Faculdade de Medicina de São José do Rio Preto

Programa de Pós-Graduação em Ciências da Saúde

Fabiana de Campos Gomes

Síndrome de Down: Aspectos Epidemiológicos, Genéticos e Experimentais

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FACULDADE DE MEDICINA DE SÃO JOSÉ DO RIO PRETO

Fabiana de Campos Gomes

Síndrome de Down: Aspectos Epidemiológicos, Genéticos e Experimentais

Tese apresentada à Faculdade de Medicina de São José do Rio Preto para obtenção do Título de Doutor no curso de Pós-graduação em Ciências da Saúde, Eixo Temático: Medicina e Áreas Correlatas.

Orientadora: Profa. Dra. Érika Cristina Pavarino

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SUMÁRIO

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Epígrafe

"Treine enquanto eles dormem, estude enquanto eles se divertem, persista enquanto eles descansam, e então viva o que eles sonham. "

Provérbio Japonês

"O que eu faço é uma gota no meio de um oceano. Mas sem ela, o oceano será menor"

Madre Teresa de Calcutá

".... Nada nessa vida é por acaso. Absolutamente nada. Por isso, temos que nos preocupar em fazer a nossa parte, da melhor forma possível. A vida nem sempre segue a nossa vontade, mas ela é perfeita naquilo que tem que ser."

Chico Xavier

"O SENHOR é o meu pastor, nada me faltará". Deitar-me faz em verdes pastos, guia-me mansamente a águas tranquilas. "

Salmo 23:1,2

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Figure 6. Scheme proposed for the interactions between Pgp and MTHFR proteins in kidney of Ts and Wild-type mice. The VD_3 from a standard diet or supplementation are incorporated into cytochrome and transported through the blood vessels. VD3 can be stored and released by adipocytes. Circulating VD_3 is transported to the liver and converted into 25-hydroxyvitamin D3 [25(OH)D3] by the enzyme, 25-hydroxylase. 25(OH)D3 is the primary circulating metabolite. In kidneys, specifically in the mitochondria of proximal tubule epithelial cells, 25(OH)D3 is converted by the enzyme 1-alpha hydroxylase into its active form 1,25(OH)2D3, that has biological action [18, 58]. Our findings suggest that VD_3 supplementation causes a high availability of VD3 and reduces the need for storage, leading to increased lipolysis and thus resulting in

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Lista de Abreviaturas e Símbolos

AD	*Alzheimer's disease*
APP	*Amyloid precursor protein ou* Proteína precursora de amiloide
βA	Beta amiloide
Aβ	*amyloid beta*
BO	Bulbo olfatório
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
Chr21	Cromossomo 21
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CNS	*Central nervous system*
DA	Doença de Alzheimer
DATASUS	Dados do departamento de informática do Sistema Único de Saúde do Brasil ou *Department of the Brazilian Unified Health System*
DAVID	*Database for Annotation Visualization and Integrated Discovery*
DS	*Down syndrome*
FACERES	Faculdade de Medicina Ceres
FAMERP	Faculdade de Medicina de São José do Rio Preto
GO	*Gene Ontology*
HSA21	*chromosome human 21* ou Cromossomo humano 21
LITEX	Laboratório de Imunologia e Transplante Experimental
MTHFR	*Methylenetetrahydrofolate Reductase* ou metilenotetrahidrofolato redutase

NeuN	*neuronal nuclear antigen* ou antígeno nucleo neuronal
OB	*olfactory bulb*
SD	Síndrome de Down
SNC	Sistema nervoso central
TCr21	trissomia do cromossomo 21
UNESP	Universidade Estadual Paulista
UPGEM	Unidade de Pesquisa em Genética e Biologia Molecular
USP	Universidade de São Paulo
VD_3	Vitamina D_3 ou *vitamin* D_3

RESUMO

Introdução: Indivíduos com síndrome de Down (SD) apresentam menor expectativa de vida. Na fase adulta, o surgimento da Doença de Alzheimer (DA) contribui para mortalidade precoce. O comprometimento da função olfatória e a disfunção renal podem estar associados com a DA. A principal causa da DA na SD é a triplicação do gene *Amyloid precursor protein (APP*) que resulta no aumento de beta amiloide (βA). A vitamina D_3 (VD_3) pode minimizar os efeitos patogênicos de βA aumentado a depuração em tecidos periféricos e no sistema nervoso central (SNC). **Objetivo:** Identificar o perfil epidemiológico de indivíduos com SD no Brasil e os genes, localizados na região critíca do cromossomo 21, envolvidos com os fenótipos neurológicos e, avaliar os efeitos da suplementação de VD_3 no rim e bulbo olfatório (BO) em um modelo de camundongo para SD. **Métodos:** Foram coletados 10.028 registros de óbitos (1996-2016) no banco de dados do departamento de informática do Sistema Único de Saúde do Brasil (DATASUS). As variáveis sexo, etnia e escolaridade foram definidas para verificar o nível de associação com a mortalidade. Na análise de bioinformática o banco de dados PUBMED foi utilizado para selecionar genes alvos que foram analisados no *Database for Annotation Visualization and Integrated Discovery (DAVID)* e *Gene Ontology* (GO). Para pesquisa experimental 20 camundongos fêmeas da linhagem B6EiC3Sn-Rb(12.Ts171665Dn)2Cje/CjeDnJ foram divididos em quatro grupos experimentais: controle com e sem trissomia; VD_3 com trissomia e sem trissomia. O tratamento foi realizado por 10 semanas; na 24ª o protocolo experimental foi interrompido. O rim e o BO foram coletados e processados para análise histológica, imunoistoquímica e *Western blotting*. **Resultados:** Foi observada correlação positiva entre número de registro de óbitos e anos analisados.

Crianças com idade inferior a dois anos, independente das regiões administrativas, foram mais susceptíveis ao óbito. As variáveis escolaridade e etnia foram associadas com mortalidade. Nas regiões Sudeste e Sul, os indivíduos de etnia amarela e branca e que apresentam algum nível de educação apresentaram maior sobrevida. Na predição de bioinformática, 19 genes localizados na região crítica mostraram envolvimento com disfunção e doença neurológica. O modelo de camundongo para SD apresentou alterações morfofuncionais no rim e BO que foram amenizados após suplementação com VD_3. O tratamento também aumentou a imunomarcação das proteínas metilenotetrahidrofolato redudase (MTHFR), caspase-3 p12 e glicoproteína-P (Pgp); e reduziu a βA_{42} no rim. No BO a VD_3 foi um fator importante para redução de caspase-3 p12 e MTHFR. **Conclusão:** O registro de mortalidade na população com SD tem aumentado na última década e, as variáveis sociodemográficas estão associadas com a mortalidade e repercutem na sobrevida. Alguns genes localizados na região crítica do cromossomo 21 contribuem para disfunções e doenças neurológicas. O tratamento com VD_3 reverte anormalidades morfológicas no rim e BO e afeta o nível de proteínas alvos, em camundongos modelo para SD, minimizando os efeitos causados pelo aumento de βA_{42}.

Descritores: 1. Síndrome de Down, 2. Epidemiologia, 3. Genética, 4. Vitamina D, 5. Doença de Alzheimer.

ABSTRACT

Introduction: Individuals with DS present a low life expectancy. In adulthood, the Alzheimer's disease (AD) contributes to early mortality. Impaired olfactory function and renal dysfunction may be associated with AD. The main cause of AD in DS is the triplication of gene that codes for *Amyloid precursor protein* (*APP*), which results in the increase of amyloid beta (Aβ). Vitamin D_3 (VD_3) can minimize the pathogenic effects of Aβ increasing clearance in peripheral tissues and in the central nervous system (CNS). **Objective:** To identify the epidemiological profile of individuals with DS in Brazil and genes, located in the critical region of chromosome 21, involved with neurological phenotypes. In addition to evaluate the effects of VD_3 supplementation on the kidney and olfactory bulb (OB) in a mouse model of DS. **Methods:** Data of 10,028 death records (1996-2016) were collected from the informatics Department of the Brazilian Unified Health System (DATASUS). The variables as gender, ethnicity and schooling were defined to verify the level of association with mortality. In the bioinformatics analysis, the database PUBMED was used to select target genes localized on Down syndrome critical region, of which were analyzed in the Database for Annotation Visualization and Integrated Discovery (DAVID) e Gene Ontology (GO). For experimental research 20 B6EiC3Sn Rb(12.Ts17^{16}65Dn)2Cje/CjeDnJ female mice, were divided into four experimental groups: control with and without trisomy; VD_3 with and without trisomy. Treatment was performed for 10 weeks. In 24ª week the experimental protocol was discontinued. Kidney and OB were collected and processed for histological analysis, immunohistochemistry and Western blotting. **Results:** There was a positive correlation between the number of death records and the analyzed years. Children under two years of age, regardless of administrative regions, were more

susceptible to death**.** The variables education and ethnicity were associated with mortality. In the Southeast and South regions, individuals of yellow and white ethnicity and some level of education had longer survival. According to bioinformatics prediction, 19 genes located in the critical region showed involvement with neurological disorders. The mouse model for DS presented morphofunctional changes in kidney and OB that were attenuated after VD_3 supplementation. The treatment also increased the immunostaining of the methylenetetrahydrofolate redudase (MTHFR), caspase-3 p12 and P-glycoprotein (Pgp) proteins; and reduced $A\beta_{42}$ in the kidney. In OB, VD_3 was an important factor for reducing caspase-3 p12 and MTHFR. **Conclusion:** The mortality record in the population with DS has increased in the last decade and sociodemographic variables are associated with mortality and have repercussions on survival. Some genes located in the critical region in chromosome 21 contribute to neurological dysfunctions and disorders. VD_3 supplementation attenuates morphological abnormalities in the kidney and OB affecting the expression of target proteins, in mouse model of DS, minimizing the effects caused by increase of βA_{42}.

Key words: 1. Down syndrome, 2. Epidemiology, 3. Genetics, 4. Vitamin D, 5. Alzheimer disease.

1. INTRODUÇÃO

1.1 Síndrome de Down: aspectos gerais e epidemiológicos

Síndrome de Down (SD) ou trissomia do cromossomo 21 (TCr21) é uma anormalidade cromossômica causada pela trissomia parcial ou total do cromossomo 21 (Chr 21). [1] A TCr21 é a cromossomopatia humana mais comum, com uma incidência de 1:1.000 nascidos vivos no mundo. [1,2] Aproximadamente 95% dos casos de SD são resultantes da presença de três cópias completas do Chr 21 e uma pequena porcentagem ocorre devido a translocação (3-4%) ou mosaicismo (menos de 2% dos casos). [3,4] Na translocação, o Chr21 é translocado preferencialmente com o cromossomo 13 ou 14; no mosaicismo algumas células dos tecidos têm 46 e outras 47 cromossomos. [5]

Embora a SD seja considerada uma doença genética comum entre os nascidos vivos [1,2], no Brasil são escassos estudos epidemiológicos referentes à estimativa de taxa de natalidade ou que contenham dados relacionados à sobrevida e mortalidade de indivíduos com a síndrome. As informações sobre o número de nascidos com SD são registrados como anomalia congênita no banco de dados disponibilizado pelo departamento de informática do Sistema Único de Saúde do Brasil (DATASUS), portanto, não é possível estimar o número de nascidos vivos a nível nacional. Ao contrário, os dados sobre mortalidade são mais específicos e disponibilizados em âmbito nacional pelo Sistema de Informações sobre Mortalidade (SIM) integrado ao DATASUS.

