potential of ALA as an antifibrotic agent

Currently, several in vivo studies support the hypothesis that ALA could be a powerful alternative to treat fibrotic diseases [37,40,41,69]. Thus, we present below some evidence for these protective actions in classical molecular pathways such as inflammation and oxidative stress (Fig. 2). Subsequently, we highlight preclinical investigations indicating the potential antifibrotic actions of ALA (Fig. 3) in the cardiovascular system, liver, and lung.

5.1. Inflammatory, apoptotic, and oxidative pathways opposed by ALA

The mechanisms involved in the anti-inflammatory, antioxidant, and antifibrotic effects of ALA are still not fully understood. However, recent studies have indicated the ability of this peptide to inhibit the activation of the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) and mitogen-activated protein kinase (MAPK) pathways [38]. In humans, the PI3K/AKT pathway is an essential regulatory component for cell metabolism, growth, proliferation, survival, protein synthesis, and apoptosis [70]. Additionally, the MAPK pathways play an important role in proliferation, differentiation, migration, cell survival, apoptosis, and inflammatory responses [71]. Therefore, the inhibition of these pathways represents an important mechanism of ALA in several pathologies that could lead to fibrosis.

In a sepsis-induced myocardial dysfunction model, Li et al. [47] observed that the anti-apoptotic and anti-inflammatory participation of ALA can be explained, at least in part, by the phosphorylation inhibition of the MAPK family members, i.e. ERK, JNK, and p38. The authors showed that LPS increased the levels of p-ERK, p-JNK and p-p38, inhibited by ALA. Similar results were observed by Hu et al. [38] in a

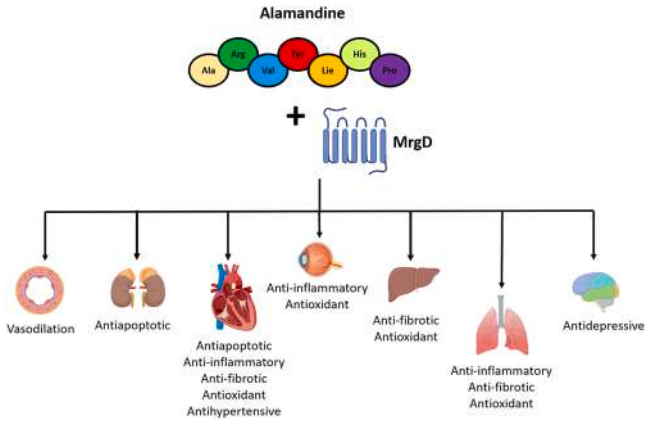

Fig. 2. Protective actions of alamandine on the cardiovascular, renal, hepatic, pulmonary, and nervous systems. MrgD = Mas-related G-protein coupled receptor type D.

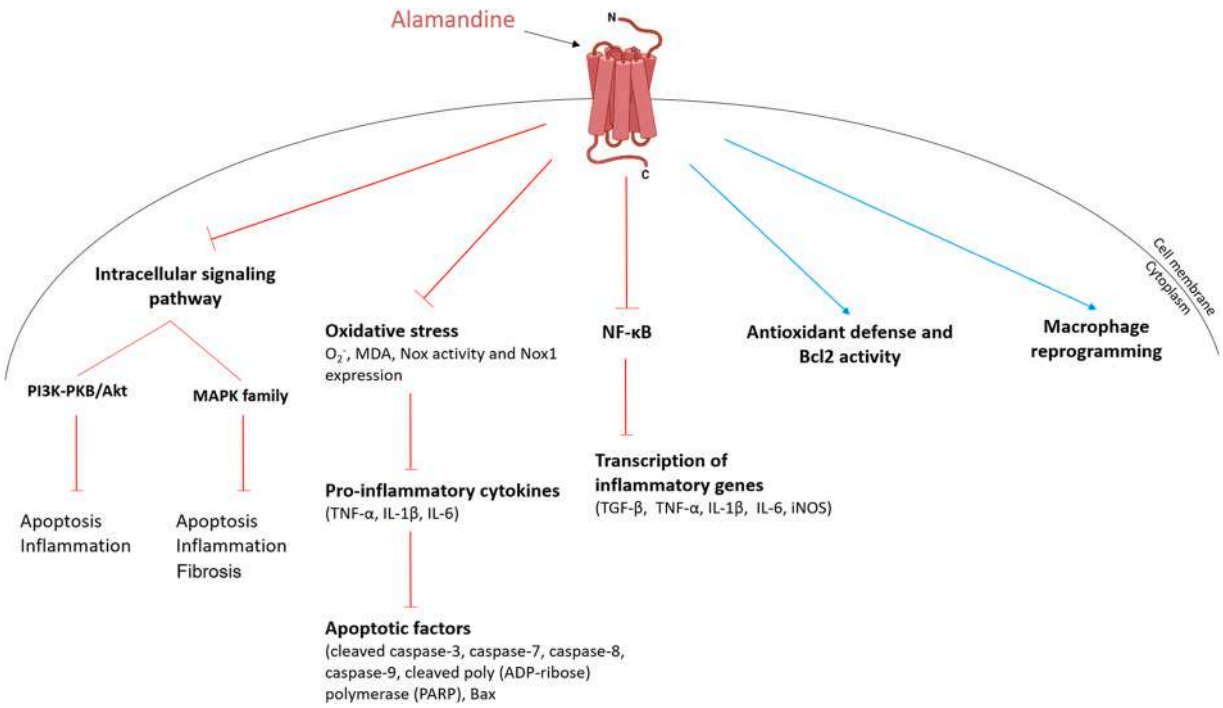

Fig. 3. Alamandine potential therapeutic targets for fibrotic disease. PI3K = Phosphoinositide 3-kinase; PKB = protein kinase B; Akt = protein kinase B; MAPK = mitogen-activated protein kinase; O_2 = Superoxide; MDA = Malondialdehyde; TNF-α = Tumor Necrosis Factor alpha; IL = Interleukin; NF-κB= Nuclear factor kappa B; TGF-β = Transforming growth factor beta; iNOS = Inducible nitric oxide synthetase; Bcl-2 = B-cell lymphoma 2.

sepsis-associated kidney injury model. The authors showed that the ALA inhibits the phosphorylation of PI3K/Akt and MAPKs signaling, reversing the increased levels of phosphorylated PI3K, Akt, ERK, JNK, p38.

In addition, nuclear factor kappa B (NF-κB) is an essential intracellular signaling molecule that is considered to be an inhibitory target of ALA. NF-κB activation and migration to the cell nucleus stimulates the release of several pro-inflammatory agents such as cytokines, chemokines, growth factors, and mediators that contribute to apoptosis [72].

