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Victor Teatini Ribeiro

O SISTEMA RENINA-ANGIOTENSINA NA DOENÇA DE ALZHEIMER: revisão de literatura e estudo-piloto

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O SISTEMA RENINA-ANGIOTENSINA NA DOENÇA DE ALZHEIMER: revisão de literatura e estudo-piloto

VICTOR TEATINI RIBEIRO

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Aos meus pais, pelo apoio.

RESUMO

A Doença de Alzheimer (DA), principal causa de demência, desperta crescente interesse científico, uma vez que a sua fisiopatologia está, em grande parte, ainda por ser desvendada. O sistema-renina angiotensina (SRA) é um dos sistemas fisiológicos mais importantes para a homeostase. À medida que o conhecimento sobre as funções do SRA foi se acumulando, constatou-se a sua participação também em variados processos fisiopatológicos, incluindo as doenças psiquiátricas e neurodegenerativas. O envolvimento do SRA na fisiopatologia da DA, especificamente, ainda é pouco explorado. Este trabalho teve como principais objetivos: (1) realizar uma ampla revisão da literatura sobre o SRA, a DA e os possíveis pontos de interação entre ambos; (2) conduzir um estudo-piloto explorando o *status* do SRA em pacientes com DA e em indivíduos cognitivamente saudáveis, visando à geração de hipóteses fisiopatológicas. Para atender ao primeiro objetivo, realizamos pesquisa não sistemática nas bases PubMed/Medline e Google Acadêmico. Foram encontrados estudos originais pré-clínicos e clínicos que investigaram diretamente o papel do SRA na DA. Produzimos, então, um artigo de revisão narrativa com um sumário da evidência direta, acompanhada de uma discussão ampla sobre potenciais mecanismos. Para satisfazer ao segundo objetivo, conduzimos um estudo transversal, exploratório, de caso-controle, em que foram recrutados 14 pacientes com DA e 14 voluntários cognitivamente saudáveis da mesma faixa etária. De todos os participantes, obtivemos dados clínicos e neuropsicológicos, além de amostras de sangue e imagens por ressonância nuclear magnética (RNM) do encéfalo. Foram dosadas, no plasma, angiotensina II (Ang II) e angiotensina-(1-7) [Ang-(1-7)], as moléculas efetoras dos eixos clássico e alternativo do SRA, respectivamente. Constatou-se que a Ang-(1-7) está significativamente reduzida no plasma de indivíduos com DA. Nesse grupo, houve uma correlação positiva entre a Ang-(1-7) circulante e marcadores de lesão cerebrovascular na RNM. Nossos achados favorecem a tese de que o eixo alternativo do SRA está alterado na DA e indicam um possível mecanismo de interação. Os resultados fornecem subsídio para que estudos maiores, com poder estatístico adequado, testem essa hipótese.

Palavras-chave: Sistema Renina-Angiotensina. Doença de Alzheimer. Angiotensina-(1-7)

ABSTRACT

Alzheimer's Disease (AD), the leading cause of dementia worldwide, is the subject of growing scientific interest, given that part of its pathophysiology is yet to be unveiled. The renin-angiotensin system (RAS) is one of the most important physiological systems for homeostasis. As the knowledge about the RAS and its functions has accumulated, the system has been implicated in many pathological processes as well, including psychiatric and neurodegenerative diseases. However, the RAS role in AD pathophysiology, in particular, remains largely unexplored. This study addressed this gap in knowledge by targeting two main objectives: (1) to perform a comprehensive literature review about the RAS, AD and their possible intersection points; (2) to conduct a pilot-study to explore the RAS *status* in AD patients compared to cognitively healthy individuals, so as to generate hypotheses. To accomplish the first goal, we have non-systematically searched throughout PubMed/Medline and Google Scholar databases. We have found pre-clinical and clinical primary studies which directly addressed RAS participation in AD. As a result, we have written a narrative review article that summarizes all direct pieces of evidence and discusses at length the potential mechanisms behind them. In order to achieve the second objective, we have conducted a cross-sectional, case-control exploratory study which recruited 14 patients with AD and 14 cognitively healthy age-matched volunteers. Clinical data, neuropsychological test results, blood samples and magnetic resonance imaging (MRI) were obtained from all participants. We have measured plasma levels of angiotensin II (Ang II) and angiotensin-(1-7) [Ang-(1-7)], the effector peptides of RAS classical and alternative axis, respectively. Ang-(1-7) was found to be significantly reduced in AD patients. In the AD group, there was a positive correlation between circulating Ang-(1-7) and MRI markers of cerebrovascular lesions. Our findings strengthen the hypothesis that RAS alternative axis is downregulated in AD and points to a potential interaction mechanism. Moreover, these results provide a basis for conducting larger properly powered studies willing to test this hypothesis.

Keywords: Renin-Angiotensin System. Alzheimer's Disease. Angiotensin-(1-7)

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LISTA DE ABREVIATURAS E SIGLAS

Aβ – beta-amiloide

Ang II – Angiotensina II

Ang-(1-7) – Angiotensina (1-7)

BRA – Bloqueadores do Receptor de Angiotensina

DA – Doença de Alzheimer

DOI – *Direct Object Identifier*

ECA – Enzima Conversora de Angiotensina

ECA-2 – Enzima conversora de Angiotensina 2

ELISA – *Enzyme-Linked Immunosorbent Assay*

FLAIR – *Fluid-attenuated Inversion Recovery*

iECA – inibidores da Enzima Conversora de Angiotensina

p-tau –proteína tau fosforilada

RNM – Ressonância nuclear magnética

SNC – Sistema Nervoso Central

SRA – Sistema renina-angiotensina

t-tau – proteína tau total

TE: Tempo de eco

TR: Tempo de repetição

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“Porque a cabeça da gente é uma só, e as coisas que há e que estão para haver são demais de muitas, muito maiores diferentes, e a gente tem de necessitar de aumentar a cabeça, para o total.” (GUIMARÃES ROSA, 1956)

SUMÁRIO

1 INTRODUÇÃO

Hoje, em todo o mundo, 50 milhões de pessoas sofrem com demência, mais da metade delas por doença de Alzheimer (DA) [1]. Tipicamente, a DA se manifesta como perda insidiosa e progressiva da memória, que evolui para o comprometimento global da cognição [2]. Segundo a explicação mais aceita, a sua fisiopatologia envolve uma sequência de eventos iniciada com o depósito de proteína beta-amiloide (Aβ) no encéfalo, formando as placas senis. A isso, sucede-se a hiperfosforilação da proteína tau e o arranjo da tau fosforilada (p-tau) em emaranhados neurofibrilares, que, então, desencadeiam considerável neurodegeneração [3]. Recentemente, contudo, tem-se questionado se esse modelo (conhecido como "teoria da cascata amiloide") é satisfatório, em especial na DA esporádica. Na última década, foram testadas várias drogas que atuam na via da amiloide. Muitas delas, de fato, reduziram substancialmente o acúmulo de Aβ no sistema nervoso central (SNC). No entanto, sucessivos ensaios aleatorizados e controlados com o uso desses fármacos falharam em demonstrar o benefício clínico deles esperado. Apesar de não definitiva, essa evidência clínica negativa tem posto em dúvida o papel da amiloide como elemento-chave da cascata patológica que leva à DA [4,5]. Com efeito, cada vez mais pesquisas têm buscado examinar outros fatores que podem contribuir para o desenvolvimento da doença. Nessa linha, a atenção tem se voltado para aspectos até então pouco explorados da DA, como a neuroinflamação [6] e o acometimento vascular [7,8].

Em meio à busca por entender a fisiopatologia da DA de forma mais abrangente, cresce o interesse na possível participação do sistema renina-angiotensina (SRA) [9,10]. Em geral, o SRA é conhecido pelo seu papel como grande regulador da filtração glomerular, do balanço hidroeletrolítico, do tônus vascular periférico e, como resultante, da pressão arterial sistêmica [11]. No entanto, sabe-se hoje que, para além dessas funções clássicas, o SRA participa de inúmeros outros processos homeostáticos e das suas perturbações nas mais diversas doenças, incluídas aí as neurodegenerativas [12,13]. O caráter pleiotrópico do SRA decorre de suas subdivisões, resumidas a seguir:

> Primeiro, distinguem-se as ações do SRA sistêmicas, dependentes de moléculas circulantes, das ações locais, resultado da produção local de angiotensinas em alguns tecidos, inclusive o nervoso [14,15].

> Segundo e mais importante, descrevem-se hoje dois grandes eixos do SRA, o clássico e o alternativo, com funções por vezes antagônicas, levadas a cabo por peptídeos ativos distintos – quais sejam, respectivamente, a angiotensina II (Ang II) e a angiotensina-(1-7) [(Ang-(1-7)]. Principal responsável pelas funções do eixo clássico, a Ang II, agindo via receptor de Ang II do tipo 1 (AT1R), inibe a natriurese e promove vasoconstrição, inflamação e fibrose. Por outro lado, a Ang-(1-7), maior agente do eixo alternativo, ligando-se ao receptor Mas (MasR), é vasodilatadora, anti-inflamatória, antioxidante e neuroprotetora [16].

Há várias zonas de interseção entre a atividade do SRA e a fisiopatologia da DA. Em primeiro lugar, tem-se a presença de componentes do SRA no cérebro, onde eles parecem participar diretamente de processos ligados à memória e à cognição. Os efeitos da Ang II e da Ang-(1-7) na memória já foram objeto de estudos pré-clínicos, tanto em animais saudáveis quanto em modelos de DA [17–19]. A relevância fisiológica do SRA cerebral, contudo, ainda é objeto de debate [20,21]. Mais consolidado, o papel das moléculas circulantes do SRA também é investigado no contexto da DA. Como já dito, o SRA interage com as cascatas da inflamação, cujo desequilíbrio é encontrado na DA. Ademais, Ang-(1-7) e Ang II interferem no fluxo sanguíneo cerebral e na sua regulação – ao passo que, na DA, é marcante a hipoperfusão cerebral, sendo também comum a coexistência de lesões cerebrovasculares.

Apesar do interesse crescente na matéria, a literatura sobre o SRA na DA é relativamente escassa. Os artigos de revisão publicados a respeito do tema são de escopo limitado e enfatizam hipóteses específicas sobre a ação do SRA na DA. Entre os artigos originais, poucos estudos dosaram moléculas do SRA em indivíduos com DA, com resultados conflitantes. A maioria abordou o SRA cerebral e buscou a presença de seus componentes no líquor ou no tecido nervoso. Mesmo entre os trabalhos que mediram os níveis séricos, nenhum investigou os dois eixos do SRA simultaneamente.

O presente trabalho procurou suprir essas lacunas de conhecimento e ajudar na compreensão acerca do papel que o SRA pode desempenhar na DA. Com vistas a esse objetivo, realizamos: (1) uma revisão narrativa da literatura, o mais abrangente possível, que não só reuniu as evidências diretas, mas também discutiu as possibilidades de interação entre SRA e DA; (2) um estudo-piloto, que avaliou os níveis séricos de Ang-(1-7) e Ang II numa amostra

pequena, mas homogênea, de indivíduos com DA e procurou correlacioná-los com dados de neuroimagem estrutural.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Investigar a relação fisiopatológica do Sistema Renina Angiotensina (SRA) com a doença de Alzheimer (DA).

2.2 OBJETIVOS ESPECÍFICOS

1) Revisar criticamente a literatura, analisando evidências (diretas e indiretas) que apontem possibilidades de interação entre SRA e DA, com vistas a:

i. Compreender o estado atual do conhecimento sobre a fisiologia do SRA e a fisiopatologia da DA, destacando áreas de interseção;

ii. Conhecer as teses já postuladas sobre a participação do SRA na DA e quais os estudos (clínicos e pré-clínicos) em que elas se baseiam;

iii. Compilar os estudos que avaliaram diretamente o SRA em indivíduos com DA.

2) Gerar hipóteses sobre o estado dos principais eixos do SRA em portadores de DA, conduzindo, para tal, um estudo exploratório capaz de:

i. Comparar os níveis séricos de moléculas do SRA entre pacientes com DA e indivíduos cognitivamente saudáveis;

ii. Correlacionar as dosagens de moléculas do SRA com variáveis de ressonância nuclear magnética (RNM) estrutural do encéfalo.

3 MÉTODOS

3.1 A REVISÃO DE LITERATURA

Para retratar o atual estado do conhecimento e abordar, de maneira abrangente, os meios pelos quais o SRA pode estar relacionado à DA, optamos pelo método da revisão

narrativa [22]. Assim sendo, foi feita uma busca extensa, não sistemática, nas bases de dados PubMed/Medline e Google Acadêmico, usando diferentes combinações entre as seguintes palavras-chave: “Alzheimer’s Disease”, “Renin-Angiotensin System”, “Angiotensin-(1-7)”, “Angiotensin II”, “Angiotensin-Converting Enzyme inhibitors”, “Angiotensin Receptor Blockers”. Foram selecionados todos os estudos que abordaram diretamente a relação entre SRA e DA. Além desses, foram incluídos demais artigos úteis para elucidar os potenciais mecanismos de interação entre SRA e DA. O texto foi estruturado de acordo com o panorama conceitual e incluiu: (1) uma revisão sobre o SRA, sua ação no SNC e na cognição; (2) uma revisão sobre a DA e sua fisiopatologia, com destaque para os aspectos vasculares e inflamatórios; (3) uma análise sobre o papel do SRA em comorbidades associadas à DA; (4) uma discussão sobre potenciais pontos de interseção entre atividades do SRA e a fisiopatologia da DA; (5) um compilado das evidências diretas de relação entre SRA e DA, notadamente as dosagens de moléculas do SRA em pacientes com DA; (6) uma conclusão sobre a relevância dos achados e as perspectivas futuras.

3.2 O ESTUDO-PILOTO

3.2.1 Desenho do estudo

Para investigar a participação do SRA na DA, conduzimos um estudo observacional (com coleta de dados, sem intervenção); exploratório (com investigação sistemática das relações entre as variáveis coletadas); transversal; de caso-controle [23]. Como forma de avaliar o estado dos eixos clássico e alternativo do SRA em indivíduos com DA, medimos os níveis séricos de Ang II e Ang-(1-7), comparando-os com os do grupo-controle. Em cada um dos grupos, buscamos correlações entre variáveis pré-determinadas de neuroimagem e as dosagens séricas de Ang II e Ang-(1-7). O tamanho amostral foi restrito a 14 indivíduos por grupo, consequente à amostragem por conveniência. Apesar de pequena, a amostra é considerada suficiente para um estudo-piloto que visa à geração de hipóteses [24].

3.2.2 Participantes: recrutamento, inclusão, dados clínicos e avaliação cognitiva

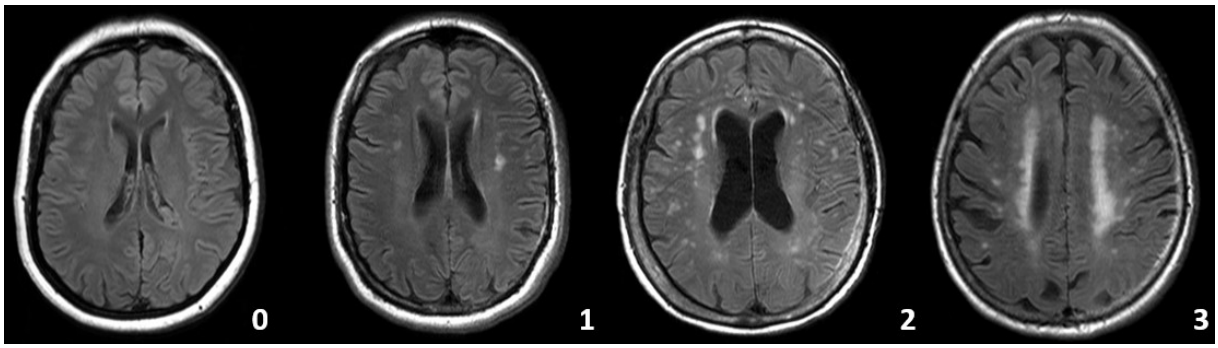
No ambulatório de Neurologia do Hospital das Clínicas da UFMG, foram recrutados, em amostragem por conveniência, 14 pacientes com DA leve a moderada. Todos apresentavam história típica de perda progressiva de memória episódica, combinada com atrofia temporal medial à RNM, satisfazendo assim os critérios diagnósticos para DA [25]. Causas alternativas de demência foram afastadas em todos os casos. Dez dos 14 pacientes haviam sido submetidos à coleta de líquor por punção lombar, e apresentaram perfil de biomarcadores compatíveis com DA, convalidando assim o diagnóstico clínico [26]. Para compor o grupo controle, 14 adultos sem queixas cognitivas, advindos da comunidade, foram convidados a participar do estudo. Nenhum dos controles apresentava pontuação menor que 28 no mini-exame do estado mental (MEEM) [27]. Não foram incluídos, em qualquer um dos grupos, indivíduos com lesões cerebrovasculares importantes, definidas como grau 3 na classificação de Fazekas (cf. seção 3.2.3) [28]. Todos os pacientes passaram por entrevista clínica não estruturada e avaliação cognitiva, que incluiu, além do MEEM, o Teste de Figuras [29], a bateria de avaliação frontal (BAF) [30] e a fluência verbal (animais) [31]. Eram critérios de exclusão, para ambos os grupos: história pregressa de acidente vascular encefálico (AVE); passado de procedimento neurocirúrgico ou de traumatismo cranioencefálico (TCE); comorbidades neuropsiquiátricas graves (esquizofrenia, transtorno afetivo bipolar); infecções atuais ou recentes (no último mês); comorbidades clínicas instáveis. Todos os participantes (ou seus responsáveis) consentiram por escrito em participar do estudo, que foi realizado com aprovação do Comitê de Ética em Pesquisa da UFMG (protocolo CAAE-17805051).

3.2.3 Neuroimagem: aquisição, processamento e variáveis de interesse

Todos os participantes realizaram RNM do encéfalo em um aparelho Intera-Achieva da Philips de 3 Tesla, seguindo o mesmo protocolo de aquisição. Foram obtidas imagens tridimensionais volumétricas ponderadas em T1 (3D-T1), usando os seguintes parâmetros: TR (tempo de repetição) 8,13 ms, TE (tempo de eco): 3,71 ms; matriz de 256 x 256; espessura do corte= 1,0 mm; 1,0 mm de intervalo entre cortes; plano de visão coronal. As imagens 3D-T1 serviram de base para as medidas de volumetria das regiões de interesse pelo software *FreeSurfer* (v. adiante). Também foram obtidas, em todos os participantes, imagens da

sequência FLAIR (*Fluid-attenuated Inversion Recovery*), ideal para visualização de lesões de substância branca, que no FLAIR aparecem hiperintensas e distintas do líquor adjacente, cujo sinal é suprimido[32]. As imagens axiais em FLAIR foram avaliadas por uma neurorradiologista, que desconhecia a identidade e o diagnóstico dos participantes. Em sua análise, a neurorradiologista classificou as lesões de substância branca usando a escala de Fazekas [28,33]. Resumidamente, de acordo com essa escala, as imagens podem ser categorizadas como: grau 0 (ausência de lesões na substância branca); grau 1 (lesões puntiformes na substância branca); grau 2 (lesões confluentes iniciais); grau 3 (grandes áreas de lesões confluentes na substância branca) – ver Figura 1.

Figura 1 Imagens representativas da classificação de Fazekas.