Informações sobre a mortalidade podem auxiliar na estimativa populacional. Recentemente um estudo realizado na América do Norte, verificou o número populacional de indivíduos com SD, utilizando dados de mortalidade, juntamente com as informações sobre natalidade e sobrevida. [6]. Além disso, o índice de mortalidade e

sobrevida podem estar associados a determinadas características sociodemográficas [7-9], as quais podem contribuir para menor expectativa de vida em comparação a população em geral. [9, 10]

Portanto, considerando estes achados e devido à escassez de informações epidemiológicas sobre a população com SD no Brasil, os dados sobre mortalidade e sobrevida associado às características sociodemográficas podem auxiliar no conhecimento do perfil populacional destes indivíduos, permitindo o direcionamento de estratégias e medidas públicas que possam fornecer assistência médica adequada e garantir uma melhor qualidade de vida em todas as faixas etárias, principalmente nos primeiros de vida, período do qual é considerado crítico. [11]

1.2 Fenótipos, utilização de ferramentas de bioinformática e modelos animais

O aumento da dosagem ou do número de genes no Chr21 resulta em um desequilíbrio genético com impacto na expressão e regulação de genes localizados ou não no Chr21.[12] A interação de genes triplicados com genes localizados em outros cromossomos pode resultar nos diferentes fenótipos, anormalidades congênitas e comorbidades observados na SD.[1]

Entre os fenótipos típicos da síndrome uma combinação de características dismórficas, hipotonia, deficiência intelectual, dificuldades no processamento sequencial auditivo, déficit de memória e aprendizagem são observados entre os indivíduos. [13,14] Com relação às anormalidades congênitas, as mais frequentes são cardíacas, gastrointestinais e distúrbios no sistema imunológico. [15] Alguns estudos também têm mostrado a presença de anormalidades no sistema urogenital, principalmente nos rins de indivíduos jovens e adultos [16-19], no entanto, estudos que abordam as causas da disfunção renal na SD são escassos.

Além dos fenótipos típicos e anormalidades congênitas, algumas neuropatologias [20], como a doença de Alzheimer (DA) em idade precoce, são comuns em indivíduos adultos com SD. [14, 21-23] A DA na SD (DA-SD) e em pessoas sem a síndrome resulta no comprometimento funcional de diversas estruturas do sistema nervoso central (SNC) incluindo o hipocampo, cerebelo e córtex pré-frontal. [15,24,25] Alguns achados também têm mostrado que mecanismos envolvidos na DA também podem acometer áreas olfatórias [26,27], entretanto, até o momento esta associação não foi investigada na SD.

Devido à complexidade e variabilidade fenotípica observada entre os indivíduos com SD [28], a utilização de métodos alternativos de estudos como ferramentas de bioinformática e modelos de animais são extremamente importantes para melhor compreensão fenotípica da síndrome. Com relação aos métodos de bioinformática, algumas ferramentas possibilitam a melhor compreensão e análise genômica em diversos aspectos incluindo: sinalização de redes, vias metabólicas e classes funcionais. [29]

Para SD, alguns estudos envolvendo recursos de bioinformática são utilizados para analisar a complexidade da expressão de genes envolvidos na patogênese da síndrome. [30,31] Além disso, a bioinformática tem possibilitado a análise e detecção de regiões específicas para fenótipos [32] e a identificação de genes localizados na região crítica do Chr21, associados aos fenótipos neurológicos [33] e de genes alvos envolvidos com a desabilidade cognitiva.[34] Assim, considerando a complexidade da SD, a utilização de ferramentas de bioinformática pode auxiliar para detecção de genes e proteínas alvos, possibilitando uma melhor compreensão sobre os efeitos do aumento da dosagem de determinados alvos em vias metabólicas e processos biológicos.

Além da importância das ferramentas de bioinformática para análises de predição [35], o uso de modelos de roedores permite compreender vários aspectos importantes envolvidos na TCr21, como por exemplo, a complexidade da desordem cromossômica humana; relação entre genótipo e fenótipo; identificação de genes envolvidos em uma determinada patofisiologia em um tecido específico; exploração do impacto da anormalidade cromossômica em todo o genoma; e investigação de possíveis alvos terapêuticos. [36-39]

Várias linhagens de camundongos têm sido desenvolvidas e utilizadas com intuito de investigar fenótipos específicos observados na SD.[40] O cromossomo humano 21 (Hsa21) apresenta três regiões ortólogas nos cromossomos 10, 16, 17 do camundongo (*Mus musculus* - *Mmu*), das quais a sequência e orientação dos genes são conservadas nestes cromossomos.[41] Devido ao fato dos genes do Hsa21 estarem localizados em três cromossomos diferentes no camundongo, a TCr21 no modelo não é completa.[2] Além disso, muitos genes triplicados nas regiões do Mmu 16 não tem homologia com o Hsa21, e, portanto, não modelam a SD da espécie humana.[38]

Geneticamente, alguns modelos apresentam uma cópia adicional de um dado segmento ortólogo do Hsa21[42], enquanto outros têm cópias extras de determinados genes homólogos ao Hsa21.[43] Entre os modelos existentes, os mais estudados são os camundongos Ts1Cje e Ts65Dn dos quais carregam um número representativo de trissomia segmentares para o cromossomo 16 do camundongo (MMU16).[13,40] O Ts65Dn é bastante utilizado em ensaios pré-clínicos de medicamentos para cognição[41] e, principalmente, em estudos que envolvem o SNC.[44,45] Outro modelo oriundo do Ts65Dn, denominado Rb(12.Ts17^{16}Dn)2Cje, apresenta as mesmas características fenotípicas do Ts65Dn e uma maior viabilidade reprodutiva em comparação a esta linhagem.[46] Desta forma, como Ts65Dn, o modelo Rb(12.Ts17^{16}Dn)2Cje também

podem carregar modificações genéticas adicionais, que não estão diretamente relacionados com a SD e que podem repercutir nos fenótipos.[46] Portanto, considerando a complexidade da SD, a utilização de modelos de animais pode possibilitar uma investigação mais detalhada e pontual de um determinado fenótipo em um órgão específico.

1.3 Fenótipos na SD: sistema periférico e sistema nervoso central

Devido ao desequilíbrio gênico causado pela triplicação de genes específicos no Hsa21 e a interação com genes localizados em outros cromossomos [2,12], na SD é observada uma ampla variabilidade fenotípica que pode afetar a morfuncionalidade de órgãos específicos localizados no sistema periférico [16] e no sistema nervoso central (SNC). [22, 47]

Com relação ao SNC, inúmeras anormalidades anatômicas e morfofuncionais são descritas no cérebro de indivíduos com SD.[48] Essas alterações são de origem embrionária e ocasionadas pelo aumento na expressão de genes que afetam o desenvolvimento normal do cérebro, e contribuem para o surgimento dos fenótipos neurológicos.[20] Além dos fenótipos neurológicos, indivíduos com SD desenvolvem precocemente a demência do tipo DA. Esta neuropatologia acomete os indivíduos entre a terceira e quarta década de vida [14,22,49,50]; a média de idade para o surgimento da demência é por volta dos 47 anos [51], com uma incidência maior em mulheres.[52]

O aumento na expressão de genes no Chr21, principalmente na região denominada como "região crítica" tem um papel importante no desenvolvimento da DA-SD.[14,53,54] Dos genes localizados nesta região, estudos têm mostrado que uma cópia extra do gene que codifica a proteína amiloide (*amyloid precursor protein -APP*) contribui com o desenvolvimento da DA-SD.[14,22] A triplicação de *APP* resulta no aumento da proteína APP e seus derivados [24,47], principalmente dos peptídeos beta-

amiloide (βA) de formas longas de 40 e 42 aminoácidos; o peptídeo βA_{42} é a forma mais patogênica.[10,51,55,56]

A βA em indivíduos com ou sem SD pode se depositar em várias regiões do cérebro [10,57,58], incluindo áreas olfatórias. [59,60] Alguns estudos, em indivíduos com DA sem SD, mostram que o comprometimento da função olfatória pode estar associado à neuropatologia e indicar um dos primeiros sinais clínicos da DA. [61-63] Indivíduos com SD apresentam um comprometimento da função olfatória, no entanto, os mecanismos envolvidos nesta anormalidade não têm sido esclarecidos.[64] Possivelmente, a presença de βA no bulbo olfatório (BO) pode ter algum envolvimento com as anormalidades olfatórias observadas em indivíduos com SD; consequentemente, a identificação de uma disfunção olfatória prévia também pode fornecer indicativos de estágio sobre a neuropatologia e auxiliar no diagnóstico precoce da DA-SD.

Ainda considerando a fisiopatologia da DA, órgãos periféricos como o rim desempenham um importante papel na depuração periférica de βA. [65,66] Alguns estudos mostram que indivíduos com doença renal crônica apresentam níveis elevados de βA_{42} no plasma [67] e estão mais susceptíveis para desenvolver DA.[68,69] O rim é um dos principais órgãos periféricos envolvidos na depuração de βA [66,70], portanto, qualquer disfunção renal pode comprometer a depuração e favorecer o acúmulo de βA no rim e em outros órgãos, tais como cérebro.

Embora indivíduos com SD apresentem várias anormalidades no sistema urogenital [19,71], principalmente nos rins [17,18], a associação entre estas alterações e βA_{42} não tem sido investigada. Portanto, analisar os aspectos morfofuncionais do rim associados à presença de peptídeos βA pode fornecer informações sobre uma possível relação entre o comprometimento da depuração periférica e a susceptibilidade associada ao desenvolvimento precoce da DA-SD.

1.4 Vitamina D_3, biomarcadores envolvidos na DA e depuração de beta amiloide

A 1,25-dihidroxivitamina D_3 ($1,25(OH)_2D_3$) ou VD_3 participa de vários processos biológicos, que incluem redução no risco de doenças autoimunes, modulação de processos inflamatórios, prevenção de doenças neurodegenerativas, regulação da absorção de cálcio no organismo, biossíntese de estrógenos e outras funções importantes a diversos tecidos [72-75], como por exemplo, o rim e o cérebro. [76,77]

No cérebro, a VD_3 é importante para o desenvolvimento do tecido atuando na biossíntese de neurotransmissores e fatores neurotróficos, participa do processo de neurogênese, sinaptogênese e tem um efeito neuroprotetivo. [78,79] Com relação ao rim, a VD_3 é essencial para a integridade morfofuncional do órgão [76,80, 81]; a deficiência de VD_3 aumenta a susceptibilidade e favorece o surgimento de doenças renais. [83,84]

Alguns estudos mostram que indivíduos com SD apresentam deficiência de VD_3.[85-88] A deficiência de VD_3 no cérebro afeta o desenvolvimento e função cerebral e, predispõe o indivíduo a uma maior susceptibilidade ao desenvolvimento da DA. [31,89-91] A suplementação de VD_3 em humanos promove um efeito benéfico ao organismo e é utilizada como método de prevenção, tratamento de doenças e manutenção de processos biológicos.[92-93] No entanto, os efeitos da suplementação de VD_3 em humanos com SD ou em modelos de camundongo não foram investigados.