Studies have shown that ALA can inhibit the expression of NF-κB and, therefore, reduce inflammatory mediator expression [53,73]. Song et al. [73] found that, by inhibiting NF-κB, ALA can significantly reduce TNF-α, IL-1β, IL-6, and nitric oxide in rats with myocardial ischemia-reperfusion injury. Importantly, this reduction of inflammatory parameters was associated with preservation of the histological architecture. Similarly, results from in vitro studies with human retinal cells showed that ALA attenuated NF-κB activation, inflammatory cytokine gene expression, and ROS production [53]. Collectively, these data indicate a useful inhibitory action of ALA that could represent an important therapeutic target in the future.

In addition to inflammation, pathological cell death is an important trigger for inadequate tissue repair [63,65]. ALA can also act in the early stages of fibrotic diseases by reducing cell death. ALA administration decreases apoptotic marker expression in the kidney [38,74] and heart [47,75], indicating tissue protection against inflammation and the exacerbation of fibrosis.

Moreover, the literature provides evidence that oxidative stress is an important component of inflammation and fibrosis. Oxidative stress is characterized by the excessive production of ROS and/or depletion of antioxidant defenses [76]. The increase in the Ang II axis is closely related to ROS production [77–81], playing an important role in oxidative stress. On the other hand, in some cases, exogenous Ang-(1–7) could represent a therapeutic strategy [77,78,82]. Likewise, recent studies have shown that ALA can modulate the redox balance by attenuating and/or inhibiting the oxidant action of Ang II [75,76,83]. Zhu et al. [74] recently observed that the inhibition of ROS formation by ALA resulted in the attenuation of pro-inflammatory cytokines and, consequently, apoptosis.

Data from the literature also indicate that ALA can inhibit immune cell function. Inflammation resolution can restore tissue homeostasis and involves macrophages and neutrophil degranulation [84], with a positive impact on fibrosis control. Promisingly, Da Silva et al. [84] demonstrated the potential of ALA to reduce granular neutrophil products in atherosclerosis. This finding was confirmed by Santuchi et al. [54], who showed that ALA reduces the number of neutrophils and inflammatory macrophages in vivo, and modulates macrophage plasticity.

In summary, the literature shows that ALA can act on several inflammatory and oxidant mechanisms that are directly or indirectly involved in the development of fibrosis. Next, preclinical studies performed in different models of fibrosis in the cardiovascular system [37,46,59], liver [41] and lung [40,83] will be discussed.

5.2. Cardiovascular, hepatic and pulmonary fibrosis opposition by ALA: what do we know?

5.2.1. Cardiovascular fibrosis

The first description of the potential antifibrotic effect of ALA was carried out in 2013, concomitantly with its discovery [37]. Lautner et al. [37] showed in aortic rings from rats that, after oral ALA administration, the collagen I, collagen III, and fibronectin content was decreased in isoproterenol-treated rats. This antifibrotic role was also demonstrated in cardiac fibrosis secondary to hypertension in a spontaneous hypertensive rat model [46] and in a rat model of 2-kidney, 1-clip hypertension (2K1C) [59].

Hekmat et al. [59] observed that systemic ALA infusion decreased cardiac fibrosis and blood pressure (BP) and increased ACE2 expression in hypertensive rats. On the other hand, studies by Wang et al. [46] and Fernandes et al. [40] found that the reduction in fibrotic parameters occurred independently of BP. The dissociation between antifibrotic action and the decrease in BP is of extreme clinical relevance because increased BP is not present in all fibrotic diseases, as seen in patients with PF.

According to the literature, at least in part, the effects of ALA in cardiovascular system fibrosis could be explained by MAPK pathway inhibition. Yang et al. [5] demonstrated that ALA blocks p38 expression and attenuates Ang II-induced arterial fibrosis. These authors observed that ALA reverses the increase in collagen I, TGF-β, and connective tissue growth factor content in the vascular smooth muscle cells of mouse and rat thoracic aortas after Ang II treatment.

It is known that there are still countless mechanisms to be elucidated about the antifibrotic action of ALA on the cardiovascular system. However, currently, strong evidence supports the rationale that ALA has high potential to be considered an effective therapy against fibrosis in this system.

5.2.2. Hepatic fibrosis

The beneficial effect of classic RAS-targeted drugs in human liver disease has already been demonstrated [85–87]. Shim et al. [85] suggested that the administration of agonizts counterregulatory to the ACE2/ALA/MrgD axis of RAS are important pharmacological molecules that can support prevention and treatment of chronic liver disease.

Recently Huang et al. [41] demonstrated, in vivo, that ALA ameliorated CCl_4-induced liver fibrosis, reduced the hydrogen peroxide (H_2O_2) content and NOX4 protein level, and compromised autophagy. In the same study, these authors reported that ALA also shifted the RAS balance towards the counterregulatory ACE2/ALA/MrgD axis, reducing the oxidative stress induced by Ang II and autophagic activation. This attenuates fibrotic processes, reinforcing the importance of ALA not only in liver pathophysiology, but also in the systemic anti-fibrotic effect of ALA.

5.2.3. Pulmonary fibrosis

Despite the efforts of the scientific community, the pathophysiology of PF remains unclear and the patient survival rate is still poor and unacceptable [88,89]. Sipriani et al. was a pioneer demonstrating by High performance liquid chromatography (HPLC) mass spectrometry approach that, compared to the control group (2,7 ± 0,47 pg/μL), the ALA plasma concentration was 3.65 times lower ($P < 0.0001$) in PF patients (0,74 ± 0,44 pg/μL) [90]. Moreover, there was no difference in the plasma concentration of Ang-(1–7) between the groups ($P = 0.684$). Our research group hypothesized that, in PF patients, there is a reduction in the participation of the ACE2/ALA/MrgD axis. Probably, the decrease in this axis is more harmful than changes in the Ang II/AT1 axis.

Recently, our group also investigated the antifibrotic actions of ALA in a bleomycin-induced experimental model of PF, considered to be one of the most similar models to PF in humans [91,92]. Fernandes et al. [40] found that ALA administration for 15 days reduced the worsening of breathing patterns in rats treated concomitantly with bleomycin and ALA. In addition, an attenuation of the histological damage to the lungs characteristic of PF was shown. Likewise, corroborating our results, Liu et al. [83] demonstrated in vivo that ALA prevented the effects of fibrosis similarly to pirfenidone, one of the current therapies for patients with PF. This protection was associated with a reduction in oxidative stress and autophagy, attenuated production of α-collagen I, connective tissue growth factor, and α-smooth muscle actin induced by Ang II in fibroblasts.

One of the main mechanisms involved in the pathophysiology of PF is MAPK signaling. However, in the lungs, this pathway could be more complex than in other tissues. The induction of TGF-β1 in PF is mediated by p38 MAPK, which up-regulates fibronectin and collagen expression and stimulates the epithelial to mesenchymal transition (EMT), giving rise to ECM-producing myofibroblasts [93,94]. Therefore, the inhibition of this pathway may attenuate the development of PF and represent a possible therapeutic target. But, myofibroblasts can also be activated by another component of the MAPK pathway, ERK 1/2, via crosstalk with p38. The authors demonstrated that phosphorylated p38 MAPK interacts with ERK1/2 to suppress its activity. Therefore, curiously, the inhibition of p38 MAPK in vitro increases the activation of ERK 1/2 in pulmonary myofibroblasts. However, the positive effects of p38 inhibition appear to outweigh the negative observations, as described by Matsuoka et al. [93]. In this context, and considering the effects of ALA on p38 in cardiovascular tissue, it is desirable that future studies should elucidate this pathway in protection against PF.