As imagens ponderadas em T1, por sua vez, foram inspecionadas visualmente para o controle de qualidade antes do processamento pelo software. As imagens de um indivíduo do grupo controle foram consideradas de qualidade insuficiente, e excluídas da análise posterior. Disponível gratuitamente on-line, o software *FreeSurfer image analysis suíte*, versão 6.0 foi utilizado para processar as imagens 3D-T1, para reconstrução cortical e segmentação volumétrica[34]. Os detalhes técnicos desses processos fogem ao escopo deste trabalho, mas foram descritos minuciosamente na literatura e sumarizados recentemente[35]. O programa é capaz de distinguir, nas imagens de cada indivíduo, substância branca, substância cinzenta e líquor. Áreas corticais são classificadas anatomicamente por um sistema automatizado. Os volumes de cada região anatômica são calculados pelo programa a partir da espessura cortical, com resultados validados por comparação a medidas manuais[36] e análise histológica[37]. Depois da primeira análise pelo software, é feita uma nova inspeção manual, com correção de erros

identificados. Em seguida, as imagens de cada participante são reprocessadas pelo *FreeSurfer* considerando as intervenções feitas manualmente. Essa sequência pode ser repetida até que se obtenha a correção mais acurada possível.

As variáveis de interesse na RNM foram definidas *a priori* de acordo com a sua relevância para a fisiopatologia da DA. Em cada indivíduo foram medidos, em mm^3, considerando a soma de ambos os hemisférios: (1) o volume do hipocampo; (2) o volume da região temporal medial, definida como uma combinação do córtex entorrinal com o giro parahippocampal; (3) o volume do pré-cuneus; (4) o volume das hipointensidades de substância branca. As primeiras duas variáveis foram escolhidas dada a importância da atrofia temporal medial para a DA.[38] O pré-cuneus foi selecionado porque parece ser uma das primeiras áreas a experimentarem hipoperfusão na DA [39]. Por fim, as hipointensidades de substância branca em T1, da forma como estimadas pelo *FreeSurfer*, podem indicar acometimento cerebrovascular. Lesões de substância branca, presumivelmente de causa vascular, podem aparecer hipointensas em T1, especialmente quando mais graves [32]. A olho nu, no entanto, a avaliação de lesões de substância branca é feita preferencialmente pelas sequências ponderadas em T2 e FLAIR[40]. Em contraste, o *FreeSurfer* usa um procedimento probabilístico[41] para distinguir, mesmo em T1, a substância branca normal das áreas incrustadas com discreta anormalidade de sinal. Dessa forma, o volume das hipointensidades de substância branca (em T1) estimado pelo *FreeSurfer* tem forte correlação com marcadores de lesão de substância branca reconhecidos, tais como a escala de Fazekas [42] e as hiperintensidades de substância branca em T2 [43]. Por fim, ainda que o volume de hipointensidades de substância branca em T1 possa subestimar a real extensão das lesões [44], ele tem sido usado consistentemente como medida de acometimento cerebrovascular em indivíduos com DA e em idosos saudáveis [45–52].

3.2.4 Dosagem sérica de angiotensinas

Coletou-se sangue venoso de todos os participantes. As amostras foram colocadas em tubos a vácuo com heparina e centrifugadas duas vezes a 1.800xg por 10 minutos a 4°C. Obtiveram-se então amostras de plasma, armazenadas a -70°C até o processamento. Utilizamos *kits* de ELISA sanduíche quantitativo para dosar os níveis plasmáticos de Ang-(1-7) (catálogo #MBS084052) e Ang II (#MBS028394), seguindo as instruções do fabricante (MyBioSource,

San Diego, CA, Estados Unidos). As concentrações foram medidas em pg/ml e a sensibilidade reportada para ambos os analitos é de 2,0 pg/ml. Todas as amostras foram analisadas em um só ensaio para evitar a influência da variabilidade entre ensaios. Nossa variabilidade intra-ensaio foi menor que 3%. Além dos níveis de Ang-(1-7) e Ang II dosados, a razão Ang-(1-7)/Ang II foi calculada como parâmetro do equilíbrio entre os eixos alternativo e clássico do SRA.

3.2.5 Análise estatística

As análises estatísticas foram feitas usando o software *GraphPad Prism 8.0.2* (GraphPad Software, San Diego, CA, Estados Unidos). Para avaliar a normalidade dos dados, inspecionamos visualmente a distribuição de cada uma das variáveis contínuas, e as submetemos ao teste de Shapiro-Wilk. Como não havia dados com distribuição normal, todas as análises usaram testes não-paramétricos. Com relação às variáveis contínuas, os dois grupos (DA e controle) foram comparados através do teste U de Mann-Whitney. O teste exato de Fisher foi empregado para comparar variáveis categóricas (binárias) entre os grupos. As correlações entre variáveis foram calculadas usando o coeficiente de Spearman (ρ). Devido à natureza exploratória do estudo, optamos por não realizar ajustes para comparações múltiplas[53]. Quando a amostra do estudo foi dividida não em dois grupos, mas em três categorias (pontuações de 0,1 e 2 na escala de Fazekas), elas foram comparadas usando a análise de variância de Kruskal-Wallis.

4 RESULTADOS

Nesta seção serão apresentados os artigos *Renin-Angiotensin System and Alzheimer's Disease Pathophysiology: From the Potential Interactions to Therapeutic Perspectives* e *Circulating Angiotensin-(1-7) is Reduced in Alzheimer's Disease Patients and Correlates With White Matter Abnormalities: Results from a Pilot Study.*

4.1 ARTIGO DE REVISÃO: *RENIN-ANGIOTENSIN SYSTEM AND ALZHEIMER'S DISEASE PATHOPHYSIOLOGY: FROM THE POTENTIAL INTERACTIONS TO THERAPEUTIC PERSPECTIVES*[*]

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ABSTRACT

New roles of the Renin-Angiotensin System (RAS), apart from fluid homeostasis and Blood Pressure (BP) regulation, are being progressively unveiled, since the discoveries of RAS alternative axes and local RAS in different tissues, including the brain. Brain RAS is reported to interact with pathophysiological mechanisms of many neurological and psychiatric diseases, including Alzheimer's Disease (AD). Even though AD is the most common cause of dementia worldwide, its pathophysiology is far from elucidated. Currently, no treatment can halt the disease course. Successive failures of amyloid-targeting drugs have challenged the amyloid hypothesis and increased the interest in the inflammatory and vascular aspects of AD. RAS compounds, both centrally and peripherally, potentially interact with neuroinflammation and cerebrovascular regulation. This narrative review discusses the AD pathophysiology and its possible interaction with RAS, looking forward to potential therapeutic approaches. RAS molecules affect BP, cerebral blood flow, neuroinflammation, and oxidative stress. Angiotensin (Ang) II, via angiotensin type 1 receptors may promote brain tissue damage, while Ang-(1-7) seems to elicit neuroprotection. Several studies dosed RAS molecules in AD patients' biological material, with heterogeneous results. The link between AD and clinical conditions related to classical RAS axis overactivation (hypertension, heart failure, and chronic kidney disease) supports the hypothesized role of this system in AD. Additionally, RAS-targeting drugs as Angiotensin Converting Enzyme inhibitors (ACEis) and Angiotensin Receptor Blockers (ARBs) seem to exert beneficial effects on AD. Results of randomized controlled trials testing ACEi or ARBs in AD are awaited to elucidate whether AD-RAS interaction has implications on AD therapeutics

KEYWORDS: Renin-angiotensin system, Alzheimer's disease, angiotensin II, angiotensin-(1-7), dementia, neuroinflammation, amyloid hypothesis.

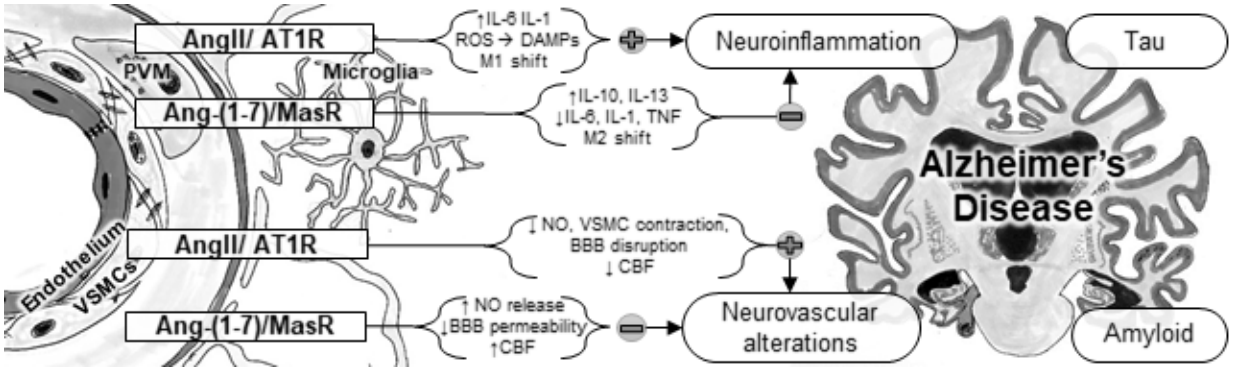

Figura 2: Graphical Abstract

1. INTRODUCTION

The Renin-Angiotensin System (RAS) is widely regarded, above all, as an important regulator of cardiovascular and renal functions. Nevertheless, RAS is also implied in many other processes, to the point that it has been called “the ubiquitous system for homeostasis and pathologies” [1]. By means of both its classical and alternative axes, RAS has been implicated in medical conditions outside the cardiovascular system and the kidneys. RAS components are believed to play a role in some neuropsychiatric disorders, including neurodegenerative diseases, such as Parkinson’s disease and dementia [2]. It has long been suggested that drugs targeting the RAS could help to prevent Alzheimer’s disease (AD) or might slow its progression [3, 4]. The biological mechanisms that lie underneath this hypothesis remain under active investigation. The aim of this article is to provide an overview of the current knowledge about the link between RAS and AD, from the pathophysiological rationale to the possible clinical implications. In order to achieve this goal, we will first explore the evidence for RAS presence and functions in the brain. Then, we will move forward to consider the most important features of AD pathogenesis and their possible relationship with RAS. Finally, the results of human and animal studies that addressed this relationship will be reviewed.

2.2. RAS, THE BRAIN AND ALZHEIMER’S DISEASE

2.1. RAS and the Brain

The classical axis of the RAS comprises the cascade of events starting with the secretion of renin by the kidneys’ juxtaglomerular cells. Renin hydrolyzes the serum angiotensinogen, releasing Angiotensin I (Ang I). Ang I is then cleaved by the Angiotensin-Converting Enzyme (ACE) to produce Angiotensin II (Ang II). The binding of Ang II to the Angiotensin type 1 receptor (AT1R) mediates most of the well-known actions of RAS, including vasoconstriction, fluid retention, and aldosterone secretion [5, 6]. ACE is a membrane-bound metalloproteinase of endothelial cells [7]. Soluble ACE forms in plasma still retain its catalytic activity [8]. Besides being the classical axis’ effector peptide, Ang II also binds to the Angiotensin type 2 receptor (AT2R), leading to counterregulatory actions (e.g.,

natriuresis and hypotension) [9]. In addition, Ang II can later be a substrate to aminopeptidases (APs). Aminopeptidase A (APA) acts upon Ang II to release Angiotensin III (Ang III), which is further metabolized to Angiotensin IV (Ang IV) by aminopeptidase N (APN). Ang IV acts through Angiotensin type 4 receptor (AT4R), whereas Ang III activates AT1R. APA and APN are type II membrane proteins, also known to have active soluble forms [10, 11]. Another peptidase, homologue to ACE, termed ACE2, removes the terminal residue of Ang II and releases Angiotensin-(1-7) [Ang-(1-7)] [12, 13]. The binding of Ang-(1-7) to the G- protein coupled receptor Mas (MasR) mediates anti- inflammatory, anti-oxidative and anti-fibrotic actions of the so-called ACE2/Ang-(1-7)/MasR axis of RAS [14-16]. In addition, Ang-(1-7) produces MasR-mediated vasodilation in several vascular territories [17, 2]. Like ACE, ACE2 is a transmembrane protein that can shed into plasma in catalytically active forms [18]. The pharmacological modulation of RAS is a successful therapeutic strategy for many conditions, including hypertension, Heart Failure (HF) and Chronic Kidney Disease (CKD). The chief drug targets are the ACE, via the ACE inhibitors (ACEi), and the AT1R, targeted by the Angiotensin Receptor Blockers (ARBs) [5].

RAS is one of the major systems involved in the regulation of blood pressure (BP). BP itself is a key factor in Neurovascular Coupling (NVC) and cerebral autoregulation. NVC refers to the interaction between neurons, vessels and glial cells to synchronize neuronal activity with perfusion. As a result of NVC, local blood flow is enhanced in metabolically active areas of the Central Nervous System (CNS), a phenomenon named functional hyperemia [19]. Mean Arterial Pressure (MAP) is an important component of Cerebral Perfusion Pressure (CPP). CPP determines Cerebrovascular Resistance (CVR), and hence inversely controls Cerebral Blood Flow (CBF). Sudden rises and falls in BP elicit vasodilatory or vasoconstrictor responses throughout the brain vascular tree. Consequently, CVR vary in face of acute MAP changes, in a way that CBF is maintained relatively constant and brain damage is prevented [20]. This process, called cerebral autoregulation, relies on intricate mechanisms, which have been reviewed elsewhere [21]. Autoregulation guarantees that CBF can resist against abrupt changes in MAP, but it cannot account for chronic changes in BP [21]. Considering the critical role BP plays in regulating brain perfusion, it is unsurprising, though, that the systemic RAS can affect cognition by controlling BP [21].

Regardless of BP regulation and its consequences for the CNS, RAS is involved in many local actions within the brain. The Blood-Brain Barrier (BBB) is impermeable for all RAS components, thus RAS molecules in circulation are prevented from reaching most brain regions [22]. Even though, RAS products are found in nearly all the CNS, composing the so-called local brain RAS. The source of RAS molecules in the brain is still a matter of debate. Local synthesis actually occurs: for instance, angiotensinogen is centrally produced mainly in astrocytes, and renin has been identified on neurons and astrocytes alike [23]. However, the relevance of locally produced RAS components in the brain has been recently questioned. In the mice's brain, the levels of prorenin are very low, so that the detected renin molecules are likely to be remnants, derived from small amounts of blood trapped within brain tissue [24]. In the CNS of spontaneously hypertensive rats, results showed either low or undetectable quantities of angiotensinogen, Ang I and Ang- (1-7) [24]. Notwithstanding, this conclusion has been criticized for not taking into account the biochemical and functional complexity of the central RAS. Questions were also raised about whether methods that measure the RAS components in the periphery can be validly used in brain tissue homogenates [25].

As for the Ang II, its presence in the CNS is a matter of fact, in spite of the ensuing controversy about in what quantity [26]. Assuming that the local synthesis of renin and angiotensinogen is really negligible, then brain angiotensins probably derive from the bloodstream. It has been proposed that a fraction of circulating Ang II can physiologically cross the BBB and thus be the source of all angiotensin peptides in the brain such as Ang IV, Ang III and Ang-(1-7) [27]. Furthermore, only in regions lacking BBB, such as the Circumventricular Organs (CVOs), peripheral RAS molecules can act directly in the brain [23]. CVOs are involved in the regulation of water and electrolyte balance, linked to the systemic RAS's classical role [28]. In contrast, some RAS components acting locally in the brain are associated with different physiological processes. Local brain RAS will be briefly considered here, regarding its main components, where they are found and their most important actions. The functions of brain RAS and systemic RAS that can be implicated in cognition will be emphasized.

2.1.1.RAS Classical ACE/Ang II/AT1R Axis and the Brain

AT1R is expressed on neurons, microglial cells, oligodendrocytes, and astrocytes [23]. The highest densities of AT1R are found in areas that regulate autonomic and hormonal

responses [29]. In neurons, AT1R hyperactivation leads to increased production of Reactive Oxygen Species (ROS), through different signaling pathways of the NADPH- oxidase (NOX) complex. The resultant oxidative stress can impair cognitive function. There is a natural upregulation of AT1R expression with aging. On cholinergic neurons, specifically, upregulated expression of ACE impaired acetylcholine (ACh) release [30]. In rats, the Intracerebroventricular (ICV) injection of Ang II reduced ACh levels after 1h, despite normal Acetylcholinesterase (AChE) activity [30]. AT1R is undetectable on microglial cells in their inactive states [23]. AT1R signaling through NOX promotes microglial activation and a shift towards the M1 phenotype, capable of releasing proinflammatory cytokines including Tumor Necrosis Factor (TNF), interleukin (IL)-1β, along with Nitric Oxide (NO) and also ROS [31, 32, 33]. In rodents, upregulation of AT1R on M1 microglia in the cortex and in the hippocampus is related to cognitive impairment, mediated by neuroinflammation [33].

Circulating Ang II modifies the BBB permeability, directly and as a result of its prooxidant and proinflammatory effects. Since the BBB integrity prevents peripheral toxins from reaching the brain, its disruption can be regarded as deleterious [34, 35]. Moreover, studies on rodents show that systemic levels of Ang II interfere with CBF regulation, independently of its effects on MAP [30, 36]. In transgenic mice, the continuous activation of systemic RAS's classical axis impaired cognitive function and reduced CBF [37]. Evidence linking Ang II and cognitive functions in humans are limited. A transversal study evaluated 33 hypertensive and cognitively healthy individuals treated with ARBs. No significant correlation was found between Ang II levels in the serum and cognitive function assessed by Standardized Mini-Mental State Examination [38]. A pilot longitudinal study followed, through 1 year, 56 non-demented older adults, who were receiving neither ARBs nor ACEi. It failed to demonstrate any correlation between cognitive tests results and the blood levels of Ang II [39].

2.1.2.RAS Ang II/AT2R Axis and the Brain

AT2R is expressed on neurons, microglial cells, oligodendrocytes, and astrocytes [23]. AT2R is found in greatest density at subcortical areas [40, 41]. AT2R is not detected on healthy microglia. As a response to inflammatory stimuli, AT2R can be upregulated to offset the pro-inflammatory actions of AT1R. AT2R shifts the microglia to the M2 phenotype, responsible for producing anti-inflammatory cytokines [23, 31, 32]. Additional investigation is still needed

to clarify whether there is a direct relation between AT2R and cognition. In an experimental study with mice, the injection of Ang II in the CA1 region of the hippocampus was shown to impair memory consolidation through an AT2R-dependent mechanism [42]. These results somehow contrast with the findings that the systemic stimulation of AT2R with its agonist, Compound 21 (C21), improved the spatial learning of mice [43].

2.1.3.ACE2/Ang-(1-7)/MasR Axis and the Brain

MasRs are expressed on neurons, microglial cells, and astrocytes [23]. Ang-(1-7) exists as an endogenous peptide in the rats' hypothalamus, medulla oblongata, and amygdala, where it was firstly described in the brain [44]. The presence of Ang-(1-7) was also demonstrated in the hippocampus, where it may be partly produced by a different pathway, independent of Ang II [45]. MasRs are present in cardiovascular-related areas in the rats' brain and also throughout the cortex, in the basal ganglia, amygdala, and hippocampus [46, 47]. Activation of MasR in neurons increases NO and decreases mitochondrial respiration, therefore reducing oxidative stress. Expression of MasRs on neurons declines with age. In microglia, MasR induces M2 polarization, during which brain-derived neurotrophic factor (BDNF) production is enhanced [23]. Centrally administered Ang-(1-7) was capable of increasing CBF, bradykinin levels, NO release, and endothelial NO synthase (eNOS) expression after cerebral ischemia-reperfusion injury in rats. [48, 49]. In the same animal model, Ang-(1-7) attenuated BBB damage. Accordingly, Ang-(1-7) decreased the barrier permeability after hypoxia in the brain endothelial cell line RBE4 [50]. In brain slices, Ang-(1-7) was found to enhance long-term potentiation (LTP) in the hippocampus. Consistently, several studies point out that Ang-(1-7) can improve cognitive performance in animal models of cognition-impairing diseases [51]. Moreover, systemic Ang- (1-7) produces vasodilation, mainly through eNOS-mediated NO release, thus increasing vascular conductance and regional blood flow in several vascular territories, including the brain [52].