Em modelo de camundongo para DA, a suplementação com VD_3 favorece a neurogênese, melhora a cognição [94-97], regula e ativa biomarcadores envolvidos com depuração de peptídeos βA, incluindo a proteína P-glycoprotein (Pgp).[95,98] A proteína Pgp no cérebro é expressa nas células endoteliais da barreira hematoencefálica e pode ser observada nos astrócitos.[99-101] Esta proteína tem um papel importante na eliminação de componentes e substância que podem causar neurotoxicidade celular [102,103], incluindo peptídeos de βA.[95,104]

A VD_3 também regula os mecanismos apoptóticos no cérebro. [105,106] Uma toxicidade causada pelo acúmulo de βA induz uma cascata de eventos que ativam vias que podem induzir a morte neuronal[58], e consequentemente afetar o número de neurônios dos quais podem ser identificados por marcadores específico, incluindo o núcleo neuronal (NeuN)[106]. Neste processo pode haver a participação de várias caspases, como a caspase-3.[107,108] A caspase-3 é um mediador importante na apoptose de neurônios em condições normais e neuropatológicas.[109,110] Em resposta a estímulos, a caspase-3 é clivada gerando as subunidades p17 e p12, formando o complexo ativo caspase-3 p17/p12 que participa de vias pró-inflamatória e apoptose.[111,112]

Recentes estudos demonstram que a presença de polimorfismos genéticos, que resultam na deficiência da enzima metilenotetrahidrofolato redudase (MTHFR), é um fator de risco para a DA.[114-115] Além disso, alguns achados têm mostrado uma associação entre a VD_3 e os níveis de folato, entretanto, esta relação ainda não é compreendida.[116,117]

A enzima MTHFR é importante para o metabolismo do folato e da homocisteina, pois participa dos processos de metilação, síntese de DNA, mecanismos de proliferação e reparo.[115] Alterações na via do folato, mediada pela MTHFR e outros metabólitos, induz a desmetilação do DNA resultando no aumento da expressão de genes envolvidos na DA, tais como *Presenilin* (*PSEN1*) e *Beta-secretase* (*BACE1*), dos quais codificam enzimas que participam do processo de clivagem de APP e, consequentemente, contribuem para o aumento da deposição de $βA_{42}$.[114] Portanto, em função da importância da enzima MTHFR nos mecanismos celulares e da contribuição da variante MTHFR para o risco da DA, avaliar esta proteína antes e após o tratamento com VD_3 pode auxiliar na obtenção de informações sobre esta associação.

Considerando os achados sobre a deficiência de VD_3 na SD e a associação com a DA [10,85,86-88] é de extrema importância analisar os efeitos da suplementação da VD_3 na imunolocalização e expressão de determinados marcadores tais como βA_{42}, Pgp, caspase-3 p12, MTHFR que participam em diferentes processos biológicos como depuração, apoptose, integridade do DNA e processos de transcrição gênica no sistema periférico e SNC. Além disso, não há estudos que abordem investigações em humanos ou modelos experimentais para SD sobre os mecanismos fisiopatológicos associados à presença de βA_{42} e o seu envolvimento com as anormalidades observadas no sistema periférico e SNC, especificamente, no rim e no bulbo olfatório. O rim é o principal órgão envolvido na depuração periférica de peptídeos de $\beta A^{(65-67)}$, portanto, o comprometimento morfofuncional do órgão pode afetar a depuração deste peptídeo e contribuir para o desenvolvimento precoce da DA-SD. Com relação ao bulbo olfatório, devido à comunicação com outras regiões no cérebro, incluindo o córtex entorrinal e hipocampo, uma disfunção olfatória pode indicar um dos primeiros sinais clínicos da $DA^{(59-63)}$.

1.5. Objetivo geral

Identificar o perfil epidemiológico de indivíduos com SD no Brasil e os genes, localizados na região critíca do cromossomo 21, envolvidos com os fenótipos neurológicos e, avaliar os efeitos da suplementação de VD_3 no rim e bulbo olfatório (BO) em um modelo de camundongo para SD.

1.5. 1. Objetivos específicos

- Analisar o perfil epidemiológico de indivíduos com SD associado à mortalidade e sobrevida e verificar o nível de associação entre a mortalidade e as variáveis sociodemográficas nas cinco regiões administrativas do Brasil.

- Identificar, por meio de análise de bioinformática, genes envolvidos com os fenótipos neurológicos localizados na região cromossômica 21q22.11-21q22.3, considerada como crítica para muitos fenótipos da SD.
- Avaliar os efeitos da suplementação da 1,25-dihidroxivitamina D (vitamina D_3) sobre os aspectos morfofuncionais no rim e BO em um modelo de camundongo para SD.
- Investigar as repercussões do tratamento com VD_3 no rim e BO na expressão das proteínas βA_{42}, caspase-3 p12, MTHFR, Pgp e NeuN.

2. ARTIGOS CIENTÍFICOS

Artigos Científicos

ARTIGO 1

Título: *Trends and Predictions for Survival and Mortality in Individuals with Down Syndrome in Brazil: A 21-Year Analysis.*

Autores: Fabiana de Campos Gomes, João Simão de Melo-Neto, Eny Maria Goloni-Bertollo, Érika Cristina Pavarino.

Periódico: *Journal of Intellectual Disability Research*, submetido em 24/08/2019

ARTIGO 2

Título: *Alzheimer's disease in the Down syndrome: An overview of genetics and molecular aspects.*

Autores: Fabiana de C. Gomes, Marlon F. Mattos, Eny M. Goloni-Bertollo, Érika C. Pavarino.

Periódico: *Neurology India*, status: Pre-Acceptance proof em 23/09/2018

ARTIGO 3

Título: *Vitamin D3 increases the Caspase-3 p12, MTHFR, and P-glycoprotein reducing amyloid-β42 in the kidney of a mouse model for Down syndrome.*

Autores: Fabiana de Campos Gomes, João Simão de Melo-Neto, Merari de Fátima Ramires Ferrari, Carla Patrícia Carlos, Eny Maria Goloni-Bertollo, Érika Cristina Pavarino.

Periódico: *Life Sciences*, status: Publicado/doi: 10.1016/j.lfs.2019.06.012.

ARTIGO 4

Título: *Vitamin D_3 supplementation affects the morphology and MTHFR and caspase-3 p12 expression in the olfactory bulb of a mouse model for Down syndrome*

Autores: Fabiana de Campos Gomes, João Simão de Melo-Neto, Merari de Fátima Ramires Ferrari, Orfa Yineth Galvis-Alonso, Eny Maria Goloni-Bertollo, Érika Cristina Pavarino.

Periódico: *Brain Research*, submetido em 09/10/2019.

ARTIGO 1

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Authors
Gomes, Fabiana
de Melo Neto, João Simão
Goloni-Bertollo, Eny Maria
Pavarino, Érika Cristina

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Author Dashboard

RESEARCH REPORT

Trends and predictions for survival and mortality in individuals with Down syndrome in Brazil: a 21-year analysis

Fabiana de Campos Gomes,[1] João Simão de Melo-Neto,[2] Eny Maria Goloni-Bertollo,[1] Érika Cristina Pavarino[1]

Author affiliation: [1]Genetics and Molecular Biology Research Unit (UPGEM), São José do Rio Preto Medical School (FAMERP), São José do Rio Preto, SP, Brazil (Fabiana de C. Gomes, Eny M. Goloni-Bertollo and Érika C. Pavarino). [2]Institute of Health Sciences, Federal University of Pará (UFPA), Belém, PA, Brazil (João S de Melo-Neto).

Correspondence to Dra Érika Cristina Pavarino. Genetics and Molecular Biology Research Unit (UPGEM), São José do Rio Preto Medical School (FAMERP). Av. Brigadeiro Faria Lima, 554, São José do Rio Preto, SP, Brazil. CEP: 15090-000 (E-mail: erika@famerp.br)

Running head: Survival, mortality & Down syndrome.

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Conflict of Interest

The authors declare no conflict of interest.

ABSTRACT

Background Regional heterogeneities and sociodemographic characteristics affect mortality and population survival in Brazil. However, for individuals with Down syndrome (DS) this information remains unknown. In this study, we analyzed survival and mortality rates among DS individuals in the five Brazilian geographic regions. In addition, we investigated whether there is an association between mortality and sociodemographic factors across administrative regions. **Methods** Data between 1996 and 2016, comprising 10,028 records of deaths of individuals with DS, were collected from database records of the Department of Informatics of the Unified Health System (DATASUS). Data on race/ethnicity, sex, age and educational level were defined for the association analyses. Survival data were analyzed according to the Kaplan-Meyer method and cox regression model. Number of deaths among people with DS has increased in recent years. Children are more susceptible to death, especially in the first years of life. Individuals living in the norther region, indigenous women and people with no educational level have higher mortality. In the Southeast and South region; for White, Yellow; survival is related to a higher level of schooling. Ethnic factors and educational levels influence risk for mortality across the administrative regions. **Conclusion** These findings show that sociodemographic characteristics affect survival and are associated with the risk of mortality for people with DS. In addition, this suggests that differences in access to health services among Brazilian regions, especially in the first years of life, may affect the survival of individuals with DS.

Key words: Down syndrome; epidemiology; mortality; survival.

INTRODUCTION

Down syndrome (DS) or Trisomy 21 is the most common chromosomal abnormality in humans, with an incidence of about 1:1000 live births worldwide (Akhtar et al. 2008). The extra copy of genes on the human chromosome 21 (HSA21) results in the imbalance of genetic dosage, and thus has impact on expression and regulation throughout the genome (Antonarakis et al. 2004). This genetic imbalance results in multiple phenotypes and clinical complications observed in individuals with DS (Korenberg et al. 1994).

Due to comorbidities (Baird & Sadovnick, 1998; Boghossian et al. 2010) individuals with DS have a lower life expectancy as compared to the general population (Head et al. 2012; O'Leary et al. 2018). Furthermore, sociodemographic characteristics, such as sex, age, race/ethnicity are also associated with mortality and can affect survival rates of people with DS (Day et al. 2005; Presson et al. 2013; O'Leary et al. 2018). Although some variables such as education level and regional differences are associated with mortality among the general population (Krueger et al. 2015; França et al. 2017) for DS these variables remain indescribable.

In addition, in Brazil the population size of persons with DS is unknown. A survey on the number of death records and survival can help estimate the number of people with DS (de Graaf et al. 2017) Considering that the health system is a relevant problem in Brazil (Barreto et al. 2016), knowledge on survival of individuals with DS across the different Brazilian regions, may aid in the development or restructuring of new national health guidelines to meet the needs of the specific population (de Melo-Neto et al. 2016). Based on these findings, the aim of this study was to analyze the mortality and survival trends of individuals with DS in five administrative regions (North, Northeast, Midwest, Southeast and South) of Brazil. In addition, we

investigated the influence of age, sex, race/ethnicity, and level of schooling on mortality between 1996 and 2016.

METHODS

Study design

A longitudinal Brazilian ecological study based on secondary data on mortality among individuals with DS, between the years of 1996 and 2016.

Data source

Data were obtained through the web platform of the Department of Informatics of the Unified Health System (DATASUS). The DATASUS is a database with public open access, maintained by the Brazilian Ministry of Health. This system of records includes a variety of information, such as mortality and sociodemographic variables that are provided by hospitals of the Unified Health System (SUS). Hospitals across Brazil collect the data and transmit it electronically to the Hospital Information System (SIH) and Mortality Information System (SIM). The SIM is an uninterrupted database of data provided by the State and Municipal health department, coming from the declaration of death (http://tabnet.datasus.gov.br). Moreover, there is an Auditing National System to verify the authenticity of the data collected by the SIH-SUS (Barros et al. 2019).

Data Extraction and Variables

After determining the variables to be analyzed in relation to mortality records, the data sources were made available for download (http:// datasus. saude. gov. br/sistemas-e-aplicativos/hospitalares/sihsus). To study the variables, data on the number of deaths in individuals with DS was collected by two researchers independently using the Mortality Information System (SIM). DS was defined according to the 10th revision of the International Classification of Diseases (ICD10) by code Q90 (14) (World Health Organization).