Due to the absence of a described human enzyme that converts aspartate to alanine, Jha et al. [43] hypothesized the involvement of an intestinal or systemic bacterial β-decarboxylase. Considering that one of the most serious side effects described for antifibrotic therapy in patients with PF is diarrhea [95,96], the dysbiosis in these patients could explain the low plasma concentration of ALA found by Sipriani et al. [90]. Bacterial translocation, a process by which intestinal bacteria cross the intestinal epithelium and enter the systemic circulation, could be responsible for the formation of protective substances such as ALA [43] in the circulation. However, diarrhea may provoke a reduction in these bacterial enzymes, which would compromise systemic ALA formation by decarboxylation.

Collectively, the evidence reinforces that ALA could be beneficial for PF patients. Nonetheless, it is important to note that there are no studies being conducted to understand whether ALA can protect the lungs with or without established fibrosis. Additional studies on this issue are necessary.

6. ALA/MrgD activation as a possible strategy to prevent coronavirus (SARS-CoV-2) pulmonary fibrosis

The first case of COVID-19, i.e. severe acute respiratory syndrome caused by the coronavirus SARS-CoV-2, was reported in Wuhan, China in December 2019. In March 2020, it was recognized as a pandemic by the World Health Organization [97]. Worldwide efforts allowed for the rapid identification that the pathophysiology of this disease is associated with RAS [98,99] and its therapeutic potential in COVID-19 [100,101]. Studies have shown that SARS-CoV-2 infection is dependent, at least in part, on binding with ACE2, leading to a reduction in its activity [98,99].

The wide expression of ACE2 in the membrane of alveolar epithelial type II cells and in pulmonary endothelial cells could justify the initial respiratory symptoms and subsequent vascular endothelial involvement [100]. However, a recent study by McCracken et al. [102] demonstrated in vitro that even when exposing ECs to high concentrations of SARS-CoV-2, the replication was extremely low. The authors attributed this result to an independent ACE2 route, indicating that endothelial infection by SARS-CoV-2 is unlikely to occur in vitro [102].

The data presented in this review allow to believe that the down-regulation of ACE2 activity could cause an imbalance of the RAS and reduce the protective effects of the Ang-(1–7)/Mas and ALA/MrgD pathways in different diseases. Furthermore, according to the literature, the reduction in ACE2-cell membrane interactions can push the RAS toward the Ang II/AT1 axis [97–100]. This could be considered an important component of COVID-19 pathogenesis. However, few studies have investigated the plasma relationship of RAS components, and most show controversial results [99,103–107]. Recently, Camargo et al. [105] showed that patients who stayed longer in the hospital were also those who had a higher plasma concentration of Ang II measured at the time of admission. According to these authors, although more studies are required, this data may contribute to understand the mechanisms involved in the disease and also to the identification of Ang II as a potential biomarker of the COVID-19 severity [105].

Future studies are clearly needed to understand the effects of this disease on RAS. However, considering the many beneficial effects, it is possible that the administration of ALA could improve the clinical outcome of patients with COVID-19.

Despite major efforts directed towards treating the acute illness and developing vaccines, there are increasing reports of persistent and prolonged effects after acute COVID-19 [108–110]. Although there are several uncertainties regarding PF post-COVID-19, some patients, upon

recovery from COVID-19 may have fibrotic sequelae [110–116]. Zhao et al. [117] showed residual abnormalities of lung function and chest radiography of COVID-19 survivors three months after hospital discharge. Nabahati et al. prospectively evaluated post-COVID-19 fibrotic lung changes. They observed that PF was present in about half of the survivors and some of these patients had fibrotic changes even after six months of follow-up [118]. There is no doubt that PF can be observed in the initial phase of COVID-19 [118–120]. Although sometimes asymptomatic, the clinical relevance and PF prognosis [121,122] remain important. The decrease in patient quality of life, the sequelae, and the high costs to the public health system justify more studies and a better understanding of this complication of COVID-19.

Currently, two clinical trials, NCT04605887 and NCT04633772, employing therapy with Ang-(1–7) are being conducted; they will open up new perspectives on the understanding of RAS. The clinical trial NCT04924660 was recently finished because the treatment benefits were inferior to the placebo group. Due to the antioxidant, anti-inflammatory, and antifibrotic actions already reported for ALA in this review, it is possible to believe that the ALA/MrgD pathway may add to the beneficial effects already reported for Ang(1–7)/Mas.

Despite being the most recently discovered peptide, the actions already described for ALA indicate that it may be an important ally of the protective arm of RAS in COVID-19 and in all diseases that culminate in fibrosis. This proposal was first reported by Hekmat and Javanmardi [123] and is reinforced with this review. Here, we explored the anti-fibrotic and anti-inflammatory properties of ALA mentioned by these authors in 2021. ALA may also act on several critical molecular pathways involved in the pathogenesis of COVID-19, so better knowledge of this mediator could contribute to a reduction in the high mortality and morbidity rates associated with this disease. Briefly, the evidence indicates that the exaggerated inflammatory response and the fibrotic process induced by SARS-CoV-2 infection can be controlled, at least in part, by correcting a possible ALA deficiency. This brings the attention to the need for a new look at the RAS. More research is needed to elucidate the mechanisms involved in this promising therapeutic peptide.

7. Conclusion

Our comprehension of the RAS is far from being complete. However, findings from the last decade have contributed to a better understanding of the role of this system in many pathophysiological processes. The challenge is enormous, but it fills physicians and researchers with hope in terms of finding a treatment, adjuvant or not, that will increase the survival and/or improve the quality of life of all patients affected by fibrosis.

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Research Article

Assessment of Alamandine in Pulmonary Fibrosis and Respiratory Mechanics in Rodents

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Introduction. Pulmonary fibrosis (PF) is characterized by an accelerated decline in pulmonary function and has limited treatment options. Alamandine (ALA) is a recently described protective peptide of the renin-angiotensin system (RAS) with essential tasks in several conditions. Our group previously demonstrated that ALA is reduced by 365% in the plasma of patients with idiopathic PF, and thus, it is plausible to believe that stimulation of this peptide could represent an important therapeutic target. In this sense, this study investigates the effects of ALA in an experimental model of PF. *Materials and Methods*. Bleomycin (BLM) was administrated in Wistar rats, and these fibrotic animals were treated with ALA for 14 days. Body weight, histology, respiratory, and hemodynamic parameters were analyzed to study the effects of ALA. *Results*. ALA treatment attenuated the development of fibrosis ($P < 0.0001$), reduced respiratory system elastance ($P < 0.0001$), and preserved weight gain ($P < 0.0001$) in fibrotic animals without affecting the autonomic control of blood pressure and heart rate. *Conclusion*. The data from this study demonstrate the potential of ALA to alleviate pulmonary fibrosis and improve respiratory system mechanics *in vivo*. The promising results encourage more detailed investigations of the potential of ALA as a future and efficient antifibrotic.