2.2.Ang IV/AT4R Axis and the Brain

In the CNS, AT4Rs are expressed on neurons and astrocytes [53]. In terms of anatomical location, those receptors are widely distributed, showing high abundance throughout the neocortex, in the basal nucleus of Meynert, and within the hippocampus, especially in the CA2 region and in the dentate gyrus. This pattern of distribution is conserved

among the studied mammal species, and closely resembles the pattern of the cholinergic projections in the brain [54]. Consistently with that, Ang IV was actually shown to potentiate cholinergic transmission in the rat hippocampus [55]. The recognized role of the Ang IV/AT4R axis in the memory process is even more intriguing: Ang IV was found to enhance LTP in the CA1 region in vitro [56]. The same positive effect was demonstrated in the rat's dentate gyrus [57]. The Ang IV/AT4R action on LTP could represent a distinct pathway, different from the glutamatergic pathway of NMDA-dependent LTP [40]. Synaptogenesis can also be favored by Ang IV in the hippocampus [58]. Additionally, systemic Ang IV leads to vasodilation through a NO-dependent mechanism and enhances CBF [59].

2.3.Alzheimer's Disease: Essentials

Around 44 million people worldwide suffer from dementia, defined as an acquired progressive cognitive impairment sufficient to impact on activities of daily living (ADL). AD is by far the most prevalent cause of dementia, accounting for 50-75% of the cases [60]. To this day, no treatment can halt the downhill course of AD [61]. Local brain RAS and systemic RAS are believed to play a role in AD pathogenesis, hence standing as a potential drug target. [62]. Before actually reviewing the evidence that supports this claim, we shall present some information about AD at a deeper level. After giving a short account of AD definition and diagnosis, we will explore some relevant issues necessary to achieve a better understanding of the relationship between AD and RAS. In this regard, some key features of AD's pathophysiology have to be addressed, notably: the aggregation of misfolded proteins (amyloid and tau), the role of inflammation, and the vascular alterations.

2.3.1. AD Clinicopathological Characteristics and Diagnosis

Clinically, AD typical presentation comprises a progressive amnesic disorder with the ensuing development of other cognitive, behavioral and neuropsychiatric changes[63]. AD can be regarded as a disease of the elderly, at least in its preponderant sporadic form (sAD) [60]. Indeed, sAD can be also referred to as late-onset AD (loAD) [61]. Only a small minority of patients (<0,5%) have the familial form (fAD), which can manifest as early as 30 years-old [60]. Most cases of fAD are inherited in an autosomal dominant fashion. Three loci can carry mutations that lead to fAD, in the genes encoding amyloid precursor protein (APP),

presenilin(PSEN)-1, and PSEN-2 [60, 61]. As for sAD, there is no determinant genotype, although inherited variants of several genes are associated with an increased risk, the strongest of which is the apolipoprotein E (ApoE), isoform ApoE ε4 [64].

The histopathological hallmarks of AD in the brain are the senile plaques (neuritic plaques), formed by peptide amyloid-β (Aβ), and the Neurofibrillary Tangles (NFTs), formed by protein tau aggregates [65]. Other common findings in the AD brain include Cerebral Amyloid Angiopathy (CAA), glial response, synapse loss and more importantly, neuronal loss resulting in cortical atrophy. Marks of cerebrovascular disease are frequent in the aged brain, and remain so in the presence of AD. Thus, cortical microinfarcts, subcortical lacunar infarcts, and demyelination of the periventricular white matter are usually found in AD patients. Aβ plaques and NFTs are more disease-specific and withstand the pathological diagnosis [66]. Extracellular Aβ accumulates in amorphous fibrillar amyloid deposits with a compact center (dense-core plaques), usually surrounded by dystrophic neurites (hence the name neuritic plaques). Reactive astrocytes, activated microglia, and synaptic loss accompany the dense-core plaques [66]. In the AD brain, neuritic plaques are found mainly in the isocortex [67]. The pathological assessment of plaques is of little use to predict the clinical status of AD, and the amyloid burden correlates neither with the severity nor with the duration of dementia [66]. Distribution of NFTs, unlike that of plaques, show a pattern of spatiotemporal progression that runs in parallel with cognitive decline. NFTs are intraneuronal filamentous inclusions of misfolded and hyperphosphorylated tau, which remain visible even after the tau-induced neuronal death [66]. One of the first regions to be affected by NFTs is precisely the entorhinal cortex along with the CA1 region of the hippocampus. NFTs then develop in other subcortical structures, before spreading to the isocortex, first to associative areas and lately to primary sensory areas [68]. Remarkably, the typical progression of AD symptoms matches the topographical distributions of NFTs over time. Nonetheless, it is worth noting that the best correlates of cognitive decline in AD are the degree of neuronal loss and the decrease in synaptic density. Although those findings often come with NFTs, they seem to be more clinically relevant than the tangles themselves [66].

Until recently, the unequivocal diagnosis of AD was considered possible only post-mortem, through histopathological analysis. In vivo, after excluding other causes, demented

patients* with the AD's clinical pattern were told to have "probable AD" (for details, see the 1984 NINCDS-ADRDA criteria) [69]. Before the onset of dementia, cognitively impaired patients would only fit in the classification of a syndromic clinical entity. Consistent with this notion, the term Mild Cognitive Impairment (MCI) refers to the presence of cognitive decline above what is expected in normal aging, but not severe enough to harm the ability to perform ADL [70]. Under this definition, MCI is one of the main risk factors for AD [71]. In the past two decades, accumulating evidence has shown the accuracy of biochemical and neuroimaging biomarkers to predict the pathological features of AD [72]. At least in the research setting, those biomarkers are now combined with the clinical findings to establish the diagnosis of AD. In this perspective, not only the term "probable AD" was abandoned, but also the category of "prodromal (predementia) AD" was introduced [63]. For a complete description of these definitions, see the IWG-2 diagnostic criteria for AD [73]. The use of biomarkers has also led to a profoundly distinct position concerning the concept of AD: the will to define AD solely by the presence of Aβ and tau biomarkers, regardless of clinical symptoms, as proposed by the National Institute on Aging - Alzheimer's Association (NIA-AA) [74]. The different sets of diagnostic criteria for AD in research pose additional challenges when it comes to interpreting the results provided by clinical studies [75].

Currently, few drugs are approved for AD treatment, showing modest benefits on cognition, without any accompanying changes in the disease course. AChE inhibitors (AChEIs), and memantine, an NMDA receptor blocker, are intended to correct AD-related abnormalities in the cholinergic and glutamatergic systems, respectively [76]. The severe ACh deficit in cerebral cortex correlates to clinical dementia in AD. This observation contributed to elucidate the AD-induced memory loss, which is in part attributable to ACh deficiency, as stated by the "cholinergic hypothesis of AD". Cholinergic neurotransmission might be functionally impaired as early as the MCI stage of AD, even in the absence of neurodegeneration [77]. How amyloid and tau pathologies interact with cholinergic and glutamatergic dysfunctions in AD is a matter of ongoing investigation [77, 78].

* NOTA: Posteriormente à publicação do estudo, tomamos conhecimento de que o termo *demented*, em língua inglesa, tem conotação pejorativa e deve ser abandonado [54].

2.3.2. AD Pathophysiology: Amyloid Pathology and the Amyloid Cascade Hypothesis

In the path to AD, it is generally accepted that amyloid deposition precedes brain atrophy and cognitive decline. The current understanding is that Aβ accumulation occurs at very slow rates, beginning around two decades before the earliest signs of cognitive decline [79]. Aβ peptides are natural products of the metabolism derived from APP, a type I transmembrane protein expressed in many organs [65]. Less is known about APP physiological functions than about its relevance to disease pathogenesis. Roughly, APP can be processed through two different pathways: nonamyloidogenic and amyloidogenic [65, 80]. In the nonamyloidogenic processing, APP is cleaved by the α- secretase right within its Aβ region, and thus Aβ formation is precluded [65, 80]. Amyloidogenic pathway of APP processing starts with the action of a β-secretase, β-site APP- cleaving enzyme 1 (BACE1). Further γ-secretase cleavage of the remaining membrane domain produces Aβ peptides with varying number of amino acids [65, 80]. Amongst all the Aβ species, two appear to be the most relevant to AD pathogenesis: the 40 amino acids monomer Aβ40, and the 42 amino acids peptide Aβ42. Aβ40 is more prevalent than Aβ42, which is more toxic and aggregation-prone [65, 80]. Aβ peptides spontaneously self-aggregate into multiple compounds of different sizes: 2 to 6 peptides combine to form oligomers. These can assembly and generate intermediate forms. Aβ can also give rise to fibrils, which can be arranged to form fibers of advanced amyloid plaques [80]. Allegedly, soluble toxic Aβ oligomers, rather than plaques, can damage synaptic function. Plaques may be regarded as reservoirs of toxic species and triggers of a local inflammatory response, with microglial recruitment [65, 81]. Additionally, Aβ could drive tau pathology and synergistically collaborate in tau-driven neurotoxicity [82]. However, it is still to be determined whether Aβ directly induces tau misfolding in humans.

The amyloid cascade hypothesis states that Aβ dyshomeostasis is the initiating event of AD pathogenesis. Tauopathy, neurodegeneration, neuroinflammation, oxidative stress, vascular alterations, and all other elements of AD pathology are considered to be downstream of the Aβ dysequilibrium in the disease's pathogenetic cascade and to be caused by Aβ, either directly or indirectly [83, 84]. The strongest evidence supporting the amyloid hypothesis comes from individuals with fAD, in which all underlying mutations are related to amyloid processing.

APP, PSEN-1 and PSEN-2 mutations lead to an unbalance between the different Aβ subsets. Thus, in fAD, Aβ oligomerization and accumulation are favored, because of the relative increase in the levels of aggregation-prone Aβ species, mainly Aβ42 and Aβ43. As for sAD, the reason why brain Aβ42 levels rise is far less clear. The imbalance between Aβ production and clearance allegedly contributes early to sAD pathogenesis [83]. Aβ clearance depend on complex processes in the brain and the periphery. The amyloidogenic APP processing seems to be far more active in the CNS, which is why Aβ aggregates are found almost exclusively on the brain (as plaques) and on cerebral vessel walls (as CAA) [85]. Several pathways potentially participate in the brain clearance of Aβ, notably: Aβ transport across the BBB; cellular Aβ clearance accomplished by different cell types (microglia, perivascular macrophages, astrocytes, oligodendroglia, and neurons); and proteolytic degradation by various enzymes [85]. There are still doubts about the relevance of these paths to Aβ clearance in AD, as well as the relative contributions of each one [86].

Such a considerable knowledge about Aβ physiology was achieved mostly due to the widespread acceptance of the amyloid hypothesis. Naturally, each step of the Aβ metabolism was seen as a potential drug target, in an attempt to reduce Aβ pathology and thus prevent AD or slow its progression [83, 87]. Up to now, however, no drug targeting Aβ has been proven effective in a phase III trial with AD patients [87-89][*]. Methodological limitations in the trials' design were pointed out as possible reasons for the negative results, and have been widely discussed in the literature [88, 90-92]. Even if accurate, this criticism does not seem to cover all the evidence obtained from the trials. Some findings remain largely unexplained under the amyloid hypothesis paradigm. For instance, aducanumab, a monoclonal antibody against Aβ oligomers and plaques, promoted a robust plaque clearance, but still no change in the rate of cognitive decline after 18 months of administration in individuals with mild and very mild symptomatic AD [93]. After the consecutive disappointing results with Aβ-targeting therapeutic strategies, the amyloid hypothesis itself has been challenged [87, 94-97]. More attention has been paid to alternative hypotheses for AD pathogenesis ever since [87, 94]. For

[*] NOTA: Após a publicação deste artigo, a Food and Drug Administration (FDA) concedeu aprovação acelerada ao anticorpo monoclonal aducanumab, numa decisão não isenta de controvérsia. Esse acontecimento não altera a essência da discussão sobre a hipótese amiloide.[55]

instance, the adaptive response hypothesis posits that, prior to Aβ deposition, another pathological process takes place that could drive the changes in amyloid physiology. Disruption of normal APP and Aβ metabolism is regarded as being part of a reaction to such primary underlying process. These earliest phenomena could by themselves determine the other abnormalities happening downstream in the disease progression, including tau pathology [94]. In this regard, AD pathological cascade might be prompted by one or more of the following states: oxidative stress, local immune response, lipid metabolism unbalance, and vascular disease [94, 98, 99].

2.3.3.AD Pathophysiology: Tau Pathology

The weakening of the amyloid hypothesis has also turned the attention to the role of tau pathology in AD [86]. Tau is a soluble, microtubule-associated protein involved in a wide range of physiological processes and disease states, collectively known as tauopathies [100, 101]. In the normal adult brain, tau is found almost exclusively in neurons (mainly in the axons) [100, 102]. Tau can undergo various post-translational modifications, including phosphorylation, glycosylation, nitration, and methylation [102]. In the axon, tau normally promotes microtubules assembly, stability, and reorganization [100, 102]. Under pathological conditions such as AD, tau binding to microtubules is disrupted, resulting in increased levels of free tau [101]. Tau hyperphosphorylation, due to an unbalance between phosphatase and kinase activities, is critical in reducing tau affinity for microtubules [65, 101]. Unbound tau is more prone to conformational changes (misfolding), aggregation and fibrillization [101]. Moreover, in higher cytosolic concentrations, tau leaks out of the axon to other cellular compartments. Because of its low affinity to microtubules, hyperphosphorylated tau (p-tau) passes through the axon's initial segment even more readily [65]. Within the soma, small nonfibrillary tau deposits (pretangles) can sequester other proteins and therefore hamper a series of cellular processes. Eventually, misfolded tau generates insoluble filaments that fill the entire soma, initially under the form of Paired Helical Filaments (PHFs) and ultimately as NFTs [65, 101].

Loss of tau function itself may lead to axonal transport deficits due to microtubule disassembly [100]. However, other microtubule-associated proteins likely compensate for the tau absence [65]. Although the presence of NFTs can lead to neuronal death, the extension of neuronal loss exceeds by large the number of tangle-bearing neurons in AD, whilst some

neurons can survive and function even in the presence of NFTs [100]. Tau toxicity is believed to be largely due to p-tau, which can impair synaptic function and neuron excitability (and consequently LTP) [65]. The relationship between tau pathology and other common AD features (e.g., microglial activation and neuroinflammation) is not yet fully comprehended [65,100].

2.3.4.AD Immunopathology

The immune system was once regarded as a passive player in AD pathogenesis, activated by emerging plaques and tangles. This notion has long been challenged by mounting evidence that neuroinflammation contributes substantially to neurodegeneration in AD [103-105]. Pro- inflammatory cytokines (e.g. IL-1β) show increased concentrations in AD patients' cerebrospinal fluid (CSF). The IL-1β expression is also high in microglial cells surrounding Aβ plaques. Patients in the dementia stage have higher blood and CSF levels of macrophage colony stimulating factor. MCI patients with elevated CSF cytokines are at increased risk for developing AD [104]. Furthermore, the levels of soluble triggering receptor expressed on myeloid cells (sTREM-2) were also higher in AD patients' CSF, possibly indicating microglial activation [106].

Within the CNS, microglia are the chief local cellular components of the innate immune system [107, 108]. In AD and other neurodegenerative diseases, neuroinflammation is the result of innate immune response driven by brain microglia, astrocytes and possibly Perivascular Macrophages (PVMs). The reaction of these cells is probably triggered by Damage-Associated Molecular Patterns (DAMPs) within the brain, although systemic inflammation might contribute as well [103, 104]. Non-stimulated microglia exhibit prominent ramified processes that continuously screen the surroundings [109]. These environment-sensing microglia can become reactive after exposure to stimuli (e.g. DAMPs, proinflammatory cytokines), an event referred to as priming or sensitization [103, 110]. Sensitized microglia can remove the source of priming-triggers (e.g. cellular debris) through phagocytosis [110, 111]. If the stimulus ceases, microglia can return to the quiescent state. Continuous stimuli, otherwise, maintain microglial activation [110]. The standard description of two activated microglia phenotypes – M1 and M2 – is an oversimplification, but remains useful to understand the activation phenomenon [103, 112]. M1 microglia originate from the classical activation

pathway, set in motion by injury signals. M1 cells have reduced phagocytic activity and their main function is to produce cytokines (e.g. IL-1β, IL-6) plus NO and ROS, which, in excess, are cytotoxic to surrounding neurons [104, 109]. Conversely, the alternative pathway gives origin to the M2 subset. M2 cells are responsible for engulfing damaged tissue and producing anti-inflammatory mediators (e.g. IL-10) [109]. Pharmacological transition to the alternative activation state is now pursued, and could possibly be achieved by targeting the nuclear receptor peroxisome proliferator activated receptor gamma (PPAR-γ) [104, 113].

Microglia are major players in AD neuroinflammatory state. The greatest risk factor for sAD, aging, by itself, enhances microglial sensitivity to priming-triggers, either due to intrinsic cellular senescence or to accumulated exposure to stimuli during life [104]. In the AD setting, different Aβ types induce microglial activation. Under the amyloid hypothesis paradigm, this is supposed to be protective at first, since the microglia are able to phagocytize and clear amyloid, reducing existent plaques and preventing the formation of new ones. As the disease progresses, though, the continuous Aβ burden ensues activation, until a threshold is reached after which Aβ load surpasses the microglial clearance capacity. From this point on, microglial activation becomes maladaptive, and full-blown neuroinflammation takes place. Pro-inflammatory cytokines may then mediate neuronal damage and, possibly, tau pathology [103, 114]. Microglia could be part of the missing link between Aβ load, tau burden, and neurodegeneration in AD. TREM-2 is likely to influence the extent to which microglia can remove damaged synapses around Aβ plaques. When the microglia begin to fail in this task, dystrophic neurites may induce tau phosphorylation and trigger tau pathology [115]. Blood-borne monocytes play a minor role, if any, in AD pathogenesis - except, perhaps, as precursors of the resident PVMs, which go through continuous replacement [103, 104]. Unlike blood-derived monocytes, PVMs might help in removing Aβ depositions from the CNS [104]. The overall proinflammatory environment in the AD's brain reaches damaging proportions, hampering neuronal function and suppressing LTP [104].

2.3.5. Vascular Pathology and AD

Given that, unlike fAD, sAD is a disease of the elderly, cerebrovascular commitment is expected to be found in sAD patients [116]. The most commonly found Cerebrovascular Lesions (CVLs) in both aged and AD brains are CAA, atherosclerosis, Small Vessel Disease

(SVD), White Matter Lesions (WMLs), microinfarcts (MI), lacunar infarcts and microbleeds [117]. SVD, along with silent infarctions from other causes, can lead to the slowly accumulating brain damage that characterizes chronic cerebrovascular disease (CVD) [118]. In CVD patients, the risk of dementia was associated with the presence of SVD and cortical MI, as well as with White Matter Hyperintensities (WMH) [118]. WMH are the radiological correlates of WMLs in Magnetic Resonance Imaging (MRI) [119].