The independent variables were administrative regions (North, Northeast, South, Southeast and Midwest), sex (female and male), age (<01 year, 01-04 years, 05-09 years, 10-14 years, 15-19 years, 20-29 years, 30-39 years, 40-49 years, 50-59 years, 60-69 years, 70-79 years and ≥80 years), self-reported ethnicity (White, Yellow, Brown, Black and Indigenous), level of education (study time in years). Unidentified data were excluded from the analysis.

Statistical analysis

Descriptive results were expressed as measures of central tendency and dispersion, and absolute and relative frequencies. To verify the normality of the number of deaths over the years, the data were submitted to the Kolmogorov–Smirnov test. Linear regression analysis, Pearson´s correlation coefficient (Pearson´s r) and determination coefficient (R^2) were performed to analyze the number of deaths during the period chosen for the study. Pearson's r was classified as weak ($r < 0.33$), moderate ($r = 0.34$ to 0.66) and strong ($r > 0.67$). In addition, the Log-rank, Breslow and Tarone-Ware test were used to analyze each independent variable along with the Kaplan-Meier survival curves. The variables with a statistically significant level were included in a Cox Regression analysis to estimate the proportionality of the event over the observation period by means of the Exp (B) analysis and 95% confidence interval (95% CI). Fisher's test (p) was used to analyze the association among categorical variables. In order to verify the level of the association, Odds Ratio (OR) was used with a 95% CI. The significance level of p-value <0.05 were considered for analyses

RESULTS

Population characteristics

Population characteristics of 10,028 mortality records of individuals with DS, aged between <1-79 years, during the period of 1996-2016 were analyzed. Out of the

10,028 records, 5,104 were male and 4,916 women i.e. 51% were male and the rest were female. About 43% of the deaths occurred in Southeast region; 42% of children died before completing one year; 46% of the individuals with DS did not attain any level of education; and 58% were White.

Mortality over the years and the relationship between mortality and age range of individuals with DS

The number of deaths of individuals with DS per year is presented in the figure 1A. Through estimating by means of 95% CI, the annual mortality was between 444.89 to 510.16 events. The data showed that there was a strong positive correlation, indicating an increase in the number of deaths over the years. In addition, 75% of the increase in mortality can be explained by the advancing years (Figure 1A). With regard to mortality rates in the different administrative regions; it was possible to verify that the Southeast region had a higher number of deaths regardless of the age range. However, considering all administrative regions, children under one year of age were more susceptible to death, as shown and geo-referenced in the figure 1B and 1C. Data from individuals with > 70 years of age (n = 62) were not shown in figure 1B due to the sample size.

Survival rate according to the sociodemographic variables

According to the analysis of survival curve, we observed that differences exist among administrative regions (Figure 2.1A), ethnic groups (Figure 2.1B), and levels of education (Figure 2.2C). However, no gender/sex differences were observed between regions (figure 2.2D).

The analysis of survival in accordance with geographic regions and sociodemographic variables (Table 1) show that the survival rate throughout the aging process is higher for the Northeast, South, Southeast and Midwest regions than for the

North. In addition, Northeast and Midwest show a lower survival rate compared to the others regions. Regarding the ethnic groups, the survival rate is higher among White. Black individuals present greater survival as compared to the Brown and Indigenous (population with lower survival). While Indigenous showed a lower survival rate compared to the Yellow and Brown. For the level of schooling, we observed that individuals without schooling have a lower rate of survival, while individuals with level of education between 01 to 11 years had a higher survival rate (Table 1).

Risk of mortality associated with administrative regions and sociodemographic variables

Analysis of the association between mortality rates across the different administrative regions and sociodemographic variables showed that ethnicity and educational levels are associated with the risk of death in the different administrative regions. In figure 3 it is possible to observe the main characteristics in Brazil and administrative regions.

There were significant differences found between the administrative regions with regard to the number of deaths by ethnicity and level of schooling (Table 2). In the North region, Brown individuals and Indigenous have a greater association with mortality versus others ethnic groups. In the Northeast region, Black and Brown people has greater risk of death. In contrast, White individuals have a weak association with mortality in the North and Northeast regions. For Southeast region, White, Black, and Yellow persons have a greater association with death, while Brown and Indigenous individuals have a weak association with mortality. In the South, the highest number of deaths also occurs among White individuals, while Black and Brown individuals record a low number of deaths. However, in the Midwest region White individuals have a weak

association with mortality, and Brown and Indigenous have a greater association with mortality (Table 2).

With regard to the association between risk of death in the administrative regions and level of schooling (Table 2), a greater association was observed with the Southeast and less in the North and Northeast regions in individuals without schooling. Individuals with schooling between 04 to 07 years presented higher mortality in the North and South regions (Table 2). A potential association with level of schooling with ethnicity was not found (Table 2). We also did not observe any relationship between sex and administrative regions (Table 3).

However, the analysis between sex and ethnicity shows a higher risk of death among indigenous women (Table 4).

DISCUSSION

Studies in Brazil have shown that regional differences social related to health care, specific causes of death associated with diseases and public safety affect the number of deaths in the general population (Szwarcwald et al. 2013; França et al. 2015). However, specifically, for people with DS this information remains unknown. Given that individuals with DS present a higher risk for early mortality (O'Leary et al. 2018) associated with several morbidities at birth and throughout life (Castilla et al. 1998; Head et al. 2016) and that sex and ethnicity/race are also associated with the number of deaths in this population (Day et al. 2005; O'Leary et al. 2018) to know the mortality profile associated with sociodemographic variables is extremely relevant. This study analyzed the death records of individuals with DS in Brazil and in their five administrative regions North, Northeast, Midwest, South and Southeast between 1996

and 2016. In addition, we evaluated the survival rate and mortality in association with the variables of age, sex, race/ethnicity and schooling level.

A high correlation was observed between period of time and number of deaths, indicating that mortality among DS individuals has increased over the years in the Brazil. Some studies have shown that neonates, children, and adult individuals with DS are more susceptible to death compared to individuals without the syndrome (Castilla et al. 1998; Cua et al. 2017; O'Leary et al. 2018). The causes of mortality are associated with several factors such as congenital heart defects in the first years of life (Day et al. 2005; Kucik et al. 2013), respiratory problems and dementia in adulthood (Uppal et al. 2015). A study based on health data information from healthy people show that regional disparities related to health care issues are the main factors affecting the life expectancy of individuals living in the north and northeast regions of Brazil (França et al. 2017). For individuals with DS although studies show that life expectancy has increased (Asim et al. 2015), in according data provided by Datasus, the increase in mortality of this population in Brazil, is probably linked to the differences in health care among the Brazilian regions e seems to reflect the general profile of the Brazilian population.

Additionally, in this study higher number of deaths of children in the first years of life was observed. Other studies have also observed mortality in neonates and children with DS (Castilla et al. 1998; Kucik et al. 2013; Cua et al. 2017). Possibly, the highest infant mortality in all administrative regions is related to comorbidities and complications at this stage. Unfortunately, the causes of death made available in the DATASUS are grouped into categories, and it is not possible to obtain more detailed information. However, a recent study evaluated mortality in neonates with DS, in

conclusion the authors emphasize that the fact of having the syndrome is already an important risk factor (Cua et al. 2017).

For survival, a lower and higher rate was observed in the North and Southeast regions, respectively. These findings suggest that the lack of health centers specialized in the care of persons with developmental disabilities may be related to lower chances of survival in the North region. Some studies show that there is a scarcity of genetic service centers in North, while in contrast, the largest number of medical genetics services of the Brazil seem to be concentrated in the Southeast region (Horovitz et al. 2012; Passos-Bueno et al. 2012). The genetic centers offer a family orientation on health care and appropriate guidance by varied health professionals to help them assess differential needs of individuals with DS (Sobering et al. 2018).

With respect to ethnicity, a longer life span was observed among White and Yellow people. Some studies in North America (CDC 1968-1997; Krivchenia et al. 1993; Kucik et al. 2013; Presson et al. 2013) and Japan (Masaki et al. 1981) also found a similar relationship among these ethnic groups for DS. In addition, the association between ethnicity and administrative regions are also risk factors. Brazil is a country that encompasses different ethnic groups and the distribution profile of the population in general is concentrated in certain regions of Brazil (Carvalho et al. 2017; Carvalho et al. 2018). Although some findings have shown that there is a racial disparity in number of death record between person with DS (Shin et al. 2009; kucik et al. 2013) the causes for the predominance of the mortality in certain ethnic groups need further investigations (Santoro et al. 2016). In Brazil, unfortunately there are no data on the ethnic profile of people with DS.

Beyond race/ethnicity, educational level is also associated with the survival and mortality in the Brazilian regions. Individuals with longer schooling have a higher

survival rate. A recent systematic review showed that there is a relationship between the increase in schooling and the reduction of mortality (Byhoff et al. 2017). Several studies also pointed out that low educational level is associated with higher risk for dementia (Sharp et al. 2011; Krueger et al. 2015; Bento-Torres et al. 2017) and lower survival (Rogers et al. 2010). For DS there are no studies that relate schooling to mortality, however, as people with DS develop early dementia of the type Alzheimer's disease (AD) (Head et al. 2016), knowledge about this theme can direct future studies attempting to understand the association between level of schooling and the DA in the DS.

Although for individual with DS there is a need for special assistance in the school (Luiz et al. 2012) in Brazil, a study conducted in a city located in the Southeastern region evaluating the experience of parents and children with DS during the process of inclusion in a traditional school, found that teachers were not satisfactorily prepared for inclusion (Lautarescu et al. 2017). Our findings may be related to the results on the level of schooling associated with mortality in the South and Southwest regions, which is known to have the greatest number of death records.

Nevertheless, this fact is very pertinent for the North and Northeast regions where there is a lower risk of death associated with the none level of schooling. Indeed, it is necessary to consider the predominance in the number of deaths of children under one year of age, mainly, in the North and Northeast regions. In addition, it is necessary for policies to be implemented to stimulate the inclusion of this population in the education and training system of the team-teaching to reduce school evasion. Given that DS individuals have a cognitive impairment, an increased risk for developing AD (Cua et al. 2017); and that low level of schooling may accentuate cognitive decline (Bento-Torres et al. 2017) measures that enable the full inclusion of children with DS in school

represents an important strategy to increase the quality of life of these individuals (Santos et al. 2018).

Besides the fact that our study contributes to new epidemiological information on the population profile of individuals with DS; this study presents some limitations that should be highlighted. First, the database used in this research is from public hospitals, and therefore does not encompass all health centers. Second, depending on the cause of death there may be negligence, which could therefore culminate in errors in the overall estimate. Moreover, the dearth of studies in Brazil on the mortality estimates by ethnicity and education level limits a better understanding of our findings. Another limitation is the lack of data about the causes of death that could drive strategies of prevention and treatment of comorbidities associated the trisomy 21, thus avoiding the risk of complications and deaths in the DS population.

CONCLUSION

The number of deaths of individuals with DS has shown steady increases over the years in Brazil. Race/ethnicity and level of schooling directly influence the survival rate, while sex does not affect survival. Administrative regions are associated with the risk of death by ethnicity and educational level. These results suggest that socioeconomic aspects and the quality of health services available may represent a risk factor for the increase in the number of deaths, mainly among children. In this context, the findings presented can be used for the planning of health strategies and specific public policies to reduce the number of deaths of persons with DS associated the sociodemographic and regional inequalities.