1. Introduction

Pulmonary fibrosis (PF) is characterized by the excessive extracellular matrix (ECM) deposition and frequently evolve to death [1]. In these patients, the elastance of the respiratory system are significantly increased [2], requiring greater respiratory work. Therefore, it is essential to find effective therapeutic strategies that facilitate lung expansion, reducing episodes of respiratory failure.

The participation of renin-angiotensin system (RAS) in PF has been well described [3–5]. The angiotensin-converting enzyme 2 (ACE2) has shown been an important counter-regulatory axis in several different conditions [6–8]. The alamandine (ALA), generated by ACE2 axis, although recently discovered [9], is well-known due its protective action in the cardiovascular system, i.e., vasodilation [9] and antifibrotic effects [10]. In addition, our previous study showed that patients with idiopathic PF present 365% less ALA in plasma [11], which probably indicates that the exogenous administration of this peptide may attenuate the development of PF and, consequently, reduce the decline in lung function.

Therefore, since bleomycin (BLM) is the best characterized PF model [12, 13], this study is aimed at evaluating the protective effect of ALA on the development of PF in BLM-induced rats.

2. Materials and Methods

2.1. Animals. All procedures followed the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health [14]. Ethics Research Committee of the Universidade Federal de Ciências da Saúde de Porto Alegre

approved the study (protocol 17/207). Five-week-old male Wistar rats were housed in a room (25 ± 2°C) on a 12 : 12 h dark/light circadian rhythm with access to standard diet and water *ad libitum*.

2.2. Pulmonary Fibrosis Protocol. Rats were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg), and BLM (2.5 mg/kg, Bonar, Ache) or saline (0.9%) was administered by oropharyngeal aspiration (OA). On the same day, the miniosmotic pumps (OM; Alzet 2004) containing saline or ALA (Sigma Aldrich, St. Louis, MO, USA) solution were introduced subcutaneously onto the animal's back to deliver 0.25 μl/hour (ALA; 50 μg/kg or saline = 0.9% a day) for 14 days, respectively. The rats' health status and body weight were monitored daily.

Groups ($N = 7$/per group) are as follows: (1) CO: saline by OA and OM. (2) ALA: saline by OA and ALA (50 μg/kg/day) in the OM. (3) BLM: BLM (2.5 mg/kg) by OA and saline in the OM. (4) BLM+ALA: BLM (2.5 mg/kg) by OA and ALA (50 μg/kg/day) in the OM.

2.3. Histopathology. The lung was inflated and fixed in 10% phosphate-buffered formalin, and 5 μm thickness sections were stained with hematoxylin and eosin (HE) and Masson's trichrome (TM). 20 fields of each slide were examined at a magnification of 400x. The fibrosis classification was according to the modified Ashcroft score [15]. Quantitative analysis of stained collagen area was performed using the Image Pro-Plus® 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

2.4. Assessment of Respiratory Function. On day 14, animals were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg), positioned on a plane surgical table and tracheostomized to introduce a rigid-type cannula (2-mm ID). The cannula was fixed to the trachea by a silk thread and connected to the mechanical ventilator. Respiratory function was analyzed using a mechanical ventilator for small animals (FlexiVent, Scireq, Montréal, Canada) [16].

2.5. Hemodynamic Analyses and Autonomic Evaluation. After respiratory mechanics data collection, still under anesthesia, a polyethylene catheter (PE-50) was inserted into the right carotid to record arterial blood pressure (ABP) for 10 minutes (sample rate = 2000 Hz/channel). The analogical signals were digitalized by a data acquisition system (Windaq-AT/CODAS, Dataq 143 Instruments Inc., OH, USA). The data were analyzed by spectral analysis to assess the sympathovagal balance in the cardiovascular system [17, 18]. At the end of the experiment, animals were euthanized by intramuscular anasthetic overdose (240 mg/kg of ketamine and 30 mg/kg of xylazine) for lung collection.

2.6. Statistical Analysis. The normal distribution was tested by the Shapiro-Wilk test. Parametric data analysis was performed using one-way or two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. For data with nonnormal distribution, the Kruskal-Wallis test was used, followed by the Dunn *post hoc* test. The associations between data were demonstrated through Pearson's correlation test. Data analysis was performed using the GraphPad Prism 8 software and presented as mean ± SEM. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Alamandine Attenuates Loss Weight of Pulmonary Fibrosis. From day 6, the BLM group gained less weight onwards when compared to the CO and ALA groups ($P < 0.01$). ALA treatment had a protective effect starting on day 8 ($P < 0.05$). By day 14, PF rats gained significantly less body weight compared to the CO and ALA groups ($P < 0.0001$). However, animals treated with ALA maintained a similar weight to healthy animals ($P < 0.0001$, vs. BLM group; Figure 1(a)).

3.2. Alamandine Attenuates the Development of Pulmonary Fibrosis. The lung parenchyma was preserved in the CO and ALA groups (score = 0) compared to the BLM group (score = 3.6). ALA treatment had a potent effect (Figure 1(b)) by attenuating fibrosis (BLM + ALA score = 1.3; $P < 0.0001$; $F = 33.10$). Moreover, collagen deposition was confined to the regions around blood vessels and airways in the CO and ALA groups. As expected, there was considerable lung interstitial collagen deposition in the BLM group ($P < 0.05$ vs. CO and ALA). ALA treatment reduced significantly the collagen deposition compared to the BLM group ($P < 0.001$; $F = 8.285$). Cellular and alveolar areas were similar among groups in (Figure 1(b)). Images of lung histology are shown in Figure 1(c).

3.3. Alamandine Treatment Improves the Respiratory Mechanics in Pulmonary Fibrosis. There was significant increase in the dynamic elastance (Edyn) in the BLM group (7.27 ± 1.6 cmH_2O/ml) compared to the CO (2.13 ± 0.10 cmH_2O/ml) and ALA (1.92 ± 0.08 cmH_2O/ml) groups (Figure 2(a)). The BLM+ALA group demonstrated a significant attenuation in Edyn (2.22 ± 0.18 cmH_2O/ml) compared to the BLM group ($P < 0.001$; $F = 11.28$; Figure 2(a)).

As expected, the dynamic compliance was lower in the BLM group (0.18 ± 0.04 cmH_2O/ml) vs. CO (0.48 ± 0.02 cmH_2O/ml) and ALA (0.53 ± 0.02 cmH_2O/ml) groups. The results from the BLM+ALA (0.47 ± 0.04 cmH_2O/ml) demonstrated a protective role of ALA on respiratory mechanics ($P < 0.0001$ vs. BLM; $F = 20.50$; Figure 2(b)). Moreover, the BLM group showed significantly higher respiratory dynamic resistance ($P < 0.001$ vs. CO; $F = 8.672$; Figure 2(c)), indicating the protective effect ALA in the BLM+ALA group ($P < 0.001$) to prevent loss of tissue function.