CVLs were consistently shown to be more frequent in patients with pathologically confirmed AD than in controls with normal cognition and no AD pathology, matched by age [120]. These alterations are typically attributed to coexisting CVD, which was considered to be completely independent of AD pathology [121]. Assuming such independence, CVD would contribute to cognitive decline in AD patients only through reducing the so-called 'brain reserve', thus allowing an earlier clinical manifestation of the damage induced by AD's own pathological substrate (i.e., amyloid and tangles) [121-123]. Vascular Dementia (VaD) and the broader term Vascular Cognitive Impairment (VCI) generally designate the dementia-MCI syndrome presumably caused by vascular pathology [124-126]. The term mixed dementia refers to the cases in which both CVLs and AD pathology are preeminent and concur to cause the dementia syndrome [124]. Under this definition, mixed dementia, in fact, could be the most common type among the elders [127]. Against this background, AD and VCI are often assumed to be at best concurrent, if not mutually exclusive [128, 129]. Conventionally, signs of altered vascular physiology in AD, can be understood under this perspective. For instance, average CBF is shown to be lower in AD patients compared to age-matched controls and continually decreases during disease progression. This was long seen as a response to the cerebral hypometabolism that results from neurodegeneration [130, 131].

CBF and CBF regulation have been extensively studied in AD, MCI, and normal aging. Steady-state CBF progressively decreases with aging, as do the metabolic needs for glucose and oxygen in the brain. Elderly people more frequently have a higher MAP, a lower Cardiac output (CO), and increased pulse pressure (PP). The latter is commonly outside the range of cerebral autoregulation [132]. Among cognitively normal elders, reduced baseline CBF was associated with a greater risk of developing MCI. As for MCI subjects, albeit total CBF may not be reduced, some early signs of dysregulation may be seen, with differences in

regional CBF (rCBF) among brain areas becoming evident. This could be the result of early neurodegeneration driving changes in metabolic demands. There is not enough evidence supporting the validity of altered CBF as a biomarker of preclinical AD, despite some interesting findings. For example, early local hypoperfusion was associated with a correspondent increase in Aβ deposition in some brain areas, in both AD and MCI patients. It was actually suggested that hypoperfusion could precede (or even induce) amyloid pathology [133]. Cerebral hypoperfusion is an early AD feature, beginning in preclinical phases when the decline in blood supply can actually exceed the fall in metabolic demand [131]. Hypoperfusion is more likely to be the result of functional changes in vascular regulation than of structural vascular disease (e.g. atherosclerosis, CAA) [134, 135].

Mechanisms underlying CBF dysregulation in AD can be studied at a cellular level in the cells that compose the Neurovascular Unit (NVU): endothelium, pericytes, Vascular Smooth Muscle Cells (VSMCs), PVMs, glial cells (mainly astrocytes) and neurons. All NVU cells operate collectively to control the vascular tone. The function of each cell type is affected at some stage during the AD course [136-138]. Contractile VSMCs control the vessel diameter and blood flow to match the metabolic demands of neurons (i.e., the NVC) [137]. VSMC contractility is responsible for the increase in arteriolar diameter at higher carbon dioxide (CO2) concentrations, a mechanism known as cerebral Vasomotor Reactivity (CVMR) [139]. Likewise, cerebral autoregulation is determined by the VSMC myogenic response to fluctuations in BP, aimed to induce changes in the arterial vessel lumen. [140]. Impaired NVC occurs early in AD and results in a disequilibrium between CBF, oxygen (O2) delivery and neuron activity [137]. Accordingly, CVMR impairment is already evident at the MCI stage, when it represents an increase in the risk of conversion to AD [139]. Cerebral autoregulation deteriorates with age, but whether AD results in additional loss of function remain elusive [140].

Neuron-mediated regulation of VSMC tone is well- known and depends on either NO synthesis or vasoactive neurotransmitters (e.g., ACh). Neuronal loss can influence CBF decline in AD patients, essentially by lessening ACh- induced vasodilation. In brain vessels, perivascular cholinergic nerve terminals can originate extrinsically or intrinsically. Extrinsic innervation, i.e., derived from parasympathetic fibers, is the main source of ACh to the vessels at the brain surface. Downstream in the brain vasculature, intrinsic CNS cholinergic neurons

release most of the vasoactive ACh. In the cortex, part of these perivascular fibers stems from the basal forebrain cholinergic complex, which is substantially impaired in AD. Consistently, AD brains show extensive denervation of cortical microvessels, with particularly severe loss of cholinergic terminals. The "cholinergic vascular-hypothesis" posits that insufficient cholinergic innervation to brain vessels is responsible for much of the CBF impairment observed in AD. The administration of AChEIs has positive effects on CBF among treatment-responders, therefore providing clinical evidence in favor of the cholinergic- vascular hypothesis [141, 142].

Endothelial dysfunction is believed to play a role in AD pathogenesis, although confirmation is still required [135]. The BBB lies on a monolayer of tightly-sealed endothelial cells, with low paracellular and transcellular permeability. BBB breakdown is demonstrable in several AD animal models, where it precedes any visible Aβ and tau pathology. Accordingly, subtle increases in BBB permeability can be seen in patients with MCI and early AD, through Dynamic Contrast-Enhanced MRI (DCE-MRI) [143]. The CSF to serum albumin ratio, named albumin quotient (Qalb), also reflects BBB permeability. Qalb is reported to be higher in MCI and AD patients [144]. Albumin extravasation induces perivascular edema and subsequent local hypoperfusion [139]. Chronic hypoperfusion, in its turn, contributes to further BBB damage [145]. Additionally, BBB dysfunction might reduce Aβ clearance, partly via increasing Receptor for Advanced Glycosylation End products (RAGE)-mediated Aβ uptake from the blood. The permeable BBB also allows potentially harmful molecules to reach the brain [144].

It was suggested that vascular pathology, in general, and CBF dysregulation, in particular, are part of the causal pathways of AD pathogenesis, rather than a consequence of underlying neurodegeneration. This claim became known as the vascular hypothesis of AD [146]. Classically, it posits that AD develops when, due to a vascular cause, chronical declines in CBF surpass a critical threshold. The mismatch between blood supply and neuronal demand could itself become clinically evident as MCI. Additionally, such chronic ischemic-hypoxic state would induce Aβ deposition, for example through hypoxia-inducible factor 1α (HIF-1α)-mediated BACE-1 overactivity [147]. Instead, hypoperfusion can be interpreted as a result of amyloid dyshomeostasis, since Aβ induces structural abnormalities in the brain vasculature (i.e. CAA), and what is more important, because Aβ interferes with the vascular tone. For instance, Aβ40 and Aβ42 are deemed to have vasoconstrictor effects, through upregulating endothelin

production, inhibiting eNOS, and possibly enhancing ACE activity [131]. As an alternative, the two-hit vascular hypothesis acknowledges the contributions of both amyloid pathology and vascular changes, synergistically leading to AD's onset and progression. The first hit is cerebrovascular damage. In addition to low CBF and hypoperfusion, it induces BBB breakdown and accumulation of neurotoxic circulating molecules (e.g. thrombin, plasminogen, fibrinogen). Hypoperfusion and blood-borne neurotoxic molecules might directly cause neuronal injury. Additionally, vascular dysfunction could promote amyloid burden (hit 2), by diminishing Aβ clearance (through the impaired BBB) and increasing Aβ production (because of oligemia) [136, 148, 149].

3.RAS AND AD: INTERACTIONS

Different lines of research attempted to establish a connection between RAS compounds and distinct aspects of AD pathogenesis. In this section, possible intersection points will be presented. There are several prevalent diseases that combine an altered RAS function and a higher AD risk (see section 3.1). Some of the questions that drove pioneer investigative studies about RAS-AD interaction seem to be now surpassed. In the mid-1990s, clinical studies addressed the question of whether inherited variants of the ACE gene were related to changes in AD risk (see section 3.2). At the same time, several *in vitro* studies focused on ACE alleged amyloid-degrading properties (further discussed in section 3.2). Since then, the field has moved its efforts towards identifying links between RAS molecules and the vascular and immunopathological and vascular aspects of AD pathophysiology (reviewed in sections 3.3 and 3.4).

3.1.Risk Factors for AD and RAS

The argument that vasculopathy substantially contributes to AD pathogenesis and progression, reviewed above, derives much of its strength from the fact that some diseases that primarily affect the cardiovascular system seem to favor AD development [148]. In this section, three cardiovascular diseases will be addressed with respect to their association with AD and the contribution of RAS to their pathogenesis: hypertension, type 2 diabetes, and HF.

Afterward, the less well-known relationship between CKD and AD, as well as the RAS status in CKD, will be reviewed. Given its importance to comprehend the relation between RAS and AD, hypertension deserves a more detailed appraisal.

3.1.1.Hypertension, RAS and AD

Classically, BP is determined by the CO and peripheral vascular resistance. Therefore, BP regulation depends on intricate processes, involving heart functioning, kidney handling of sodium-water retention, and vascular tone control by endocrine and nervous stimuli [150]. Short and long-term BP regulation rely on different mechanisms. The Autonomic Nervous System (ANS) plays a key role in moment-to-moment BP regulation, through the baroreflex [151, 152]. Long-term BP regulation relies on the kidneys' capacity to increase sodium and water excretion in response to high BP levels, which is highly dependent on adequate production of natriuretic hormones, including Ang II and aldosterone [151]. Ang II, binding to AT1R, mediates most RAS actions over BP: as a vasoconstrictor, Ang II elevates peripheral vascular resistance. In the kidneys, Ang II increases the driving forces for fluid reabsorption across the nephron tubule. In the adrenal cortex, Ang II induces aldosterone release, in order to enhance aldosterone-mediated sodium retention [151].

The presence of essential hypertension is characterized when BP is chronically elevated, due to an altered state of the long-term regulatory mechanisms. Primary hypertension can be a result of continuous sympathetic overactivity, impairment of pressure natriuresis mechanisms in the kidneys, and RAS classical axis' upregulation. The rate- limiting step of Ang II synthesis depends on renin, which is present at higher levels, on average, among hypertensive patients [150]. Targeting RAS is an effective strategy for treating hypertension and preventing its complications. In fact, ACEi and ARBs are first-line options for essential hypertension. [153]. Persistently elevated BP elicits adaptive responses throughout the vascular tree: Arteries and larger arterioles face structural changes, (e.g. hypertrophy of tunica media); Small resistance arterioles, otherwise, rely on myogenic constriction to reduce the lumen, resulting in capillary rarefaction. Endothelial cells in capillaries face oxidative stress, following ROS-mediated eNOS downregulation, and apoptosis. Ensuing reduced tissue perfusion is especially detrimental to organs adapted to high blood flow, such as the brain [154]. The Ang II/AT1R axis directly contributes to vascular remodeling and endothelial dysfunction in

hypertension. Circulating AngII binds to AT1R on fibroblasts to promote the hypertrophy of great vessels and induce vascular profibrotic responses [155, 156]. AT1R activation on the endothelium elicits ROS overproduction, undermining eNOS function [155]. Furthermore, hypertension-related DAMPs and ROS trigger innate immune responses, whilst Ang II directly mediates adaptive immune cells' activation [157].

Hypertension-related damages do not spare the cerebral vasculature. Chronically elevated BP reduces resting CBF and shifts the cerebral autoregulation curve to the right, i.e., higher pressures are required to maintain adequate blood supply to the brain. Hypertension-induced endothelial dysfunction interferes with myogenic tone responses, breaks NVC, and disrupts endothelial barriers, in general, and BBB, in particular. In hypertension, BBB disruption can be partly attributed to endothelial AT1R-mediated signaling [158]. Also, the chronic, low-grade systemic inflammation that happens in hypertension induces changes in BBB permeability, elicits leucocyte extravasation and promotes CNS inflammation [159]. In larger arteries, injured endothelium permits egress of lipids in tunica intima, prompting the atherosclerotic process. Atherosclerosis affects cerebral perfusion by various mechanisms. Smaller cerebral arteries may suffer direct hypertensive damage. SVD predisposes lacunar infarctions as a consequence of either small vessels' occlusion or non-occlusive post-stenotic hypoperfusion. In hypertensive patients' brain, hypoperfused deep arterial territories induce white matter rarefaction, named leukoaraiosis. Moreover, hypertension- related vascular remodeling favors spontaneous intracerebral hemorrhage (ICH), mainly deep ICH. Hypertension-related CVLs might be associated with cognitive decline in MCI and AD [160-162].

Approximately one-third of the adult population is hypertensive, more than 90% of the times due to essential (primary) hypertension [163, 164]. Chronic hypertensive disease is even more common in geriatric patients, with an overall prevalence greater than 50% among those aged over 65 years. In general, age is associated with increased arterial stiffness (reducing compliance), a higher incidence of HF, and renal dysfunction (increasing circulatory volume), along with ANS dysregulation. Typically, hypertension manifests in the elderly with high Systolic Blood Pressure (SBP) and normal-to-low Diastolic Blood Pressure (DBP) (i.e., high PP), as a result of the reduced vascular compliance. In contrast, hypertensive patients aged up

to 50-55 years old usually show parallel rises in SBP and DBP, as a result of increased peripheral vascular resistance. Comprehensibly, renin activity may not be as increased among hypertensive old adults (excluding those with end- stage CKD), when compared to their mid-aged counterparts [164-166].

Hypertension, especially when it occurs in midlife, is frequently mentioned as a reversible risk factor for AD. [60, 167-169] It may be difficult to verify whether this association stands firm by searching the literature, mainly because primary studies are highly heterogeneous in terms of methodological quality, AD diagnostic criteria, selected population, hypertension treatment status, and reported dependent variable [71]. For instance, different studies evaluated correlations between AD onset and diverse threshold-values of one or more among the following parameters: SBP, DBP, MAP, PP, and BP variability. Similarly, studies widely vary regarding how BP was measured. There are reports of serial or single measurements, taken in the ambulatory or outpatient clinic, and so forth [170]. Autonomic dysfunction might impact BP regulation in AD, posing additional challenges to the interpretation of clinical findings [171]. Long-term changes in BP levels, observed in AD patients, could be a result of baroreflex alterations [172]. Baroreflex response was actually found to be impaired in AD and might partially recover with AChEI treatment [173]. Cholinergic system hypoactivity provides physiological grounds to the presumed ANS in AD [174]. Parasympathetic dysfunction in MCI has been hypothesized to be a result of early cholinergic impairment [175]. Studies addressing ANS function in AD, however, have shown conflicting results, and patients rarely report symptoms of dysautonomia [171, 176].

Consistently, longitudinal studies have shown that high BP at baseline implicates a higher risk of presenting dementia after long-term follow-up. This effect was regularly present in male, female and mixed cohorts [172, 177, 178]. Such increase in risk is unequivocally demonstrated for VaD, and less evident for AD [162, 179]. Elevated BP variability, rather than high BP levels, might be a risk factor for AD [180]. Whilst midlife hypertension possibly predisposes AD development, BP actually seems to fall once AD pathological cascade has been set in motion. Accordingly, the prevalence of hypertension can, in fact, be lower among AD patients compared to controls [170, 181]. After AD onset, enduring hypertension is related to worse cognitive function and hippocampal hypometabolism, but not with amyloid and tau

biomarkers [182]. In clinically diagnosed AD patients, increased antemortem PP predicted CVLs, but not the extent of AD pathology, at autopsy [183]. Comprehensibly, consequences of hypertension to the cerebral vasculature can impact AD course. Hypertension- induced oxidative stress and inflammation have been highlighted as agents of AD pathogenesis, by promoting RAGE upregulation and consequently, CNS Aβ influx [184]. Ang II, acting upon endothelial AT1R, might directly increase RAGE expression, providing an additional mechanism for increased brain Aβ uptake during hypertension [185].

3.1.2 Heart Failure, RAS and AD

Prevalence of HF increases with age, and it is a common condition (>5%) over 75 years old [186]. HF is a progressive disorder of cardiac pumping function, prompted either by an acute injury or by progressive hemodynamic overload (e.g., in hypertension) [187]. Myocardial impairment leads to changes in the hemodynamic status that are sensed by arterial baroreceptors and interpreted as "underfilling" (low effective circulatory volume), triggering compensatory mechanisms aimed at maintaining CO [188]. Increased peripheral arterial vasoconstriction, salt and water retention, and increased heart contractility are achieved by over- activating the sympathetic nervous system (SNS) and RAS. Adaptative at first, this neurohumoral activation promotes progressive heart remodeling. Antagonism of these systems is the basis of contemporary treatment for HF, which comprises a pivotal role for RAS-targeting drugs [188]. A number of patients show persistently high Ang II levels, despite ACEi use ("ACE escape") [188, 189].

Apart from hypertension, which is an important HF cause, few studies have addressed specifically the link between developed HF and AD. If CO is reduced during HF, CBF would also decrease, theoretically contributing to AD symptoms display, set aside AD pathogenesis. Reduced ejection fraction has actually been associated with impaired cognition in some studies. In addition, lower values of cardiac indexes positively correlate with smaller brain volumes in HF cohorts. HF increases the incidence of WMH, lacunar infarcts and microbleeds. There seems to be a higher overall prevalence of dementia among HF patients, but the specific rate of AD in this population remains unknown [190, 191].

3.1.3Diabetes, RAS, and AD

Type 2 diabetes mellitus (T2D), or adult-onset diabetes, accounts for over 90% of all diabetes cases, and is the result of progressive loss of pancreatic β-cell insulin secretion, on the background of insulin resistance [192]. RAS compounds possibly participate in the poorly understood mechanisms behind insulin resistance. In peripheral tissues, Ang II binding to AT1R triggers intracellular signals that hinder the insulin receptor signaling pathways. Ang-(1-7) possibly counteracts these Ang II negative effects over insulin functioning [193]. In addition, RAS influences the occurrence of microangiopathy and end-organ damage (especially kidney disease) in diabetic patients. Diabetic nephropathy is associated with a higher ACE/ACE2 ratio, and the prescription of ACEi or ARBs is mandatory in diabetic patients with hypertension plus albuminuria (but not albuminuria alone) to prevent CKD [194-196]. Serum ACE activity and levels were reported to be significantly higher in patients with T2D plus MCI than in cognitively normal diabetic controls [197].

T2D is commonly mentioned as a risk factor for AD [60, 167-169, 190]. Unlike peripheral tissues, the brain does not depend on insulin to harvest glucose from the bloodstream. Glucose freely diffuses across the BBB, whereas insulin cannot move passively through the BBB. Comprehensively, though, research has long focused on direct effects of altered glycemia in the brain while exploring the link between T2D and dementia. Chronic hyperglycemia could affect synaptic plasticity, whereas recurrent hypoglycemia might compromise neuronal energy supply. Discovery of insulin receptors in the brain and BBB endothelium brought to light the possibility of insulin resistance in the (pre)diabetic brain. Mounting evidence suggests that brain insulin resistance could impair normal CNS activity and damage Aβ turnover, thus promoting cognitive decline and AD pathology. Additionally, T2D-associated metabolic changes might be deleterious to the brain by inducing inflammation [198-200]. Above all, however, diabetic vascular pathology and SVD may contribute to the worsening of cognitive function in AD (or mixed dementia). In fact, diabetes was associated with an increased presence of CVLs, but not of AD pathological hallmarks, in clinicopathological studies [201, 202].