Table 1 Survival in accordance with aged associated administrative regions, ethnic groups and level of education in the Brazil, between the years of 1996 and 2016, using Cox Proportional.

		P	Exp(B)	CI 95% for Exp(B)
Administrative regions				
North	Northeast	0.015	0.895	0.818, 0.979
	Southeast	<0.0001	0.697	0.640, 0.759
	South	<0.0001	0.702	0.641, 0.769
	Midwest	<0.0001	0.802	0.719, 0.895
Northeast	Southeast	<0.0001	0.778	0.740, 0.819
	South	<0.0001	0.784	0.739, 0.832
	Midwest	0.012	0.896	0.823, 0.976
Southeast	South	0.778	1.008	0.956, 1.062
	Midwest	0.001	1.152	1.062, 1.248
South	Midwest	0.002	1.143	1.048, 1.246
Ethnic groups				
White	Black	0.001	1.222	1.084, 1.379
	Yellow	0.829	0.970	0.736, 1.278
	Brown	<0.0001	1.399	1.331, 1.470
	Indigenous	<0.0001	2.192	1.663, 2.890
Black	Yellow	0.130	0.794	0.589, 1.070
	Brown	0.034	1.144	0.101, 1.297
	Indigenous	<0.0001	1.794	1.330, 2.419
Yellow	Brown	0.010	1.442	1.092, 1.904
	Indigenous	<0.0001	2.260	1.532, 3.334
Indigenous	Brown	0.002	0.638	0.483, 0.843
Level of education				
None	01 to 03 years	0.003	0.834	0.738, 0.942
	04 to 07 years	0.045	0.823	0.681, 0.995
	08 to 11 years	0.027	0.690	0.497, 0.959
	≥ 12 years	0.597	1.303	0.489, 3.474
01 to 03 years	04 to 07 years	0.909	0.987	0.791, 1.233
	08 to 11 years	0.288	1.207	0.853, 1.709
	≥ 12 years	0.375	0.640	0.238, 1.717
04 to 07 years	08 to 11 years	0.361	1.192	0.818, 1.738
	≥ 12 years	0.367	0.632	0.233, 1.714
08 to 11 years	≥ 12 years	0.228	0.530	0.189, 1.489

CI: 95% Confidence interval; P (Cox Proportional).

Table 2 Association of the mortality in the administrative regions in relation to ethnic groups and level of education in the Brazil, between the years of 1996 and 2016.

	North	Northeast	Southeast	South	Midwest	Total
Ethnic groups						
White	N = 186 P < 0.0001 OR: 0.216 95% CI: 0.179, 0.259	N = 776 P < 0.0001 OR: 0.236 95% CI: 0.211, 0.263	N = 2867 P < 0.0001 OR: 1.931 95% CI: 1.754, 2.127	N = 1659 P < 0.0001 OR: 7.512 95% CI: 6.241, 9.042	N = 355 P < 0.0001 OR: 0.646 95% CI: 0.544, 0.767	N = 5843
Black	N = 11 P = 0.136 OR: 0.608 95% CI: 0.330, 1.118	N = 73 P = 0.048 OR: 1.329 95% CI: 1.012, 1.745	N = 141 P = 0.031 OR: 1.310 95% CI: 1.031, 1.664	N = 32 P < 0.0001 OR: 0.474 95% CI: 0.327, 0.688	N = 21 P = 0.807 OR: 1.088 95% CI: 0.691, 1.711	N = 278
Yellow	N = 04 P = 0.861 OR: 1.274 95% CI: 0.457, 3.551	N = 08 P = 0.417 OR: 0.686 95% CI: 0.322, 1.462	N = 33 P = 0.005 OR: 2.325 95% CI: 1.307, 4.136	N = 06 P = 0.138 OR: 0.494 95% CI: 0.210, 1.159	N = 00 P = 0.091 OR: 0.128 95% CI: 0.008, 2.077	N = 51
Brown	N = 313 P < 0.0001 OR: 4.581 95% CI: 3.823, 5.490	N = 934 P < 0.0001 OR: 4.536 95% CI: 4.060, 5.067	N = 686 P < 0.0001 OR: 0.469 95% CI: 0.423, 0.520	N = 85 P < 0.0001 OR: 0.106 95% CI: 0.085, 0.132	N =195 P < 0.0001 OR: 1.444 95% CI: 1.208, 1.725	N = 2213
Indigenous	N = 15 P < 0.0001 OR: 6.381 95% CI: 3.470, 11.732	N = 06 P = 0.134 OR: 0.491 95% CI: 0.209, 1.153	N = 03 P < 0.0001 OR: 0.078 95% CI: 0.024, 0.251	N = 07 P = 0.255 OR: 0.589 95% CI: 0.265, 1.311	N = 20 P < 0.0001 OR: 8.829 95% CI: 5.000, 15.591	N = 51
Unidentified	N = 76	N = 508	N = 618	N = 293	N = 97	N = 1592
Total	N = 605	N = 2305	N = 4348	N = 2082	N = 688	N = 10028

N: number of individuals; OR: Odds Ratio; 95% CI: 95% Confidence interval; P (Fisher test).

Table 2 continuation.

	North	Northeast	Southeast	South	Midwest	Total
Level of education						
None	N = 198 P = 0.027 OR: 0.621 95% CI: 0.415, 0.929	N = 805 P < 0.0001 OR: 0.615 95% CI: 0.489, 0.774	N = 2211 P = 0.022 OR: 1.271 95% CI: 1.040, 1.553	N = 1044 P = 0.062 OR: 0.802 95% CI: 0.640, 1.004	N = 318 P = 0.222 OR: 0.787 95% CI: 0.551, 1.124	N = 4576
01 to 03 years	N = 16 P = 0.376 OR: 1.317 95% CI: 0.780, 2.223	N = 45 P = 0.425 OR: 0.863 95% CI: 0.622, 1.199	N = 120 P = 0.155 OR: 0.831 95% CI: 0.651, 1.062	N = 68 P = 0.601 OR: 1.090 95% CI: 0.822, 1.445	N = 27 P = 0.095 OR: 1.454 95% CI: 0.962, 2.197	N = 276
04 to 07 years	N = 10 P = 0.037 OR: 2.155 95% CI: 1.109, 4.187	N = 13 P = 0.099 OR: 0.530 95% CI: 0.331, 1.106	N = 45 P = 0.171 OR: 0.751 95% CI: 0.511, 1.103	N = 35 P = 0.039 OR: 1.565 95% CI: 1.042, 2.350	N = 07 P = 0.909 OR: 0.888 95% CI: 0.409, 1.924	N = 110
08 to 11 years	N = 02 P = 0.799 OR: 1.204 95% CI: 0.288, 5.039	N = 06 P = 0.909 OR: 0.864 95% CI: 0.359, 2.077	N = 14 P = 0.292 OR: 0.662 95% CI: 0.340, 1.291	N = 12 P = 0.252 OR: 1.598 95% CI: 0.800, 3.192	N = 03 P = 0.810 OR: 1.157 95% CI: 0.353, 3.786	N = 37
≥ 12 years	N = 01 P = 0.444 OR: 7.040 95% CI: 0.729, 67.982	N = 01 P = 0.728 OR: 1.490 95% CI: 0.155, 14.346	N = 02 P = 0.930 OR: 1.092 95% CI: 0.154, 7.759	N = 00 P = 0.613 OR: 0.368 95% CI: 0.020, 6.846	N = 00 P = 0.580 OR: 1.452 95% CI: 0.078, 27.035	N = 04
Unidentified	N = 378	N = 1435	N = 1956	N = 923	N = 333	N = 5025
Total	N = 605	N = 2350	N = 4348	N = 2082	N = 688	N = 10028

Table 3 Association of the mortality between male and female sex in the administrative regions of the Brazil, between the years of 1996 and 2016.

Region	Male (n = 5104)	Female (n = 4916)	OR	CI 95%	P
Northeast	1143	1156	1.053	0.880, 1.260	0.604
North	293	312			
Southeast	2253	2094	1.146	0.966, 1.358	0.127
North	293	312			
South	1051	1030	1.087	0.907, 1.302	0.394
North	293	312			
Midwest	364	324	1.196	0.961, 1.489	0.119
North	293	312			
Southeast	2253	2094	1.088	0.983, 1.204	0.107
Northeast	1143	1156			
South	1051	1030	1.032	0.916, 1.162	0.624
Northeast	1143	1156			
Midwest	364	324	1.136	0.958, 1.348	0.154
Northeast	1143	1156			
South	1051	1030	0.948	0.854, 1.053	0.154
Southeast	2253	2094			
Midwest	364	324	1.044	0.889, 1.227	0.628
Southeast	2253	2094			
Midwest	364	324	1.101	0.926, 1.308	0.294
South	1051	1030			

N: number of individuals; OR: Odds Ratio; 95% CI: 95% Confidence interval; P (Fisher test).

Table 4 Mortality in accordance with sex associated ethnic groups and level of education in the Brazil, between the years of 1996 and 2016.

	Male	**Female**	**Unidentified**	**OR**	**CI 95%**	**P**
Ethnic groups						
White	2944	2898	01	0.915	0.834, 1.003	0.062
Black	148	130	-	1.094	0.867, 1.390	0.502
Yellow	29	22	-	1.264	0.725, 2.204	0.491
Brown	1169	1043	01	1.101	0.999, 1.213	0.056
Indigenous	18	33	-	0.520	0.292, 0.926	0.034
Unidentified	796	790	06			
Level of education						
None	2388	2187	01	1.145	0.938, 1.397	0.200
01 to 03 years	130	144	-	0.828	0.648, 1.056	0.144
04 to 07 years	55	54	-	0.942	0.645, 1.378	0.834
08 to 11 years	21	16	-	1.218	0.634, 2.339	0.669
≥ 12 years	01	03	-	0.308	0.032, 2.970	0.564
Unidentified	2509	2512	07	-	-	-

OR: Odds Ratio; 95% CI: 95% Confidence interval; P (Fisher test) Funding statement