The correlation between the Ashcroft score and pulmonary elastance is shown in Figure 3. There was a strong positive correlation between the BLM and BLM+ALA groups ($r = 0.8452$; $R^2 = 0.7143$; $P = 0.0005$; Figure 3(a)) probably due to the data from the BLM group alone ($r = 0.9295$; $R^2 = 0.8640$; $P = 0.0073$; Figure 3(b)). Observing the correlation between those parameters in the BLM+ALA group, it is

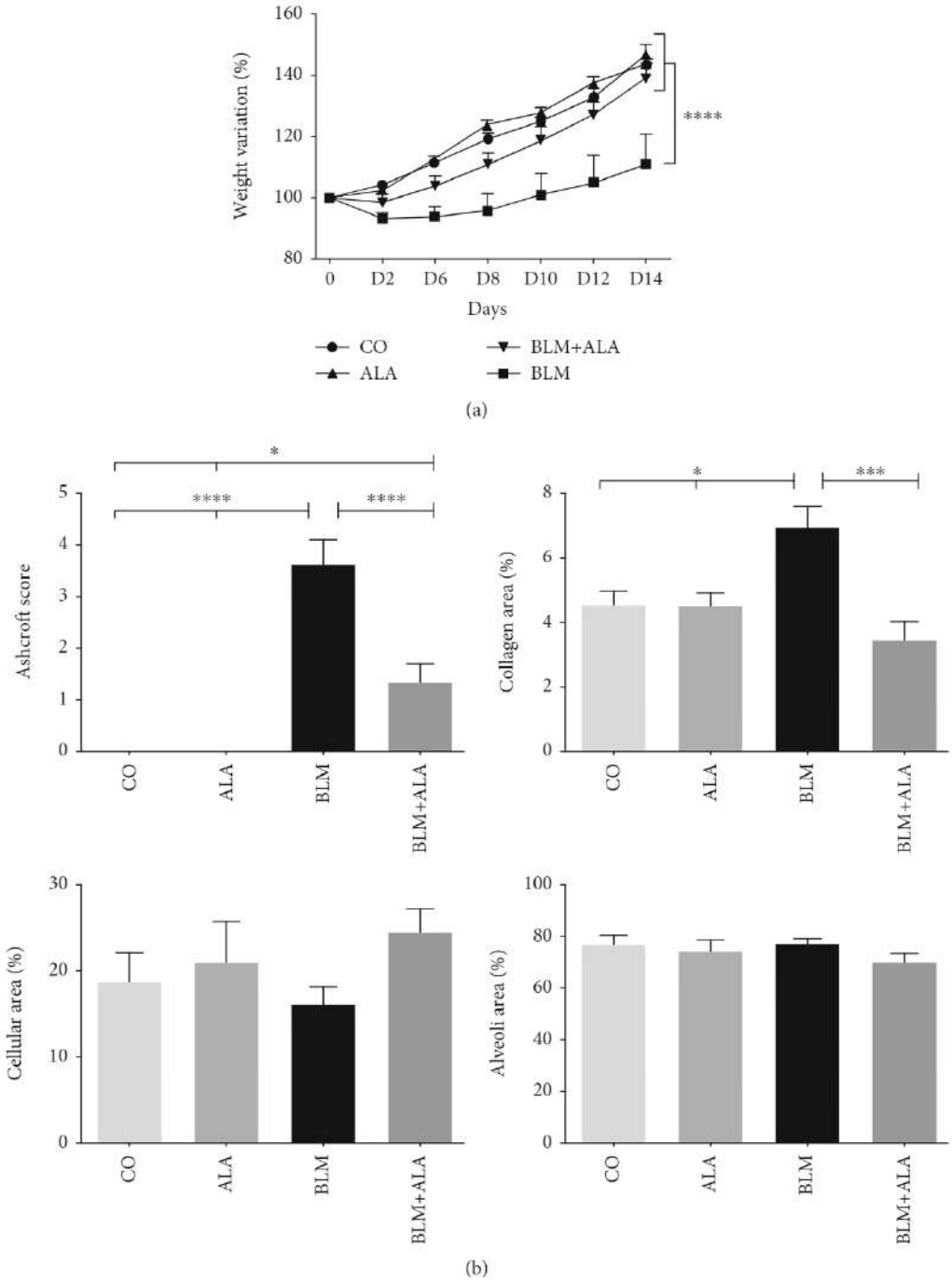
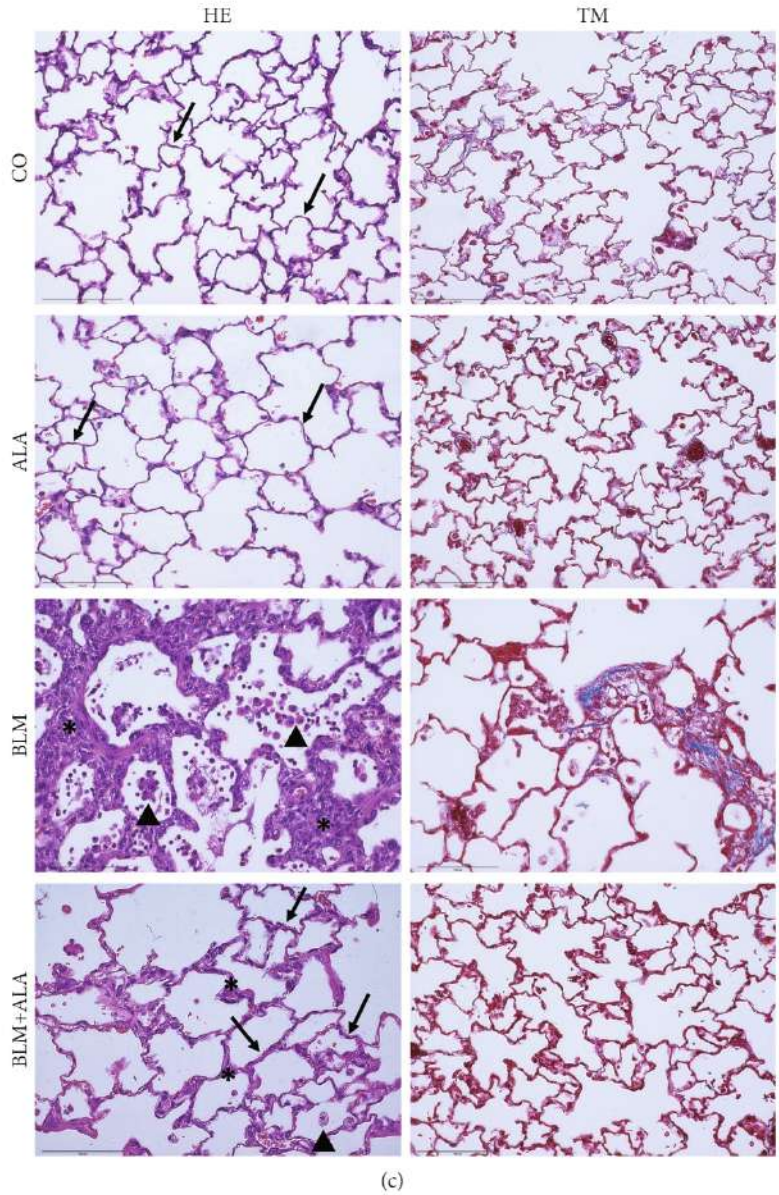


Figure 1: Continued.