3.1.4 CKD, RAS and AD

CKD is defined by the longstanding (>3 months) presence of relevant abnormalities in kidney structure or function. Clinically, CKD usually becomes evident when the insufficiency of kidney excretory function can be revealed by low Glomerular Filtration Rate (GFR) or high

serum creatinine (Cr) levels. Even earlier, albuminuria can mark kidney damage, for being a consequence of reminiscent nephrons' overload. CKD is a very common condition, which presents increasing prevalence with age, showing nearly 30% rates among those over 60 years old [203-205]. Diabetes and hypertension are the main causes of CKD. Hypertension may be both a risk factor for and a consequence of CKD [204, 206]. RAS is essential in CKD pathogenesis. Characteristically, Ang II induces podocyte hypertrophy and mediates sustained glomerular hyperfiltration, ultimately aggravating podocyte loss and albuminuria [207]. Additionally, Ang II promotes renal tissue fibrosis and inflammation in CKD. Ang-(1-7), in contrast, might exert a protective role [208]. In fact, plasma ACE2 activity is lower in patients with end-stage CKD than in earlier stages of the disease, although it is in normal kidney functioning that ACE2 has the lowest activity [209, 210].

Even though AD and CKD are very common diseases of later life, the relation between the two remains largely unexplored. CKD is often mentioned as an independent risk factor for AD, notwithstanding diabetes and hypertension being possible confounders [190, 211]. A recent meta- analysis concluded that albuminuria imparts a 35% increase in the risk of cognitive impairment and dementia, whereas there is no sound evidence that high serum Cr and low GFR have the same effect on cognitive decline [212]. Hypotheses of how CKD could interfere in AD pathogenesis concentrated in the role that kidneys might play in peripheral Aβ clearance. Several studies showed that CKD patients have higher Aβ plasma levels than controls and that in (normal or MCI) CKD patients, hemodialysis may reduce Aβ concentrations in the blood, decrease plaque deposition in the brain, and improve cognitive tests' results. It was often inferred, then, that CKD might contribute to dementia of the AD-type because of reduced amyloid excretion in the urine [213-217]. Nevertheless, it remains to be determined whether these conclusions are applicable to AD patients.

3.2 RAS and AD Genetics

The ACE encoding gene's first identified polymorphism with phenotypic implications was an insertion (I) or deletion (D) of a 287 base pairs (bp) sequence within intron 16 (NCBI ref. SNP ID: rs1799752). In individuals with the DD genotype, ACE levels are increased in comparison with II carriers. Until the early 2000s, many linkage and case- control studies

attempted to associate the incidence of several diseases with different frequencies of I/D alleles. Results of those initial studies pointed to possible implications of ACE genetic variants in many conditions, including AD [218-221]. Reports of case-control studies addressing the ACE gene polymorphisms relation with AD showed that II and ID genotypes, combined, were significantly more frequent among AD patients than in age- matched controls, amid which the DD variant prevailed [222]. Further studies with smaller sample sizes did not show any association between ACE I/D genotypes and AD, except perhaps after subgroup analyses [223, 224]. Another larger study used five independent case-control samples to confirm the initial findings and went further as to investigate specific Single Nucleotide Polymorphisms (SNPs) in respect to their relationships with the I/D ACE haplotype and with AD risk [225]. A subsequent meta-analysis favored the hypothesis that the I allele is associated with increased AD risk [226]. From the time when these studies were published, however, the field of human genetics went through major advances towards a deep understanding of the genetic basis behind complex disorders, like AD. It became feasible to genotype large groups of patients with a given disease and age- matched controls in order to evaluate associations between genotype SNPs and phenotypes of interest. These so-called Genome-Wide Association Studies (GWAS) proven to be more effective at identifying genetic risk factors for complex diseases, undermining many conclusions from previous small-scale studies directed at certain candidate genes [227- 230].

GWAS have identified at least 20 loci as being genome- wide significant to AD risk. ACE gene-related SNPs have hitherto failed to reach the GWAS-required level of significance [231, 232]. In fact, no SNP in any of the 14 RAS-related genes was reported to be associated with AD or other neurodegenerative diseases [233]. One of the scholars involved in the earliest studies linking ACE gene polymorphisms and AD, P.G. Kehoe argues that GWAS use a stringent significance threshold. For this reason, the ACE gene might still be considered of interest in AD, even after the negative GWAS results [234]. Contrariwise, GWAS p- values have been objected not for being too narrow, but for being too broad, leading to false-positive associations when applied to low-frequency variants, i.e. those with Minor Allele Frequency (MAF) < 5%. The p-value for genome- wide significance is considered valid for common (i.e., MAF > 5%) genetic variations [235]. ACE gene-related SNPs are in fact very common,

showing MAFs that range from 37% to 49% [236]. It is generally accepted that such common variants may actually account for much of the observed variance in a given trait or disease, which remain unexplained by combined genome-wide significant SNPs. However, the effect size of any individual SNP is usually modest, even for those proven significant in GWAS. Genome-wide non-significant variants are expected to contribute even less to the phenotype of interest [227]. Furthermore, a more recent multi-center, case-control study was specifically designed to test for ACE gene haplotypes in 10 large samples (totalizing 3,930 loAD patients and 4,282 controls). It was not able to demonstrate any association with AD risk for neither one of the tested variants (all $p>0.09$) [237]. Therefore, it seems unlikely that ACE gene SNPs have any detectable influence over AD biological pathways.

3.3 RAS and AD: Amyloid and Tau Pathologies

A controversial question, subject to extensive investigation, is whether ACE can degrade Aβ and, if so, to what extent and by which means. In vitro, it was demonstrated that ACE can cleave the aggregation-prone peptide Aβ40, releasing Aβ1-7 and Aβ8-40, which do not form plaques and are allegedly less cytotoxic [238]. ACE Aβ catalysis was reported to be accomplished by the enzyme's N-terminal active site, contrasting with the greater Ang I affinity shown by the C-terminus domain [239]. Notwithstanding, the exact nature of Aβ cleavage sites in ACE is still a matter of debate [240-242]. In a culture of human neuroblastoma cells, ACE overexpression inhibited Aβ40 and Aβ42 secretion [243]. ACE was also shown to enhance the formation of Aβ40 from Aβ42 in Tg2576 mice [244]. The two active ACE domains seemed to convert Aβ43 into Aβ41 in APP transgenic mice [245]. In mice brain tissue homogenate, ACE and ACE2 were reported to sequentially transform the more soluble, but highly toxic Aβ43 into the plaque-builder Aβ40 [246, 247]. A study assessed the effects for Aβ deposition that could be achieved by crossing an AD model (APP mice) with a mice lineage characterized by ACE overexpression in myelomonocytes. Descendant animals had reduced soluble and insoluble brain Aβ42 levels, which partially increased after administrating the ACEi ramipril [248].

Despite the body of evidence that, under experimental settings, ACE is capable of degrading Aβ both in vitro and in vivo, such ACE function has not been yet demonstrated in physiological conditions. Some animal studies, too, presented contradictory findings. For

instance, when APP transgenic mice were crossed with ACE knock-out mice, no increase in Aβ brain concentrations was observed in the progeny [249]. Additionally, ACEi captopril did not enhance amyloid accumulation in APP nor triple transgenic mice lineages [250]. In conclusion, ACE action over Aβ is generally considered not to contribute significantly to the clearance of cerebral amyloid in AD [251,252].

In respect to other RAS compounds, evidence for a role in Aβ metabolism is inconsistent. For instance, ICV Ang II injection impaired cognitive function in rats, whereas Aβ pathology increased in parallel, supposedly due to upregulated β- and γ-secretase activities [253]. Ang II effects over APP processing were investigated in cultured primary hippocampal neurons and human embryonic kidney (HEK) cells transfected with AT1R, APP, and PSEN-1 genes. The results showed no evidence of any change of β-/γ-secretase functions directly induced by Ang II/AT1R [254]. In contrast, a different study using HEK cells showed that Ang II enhances the α-secretase activity, when binding to AT1R (and not to AT2R). The finding that Ang II/AT1R signals might favor the non-amyloidogenic APP-processing pathway contrasts with previous reports of Ang II-induced amyloid burden in rodents [255].

Very few studies have related RAS compounds and tau pathology. For instance, Ang II ICV infusion in rats was shown to significantly increase brain p-tau levels without a concurrent rise in the animals' BP [256]. Ang-(1-7) levels were reported to inversely correlate with tau hyper- phosphorylation in the cortex of senescence-accelerated mouse prone 8 (SAMP8), but whether this finding provides enough grounds to infer causality remains uncertain [257]. Further in vitro and in vivo studies are needed to determine whether RAS molecules directly interfere with tau protein processing.

3.4 RAS and Neuroinflammation in AD

Neuroinflammation is an important feature of AD pathogenesis (see section 2.3.4). It is widely accepted that RAS compounds modulate peripheral inflammatory response and neuroinflammation alike [258]. Considerable evidence in this regard comes from studies addressing the role of RAS in the classic neuroinflammatory diseases and in aging. As previously discussed (section 2.1.1), ACE/AngII/AT1R axis induces microglial activation, upregulating NOX complex and increasing ROS production. Besides triggering signals that

actively induce the M1 phenotype in microglia, the AT1R activation may also prevent microglial conversion to the immunoregulatory M2 state, partly by inhibiting PPAR-γ functions [32, 259].

The pro-oxidant, pro-inflammatory environment typical of aged tissues (including brain tissue) favors the increased vulnerability to neurodegeneration in the elderly (including AD-induced neurodegeneration). To what extent age-related AngII/AT1R overactivity contributes to this condition is still a matter of debate [32, 260]. Peripheral macrophage senescence was proposed to favor monocyte infiltration in the CNS and contribute to AD pathogenesis, inducing amyloid pathology [261]. As aforementioned (section 2.3.4), however, the role of blood-borne monocytes in AD pathogenesis is speculative. PVMs, otherwise, protect the brain environment from systemic-borne harmful factors. Direct AngII/AT1R activation disrupts PVMs function, inducing NOX2-mediated ROS release, which reduces eNOS-dependent NO production in surrounding endothelial cells. In hypertension, vasoconstriction of brain small vessels partly relies on such BP-independent mechanism, mediated by AngII/AT1R signals in macrophages [262-264]. Hypertension-induced neuroinflammation is one of the putative intersection points between that condition and AD. In this regard, Ang II/AT1R activity might not only directly promote an inflammatory state in the hypertensive patients' CNS, but also increase the BBB permeability, exposing the brain to systemic pro-inflammatory signals [265].

RAS counter-regulatory axes' compounds are reputed to be neuroprotective also by reducing CNS inflammation. ICV Ang IV infusion suppressed the levels of pro-inflammatory cytokines in rats with cerebral hypoperfusion [266]. In a culture of rats' microglial cells, Ang-(1-7), acting upon MasR, was shown to directly reduce the transcription of pro- inflammatory factors IL-1β and TNF, while increasing the levels of the anti-inflammatory IL-10 [267]. AVE0091, a MasR-binder Ang-(1-7) analogue, attenuate neuro- inflammation in the SAMP8 mouse, by inducing microglial M2 shift [268].

In conclusion, RAS-innate immune system crosstalk in the brain represents an interesting possible link between RAS regulation and AD pathophysiology. Evidence directly linking RAS and neuroinflammation in AD animal models, set aside humans, is still scarce.

3.5 RAS and Cerebrovascular Pathology

As reviewed in 2.3.5, mounting evidence points out that vascular alterations do not merely contribute to exacerbating AD symptoms. CBF dysregulation can be, per se, an important element of AD pathogenesis (the "vascular hypothesis"). Conversely, AD-induced cholinergic system hypofunction may reduce CBF (the "cholinergic-vascular hypothesis"). Evidence supporting these two hypotheses provides a compelling argument in favor of a distinct role for RAS in AD pathogenesis. RAS classical axis mediates vascular remodeling independent of BP, in the brain and elsewhere (3.1.1); induces ROS production; diminishes NO synthesis; induces vasoconstriction and reduces CNS ACh levels (see 2.1.1). RAS alternative axes may counteract these deleterious AT1R effects, contributing to neuroprotection. AT2R activation may upregulate BDNF release and may increase brain angiogenesis. Additionally, AT2R signaling enhances ACh production in specific CNS areas, contributing to vasodilation [23, 269-271]. Systemic Ang-(1-7) infusion improved cognitive performance in a mouse model of HF [272]. In an AD transgenic mouse model, constant peripheral Ang-(1-7) administration counteracted Ang II, preventing cognitive deficits [273]. Centrally administered, Ang-(1-7) attenuated cognitive deficits in mice subjected to chronic cerebral hypoperfusion, partly as a result of increased NO release [274]. To summarize, RAS vascular actions interfere with CBF regulation, which may be an essential part of AD pathogenesis ("vascular hypothesis"). Brain RAS, in turn, seems to modulate ACh metabolism, which affects cerebral perfusion, possibly contributing to AD development ("cholinergic-vascular hypothesis"). Systemically, Ang II binding to AT1R tends to favor CBF decline. Locally, AngII, via AT1R signaling, reduces ACh release. Conversely, Ang II binding to AT2R and Ang-(1-7) via MasR activation present opposite effects.

3.6 RAS Components in AD Patients

Numerous studies tried to identify RAS components in AD patients' biological material, in a wide range of manners: direct dosage of Ang peptides in CSF and blood; histopathological analysis of brain tissue after immunohistochemical staining of angiotensin receptors; measurement of the levels and activities of ACE, ACE2, and APs in brain homogenates, plasma, and CSF. Results have been inconsistent, in part due to methodological

differences between the studies with respect to important features, notably: (i) the diverse nature of the material and the techniques of measurement; (ii) the baseline characteristics of the sample (e.g. AD stage, prevalence of cardiovascular comorbidities); (iii) the heterogeneous inclusion and exclusion criteria (e.g. use of different AD diagnostic tools, different decisions regarding patients in use of ACEi/ARB). For the aforementioned reasons, the results of these studies are usually non-comparable. CSF and plasma analyses are vulnerable to multiple interferences [275]. Accordingly, measurements in these samples vary widely across studies. In brain tissue, RAS classical axis' compounds are regularly reported to be upregulated in AD's brain, whereas alternative axes' molecules have not shown a discernible pattern so far. Results of the individual studies in which RAS compounds were compared in AD and controls are condensed in Table 1.

Besides comparison with controls, several studies went further and attempted to correlate RAS compounds with cardiovascular comorbidities or with pathological features in AD. In one study, brain ACE levels were evaluated in hypertensive and non-hypertensive AD patients. Surprisingly, ACE expression was higher in the frontal cortex of normotensive AD patients compared to their hypertensive counterparts [276]. ACE activity in the frontal cortex correlated with amyloid pathology, and vascular ACE was more frequent in the presence of CAA [277]. ACE levels and activity in CSF correlated with Aβ burden in several studies [278-280]. Higher ACE activity in CSF was associated with reduced risk of brain atrophy, but not with markers of SVD [281]. Serum ACE does not show a good correlation with WMLs on MRI [282]. Further studies are needed to elucidate the state of RAS compounds in AD patients.

4. PERSPECTIVES: TARGETING RAS IN AD

Whether controlling hypertension could prevent cognition impairment and all-cause dementia remain a controversial issue. Longitudinal studies consistently have shown a significant, albeit small, decrease in dementia onset among treated hypertensive patients compared to nontreated hypertensives, after adjusting for confounding factors. The effect of hypertension management on AD risk, in particular, remain highly controversial [283-288]. Currently, the American Heart Association and the American Stroke Association recommend

optimal anti-hypertensive treatment as a meaningful strategy for preventing CVD and CVD-associated cognitive decline, acknowledging a possible impact on AD incidence specifically [289-291]. One study attempted to evaluate the effects of anti-hypertensive treatment over AD pathology and RAS compounds. In treated hypertensive AD patients, brain Aβ levels were decreased, and ACE levels were higher [276].

Several studies explored the possibility of a differential effect on dementia prevention between distinct antihypertensive classes. Although results have been inconsistent, ARBs (and ACEi not as often) are reported to have a positive class effect upon the incidence of dementia. The reader is reported to published meta-analyses and reviews that addressed differential effects of antihypertensive classes over AD, including the distinct role of RAS-targeting drugs [292-296]. It has been hypothesized that ARBs showed better results than ACEi for two main reasons: (i) differential effects on the RAS alternative axes, which are upregulated by ARBs, but not as much by ACEi; (ii) the fact that a widely used ACEi, enalapril, does not cross the BBB, whilst roughly all ARBs have good CNS penetration [297]. This second explanation raised the probability of brain RAS being implicated in AD pathogenesis, possibly by deleterious local effects of RAS classical axis and/or protective effects of the alternative axes in CNS, particularly in the hippocampus. In fact, several observational studies and pilot trials showed positive effects of centrally active ACEis over the incidence of AD, independent of BP lowering effect [298-302]. The literature on the matter also comprises studies focusing on AD prevention by ARBs and RAS-targeting drugs independent of class [303-305]. There is an ongoing randomized, placebo-controlled phase 2 clinical trial, conducted on mild-to-moderate AD patients, intended to evaluate the efficacy of the ARB losartan in reducing AD- related pathology [306].

One widely accepted view thus far is that the BP- independent AD-preventing effect of ACEi and ARBs can be attributed to these drugs' actions upon the vascular and immune systems, both centrally and peripherally, e.g., inhibiting Ang II/AT1R-mediated profibrotic effects over the brain vessels wall, and counteracting Ang II/AT1R proinflammatory actions upon microglia. These possible mechanisms have been the addressed by recent narrative reviews [4, 62, 307-310]. ARBs also might shift microglia towards the M1 phenotype independently of AT1R antagonism, by directly activating the PPAR-γ [113, 311, 312].