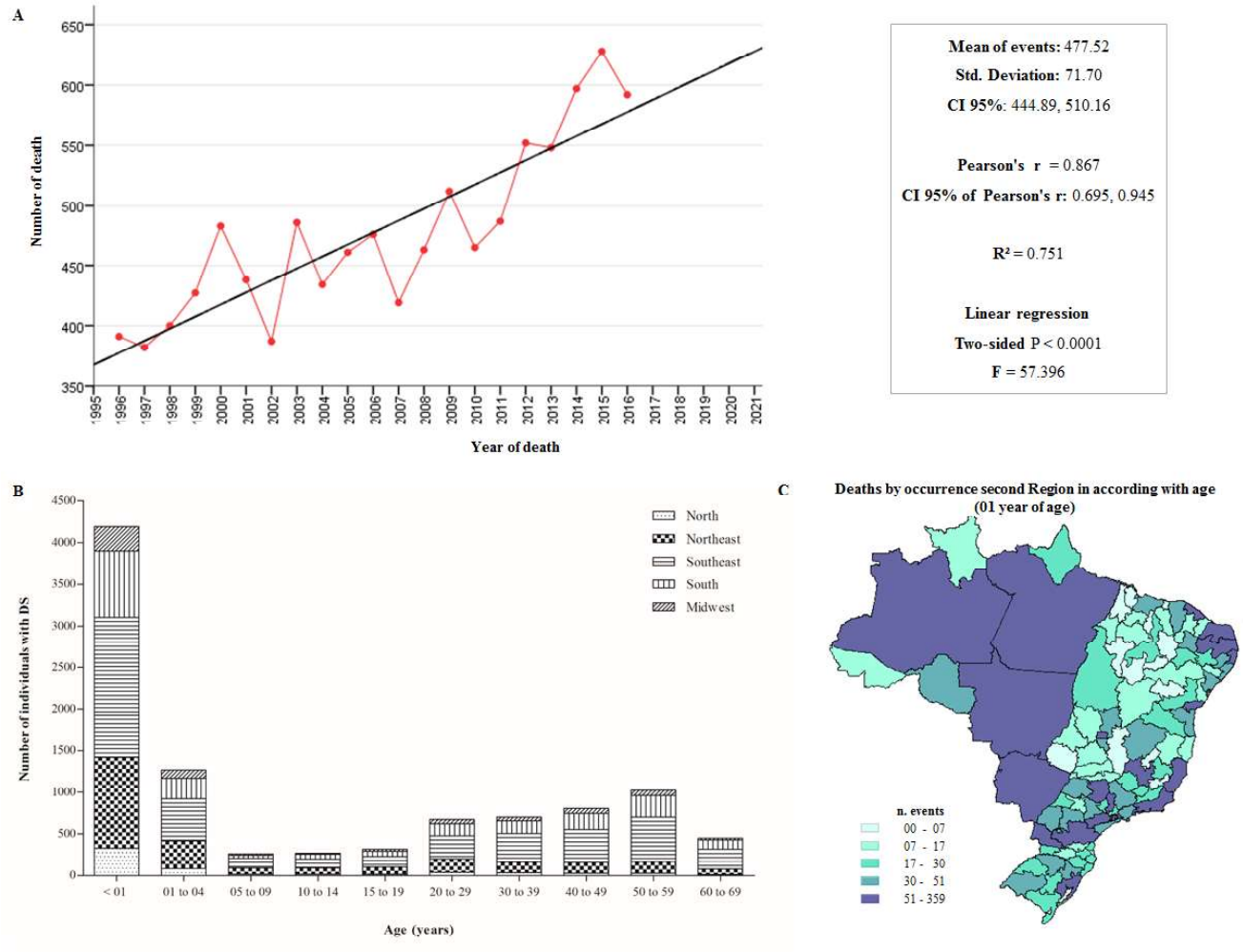

Figure 1 Number of death over a 21 years period with linear regression (A), distribution of the mortality in accordance with administrative regions and years of life in the Brazil (B) and number of death in individuals with DS and <01 years of age (C), between the years of 1996 and 2016.

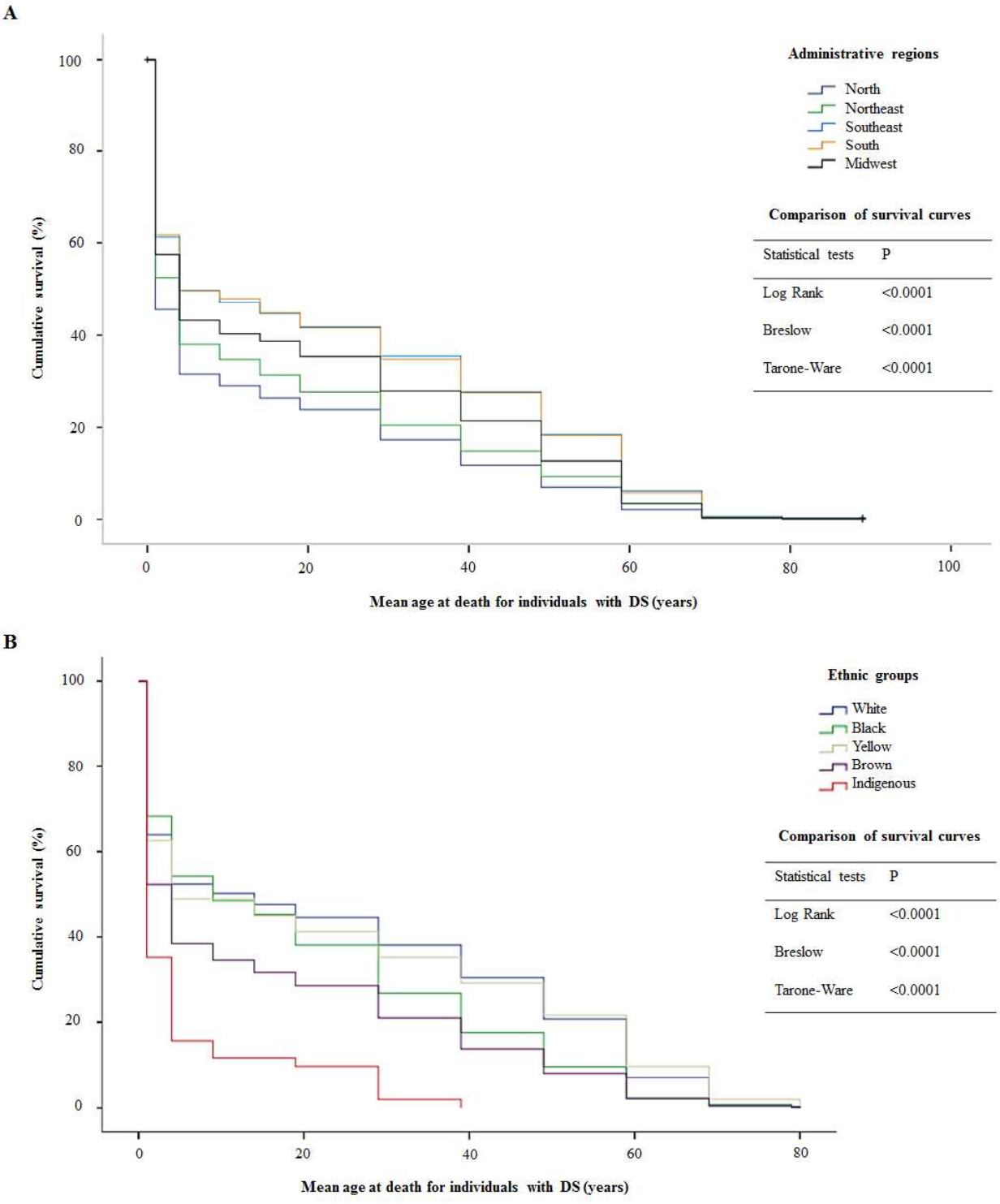

Figure 2.1 Kaplan-Meier survival curves for individuals with DS in accordance with administrative regions (A) and ethinic groups (B).

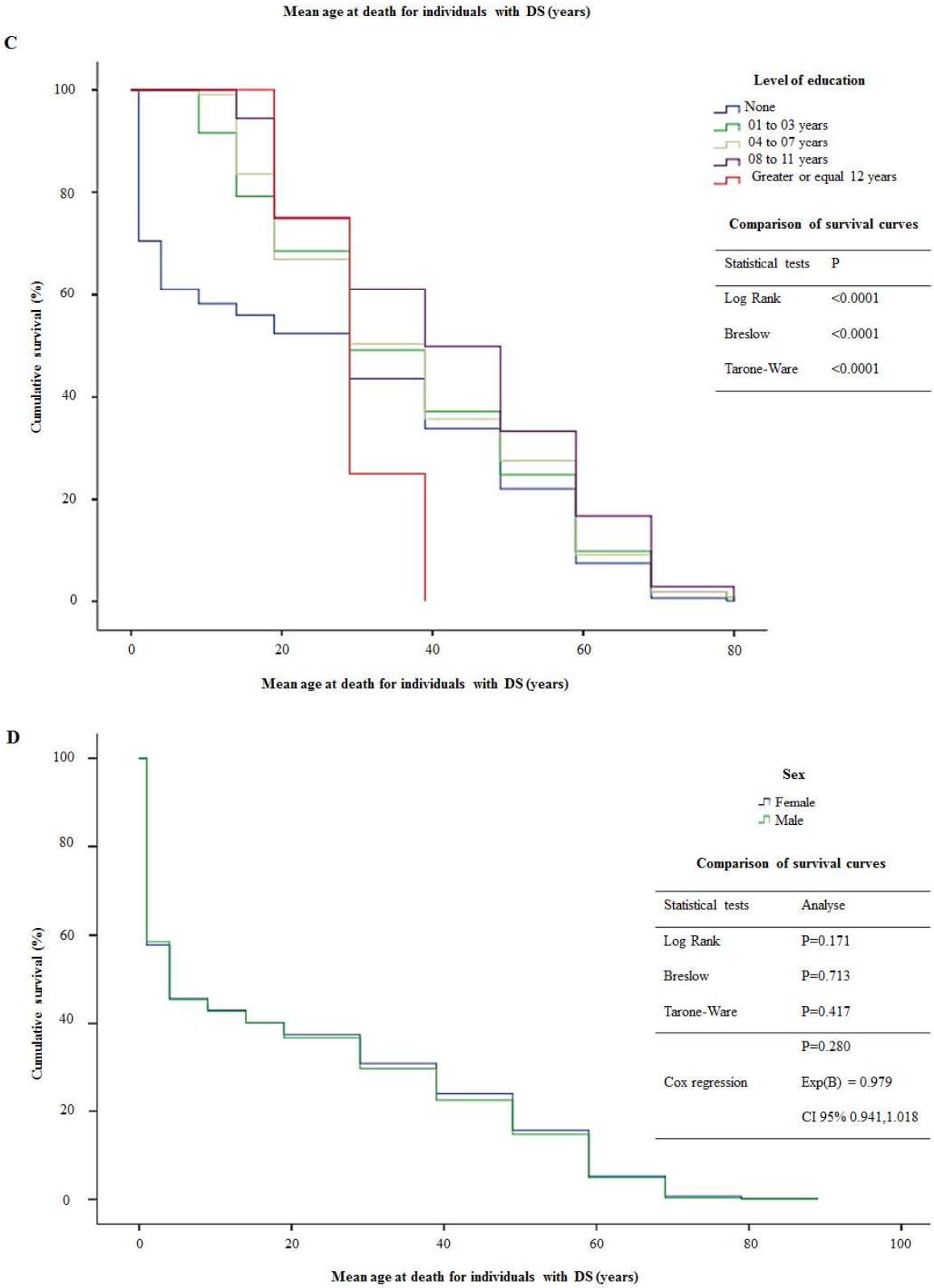

Figure 2.2 Kaplan-Meier survival curves for individuals with DS in accordance with level of educatition (C) and sex (D).