(c)

Figure 1: Effect of alamandine on development of pulmonary fibrosis. (a) Bodyweight variation (%) analyzed by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison posttest. (b) Ashcroft modified score ($F = 33.10$) and collagen area quantification in the lungs ($F = 8.285$). (c) Representative images of effects at two weeks after alamandine treatment on histological findings and collagen deposition in the lungs. Hematoxylin and eosin (HE) and Masson's trichrome (TM) staining. Magnification at 400x. CO: animals that received only saline; ALA: saline intratracheally and alamandine in the osmotic minipumps; BLM: bleomycin intratracheally and saline in the osmotic minipumps; BLM+ALA: bleomycin intratracheally and alamandine in the osmotic minipumps. Arrows: alveolar septa; asterisks: fibrous bands or fibrous masses; arrowhead: inflammatory cells. Tissue changes were analyzed by one-way ANOVA, followed by Tukey's multiple comparison posttest. All data represent mean ± SEM; $n = 7 - 9$. $P < 0.05$ was considered statistically significant. $^{*}P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

possible to verify that this association was weak ($r = 0.3670$; $R^2 = 0.1347$; $P = 0.4742$; Figure 3(c)).

3.4. Hemodynamic Analyses. Table 1 shows that there were no differences among the hemodynamic parameters. The ABP ($P = 0.8121$) and heart rate ($P = 0.1432$) were not different among groups. This result was confirmed by spectral analysis showing that the autonomic nervous system participation in the heart was also not different among groups. The sympathetic (LF) and parasympathetic components (HF) were similar in absolute and normalized units. These results were confirmed by the LF/HF ratio. In addition, the 0 V, which represents the sympathetic participation to the heart through symbolic analysis, also indicates that there was no significant difference among groups.

4. Discussion

We have demonstrated for the first time the protective effect of ALA in attenuating PF and preserving respiratory mechanics. In addition, BLM administration significantly induced weight loss, which was prevented by ALA treatment.

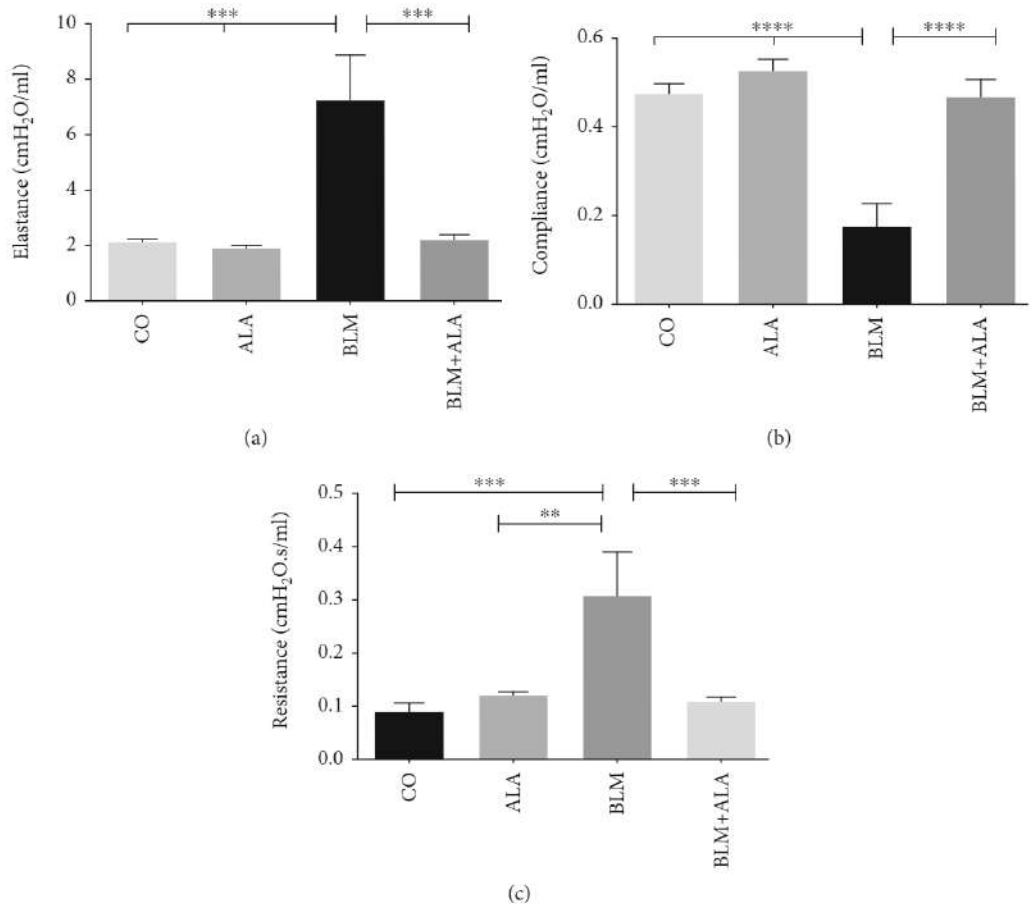


FIGURE 2: Lung mechanics on day 14. (a) Elastance, $F = 11.28$. (b) Compliance, $F = 20.50$. (c) Resistance: level of constriction, $F = 8.672$. CO: animals that received only saline; ALA: saline intratracheally and alamandine in the osmotic minipumps; BLM: bleomycin intratracheally and saline in the osmotic minipumps; BLM+ALA: bleomycin intratracheally and alamandine in the osmotic minipumps. One-way ANOVA followed by Tukey's multiple comparison test was used. Data represent mean ± SEM; $n = 6 - 7$; $P < 0.05$ was considered statistically significant. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

According to American Thoracic Society recommendations, induction by intratracheal BLM in male rats is the one that best mimics the disease in humans and is the most suitable for initial preclinical tests [19]. Kilic et al. [20] showed that 2.5 mg/kg of BLM in Wistar rats causes histological changes compatible with PF. Our study also demonstrated that this dose is sufficient to study the effects of antifibrotic substances without causing lethal damage to animals. In addition, George et al. [21]describes that studies for antifibrotic therapies using the BLM animal model of PF can be beneficial also to COVID-19. Even in patients recovered from COVID-19, the virus elimination does not preclude the development of progressive fibrosis. Thus, the promising results of ALA obtained in our study might encourage investigations of preventive fibrosis therapies after SARS-CoV-2 infection.