Studies in animal models of AD have consistently shown positive effects of ARBs and ACEi (especially brain- penetrating ACEi) over memory and cognition, as assessed by specific tests, and over AD pathological features (e.g. amyloid burden, neuroinflammation, vascular dysfunction, brain atrophy) [250, 313-319]. The relevance of these findings to comprehend the pathogenesis of human AD is not well-defined, given that all the animal models developed so far have failed to credibly reproduce the most important features of AD pathology and manifestations altogether. Apparently, no other mammal exhibits a disorder with the distinct AD characteristics, notably amyloid plaques, NFTs, and progressive cognitive decline. "Natural" mice models of accelerated aging (e.g. SAMP8) demonstrate the typical cognitive and memory dysfunction, but not the histopathological marks of AD. Transgenic mice models were produced to mimic amyloid plaques, e.g. by the introduction of the human mutated APP gene, PSEN gene, or both. Developed under the paradigm of the amyloid hypothesis, transgenic mice better represent the fAD pathogenesis. For instance, cerebrovascular dysfunction observed in AD transgenic mice can be explained largely by direct effects of Aβ oligomers, given that impaired amyloid processing is the basic alteration in these models. Taking this into account, inferences about AD pathogenetic mechanisms derived from animal models of AD should be interpreted with caution. This caveat is especially true when addressing tightly regulated systems like RAS [320-328]

Table 1.Reports of RAS components measurements in AD patients compared to controls.				
AD Diagnostic Criteria	**Material**	**Compounds**	**Findings**	**References**
NIA-AA, neuropathological [330]	Brain tissue, mid-fontal cortex	APA, APN, Ang III, Ang II	Increased Ang II and Ang III levels; reduced APA levels, unchanged APA activity; reduced APN activity, unchanged APN levels.	Kehoe *et al*., 2017 [329]
NIA-AA, neuropathological [330]	Brain tissue, mid- frontal cortex	ACE2 Ang II, Ang-(1-7)	Reduced ACE2 activity, increased Ang II/Ang-(1-7) ratio	Kehoe *et al.* 2016 [331]
CERAD, neuropathological [333]	Brain tissue, mid- frontal cortex	ACE	Increased ACE activity	Miners *et al.* 2008 [332]
CERAD, neuropathological [333]	Brain tissue, cortex	ACE, Ang II, AT1R	Increased ACE, Ang II, and AT1R staining	Savaskan *et al*., 2001 [334]
"Clinically and neuropathologically diagnosed"	Brain tissue homogenates from cortical and subcortical regions	AT1R, AT2R	AT1R and AT2R levels not significantly different from controls; alterations in Ang II differential binding affinity to the receptors	Ge & Barnes, 1996 [335]
"Histologically verified"	Brain tissue, temporal cortex, frontal cortex and cerebellum	ACE	Increased ACE density in temporal cortex; similar ACE density in frontal cortex and cerebellum.	Barnes *et al*., 1991 [336]
Neuropathological analysis, described in Perry, 1978 [338]	Brain tissue homogenates from cortical and subcortical regions	ACE	Increased ACE activity in caudate nucleus, medial hippocampus, parahippocampal gyrus and frontal cortex	Arregui *et al*.,1982 [337]
CERAD, neuropathological: Included definite, probable and possible AD [333]	Brain tissue homogenates and CSF	ACE	In homogenates, similar ACE levels and increased ACE activity. In CSF, decreased ACE levels and increased ACE activity	Miners *et al*., 2009 [277]
NIA-AA 2011, clinical [339]	CSF	ACE, ACE2, Ang II, Ang-(1-7)	Decreased ACE levels in AD. Positive correlation between ACE and Aβ42 levels	Rocha *et al*., 2018 [279]
NINCDS-ADRDA [69]	CSF	ACE	No difference in ACE levels	Nielsen *et al.* [340]
MCI (Petersen *et al*., 2001) [342] AD (NINCDS-ADRDA) [69]	CSF	ACE	Increased ACE activity: AD > MCI > controls	He *et al*., 2006 [341]
NINCDS-ADRDA [69]	CSF	ACE	Decreased ACE activity	Zubenko *et al*., 1986 [343]
Clinical AD (*Wells,* 1977) [344]	CSF	ACE	Decreased ACE activity	Zubenko *et al*., 1985 [345]

NINCDS-ADRDA [69]	CSF and serum	ACE	Decreased ACE levels and activity in serum and CSF in AD groups. Increased BP in AD group.	Jochemsen *et al*., 2014 [280]
NINCDS-ADRDA [69]	Serum	Ang-(1-7)	Decreased Ang-(1-7) concentrations	Jiang *et al*., 2018 [346].
NINCDS-ADRDA [69]	Serum	APA, APN, and APB	Decreased APA, APN, and APB activities	Gard *et al*., 2017 [347]
MCI (Petersen *et al*., 2004) [349] AD NINCDS-ADRDA [69]	Serum	ACE	Higher ACE activity moderate-severe AD > MCI and controls	Zhuang *et al*., 2016 [348]
Clinical dementia (DSM-IV) + possible or probable AD (NINCDS-ADRDA) [69, 351]	Plasma	APA, APN, APB	Decreased APN and APB in all patients, APA lower only in men	Puertas *et al*., 2013 [350]
Clinical (CERAD + ICD-10) [353]	Plasma	ACE	Higher ACE activity. AD patients were older compared to controls.	Akatsu *et al*., 2010 [352]
NINCDS-ADRDA [69]	Plasma	ACE	Decreased plasma ACE activity in AD group.	Vardy *et al*., 2009 [354]

CONCLUSION

In summary, AD pathophysiology is far from elucidated. Since the successive failures of amyloid-targeting drugs in preclude AD, extensive research is turning to the inflammatory and vascular aspects of AD. RAS compounds, both centrally and peripherally, potentially interact with neuroinflammation and cerebrovascular regulation. Clinical evidence points to possible positive effects of ACEi and ARBs over the AD incidence. In order to confirm this claim, results of clinical trials of RAS-targeting drugs in AD are awaited.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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LIST OF ABBREVIATIONS

ACE: Angiotensin-Converting Enzyme

ACE2: Angiotensin-Converting Enzyme 2

ACEi: Angiotensin-Converting Enzyme inhibitors

ACh: Acetylcholine

AChE: Acetylcholinesterase

AChEIs: Acetylcholinesterase inhibitors

AD: Alzheimer's disease

ADL: activities of daily living

Ang I: Angiotensin I

Ang II: Angiotensin II

Ang III: Angiotensin III

Ang IV: Angiotensin IV

Ang-(1-7): Angiotensin 1-7

ANS: Autonomic Nervous System

APA: Aminopeptidase A

APN: Aminopeptidase N

ApoE: Apolipoprotein E

APP: Amyloid precursor protein

APs: Aminopeptidases

ARBs: Angiotensin Receptor Blockers

AT1R: Angiotensin type I Receptor

AT2R: Angiotensin type II Receptor

AT4R: Angiotensin type 4 Receptor

Aβ: Amyloid Beta peptide

BACE1: Beta-site APP-cleaving enzyme 1

BBB: Blood-brain barrier

BDNF: Brain-derived neurotrophic factor

BP: Blood pressure

bp: base pairs

CAA: Cerebral amyloid angiopathy

CBF: Cerebral Blood Flow

CERAD: Consortium to Establish a Registry for Alzheimer's Disease

CKD: Chronic Kidney Disease

CNS: Central Nervous System

CO: Cardiac output

CO2: Carbon dioxide

CPP: Cerebral perfusion pressure

Cr: Creatinine

CSF: Cerebrospinal fluid

CVD: Cerebrovascular disease

CVLs: Cerebrovascular lesions

CVMR: Cerebral vasomotor reactivity

CVOs: Circumventricular organs

CVR: Cerebrovascular resistance

D: Deletion

DAMPs: Damage-associated molecular patterns

DBP: Diastolic blood pressure

DCE-MRI: Dynamic contrast-enhanced magnetic resonance imaging

DSM-IV: Diagnostic and Statistical Manual of Mental Disorders IV
eNOS: endothelial NO synthase
fAD: familial Alzheimer's disease
GFR: Glomerular filtration rate
GWAS: Genome-wide association studies
HEK: Human embryonic kidney
HF: Heart Failure
HIF-1α: Hypoxia-inducible factor 1α
I : Insertion
ICD-10: International Classification of Diseases 10
ICH: Intracerebral hemorrhage
ICV: Intracerebroventricular
IL: Interleukin
IWG-2: International Working Group
loAD: late onset Alzheimer's disease
LTP: Long term potentiation
MAF: Minor allele frequency
MAP: Mean arterial pressure
MasR: G-protein coupled receptor Mas
MCI: Mild Cognitive Impairment
MI: microinfarcts
MRI: Magnetic resonance imaging
NADPH: Nicotinamide adenine dinucleotide phosphate
NFTs: Neurofibrillary tangles
NIA-AA: National Institute of Aging – Alzheimer's Association
NINCDS-ADRDA: National Institute of Neurological and Communicative Disorders and Stroke - Alzheimer's Disease and Related Disorders Association
NMDA: N-Methyl-d-aspartate
NO: Nitric oxide
NOS: Nitric oxide synthase
NOX: NADPH oxidase
NVC: Neurovascular coupling
NVU: Neurovascular unit
O2: Oxygen
PHFs: Paired helicoidal filaments
PP: Pulse pressure
PPAR-γ: Peroxisome proliferator-activated receptor gamma
PSEN: presenilin
p-tau: hyperphosphorylated tau
PVMs: Perivascular macrophages
Qalb: Albumin quotient
RAGE: Receptor for advanced glycosylation end products
RAS: Renin angiotensin system
RBE4: Rat brain endothelial 4
ROS: Reactive oxygen species
sAD: sporadic Alzheimer's disease
SAMP8: senescence accelerated mouse prone 8
SBP: systolic blood pressure
SNP: Single nucleotide polymorphism
SNS: Sympathetic Nervous System

sTREM-2: soluble triggering receptor expresses on myeloid cells-2
SVD: small vessels disease
T2D: Type 2 Diabetes
TNF: Tumor necrosis factor
VaD: Vascular Dementia
VCI: Vascular cognitive impairment
VSMCs: Vascular smooth muscle cells
WMH: White matter hyperintensities
WMLs: white matter lesions

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4.2 ARTIGO ORIGINAL: *CIRCULATING ANGIOTENSIN-(1-7) IS REDUCED IN ALZHEIMER'S DISEASE PATIENTS AND CORRELATES WITH WHITE MATTER ABNORMALITIES: RESULTS FROM A PILOT STUDY*[*].

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ABSTRACT

Introduction: Alzheimer's disease (AD) is the leading cause of dementia worldwide. Despite the extensive research, its pathophysiology remains largely unelucidated. Currently, more attention is being given to the disease's vascular and inflammatory aspects. In this context, the renin-angiotensin system (RAS) emerges as a credible player in AD pathogenesis. RAS has multiple physiological functions, conducted by its two opposing axes: the classical, led by Angiotensin II (Ang II), and the alternative, driven by Angiotensin-(1-7) [Ang-(1-7)]. These peptides were shown to interact with AD pathology in animal studies, but evidence from humans is scarce. Only 20 studies dosed RAS molecules in AD patients' bloodstream, none of which assessed both axes simultaneously. Therefore, we conducted a cross-sectional, case-control exploratory study to compare plasma levels of Ang II and Ang-(1-7) in AD patients vs. age-matched controls. Within each group, we searched for correlations between RAS biomarkers and measures from magnetic resonance imaging (MRI).

Methods: We evaluated patients with AD (n=14) and aged-matched controls (n=14). Plasma Ang II and Ang-(1-7) were dosed using ELISA. Brain MRI was performed in a 3 Tesla scan, and a three-dimensional T1-weighted volumetric sequence was obtained. Images were then processed with FreeSurfer to calculate: 1) white matter hypointensities (WMH) volume; 2) volumes of hippocampus, medial temporal cortex, and precuneus. Statistical analyses used non-parametrical tests (Mann-Whitney and Spearman)

Results: Ang-(1-7) levels in plasma were significantly lower in the AD patients than in controls [median (25th – 75th percentiles)]: AD [101.5 (62.43 – 126.4)] vs. controls [209.3 (72 – 419.1)], p = 0.014. There was no significant difference in circulating Ang II. In the AD patients, but not in controls, there was a positive and significant correlation between Ang-(1-7) values and WMH volumes in AD (Spearman's rho =0.56, p = 0.038). Ang-(1-7) did not correlate with cortical volumes in AD or in controls. Ang II did not correlate with any MRI variable in none of the groups.

Conclusions: If confirmed, our results strengthen the hypothesis that RAS alternative axis is downregulated in AD, and points to a possible interaction between Ang-(1-7) and cerebrovascular lesions in AD.

KEYWORDS: Alzheimer's disease, Renin Angiotensin System, Angiotensin-(1-7), Angiotensin II, white matter hypointensities, cerebrovascular lesions

INTRODUCTION

Worldwide, more than 50 million people suffer from dementia, in 60-70% of the cases caused by Alzheimer's disease (AD) (WHO, 2018). The social burden posed by AD places it amid the top priorities for medical research: studies on the subject receive billions of dollars each year in the United States alone (Alzheimer's association, 2020). Even with the extensive scientific efforts taking place, AD's pathophysiology remains far from elucidated. Currently, our comprehension of the disease's mechanisms may be about to face a turning point. So far, most attempts to explain AD's onset and progression have focused on the brain deposits of beta-amyloid (Aβ) protein. Lately, though, amyloid-targeting drugs have failed to show clinical benefits in successive trials. Such mounting high-quality evidence fuel an active debate around the "amyloid hypothesis" of AD and the limits of its explanatory power (Makin, 2018). Ever more attention is shifting to the disease's vascular and inflammatory features, encouraging new models to come forward. For instance, one theory suggests that concurrent cerebrovascular dysfunction could prompt AD onset, or synergistically contribute to its progression (Solis, et al., 2020). Another hypothesis points to neuroinflammation as a major component of AD's cognitive decline (Heneka et al., 2015). In this context, the renin-angiotensin system (RAS) emerges as a credible player in AD's pathogenesis, particularly the RAS' components involved in cerebrovascular regulation and brain inflammation (Kehoe, 2018).

Primarily remembered as a blood pressure controller, the RAS is in fact a multifaceted system for homeostasis, carrying out diverse and intricate functions. In the past decades, important discoveries transformed the way we think about the RAS. First, active peptides were described and added to the angiotensins' family, leading to the RAS' division in two main components: the classical axis, led by its main effector molecule, angiotensin II (Ang II), and the alternative axis, driven by angiotensin (1-7) [Ang-(1-7)]. Second, the concept of "local RAS" (in opposition to systemic RAS) was coined after RAS compounds were found in

different organs and tissues, including the central nervous system (CNS) (Mascolo et al., 2017). Following these developments, the RAS has been implicated in medical conditions outside the heart and the kidneys, including neuropsychiatric disorders, AD among them (Rocha et al., 2018). Brain RAS is present to some extent in the hippocampus and other areas affected by AD pathology, where an interaction could take place. More significant, though, are the postulated relations between systemic RAS and AD. At the neurovascular unit level, circulating Ang II deregulates the cerebral blood flow, weakens the blood-brain barrier, and promotes neuroinflammation – all actions that might contribute to AD onset and progression. Plasma Ang-(1-7), on the contrary, might protect against AD-related damages, once it increases cerebral blood flow, reduces blood-brain barrier permeability, and inhibits inflammation. These theoretical perspectives are extensively discussed in our recent review (Ribeiro et al., 2020).

Literature on RAS-AD interaction is profuse and goes beyond theoretical speculation. Experiments with animal models help to build the case for a distinct role for RAS in AD pathogenesis, many even testing RAS as drug target in AD (Saavedra, 2016). Evidence from humans is contrastingly scarce. A recent review found 20 reports of RAS' molecules measured in AD subjects (Ribeiro et al., 2020). Most of these studies were investigating RAS' components in the CNS and thus examined brain tissue or cerebrospinal fluid (CSF), with conflicting results. The state of systemic RAS in AD is still largely unexplored. Less than ten studies dosed RAS molecules in AD patients' blood samples, none of which assessed both RAS axes. More often the focus has been the classical axis, especially the angiotensin-converting enzyme (ACE), and even its role remains unclear. One exception is worth noting: in a case-control study with 228 participants, plasma Ang-(1-7) was significantly lower in the AD group (Jiang et al., 2016a). However, such interesting finding only describes the alternative axis, as other molecules have not been analyzed in the same sample.

Here, we aimed to help shed more light on the complex relationship between AD and systemic RAS. Hence, we have conducted a cross-sectional exploratory study, comparing plasma levels of Ang II and Ang-(1-7) in AD patients vs. cognitively healthy age-matched subjects. Within each group, we searched for correlations between RAS biomarkers and relevant neuroimaging variables, particularly the cortical areas most hit by AD, and markers of cerebrovascular lesions. With our results, we expect to generate hypotheses about the state of

both systemic RAS axes in AD, which may help to build inferences about potential mechanisms of interaction.

MATERIALS AND METHODS

Participants

We included 14 patients with mild to moderate AD evaluated at the Neurology Outpatient Clinic of a University Hospital (Hospital das Clínicas da Universidade Federal de Minas Gerais, Belo Horizonte – MG, Brazil). All patients presented with a typical history of progressive episodic memory deficits and showed medial temporal atrophy in brain magnetic resonance imaging (MRI), meeting the AD diagnostic criteria (McKhann et al., 2011). Experienced neurologists and psychiatrists carefully evaluated all patients, to rule out conditions that may mimic AD cognitive impairment. In addition, 10 out of the 14 patients had their CSF analyzed for amyloid beta 42 (Aβ 42), total tau (t-tau) and phosphorylated tau (p-tau). CSF samples were collected by lumbar puncture and biomarkers were measured with a double-sandwich enzyme-linked immunosorbent assay (ELISA) kit (Innogenetics, Gent, Belgium), as previously described (Magalhães et al., 2015). Patients with marked cerebrovascular lesions on brain MRI (Fazekas grade 3) were not included. To further ensure diagnostic accuracy, we followed the participants for at least 24 months after data were collected. In all of them, the disease progressed as expected given the baseline diagnosis.

To compose the control group, 14 older adults without cognitive complaints were recruited within the local community. All participants (AD and controls) underwent a neurological assessment, which included versions of the Mini-Mental State Examination (MMSE) validated in Brazil (Folstein et al., 1975; Brucki et al., 2003). All controls scored 28 or higher in the MMSE. In the clinical interview, participants or family members were asked about time of disease, comorbidities (including hypertension, diabetes, heart failure) and use of medications, especially ACE inhibitors (ACEi) and angiotensin receptor blockers (ARBs). Additional neuropsychological assessment included the Figure Memory Test from the Brief Cognitive Screening Battery (BCSB) for visual episodic memory (Nitrini et al., 2004), the

Frontal Assessment Battery (FAB) for executive functions (Beato et al., 2012), and category fluency test (animals in one minute) for verbal fluency (Machado et al., 2009).

Exclusion criteria for both groups were: a) history or signs of previous stroke; b) past neurosurgical procedures; c) history of other neuropsychiatric conditions, including epilepsy, traumatic brain injury, demyelinating diseases, schizophrenia, bipolar disorder; d) current or recent (past month) infections; e) unstable clinical diseases. The Local Research Ethics Committee approved this study's protocol. All controls and patients (or their legal representatives) were informed about the study and agreed to participate, providing their written informed consent.

Measurement of Angiotensin Molecules

Peripheral blood was collected from all participants. Blood samples were drawn in vacuum tubes with heparin, centrifuged twice at 1800 × g for 10 minutes at 4 °C. Plasma samples were then obtained and stored at -70 °C until further processing. A quantitative sandwich ELISA was performed to assess plasma levels of Ang-(1-7) (catalog # MBS084052) and Ang II (catalog # MBS028394), following manufacturer's instructions (MyBioSource, San Diego, CA, USA). Concentrations were measured in pg/ml. The reported sensitivity of the ELISA kits is 2.0 pg/ml for both analytes. All samples were measured in a single assay to avoid inter-assay variability. Our intra-assay variability was lower than 3%. To estimate the balance between RAS alternative and classical axes, the Ang-(1-7)/ Ang II ratio was calculated for each subject (Mohite et al., 2018).

Neuroimages Acquisition and Processing

For all participants, brain MRI was performed in a 3 Tesla Intera-Achieva (Philips, Netherlands) scan. Three-dimensional 1 mm isometric T1-weighted (T1w) volumetric sequence images were acquired with the following parameters: TR: 8.13 ms, TE: 3.71 ms, 256 × 256 matrix, coronal field of view, and slice thickness of 1 mm. Fluid-attenuated inversion recovery (FLAIR) sequence was obtained in all AD subjects (n=14) as well as controls (n=14). FLAIR axial images were evaluated by a neuroradiologist, blinded to subjects' identity and diagnosis, who classified the deep white matter lesions (WMLs) in the Fazekas' scale, as

described (Fazekas et al., 1987; Kim et al., 2008). Briefly, Fazekas scores are assigned as 0 (absence of WMLs), 1 (punctate WMLs), 2 (early confluent WML), and 3 (large confluent areas of lesion in the white matter).