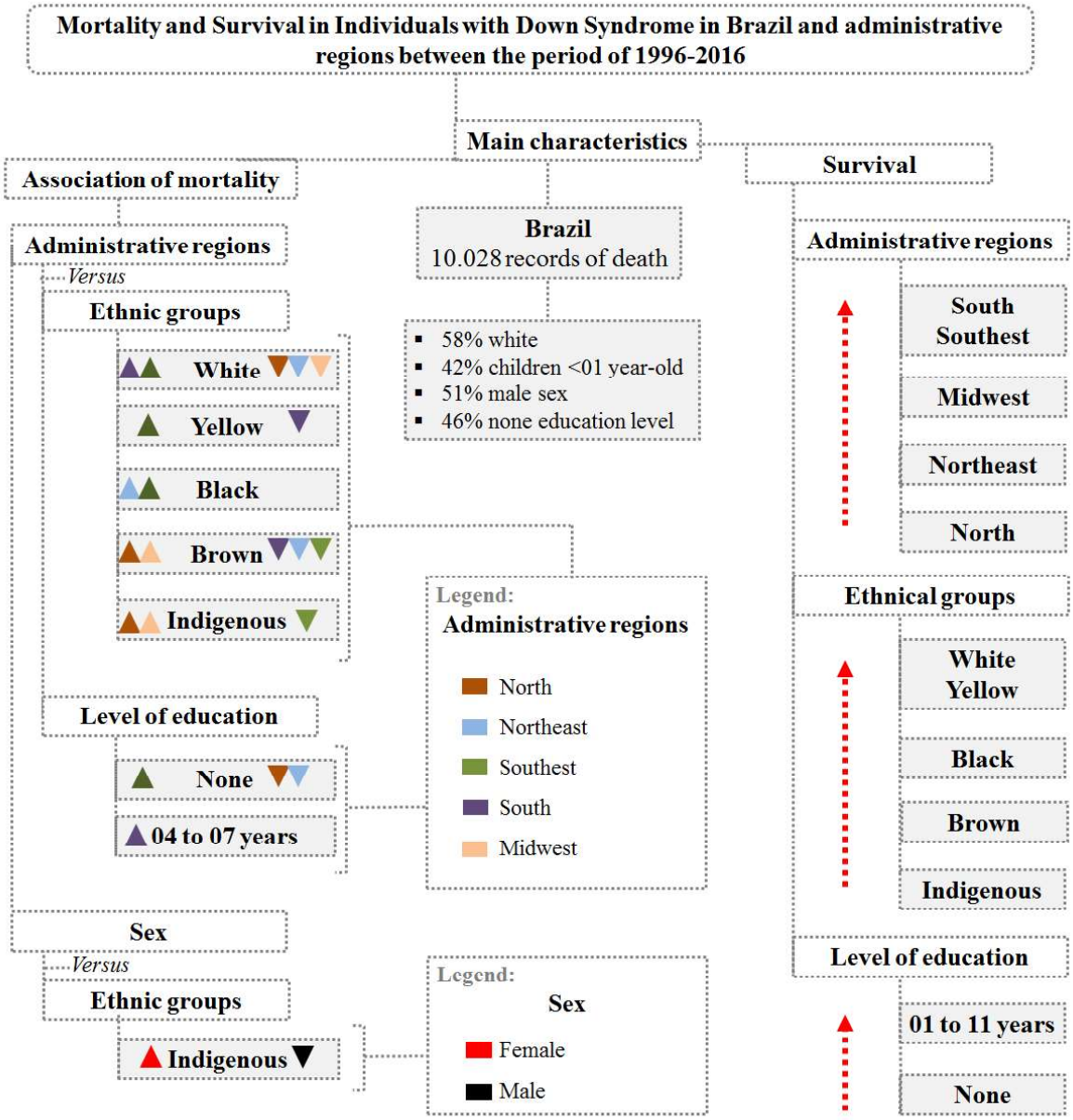

Figure 3 Association of mortality with the variable sociodemographic in individual with DS. Arrowhead pointed up or down indicates increased or decreased, respectively according to administrative regions and sex (in legend) for association of mortality. Red dotted arrow up indicates longer survival. The colors for arrowhead are explained in the legend in box.

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ARTIGO 2

Título: *Alzheimer's disease in the Down syndrome: An overview of genetics and molecular aspects.*

Autores: Fabiana de C. Gomes, Marlon F. Mattos, Eny M. Goloni-Bertollo, Érika C. Pavarino.

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Review Article

Alzheimer's disease in the Down syndrome: An overview of genetics and molecular aspects

Fabiana de C. Gomes, Marlon F. Mattos, Eny M. Goloni-Bertollo, Érika C. Pavarino

Genetics and Molecular Biology Research Unit (UPGEM), Department of Molecular Biology, São José do Rio Preto Medical School (FAMERP), São José do Rio Preto – SP, Brazil

Address for correspondence:

Dr. E. C. Pavarino, Genetics and Molecular Biology Research Unit (UPGEM) , São José do Rio Preto Medical School (FAMERP), São José do Rio Preto – SP, Brazil.

E-mail: erika@famerp.br

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Abstract

The overexpression of the amyloid precursor protein (*APP*) gene, encoded on chromosome 21, has been associated in Down syndrome (DS) with the development of early-onset Alzheimer's disease (EOAD). The increase in APP levels leads to an overproduction of amyloid-β (Aβ) peptide that accumulates in the brain. In response to this deposition, microglial cells are active and generate cascade events that include release cytokines and chemokine. The prolonged activation microglial cells induce neuronal loss, production of reactive oxygen species, neuron death, neuroinflammation, and consequently the development of Alzheimer's disease (AD). The intrinsically deficient immune systems in people with DS result in abnormalities in cytokine levels, which possibly contribute to the development of neurodegenerative disorders such as AD. Knowledge about the biomarkers involved in the process of neuroinflamation and neurodegeneration is important for understanding the mechanisms involved in the incidence and the precocity of AD in individuals with DS.

Keywords: Alzheimer's disease, amyloid, cytokines, Down syndrome

Key Messages: In DS, some peculiarities influence the neuroinflammatory response and contribute to early-onset dementia in adults with DS. This review supports the idea that development of neurodegenerative disease such as AD in people with DS is complex and involves the interaction of genes localized outside and inside of chromosome 21.

Down syndrome (DS), or trisomy 21 (T21), is a chromosomal abnormality with complete or partial copy of chromosome 21 (Hsa21),[1] with incidence estimated at 1 in 660 live births.[2,3] The extra copies of genes on Hsa21 result in gene dosage imbalances, affecting expression and regulation throughout the genome.[1] Gene overdoses are associated with the existence of a critical region, implying several phenotypes observed in the DS.[4]

It has been suggested since the 1970s that the region 21q22.11-21q22.2 of 3.8–6.5 Mb localized in the distal segment of the long arm of chromosome 21, called as "Down syndrome critical region" (DSCR), is crucial to the phenotypes of DS.[4,5] About 30 genes localized in this "critical region" are responsible for most features observed in T21.[6] However, Korenberg *et al*. suggested that there was no single region on 21q responsible for the phenotype of the syndrome, as they showed the contribution of genes outside of the DSCR.[7] Furthermore, other studies have demonstrated that multiple critical regions or critical genes can contribute to the appearance of features associated with T21.[6,8] Contrarily, Chabert *et al*.'s[9] Galois lattice analysis associated only the region between the *D21S17* and ETS proto-oncogene 2, transcription factor (*ETS2*) sequences, which lie in the proximal part of 21q22.3, with the pathogenesis of the DS.[7,10] More recently, Pelleri *et al*.[11] identified a smaller region on 21q22.13 as critical to the manifestation of typical features in the SD. Therefore, there is still no consensus about the region that is determinant for clinical manifestations.

Although several studies have been performed to identify a "critical region" that contributes to clinical manifestations, the main point is to understand the direct or indirect effects of dosage-sensitive genes on the phenotype of the syndrome and the consequences of interactions between specific genes or subsets of genes on clinical manifestations.[1,4] The genic interaction and overproduction result in deregulation of biochemical pathways that are implicated in clinical aspects and posterior complications.[12,13]

Clinical aspects of DS are complex and variable. Although some characteristic are observed in all individuals, others are seen in only some persons.[14] The individuals are characterized by a set of facial and physical features, such as muscle hypotonia, flat-looking face, cognitive impairment, and immune system defects.[7,15] In DS, the brain is morphologically and anatomically characterized by diminished volumes of hippocampus and of the temporal and frontal lobes.[16,17] Postmortem brain of DS has showed abnormal distributions of neurons in some brain regions, decreased neurogenesis, neuronal hypocellularity, and hypoplasia, resulting in reduction in the number of neurons and synaptic transmission.[16-19] In most cases, additional problems exist, such as hearing and visual defects, respiratory disorders, gastrointestinal tract anomalies, congenital heart disease, and susceptibility for developing early-onset Alzheimer's disease (EOAD).[7,15,20] It is likely that genic imbalance observed in DS contributes to development of neurodegenerative factors such as AD.[21-23]

Alzheimer's Disease and the Neuropathology in Down Syndrome

AD is a chronic neurodegenerative disease characterized by loss of memory and other cognitive abilities. It is also a main cause of dementia worldwide with an incidence of 100 person-years.[24,25] The risk of AD increases progressively in advancing age; about 25%–45% of people more than 85 years of age have dementia.[26] Based on the ages of the onset of dementia, AD is divided into two subtypes: early-onset AD (EOAD), which affects persons between 30 and 60 years old, and late-onset AD (LOAD), which is more frequent in persons older than 60 years.[24] Clinical symptoms of EOAD and LOAD are similar, including decline in memory and impairment of the cognitive functions, language, and motor skills. Over time, the severity of these symptoms increases, leading to difficulties performing physical and cognitive functions in work, home, and social situations.[24]

Several investigators have reported EOAD in adults with DS.[20,27-30] Usually, AD appears two to three decades earlier in people with DS than in people without T21.[30] The average age for diagnosis and dementia incidence in DS was reported to be around 47 years.[31] After 60 years, the incidence of neuropathology decreases, likely due to variations in diagnoses and/or the absence of information about mortality rates associated with dementia.[27]

Aberrant dosages of genes and noncoding sequences present on HSA21 may have a role in the development of AD in individuals with DS (DS-DA).[27] In HS21, specifically in the region 21q22.11-21q22.2, there are genes that contribute to many neurological features of DS; on the other hand, some findings have focused on the region 21q21–21q22.3, which contains genes important for brain development.[32,33]

In this review, we performed a search in PubMed for identification of genes located in the region 21q22.11-21q22.3, which is reported as a critical region for most neurological phenotypes of DS. After search were identified 464 genes localized that were analyzed through Database for Annotation Visualization and Integrated Discovery (DAVID) version 6.8. Further analyses excluded 23 genes due to lack of information obtained from the Gene Entrez database. After exclusion, 441 genes were subjected to multiple tests. We selected only significant results ($P < 0.05$) related to neurologic functions.

When analyzing disease classes, we observed that 25 genes showed moderate concordance ($\kappa = 0.44$, $P = 0.006$, multiple tests of DAVID) with neurological diseases and other systemic dysfunctions. We also verified that 55 genes were involved in neurological and metabolic dysfunctions; however, the concordance level was not significant ($\kappa = 0.40$, $P = 0.096$, multiple tests of DAVID). Finally, the analyses that considered only neurological

dysfunctions showed that 19 genes [Table 1] were involved in this function. These genes were selected for functional annotation of the gene ontology [Table 2].

Of these 19 genes, only 21% [superoxide dismutase 1 (*SOD1*), runt-related transcription factor 1 (*RUNX1*), dual-specificity tyrosine-phosphorylation-regulated kinase 1A (*Dyrk1A*), and lipid transporter ATP-binding cassette G1 (*ABCG1*)] have been described to be involved in AD-DS. Therefore, the role of many genes that have been presented in this bioinformatics analysis remains unknown. The knowledge about these genes may direct future research on potential targets involved in dementia.

Independent of the localization of triplicate genes in HS21, particularly for neurological phenotypes, imbalances and changes in the expression of genes located within or even outside of the DRSC affect mechanisms involved in the development of tissue and cellular differentiation, leading to neuronal reductions that favor the emergence of neurological complications, such as EOAD.[17]

Neuropathologic Mechanisms Linked to AD in DS

Clinical, histopathological, and molecular aspects of AD are observed in adults with DS.[30,34] Studies have reported that individuals with DS at age 30 years show severe cognitive deterioration, such as difficulties with verbalizing, apraxia, and other dysfunctions similar to dementia that can be linked to frontal lobe dysfunctions.[20] Current findings support the unclear association between severity of cognitive disability and development of AD in DS (AD-DS), possibly because the preexisting cognitive impairments in DS make a neuropathological diagnosis of AD difficult.[27] After 40 years of age, the neuropathology rapidly increases and AD may be diagnosed.[35] The main genetic factors involved in clinical manifestations of AD-DS are shown in Figure 1.