Our findings agree with the literature showing that PF leads to a decrease in body weight [22], a strong indication of health in animals [22]. While ALA treatment prevented the loss in body weight, the higher energy consumption to respiratory work probably explains this decrease in BLM group. This is true also for humans because treatment with nintedanib or pirfenidone normally provokes weight loss in patients [23], contributing to their poor prognosis [24].

Furthermore, the antifibrotic effect of ALA has already been described in the cardiovascular system [25] and, more recently, in the liver [26]. Although there are strong indications of the protective effects of ALA, there are no reports in the literature investigating its action on the fibrotic process in the lung. To date, there are only studies suggesting the protective role of to ACE2 and angiotensin-(1–7) in PF [27] and COVID-19 [8, 28]. In this study, ALA alleviated the lung degree of fibrosis and collagen deposition. It is established in the literature that collagen is the primary determinant of overall lung tissue elasticity [29], which therefore commits to functional capacity [30] in the same proportion as the degree of fibrosis [31]. Thus, if ALA prevent the fibrosis, it is possible that ALA can also act by improving respiratory mechanics.

Furthermore, our findings show that the degree of fibrosis was positively correlated with respiratory system elastance and that ALA treatment reduced this correlation. It is well

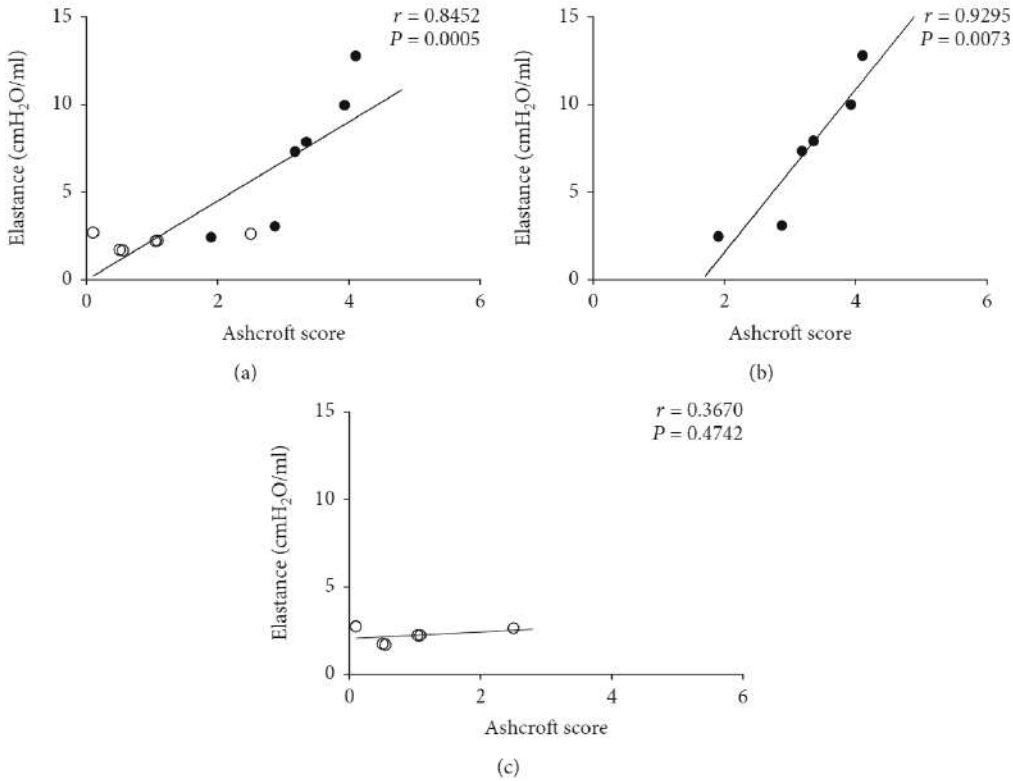


FIGURE 3: Pearson's correlation between Ashcroft score and pulmonary elastance. (a) BLM and BLM+ALA groups ($R^2 = 0.7143$). (b) BLM group ($R^2 = 0.8640$). (c) BLM+ALA group ($R^2 = 0.1347$). BLM: bleomycin intratracheally and saline in the osmotic minipumps; BLM +ALA: bleomycin intratracheally and alamandine in the osmotic minipumps; $N = 7$. •: BLM group. ▫: BLM+ALA group.

TABLE 1: Hemodynamic data and spectral and symbolic analysis results. CO: control rats; ALA: rats treated only with alamandine; BLM: rats treated with bleomycin; BLM+ALA: rats treated with bleomycin+alamandine. HR: heart rate; bpm: beats per minute; ABP: average blood pressure. HRV: heart rate variability; LF: low- and HF: high-frequency component; a: absolute and nu: normalized units. A one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison posttest was used for ABP and HR evaluation. The Kruskal-Wallis and the *post hoc* Dunn's multiple comparison tests were performed to detect differences in spectral and symbolic analysis. Data represent mean ± SEM, and a $P < 0.05$ was considered statistically significant.

	Spectral analysis				
	CO ($n = 7$)	ALA ($n = 7$)	BLM ($n = 7$)	BLM+ALA ($n = 7$)	P
ABP (mmHg)	75 ± 6	70 ± 3	74 ± 5	77 ± 3	0.81
HR (bpm)	299 ± 16	269 ± 17	251 ± 11	267 ± 10	0.14
HRV (ms^2)	9.18 ± 2.26	9.58 ± 2.81	9.43 ± 4.28	12.69 ± 6.68	0.90
LFa (ms^2)	2.12 ± 0.74	3.15 ± 1.38	1.31 ± 0.37	2.12 ± 0.86	0.68
HFa (ms^2)	5.50 ± 1.63	4.30 ± 0.98	7.07 ± 4.12	6.03 ± 2.47	0.96
LFnu	0.32 ± 0.09	0.32 ± 0.07	0.28 ± 0.08	0.30 ± 0.07	0.96
HFnu	0.68 ± 0.09	0.68 ± 0.07	0.72 ± 0.08	0.70 ± 0.07	0.96
LF/HF ratio	0.83 ± 0.38	0.62 ± 0.19	0.56 ± 0.21	0.50 ± 0.16	0.97
Symbolic analysis (%)					
0V pattern	0.101 ± 0.006	0.100 ± 0.019	0.111 ± 0.027	0.111 ± 0.010	0.99
1V pattern	0.370 ± 0.010	0.372 ± 0.021	0.361 ± 0.017	0.392 ± 0.010	0.40
2LV pattern	0.102 ± 0.014	0.091 ± 0.012	0.103 ± 0.012	0.075 ± 0.014	0.34
2UV pattern	0.418 ± 0.020	0.434 ± 0.031	0.424 ± 0.035	0.413 ± 0.020	0.94

established that fibrosis increases the elastic recoil forces of the lung and therefore reduces lung compliance. Moreover, excess ECM alters ventilation/perfusion ratios in the lung, causing hypoxemia both at rest and with effort [32]. Consequently, our results indicate that treatment with ALA might overcome these mechanical changes and could be effective in reducing respiratory work also in IPF patients. Despite evidence in the literature regarding the involvement of the RAS in PF [33], this is the first study which demonstrates the protective effect of ALA in lungs, extending the knowledge about the potential of the ACE2 axis [34]. In this sense, our data point to the possibility of using ALA to treat PF of varying etiology, mainly when involving ACE2 participation. As proposed by Wu [8], our findings indicate that the compensation of ACE2 function, with ALA administration, could be a promising alternative to treat the severe respiratory damage provoked by PF, as found in COVID-19 [21, 28].