Before processing, all MRIs were manually assessed for quality control. MRIs with low quality were excluded, e.g. significant presence of motion artefacts, blurring, ringing/truncation, susceptibility phenomenon and bad contrast to noise ratio. On this basis, we excluded from further analysis the MRI data from one of the controls (and none of the AD patients). MRI T1 images were processed for cortical reconstruction and volumetric segmentation using the Freesurfer image analysis suite version 6.0 (available at http://surfer.nmr.mgh.harvard.edu/). The technical details of these procedures are described in prior publications (Dale and Sereno, 1993; Dale et al., 1999; Fischl et al., 1999a, 1999b, 2001, 2002a, 2004b, 2004a; Fischl and Dale, 2000). Cortical areas are anatomically labelled by an automated system (Desikan et al., 2006). Using intensity and continuity information from the entire three-dimensional MR volume, the software processes it (through segmentation and deformation) and calculates cortical thickness, and then cortical volumes. The method has been validated against histological analysis (Rosas et al., 2002) and manual measurements (Han et al., 2006). Following initial automated analysis, manual inspection of the accuracy of post-processing steps was performed. Identifiable errors were corrected through the Freeview visualisation tool (from the Freesurfer image analysis tool, https://surfer.nmr.mgh.harvard.edu/fswiki/FreeviewGuide). Following manual inspection and any necessary edits, each subject was re-processed through the automated pipeline to account for manual intervention and then manually re-inspected for correction accuracy.

Neuroimaging variables of interest were pre-determined according to their relevance to AD. Hippocampus, entorhinal cortex, and parahippocampal cortex volumes were chosen considering the relevance of medial temporal atrophy for AD (Frisoni et al., 2010). Entorhinal and parahippocampal cortices were combined to compose the medial temporal cortex, as previously defined (de Souza et al., 2012). Precuneus' cortical volume was also selected given the area's relevance for disease progression: for instance, this region shows the earliest decline in cerebral perfusion in AD patients (Miners et al., 2016). Finally, the extent of cerebral small vessel disease was assessed using the volume of white matter hypointensities on T1-w images.

On T1w sequences, WMLs of presumed vascular origin can appear hypointense, especially when more severe (Wardlaw et al., 2013). Using a probabilistic procedure (Fischl et al., 2002b), FreeSurfer differentiates between normally appearing white matter and encompassed white matter signal abnormalities (i.e., hypointensities). The volume of T1w WM hypointensities strongly correlates with distinguished markers of WMLs, such as the Fazekas scale and white matter hyperintensities on T2w and FLAIR sequences (Dadar et al., 2018; Cedres et al., 2020). T1w WM hypointensities may underestimate the true extent of WMLs (Olsson et al., 2013), but have been nonetheless consistently used to measure white matter damage in AD patients and healthy elders (Burns et al., 2005; Salat et al., 2009; Jacobs et al., 2013; Leritz et al., 2014; Fischer et al., 2015; Dadar et al., 2019; Wei et al., 2019; Nemy et al., 2020). All volumes are given in mm^3 and reported as the sum of left and right hemispheres' measurements for each individual.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8.0.2 (GraphPad Software, San Diego, California, USA). To assess normality, we visually inspected the distributions of all continuous variables and run Shapiro-Wilk test. Since data were not normally distributed, non-parametrical tests were used in further analyses. Regarding continuous variables, the two groups (AD and controls) were compared using Mann-Whitney U test. Fisher's exact test was used to compare categorical (binary) variables among groups. Correlations between variables were calculated using Spearman's coefficient. Due to the exploratory nature of the study, we have chosen not to adjust for multiple comparisons (Bender and Lange, 2001). When the study sample was divided in three categories, they were compared by Kruskal–Wallis one-way analysis of variance.

RESULTS

Clinical Parameters

AD patients and controls were similar in age [(mean ± standard deviation)]: AD (69.5 ± 8.8 years-old) v. controls (66.0 ± 11.0 years-old), $p = 0.59$. As shown in **Table 1**, AD and

control groups were also balanced regarding sex, rates of hypertension and diabetes, and use of ACEi or ARB. Time since first symptoms was, on average, 3.3 years in those with AD (3.3±1.3). As expected, AD patients scored less than controls in MMSE (24.8 ± 2.2 vs. 28.8 ± 0.8, $p < 0.0001$). The results of AD patients were also lower in Figure Memory, categorical fluency (animals) and FAB tests (see **Table 1**).

Angiotensins

Ang-(1-7) levels in plasma were significantly lower in the AD patients than in controls [median (25th – 75th percentiles)]: AD [101.5 (62.43 – 126.4)] vs. controls [209.3 (72 – 419.1)], $p = 0.014$ (**Figure 1A**). There was no significant difference in circulating Ang II between AD patients [61.45 (37.52 – 88.6)] and controls [61.7 (50.3 – 94.5)], $p = 0.602$ (**Figure 1B**). The difference in Ang-(1-7) levels between groups reflected in the Ang-(1-7)/Ang II ratio, which was significantly lower in AD patients ($p = 0.044$). All results are detailed in **Table 1**.

To evaluate whether ACEi and ARB use by few patients have influenced our results, we divided the whole sample (AD and controls included) between ACEi/ARB users and non-users. These groups have shown no significant difference in Ang-(1-7) and Ang II levels (see **Supplementary Table 1**).

Neuroimaging Variables

MRI variables were first compared between groups. As expected, AD patients presented lower cortical volumes than controls in the hippocampus, medial temporal cortex, and precuneus (all $p < 0.01$). Values are reported in **Table 1**. Differences in Fazekas grades between the groups were not significant ($p = 0.21$). White matter hypointensities (WMHs) showed a significantly higher volume in AD patients (2565 ± 1775 mm^3) compared to controls (1204 ± 675 mm^3), $p = 0.007$.

To verify whether T1w WMHs reflected WMLs in our sample, we grouped all participants (AD and controls) and evaluated the correlation between the Fazekas scale and WMHs volume. Confirming previous findings (Cedres et al., 2020), there was a significant correlation between WMHs and Fazekas grade in our cohort (Spearman's rho = 0.62, $p < 0.001$). Dividing subjects in three categories according to Fazekas' grade (0, 1 and 2), we

showed WMHs volume was significantly different across three groups (Kruskal-Wallis test, $p < 0.01$). Results of this proof of concept are depicted in **Supplementary Figure 1**

Correlations Between Neuroimaging Variables and Angiotensins

In the AD group, no significant correlation was found between plasma Ang II and MRI variables, namely hippocampus, medial temporal cortex, precuneus, and WMHs (see **Table 2**). In AD patients, Ang-(1-7) levels were not associated with any cortical measure of interest. In contrast, there was a positive and significant correlation between Ang-(1-7) values and WMHs volumes in AD (Spearman's rho =0.56, $p = 0.038$). The same relationship was not observed in controls (See **Figure 2**). In fact, controls did not present any significant correlations between MRI variables and angiotensins. All analyses are detailed in **Table 2**, whereas the main findings are shown in **Figure 2**.

DISCUSSION

To find whether RAS systemic axes were unbalanced in AD, we compared Ang II and Ang-(1-7) levels between AD patients and cognitively healthy controls. Our results showed that Ang-(1-7) was reduced in AD patients, whereas no difference was found in Ang II levels. Ang-(1-7)/Ang II ratio was lower in AD patients simply reflecting the difference in Ang-(1-7). Then, to investigate if systemic RAS is linked to brain pathology, we looked for correlations between plasma angiotensins and MRI variables. In AD patients, but not in controls, plasma levels of Ang-(1-7) correlated with WMHs. No association was found between angiotensins and selected cortical volumes.

Table 1. AD patients v. controls: clinical characteristics, plasma angiotensins and neuroimaging

	AD (n=14)	Controls (n=14)	p-value
Clinical data:			
Age in years - mean ± SD	69.5 ± 8.8	66.0 ± 11.0	0.594 [a]
Sex (female) - n (%)	6 (42)	8 (57)	0.706[b]
Hypertension – n (%)	8 (57)	5 (35)	0.449[b]
ACEi or ARB use - n (%)	5 (35)	2 (14)	0.384[b]
Diabetes – n (%)	2 (14)	1 (7)	>0.999[b]
Time of disease (years) – mean ± SD	3.3 ± 1.3	-	-
CSF biomarkers			
Aβ 42 (pg/ml) - mean ± SD	572 ± 109 *	-	-
t-tau (pg/ml) - mean ± SD	742 ± 271 *	-	-
p/tau (pg/ml) - mean ± SD	96 ± 37 *	-	-
Cognitive tests			
MMSE – median (25^{th} - 75^{th} percentile)	24.5 (24 – 26)	29 (28-30)	**<0.0001** [a]
FMT - 5 min Delayed Recall (/10) – median (25^{th} – 75^{th} percentile)	4 (2.75 – 5)	9 (7.75 – 10)	**<0.0001** [a]
Categorical Fluency (Animals) - median (25^{th} – 75^{th} percentile)	12.5 (9.75 – 14.5)	17.5 (14 – 19.75)	**<0.001** [a]
FAB - median (25^{th} - 75^{th} percentile)	14 (11 – 15.25)	15.5 (14 – 17)	**0.035** [a]
Plasma molecules:			
Ang II pg/ml, median (25^{th} - 75^{th} percentile)	61.4 (37.5 -88.6)	61.7 (50.3 – 94.5)	0.602 [a]
Ang-(1-7) pg/ml, median (25^{th} - 75^{th} percentile)	101.5 (62.4-126.4)	209.3 (72.0 – 419.1)	**0.014** [a]
Ang-(1-7)/Ang II ratio, median (25^{th} – 75^{th} percentile)	1.62 (1.24 – 2.12)	2.67 (1.63 – 6.17)	**0.044** [a]
MRI measures:			
Hippocampus volume[†] mm^3 median (25^{th} - 75^{th} percentile)	5523 (5183-6504)	7771 (7303-8241)[#]	**<0.0001** [a]
Medial temporal cortex volume ⁑ [†] mm^3 median (25^{th} - 75^{th} percentile)	5283 (4322-5575)	6858 (6452 – 7258)[#]	**<0.0001** [a]
Precuneus cortical volume[†], mm^3 median (25^{th} - 75^{th} percentile)	14454 (13800-15029)	16692 (15471 - 17702)[#]	**0.004** [a]
White matter hypointensities volume, mm^3 median (25^{th} - 75^{th} percentile)	1912 (1409 – 3436)	1025 (645 – 1697)[#]	**0.007** [a]

ACEi: Angiotensin-converting enzyme inhibitors; ARB: Angiotensin Receptor Blockers; FAB: Frontal Assessment Battery; FMT: Figure Memory Test; MMSE: Mini Mental State Examination; MRI: Magnetic Resonance Imaging; SD: standard deviation;

[a] Mann Whitney U test; [b] Fisher's exact test ;

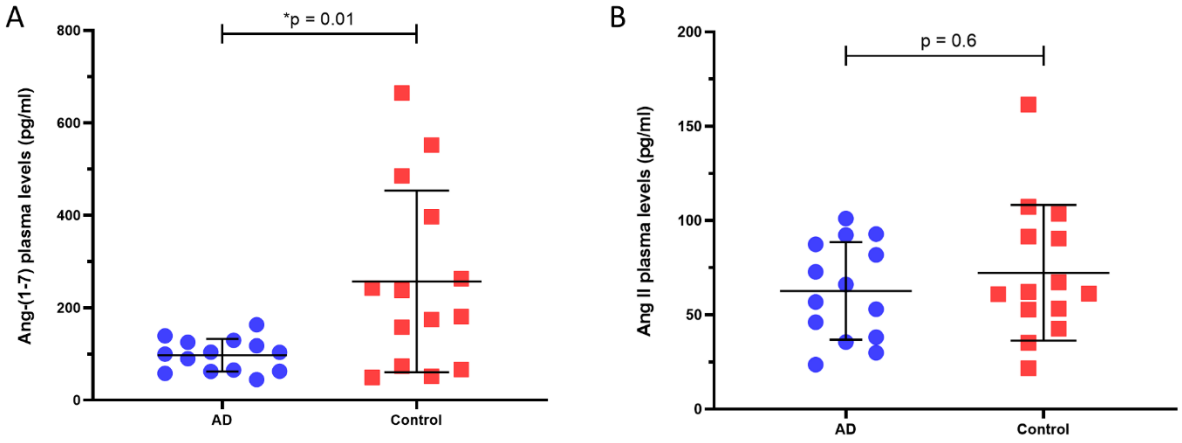
* $n = 10$

[†] Regions reported as the sum of left and right hemisphere volumes in each subject

⁑ Medial temporal cortex defined as the combination of entorhinal and parahippocampal cortices

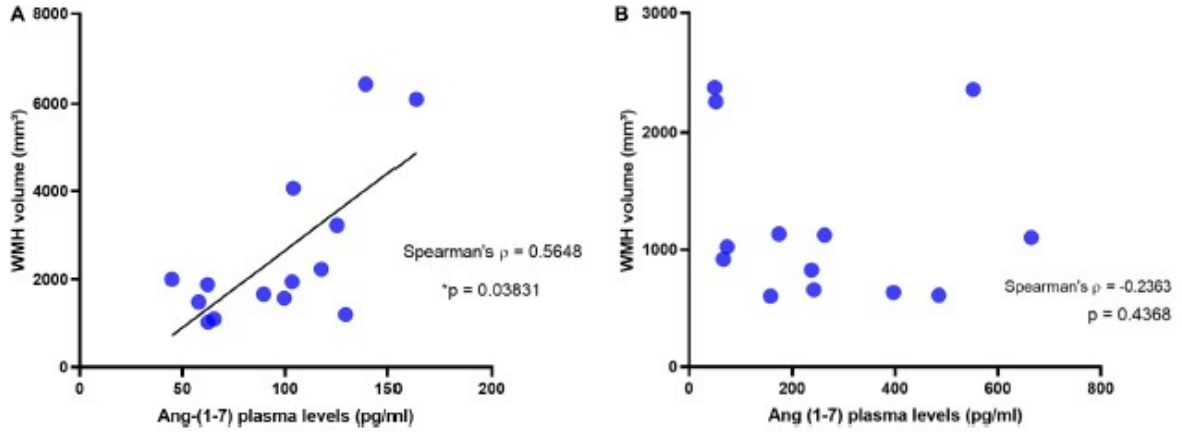
[#] $n = 13$

FIGURE 1. Ang-(1-7) and Ang II plasma levels in AD patients vs. controls



(A) AD patients had smaller levels of Ang-(1-7) compared to controls. **(B)** AD patients and controls had similar levels of Ang II. Horizontal bars represent the upper quartile, median, and lower quartile. P-values from Mann-Whitney U test.

FIGURE 2 Correlation between White Matter Hypointensities and plasma levels of Ang-(1-7) in AD patients and in controls



White matter hypointensities volumes positively correlated with Ang-(1-7) plasma levels in AD patients **(A)**, but not in controls **(B)**.

Table 2. Correlations between MRI variables and plasma angiotensins in AD patients and controls

	Plasma Ang II	Plasma Ang-(1-7)	Ang-(1-7)/Ang II ratio
	Spearman's rho coefficient (95% confidence interval)		
AD patients			
Hippocampus volume	-0.05 (-0.57 to 0.5), p=0.868	0.13 (-0.44 to 0.63), p=0.64	0.19 (-0.39 to 0.66), p=0.512
Medial temporal cortex volume	-0.21 (-0.67 to 0.37), p=0.464	0.02 (-0.52 to 0.56), p=0.928	0.26 (-0.32 to 0.7), p=0.357
Precuneus cortical volume	-0.43 (-0.79 to 0.14), p=0.124	-0.21 (-0.67 to 0.37), p=0.463	0.3 (-0.28 to 0.72), p=0.295
White matter hypointensities volume	0.464 (-0.1 to 0.8), p=0.097	**0.56 (0.03 to 0.84), p=0.038**	-0.05 (-0.58 to 0.49), p=0.844
Controls			
Hippocampus volume	0.09 (-0.49 to 0.62), p=0.751	-0.36 (-0.77 to 0.24), p=0.217	-0.38 (-0.77 to 0.22), p=0.196
Medial temporal cortex volume	-0.02 (-0.57 to 0.54), p=0.945	-0.45 (-0.8 to 0.15), p=0.123	-0.36 (-0.77 to 0.24), p=0.214
Precuneus cortical volume	0.22 (-0.39 to 0.69), p=0.47	-0.09 (-0.62 to 0.49), p=0.751	-0.32 (-0.75 to 0.29), p=0.28
White matter hypointensities volume	-0.16 (-0.66 to 0.43), p=0.591	-0.23 (-0.70 to 0.37), p=0.437	-0.4 (-0.78 to 0.21), p=0.176

Worth noting, all our significant findings regarded the RAS alternative axis' main peptide, Ang-(1-7). Before our study, Ang-(1-7) was already found to be reduced in AD patients (Jiang et al., 2016a), and mice models (Jiang et al., 2016b). The notion that AD patients may lack Ang-(1-7) is consistent with the peptide's alleged neuroprotective properties (Farag et al., 2017). To our knowledge, no prior study has evaluated Ang-(1-7) in relation to MRI measurements, neither in AD patients nor in controls. As for Ang II, however, our results contrast with a recent report by Yasar and colleagues, who found an association between Ang II levels and hippocampal atrophy in cognitively healthy elders (Yasar et al., 2020). The size of our sample and the dissimilar demographics may account for this difference. Another study (Zhuang et al., 2016) described a higher ACE activity in AD patients compared to controls.

This would presumably result in higher Ang II levels, which were not verified in our sample. Differences in the target population may help explain the disparities, as Zhuang and colleagues selected subjects with moderate-to-severe AD.

In our results, it was unsurprising that WMHs' volume was higher in AD patients. In fact, MRI and post-mortem pathological studies reveal that cerebrovascular lesions are more frequent in AD (Schneider et al., 2004, 2007; Jellinger and Attems, 2005; Attems and Jellinger, 2014; Suemoto et al., 2017; Hase et al., 2018). Classically, AD and cerebrovascular disease are considered independent entities, which often happen together only because the prevalence of both increase with age.[*] Assuming such independence, cerebrovascular lesions would contribute to cognitive decline in AD patients only by reducing the "brain reserve", thus allowing symptoms to manifest earlier (Kapasi and Schneider, 2016; Raz et al., 2016). This notion has been challenged by mounting evidence of interaction between cerebrovascular disease and AD amyloid and tau pathologies (van Norden et al., 2012; Nucera and Hachinski, 2018; Solis, et al., 2020). For instance, cerebral blood flow is dysregulated in AD (Jagust et al., 1997; Roher et al., 2012). Traditionally, ensuing brain hypoperfusion is interpreted as a late consequence of AD pathology, when neurodegeneration diminishes cerebral metabolism, and thus reduces the brain's need for blood. But there are clinical, radiological and pathological findings suggesting that the mechanisms are likely more complex: control of cerebral perfusion can be disrupted early in AD, and the reduction in blood supply may exceed the decline in metabolic demand (Ruitenberg et al., 2005; Binnewijzend et al., 2014; Hays et al., 2016; Love and Miners, 2016a). Some hypotheses go as far as to state that hypoperfusion can precede (or even induce) other key pathological events in AD (Niedermeyer, 2006; Hays et al., 2016; de la Torre, 2018). Moreover, cerebral blood flow dysregulation is possibly caused by functional changes, rather than a result of structural vascular abnormalities (e.g., atherosclerosis, cerebral amyloid angiopathy) (Kelleher and Soiza, 2013; Love and Miners, 2016b). These changes take place at the level of the neurovascular unit, which adjusts the vascular tone so as that blood supply matches energy demand in the brain (a process named neurovascular coupling). In AD,

[*] NOTA: Estudos sobre a DA familiar também desafiam noção clássica de que as lesões cerebrovasculares são prevalentes na DA apenas por terem, como fator de risco comum, a idade avançada. Já foi demonstrado que o volume das lesões cerebrovasculares é maior em indivíduos jovens, portadores de mutações determinantes de DA familiar, e que esse aumento é anterior ao início dos sintomas.[56]

a malfunctioning neurovascular unit fails to adequately regulate cerebral blood flow and weakens the blood-brain barrier (Benarroch, 2007; Zlokovic, 2011; Iadecola, 2017; Kisler et al., 2017).