Studies have also indicated that ALA has different effects on arterial blood pressure (ABP) regulation, depending on where it is administered and/or the pathophysiological condition. It causes an increase in ABP and sympathetic participation when injected into the paraventricular nucleus of spontaneously hypertensive rats [35], whereas subcutaneous infusion of ALA attenuates hypertension [36]. It was also demonstrated by Wang et al. [37] that ALA attenuates cardiac fibrosis caused by long-term hypertension independently of ABP. Similar to the report by Wang et al. in 2019 [37], our results demonstrate that ALA attenuated lung fibrosis but did not change ABP, clearly indicating the versatility of ALA's effects and confirming RAS pleiotropism.

5. Conclusion

In the future, it is possible that ALA represents an important strategy to improve IPF patient quality of life. This histological and functional study supports a significant progress and may encourage further investigation into the mechanisms of ALA in PF.

Data Availability

The experimental data that support the findings of this study are included within the article.

Conflicts of Interest

All authors declare no conflict of interest.

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CONSIDERAÇÕES FINAIS

Muitas substâncias mostraram eficácia na FP in vitro ou nos mecanismos moleculares em modelos animais. No entanto, nossos dados demonstraram, pela primeira vez, o efeito da alamandina no desenvolvimento da FP experimetnal e na proteção da função pulmonar em ratos induzidos com bleomicina.

Os promissores resultados preliminares do nosso estudo mostram que a ativação do eixo Alamandina/MrgD pode ser um potente alvo terapêutico. Estudos adicionais são, claramente, essenciais para investigar com precisão os mecanismos da alamandina e os efeitos em células humanas.

Além disso, analisar o potencial da alamandina em protocolos terapêuticos são necessários. Porém, os dados desse estudo representam um avanço importante na pesquisa básica, uma vez que há carência de abordagens terapêuticas eficazes para controlar o desenvolvimento da fibrose.

Os dados da presente tese auxiliaram na fundamentação do pedido de patente provisória internacional para o uso da ALA como medicamento para prevenir ou retardar significativamente a FP (US Provisional Patent Application No. 63/016,646 (303943/2016-5). Nesse contexto, e conhecendo a complexidade da patogênese da FP, acreditamos que a modulação do SRA possa ser utilizada isoladamente ou em combinação com outros agentes antifibróticos.

ANEXO I: Parecer de aprovação do Comitê de Ética da UFCSPA

COMISSÃO CIENTÍFICA E COMISSÃO DE PESQUISA E ÉTICA EM SAÚDE

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA
UFCSPA

A Comissão de Ética no uso de Animais, analisou o Projeto:

Projeto: 17-207 **Versão do Projeto:** **Versão do TCLE:**

Pesquisadores:
KATYA VIANNA RIGATTO
GUILHERME WATTE
FERNANDA BORDIGNON NUNES
JARBAS RODRIGUES DE OLIVEIRA
FABÍOLA ADELIA PERIN

Título: ANÁLISE TRANSLACIONAL DA PARTICIPAÇÃO DO SISTEMA RENINA ANGIOTENSINA E DE CITOCINAS INFLAMATÓRIA NA FIBROSE PULMONAR.

Este projeto foi aprovado em seus aspectos éticos e metodológicos. Todo e qualquer alteração do projeto, assim com eventos adversos graves, deverão ser comunicados a esta CEUA.

Porto Alegre, 16 de abril de 2018.

ANEXO II: PCT International Application No. PCT/US2021/029559

FLEIT Intellectual Property Law

Paul D. Bianco, Ph.D.
pbianco@FleitIP.com

VIA E-MAIL ONLY
margules@nova.edu

April 30, 2021

Gary S. Margules, Sc.D.
Vice President for Research and Technology Transfer
Nova Southeastern University
3301 College Avenue
Fort Lauderdale, FL 33314-7796

Re: PCT International Application No. PCT/US2021/029559
Filed: April 28, 2021
For: COMPOSITIONS INCLUDING ALAMANDINE PEPTIDES AND METHODS FOR TREATMENT OF PULMONARY DISEASE USING ALAMANDINE PEPTIDES
Of: K. Rigatto et al.
Your Ref.: NSU20-02; Our Ref.: 7752-P21-061

Dear Gary:

Enclosed please find the Patent Cooperation Treaty ("PCT") International Application and associated papers filed with the U.S. PCT Receiving Office on April 28, 2021. Please review the as-filed documents, including the Request, and inform us as soon as possible if any corrections are required.

Combined Declaration and Assignment

Enclosed please find a combined Declaration/Assignment (worldwide rights) document for execution by the inventors. Prior to execution, each inventor should closely review the as-filed application and the Declaration/Assignment. If any information is incorrect, please do not execute this document. We would prefer to forward you a corrected document for execution.

Please note that the USPTO does not require originals. Accordingly, please return a copy and retain the original in a safe location.

Publication

This PCT application will be published approximately eighteen (18) months from the first priority date of April 28, 2020, so that publication will be around October 28, 2021. The publication of the PCT application will act as a prior art reference against any cases later filed by you or any other parties.

Deadline to File National Stages

As you are aware, the international application does not in itself confer any patent rights. Rather, it preserves your ability to file applications in most countries throughout the world. When we filed the PCT application, we designated all available countries for possible future national stage applications. For most countries/regions typically of interest to our clients, we are required to file national/regional stage applications by **October 28, 2022**. There are a few exceptions, however. For example, national stage applications for Luxembourg, United Republic of Tanzania, and Uganda are due earlier.

Please contact us if you have any questions about a specific country. With regard to Luxembourg, United Republic of Tanzania, and Uganda, unless you inform us otherwise, we will assume that these countries are not of interest to you.

Small Entity Status

We remind you that we have filed this application under small entity status. In general, a "small entity" is an independent inventor, a nonprofit organization, or a for-profit with 500 or fewer employees (including affiliates and related entities). If any rights in the invention have been assigned, granted, conveyed, or licensed (or the owner is under an obligation to do so) to another party, then the assignee must also be a small entity for the application to qualify for small entity status.

It is extremely important that small entity status not be claimed in error. Accordingly, please inform us if you have any questions about this calculation or if at any time during the application process the application no longer qualifies for small entity status.

* * *

We will keep you informed regarding further developments in this matter.

With best and warmest regards.

Sincerely,

Paul D. Bianco, Ph. D.

PDB:df
Encs.