If AD pathophysiology is actually influenced by vascular pathology and neurovascular unit dysfunction, then systemic RAS is likely an important player, including its alternative axis (Kangussu et al., 2020). Acting upon the Mas receptor, Ang-(1-7) mediates the alternative axis' anti-inflammatory, anti-oxidative and vasodilatory properties (Santos et al., 2018). Evidence from preclinical studies suggest that Ang-(1-7) is especially important in brain response to ischemia-hypoxia, increasing cerebral blood flow and preventing blood-brain barrier breakdown (Lu et al., 2008; Zhang et al., 2008; Wu et al., 2015). In animal models of chronic cerebral hypoperfusion, Ang-(1-7) induces tolerance to ischemia and improves cognitive function (Jiang et al., 2014; Xie et al., 2014). Ang-(1-7) has also been studied in mice models of AD. In SAMP8 mice, Ang-(1-7) was reduced and inversely correlated with Tau hyperphosphorylation (Jiang et al., 2016b). When constantly given to SAMP8 mice, Ang-(1-7) counteracted Ang II and prevented cognitive decline (Cao et al., 2019). In Tg2576 mice, upregulating RAS alternative axis reduced amyloid pathology and restored cognition (Evans et al., 2020). It is worth mentioning that no mice model credibly reproduces all the key features of sporadic AD (LaFerla and Green, 2012; Neha et al., 2014). Hence, pathological inferences from animal studies should be interpreted with caution. With such caveat in mind, we can state that preclinical data support the hypothesis of an Ang-(1-7) downregulation contributing to AD.

Against this background, we risk extrapolating our findings to hypothesize that AD patients produce less Ang-(1-7), which may contribute to their disease by diminishing the magnitude of Ang-(1-7) neuroprotective effects. We also speculate that, if confirmed, the positive correlation between plasma levels of Ang-(1-7) and cerebrovascular lesions in AD might result from some sort of response mechanism: for instance, Ang-(1-7) could be upregulated in an effort to counteract the underlying cerebrovascular disease and increase tolerance to ischemia. We assume that such attempt, however, would not raise Ang-(1-7) concentrations to the same levels seen in healthy individuals.

We are aware of this study's many limitations, starting with the sample size. Besides reducing power and generalizability, having a small sample limited our capacity to adjust results

for possible confounders (e.g., hypertension, ACEi or ARB use). To minimize the chance of spurious results, we tried to keep groups balanced concerning variables that might interfere. We still recognize that when few subjects are analyzed, statistical positives can arise only by chance. Regarding the reduced Ang-(1-7) in AD, however, this possibility seems less likely in light of existing data (Jiang et al., 2016a). We also acknowledge that the study would benefit from having a second control group, ideally of patients with another dementia (e.g., vascular dementia). Had such third group been added, it would help to determine whether our findings are specific of AD or common to different dementias. Moreover, one of the inherent disadvantages of the cross-sectional design, not being able to establish causality constrained our attempts to explain mechanistically our main results. Likewise, the lack of histopathological data restricts the consistency of pathophysiological inferences we made. It should also be noticed that in this study, even the biochemical assessment of the RAS pathways was far from complete. To understand why AD patients have lower Ang-(1-7), we would have to look at the protein that produces it, angiotensin-converting enzyme 2 (ACE2). If ACE2 activity was found to be reduced in AD patients, it would explain the immediate mechanism behind their lack of Ang-(1-7). It would also be useful to measure ACE2 concentration together with its activity. Especially if the correlation between ACE2 levels and activity was strong, dosing both would guide future studies about which assays to perform (Chappell, 2015). Despite these limitations, we believe that, for an exploratory study, our methods were appropriate and achieved the goal of generating hypotheses about RAS-AD interaction.

CONCLUSION

In conclusion, our study strengthens the hypothesis that RAS alternative axis is downregulated in AD. It also points to a possible interaction between Ang-(1-7) and cerebrovascular lesions in AD patients. We hope these hypotheses will be addressed in the future by larger studies, with longitudinal follow-up and a more comprehensive assessment of the RAS molecules. We believe that, as AD pathogenesis remains largely unelucidated, it is important to follow every lead that may help to explain the disease. If confirmed, our findings corroborate the view that the RAS is a possible player in Alzheimer's disease pathophysiology.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitê de Ética em Pesquisa da Universidade Federal de Minas Gerais. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

VTR, ACSS and LCS designed the study and wrote the protocol. TMC proposed the neuroimaging protocol and conducted the MRI analysis. LGP and RSF planned and conducted the biomarker assays. LCS, PC and ALT enrolled participants and performed neurological evaluation. VTR undertook the statistical analysis, reviewed by ACSS and LCS. All authors contributed to and have approved the final manuscript.

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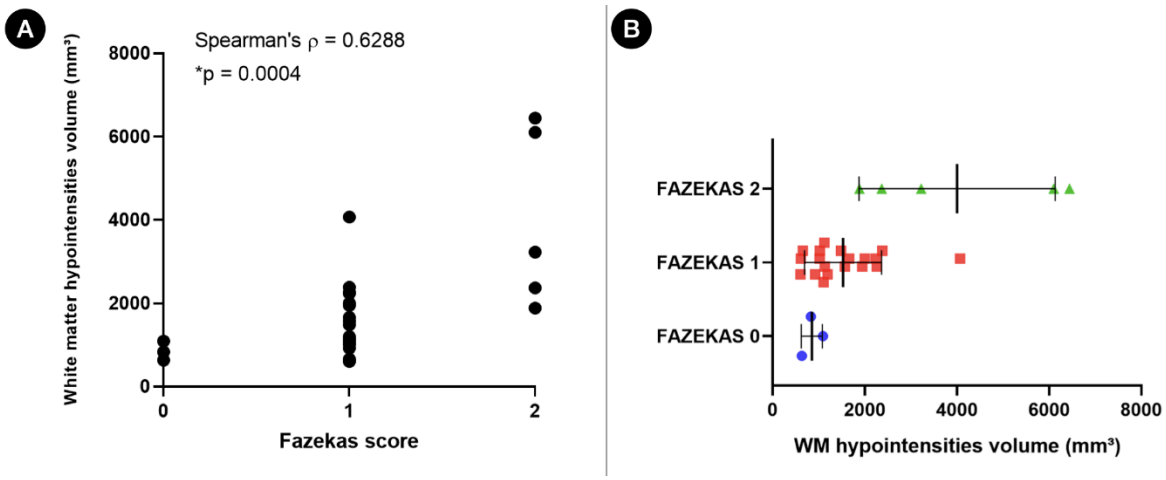
SUPPLEMENTARY MATERIAL

Supplementary Figure 1 Validation of T1-weighted White Matter Hypointensities volume against Fazekas scale in the whole sample.



White matter hypointensities volume correlated with Fazekas scale **(A)** and was different across groups defined by Fazekas score **(B)** – P=0.005 (Kruskal-Wallis).

Supplementary Table 1. Ang-(1-7) and Ang II plasma levels in ACEi/ARB users vs. non-users

	ACEi/ARB users (n=7)	**ACEi/ARB non-users (n=21)**	**p-value**
Ang II pg/ml, median (25th - 75th percentile)	72.77 (42.6 -92.3)	60.9 (42.1 – 90.9)	0.603 [a]
Ang-(1-7) pg/ml, median (25th - 75th percentile)	104.1 (62.2-129.6)	139.4 (65.9 – 250.3)	0.321 [a]
ACEi: Angiotensin-converting enzyme inhibitors; ARB: Angiotensin Receptor Blockers; **[a] Mann Whitney U test;**			

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5 . CONCLUSÃO

Em síntese, o progresso na pesquisa sobre a fisiopatologia da DA aponta para a participação de fenômenos vasculares e inflamatórios. Importante agente da homeostase vascular e imune, o SRA potencialmente interage com processos patológicos na DA. Em especial, o eixo alternativo do SRA, representado pela Ang-(1-7) circulante, aparenta estar hipofuncionante em indivíduos com DA. Ainda não é claro se e como esse desequilíbrio contribui para o desenvolvimento e progressão da doença. Especula-se que tal achado pode estar relacionado a mecanismos no nível da unidade neurovascular e da barreira hematoencefálica. Nesses sítios, a Ang-(1-7) parece atuar no controle do fluxo sanguíneo cerebral e na regulação imune do SNC. Estudos com maior tamanho amostral, idealmente prospectivos, são necessários para confirmar essas hipóteses. Diante da incerteza sobre a etiopatogênese da DA, é válido que se busquem pistas em vias ainda pouco pesquisadas. Nesse contexto, esperamos ter contribuído para o – ainda pouco explorado – campo da interação entre o SRA e a DA.

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APÊNDICE A – BANCO DE DADOS

ID	Grupo	Fazekas	Sexo	Idade	Diabetes	HAS	iECA/BRA	Tempo de doença	MEEM	Figuras	Fluência animais	FAB	Ang II	Ang-(1-7)	Ang-(1-7)/Ang II	Hipocampo	Temporal medial	Precuneus	Hipointensidades
102	DA	2	F	75	0	1	iECA	2	28	6	16	14	66,1045	62,284	0,9422	5499,8	5461	13654	1881
103	DA	1	M	54	0	0	N	5	19	0	13	11	87,3515	103,473	1,1846	6456,9	3840	13885	1943,4
104	DA	1	M	80	0	1	iECA	3	26	5	14	17	29,9	104,07	3,4806	5240,6	4361	17615	4069,3
105	DA	1	F	75	0	0	N	5	24	0	13	14	92,7785	117,766	1,2693	5507,6	5276	11343	2234,2
106	DA	2	F	77	0	1	BRA	3	24	4	12	16	72,7665	125,338	1,7225	5698,5	4996	12992	3224,8
107	DA	2	F	77	0	1	N	6	24	4	11	14	101,045	139,388	1,3795	4941,2	4206	14696	6441,2
111	DA	1	M	72	1	1	N	2	24	4	10	11	53,025	99,626	1,8788	5250,1	5422	15025	1567
113	DA	1	M	65	0	0	N	2	26	3	13	11	46,065	89,656	1,9463	6570,3	5367	13849	1658,5
117	DA	0	M	64	0	0	N	3	25	3	8	14	35,5375	65,308	1,8377	4749,6	4456	15042	1089,3
123	DA	1	F	67	1	0	N	2	24	4	11	12	23,629	62,473	2,6439	5537,4	5290	14108	1020,8
77	DA	2	M	83	0	0	N	2	27	7	9	10	56,7985	163,214	2,8736	7380,3	5918	14392	6101,6
79	DA	1	F	66	0	1	iECA	4	25	4	16	15	81,8375	44,787	0,5473	5011,9	3534	15014	1999,2
80	DA	1	M	62	0	1	N	5	28	2	18	17	38,1805	57,944	1,5176	7879,4	9188	17169	1480,9
89	DA	1	M	56	0	1	BRA	3	24	5	8	15	92,3465	129,622	1,4036	6481,6	6036	14516	1194,8
99	DA	3	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL
100	CT	0	M	50	0	0	N	nsa	30	10	17	15	61,2305	237,611	3,880598721	8348,7	7788	16546	828,1
11	CT	1	M	49	0	0	N	nsa	28	9	19	17	91,484	174,532	1,907787154	10032,4	8481	22336	1132,2
14	CT	0	F	50	0	0	N	nsa	29	8	14	15	62,2	397,056	6,383536977	7581	6452	16692	633,9
21	CT	1	F	54	0	0	N	nsa	29	10	17	14	21,717	158,071	7,278675692	7681,3	6867	13947	602,5
43	CT	1	F	68	0	1	N	nsa	29	9	19	18	67,3005	66,633	0,990081797	7025,8	6752	15027	920,2
59	CT	1	M	78	0	0	N	nsa	28	7	17	16	60,8965	263,028	4,319263012	7884,6	7172	16518	1124,3
64	CT	2	F	82	1	1	N	nsa	28	10	18	15	90,435	551,876	6,102460331	6385,1	5965	13876	2365,6
71	CT	1	F	79	0	1	BRA	nsa	30	10	13	17	42,6615	73,837	1,730764272	7855	7108	17419	1025,2
72	CT	1	M	69	0	0	N	nsa	30	9	14	17	52,842	49,289	0,932761818	8257,4	7343	20114	2382,1
83	CT	1	F	71	0	0	N	nsa	28	9	22	13	53,308	664,625	12,46764088	6722,9	5694	17359	1102,7
86	CT	1	F	68	0	0	N	nsa	29	6	13	13	107,309	180,913	1,685914909	EXCL	EXCL	EXCL	EXCL
87	CT	1	M	67	0	1	N	nsa	30	10	18	14	35,2	51,731	1,469630682	7611,1	6452	15915	2262,2
90	CT	1	F	71	0	0	N	nsa	28	9	22	16	161,461	485,294	3,005642229	7771	6803	16849	610,4
93	CT	1	M	69	0	1	iECA	nsa	28	7	23	17	103,581	242,339	2,339619909	8225,2	6858	17985	656,3
98	CT	3	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL	EXCL

ANEXO A –PRIMEIRA PÁGINA – ARTIGO DE REVISÃO

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REVIEW ARTICLE

Renin-Angiotensin System and Alzheimer's Disease Pathophysiology: From the Potential Interactions to Therapeutic Perspectives

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Abstract: New roles of the Renin-Angiotensin System (RAS), apart from fluid homeostasis and Blood Pressure (BP) regulation, are being progressively unveiled, since the discoveries of RAS alternative axes and local RAS in different tissues, including the brain. Brain RAS is reported to interact with pathophysiological mechanisms of many neurological and psychiatric diseases, including Alzheimer's Disease (AD). Even though AD is the most common cause of dementia worldwide, its pathophysiology is far from elucidated. Currently, no treatment can halt the disease course. Successive failures of amyloid-targeting drugs have challenged the amyloid hypothesis and increased the interest in the inflammatory and vascular aspects of AD. RAS compounds, both centrally and peripherally, potentially interact with neuroinflammation and cerebrovascular regulation. This narrative review discusses the AD pathophysiology and its possible interaction with RAS, looking forward to potential therapeutic approaches. RAS molecules affect BP, cerebral blood flow, neuroinflammation, and oxidative stress. Angiotensin (Ang) II, *via* angiotensin type 1 receptors may promote brain tissue damage, while Ang-(1-7) seems to elicit neuroprotection. Several studies dosed RAS molecules in AD patients' biological material, with heterogeneous results. The link between AD and clinical conditions related to classical RAS axis overactivation (hypertension, heart failure, and chronic kidney disease) supports the hypothesized role of this system in AD. Additionally, RAS-targeting drugs as Angiotensin Converting Enzyme inhibitors (ACEis) and Angiotensin Receptor Blockers (ARBs) seem to exert beneficial effects on AD. Results of randomized controlled trials testing ACEi or ARBs in AD are awaited to elucidate whether AD-RAS interaction has implications on AD therapeutics.

Keywords: Renin-angiotensin system, Alzheimer's disease, angiotensin II, angiotensin-(1-7), dementia, neuroinflammation, amyloid hypothesis.

1. INTRODUCTION

The Renin-Angiotensin System (RAS) is widely regarded, above all, as an important regulator of cardiovascular and renal functions. Nevertheless, RAS is also implied in many other processes, to the point that it has been called "the ubiquitous system for homeostasis and pathologies" [1]. By means of both its classical and alternative axes, RAS has been implicated in medical conditions outside the cardiovascular system and the kidneys. RAS components are believed to play a role in some neuropsychiatric disorders, including neurodegenerative diseases, such as Parkinson's disease and dementia [2]. It has long been suggested that drugs targeting the RAS could help to prevent Alzheimer's disease (AD) or might slow its progression [3, 4]. The biological mechanisms that lie underneath this hypothesis remain under active investigation. The aim of this article is to provide an overview of the current knowledge about the link between RAS and AD, from the pathophysiological rationale to the possible clinical implications. In order to achieve this goal, we will first explore the evidence for RAS presence and functions in the brain. Then, we will move forward to consider the most important features of AD pathogenesis and their possible relationship with RAS. Finally, the results of human and animal studies that addressed this relationship will be reviewed.

2. RAS, THE BRAIN AND ALZHEIMER'S DISEASE

2.1. RAS and the Brain

The classical axis of the RAS comprises the cascade of events starting with the secretion of renin by the kidneys' juxtaglomerular cells. Renin hydrolyzes the serum

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ANEXO B – PRIMEIRA PÁGINA – ARTIGO ORIGINAL

frontiers in Neuroscience

ORIGINAL RESEARCH
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Circulating Angiotensin-(1–7) Is Reduced in Alzheimer's Disease Patients and Correlates With White Matter Abnormalities: Results From a Pilot Study

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Introduction: Alzheimer's disease (AD) is the leading cause of dementia worldwide. Despite the extensive research, its pathophysiology remains largely unelucidated. Currently, more attention is being given to the disease's vascular and inflammatory aspects. In this context, the renin-angiotensin system (RAS) emerges as a credible player in AD pathogenesis. The RAS has multiple physiological functions, conducted by its two opposing axes: the classical, led by Angiotensin II (Ang II), and the alternative, driven by Angiotensin-(1–7) [Ang-(1–7)]. These peptides were shown to interact with AD pathology in animal studies, but evidence from humans is scarce. Only 20 studies dosed RAS molecules in AD patients' bloodstream, none of which assessed both axes simultaneously. Therefore, we conducted a cross-sectional, case-control exploratory study to compare plasma levels of Ang II and Ang-(1–7) in AD patients vs. age-matched controls. Within each group, we searched for correlations between RAS biomarkers and measures from magnetic resonance imaging (MRI).

Methods: We evaluated patients with AD (n = 14) and aged-matched controls (n = 14). Plasma Ang II and Ang-(1–7) were dosed using ELISA. Brain MRI was performed in a 3 Tesla scan, and a three-dimensional T1-weighted volumetric sequence was obtained. Images were then processed by FreeSurfer to calculate: (1) white matter hypointensities (WMH) volume; (2) volumes of hippocampus, medial temporal cortex, and precuneus. Statistical analyses used non-parametrical tests (Mann-Whitney and Spearman).

Results: Ang-(1–7) levels in plasma were significantly lower in the AD patients than in controls [median (25th–75th percentiles)]: AD [101.5 (62.43–126.4)] vs. controls [209.3 (72–419.1)], $p = 0.014$. There was no significant difference in circulating Ang II. In the AD patients, but not in controls, there was a positive and significant correlation between Ang-(1–7) values and WMH volumes (Spearman's rho = 0.56, $p = 0.038$). Ang-(1–7) did