The copy extra of amyloid precursor protein (*APP*) gene, located on chromosome 21, may have a key role in development of neuropathology in DS.[27] The overexpression of *APP* in DS results in an increase in amyloid-β (Aβ) peptides associated with dementia.[24] Histological analyses of brain tissue of individuals with DS without AD (DS-non AD) have demonstrated that Aβ can accumulate, overtime, before the EOAD.[35] This deposition occurs in the intracellular (neuron) and extracellular (around the blood vessel and neurons) spaces, forming neuritic plaques and neurofibrillary tangles (NFTs).[20,31,36]

Neuritic plaques, also called senile, dendritic, or amyloid plaques, refer to abundant depositions of insoluble Aβ in brain parenchyma.[37] The accumulation of Aβ initially forms oligomers that slowly aggregate in the form of fibrils and senile plaques.[38] Amyloid fibrils are formed by soluble proteins that agglomerate to form insoluble aggregates that are not easily degraded.[38] This aggregation generates neurotoxicity and influences kinase/phosphatase activity, inducing tau protein hyperphosphorylation and causing the formation of NFTs, consequently affecting the synaptic and neuronal functions, causing neuronal death and the eventual appearance of AD.[30,38]

NFTs are intraneuronal aggregations of hyperphosphorylated forms of the microtubule-associated protein, tau.[39] The Tau protein that is present in axon has and plays a role in stabilization of microtubules in neurons.[40] Abnormal accumulation and aggregation of the tau filaments (hyperphosphorylated form) in cell bodies and dendrites induce neurotoxicity and have been implicated in AD pathophysiology.[41,42]

Recent studies have demonstrated the participation of Tau in the development of neuropathology in the DS.[43] Some genes localized in chromosome 21 such as the regulator of regulate calcineurin 1 (*RCAN1*) and *Dyrk1A* contribute to dysregulation of tau phosphorylation, resulting in hyperphosphorylation in the adult DS brain.[44] Although tau has

been associated with the development of EOAD in individuals with DS, amyloid cascade hypothesis, due to *APP* triplication, is still the primary proposal for the cause of dementia in people with DS.[45,46]

Studies have demonstrated that the overexpression of *APP* is four to five times greater in individuals with DS.[47] In normal conditions, APP is not neurotoxic; however, errors in processing APP lead to the production and accumulation of Aβ.[48] The APP can be cleaved by three different enzymes: α, β, and γ-secretase. The α enzymes compete with the β enzymes for cleavage of APP. This competition drives APP processing through amyloidogenic and non-amyloidogenic pathways.[49]

In the non-amyloidogenic pathway, the α-secretase enzyme cleaves APP, generating fragments of amyloid precursor-protein-α (sAPPα) which have neuroprotective functions. In the amyloidogenic pathway, the cleavage of APP is mediated by β-secretase (BACE1 and BACE2) enzymes, producing *APP*-β (sAPPβ).[50] Later, γ-secretase enzyme cleaves sAPPα, producing shorter P3 fragments ($A\beta_{17-x}$), whose biological roles are unknown and also cleave sAPPβ that produce Aβ peptides.[49,51] In pathological conditions, errors in APP cleavage processing and/or imbalances between Aβ generation and clearance result in excess of long Aβ peptides (Aβ40/42) that can aggregate into amyloid plaques, giving rise to AD neuropathologies.[48,51] In individuals with DS more than 40 years old, there are observed reductions in α-secretase and increases in β-secretase. These results suggest that alterations of secretase activity may be associated with production of amyloidogenic fragments and accumulation of Aβ.[52]

The causes of modifications in the steps of APP cleaving and dysregulation between the production and degradation of Aβ may be explained by dominant autosomal mutations or genetic polymorphisms, including *APP*, Presenilin 1 and 2 (*PSEN 1/2* or *PS1/2*), *ABCG1*, and

apolipoprotein E (*APOE*) genes.[27,38] PSEN *1/2* proteins are components of the γ-secretase complex that regulate *APP* processing in normal conditions, and mutations of *PSEN 1/2* genes can induce high production of long Aβ peptides (Aβ-40/42); these peptides are known as the main pathogenic form of Aβ, associated with occurrence of EOAD and severe forms of AD.[21,48,53-55] In addition, in DS it was suggested that Dyrk1A phosphorylates the PSEN1 contributing to high γ-secretase activity, and consequently elevated amounts of Aβ40/42 in the brain.[27,55]

Regarding *APOE* in the brain, this protein participates in neuronal signaling, regulation of the cholinergic neurotransmitter system, and Aβ clearance.[56] Although it is considered a biomarker of late-stage AD,[55] studies have showed an association between APOE gene, particularly alleles ε4 (apoE4), and the development of dementia in adults with DS.[23] Still, the gene *ABCG1* is involved in the regulation of cholesterol and influences the processing of APP and clearance of Aβ.[27]

Others proteins such as BACE2 and small ubiquitin-related modifier 3 (SUMO3) are also encoded in HS21, modifying APP post-translationally, which may change Aβ production.[27] Moreover, additional factors such as microRNA-155 and ETS2 transcription factor located in HSA21 have been reported as modulating/processing APP. The acting pathways of microRNA-155 and *ETS2* in APP processing are different, but both may induce Aβ generation.[57,58] The *RUNX1*, also located in HSA21, was associated with AD in people with DS.[31] *RUNX1* is important in transcriptional regulator neural progenitor cell and has been implicated in the development of neuronal phenotypes observed in individuals with DS.[59]

For non-chromosome 21 genes, single-nucleotide polymorphisms in cystatin C (*CST3*) and microtubule affinity-regulating kinase 4 (*MARK4*) genes were related with risks of

dementia in adults with DS.[30] The *CST3* code inhibitory protein CysC binds with Aβ, inhibiting aggregation and preventing the formation of fibrils.[60] The *MARK4* encodes proteins that phosphoryl microtubule-associated protein. Errors in expressions of *MARK4* are related to increases in phosphorylation of tau and may lead to development of EOAD.[61] Thus, the interaction of genes localized outside and inside of chromosome 21 may favor aggregation of Aβ and may lead to neuroinflammation linked to DS-AD pathogenesis.[62-64]

Some Aspects of Inflammation Associated with AD in DS

In DS, some peculiarities influence neuroinflammatory response.[64] The exacerbation of amyloid deposition in DS favors a neuronal loss and consequently occurrence of EOAD.[64] Aβ accumulates in a brain's active microglial cells; these cells interact with amyloid plaques, resulting in an event known as an "activated" macrophage phenotype (M1), inducing a proinflammatory cascade and resulting in the production of chemokines and cytokines involved in Aβ clearance and preventing neuronal death.[65] However, microglial-prolonged activation and increase in proinflammatory factors favor the production of reactive oxygen species, propitiating oxidative stress, neurotoxicity, and neuron death, causing neuroinflammation.[66]

In parallel, an alternative activated macrophage phenotype (M2) is responsible for tissue reparation through the release of anti-inflammatory cytokines and reduction in proinflammatory cytokine concentrations. The release or inhibition of inflammatory mediators depends on the balance between "classical" (M1) and "alternative" (M2) activations;[67] consequently, dysregulation of pro- and anti-inflammatory molecules favors AD and DS neuropathology.[28,64]

Genes involved in the inflammatory response, located chromosome 21, associated with M1 phenotype also participate in the development of neuropathological manifestations in people

with DS. These genes act in the upregulation of glial activation.[64] More recently, it has been observed that an increase in marker M2 phenotypes (CD64 and CD86) in people with DS-AD had not been described in sporadic AD, suggesting a unique inflammatory phenotype in people with DS.[68]

In DS, the intrinsically deficient immune system results in abnormalities of cytokine levels that possibly contribute to the development of neurodegenerative factors such as AD.[17,69,70] Table 3 shows that some studies have reported associations of cytokine changes in DS with or without AD.[68,70-90] These findings indicate a possible accentuated inflammatory response before appearance of neuropathology.[68,70-88]

aThe triplicated genes in T21 accentuate the inflammatory response inducing high expressions and release of several pro-inflammatory cytokines, such as IL-1β.[30,64] Overexpression, upregulation, or liberation of IL-1β induces an increase in *APP*, contributing to Aβ deposition and activation of pathways involved in the formation of senile plaques and NFTs.[64]

Others genes involved in the inflammatory process, also located in HSA21, that participate in the formation of senile plaques and/or NFT include S100 calcium binding protein astrocyte-derived (*S100B*) and *SOD1* genes that participle in events related to oxidative stress; ADAM metalloproteinase with thrombospondin type 1 motif, 1 (*ADAMTS1*), ADAM metalloproteinase with thrombospondin type 1 motif, 5 (*ADAMTS5*), and Coxsackie virus and adenovirus receptor (*CXADR*) genes that active pathways such as mitogen-activated protein kinase (*MAPK*)-p38.[64]

The inflammatory markers in the blood, brain, and cerebrospinal fluid may indicate signs of dementia and contribute to diagnoses of AD in persons with DS; however, investigations of markers for early diagnosis are necessary.[45,91] The identification of target

biomarkers is crucial for drug development and of new therapeutic methods that may conduce a treatment of EOAD in adults with DS.[45]

Summary

In DS, some peculiarities influence the neuroinflammatory response and contribute to early-onset dementia in adults with DS. This review supports the idea that development of neurodegenerative disease such as AD in people with DS is complex and involves the interaction of genes localized outside and inside of chromosome 21.

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Conflicts of interest

There are no conflicts of interest.

Table 1: Gene ID, symbol, description, and location of genes in the critical region of the HSA21 involved in neurological disease.

Gene ID	Symbol	Description	Location
7074	*TIAM1*	T-cell lymphoma invasion and metastasis 1	21q22.11
9992	*KCNE2*	Potassium voltage-gated channel subfamily E regulatory subunit 2	21q22.11
6647	*SOD1*	Superoxide dismutase 1	21q22.11
114036	*LINC00310*	Long intergenic non-protein coding RNA 310	21q22.11
3460	*IFNGR2*	Interferon gamma receptor 2	21q22.11
861	*RUNX1*	Runt related transcription factor 1	21q22.12
80215	*RUNX1-IT1*	RUNX1 intronic transcript 1	21q22.12
3763	*KCNJ6*	Potassium voltage-gated channel subfamily J member 6	21q22.13
1859	*DYRK1A*	Dual specificity tyrosine phosphorylation regulated kinase 1A	21q22.13
150084	*IGSF5*	Immunoglobulin superfamily member 5	21q22.2
5316	*PKNOX1*	PBX/knotted 1 homeobox 1	21q22.3
9619	*ABCG1*	ATP binding cassette subfamily G member 1	21q22.3
6573	*SLC19A1*	Solute carrier family 19 member 1	21q22.3
1291	*COL6A1*	Collagen type VI alpha 1 chain	21q22.3
3689	*ITGB2*	Integrin subunit beta 2	21q22.3
875	*CBS*	Cystathionine-beta-synthase	21q22.3
5116	*PCNT*	Pericentrin	21q22.3
23181	*DIP2A*	Disco interacting protein 2 homolog A	21q22.3
4599	*MX1*	MX dynamin like GTPase 1	21q22.3

Information obtained from Gene Entrez database (http://www.ncbi.nlm.nih.gov/gene/)

Table 2: Functional annotation of the genes involved in neurological dysfunctions.

Function	Count	Genes	*P*-value *
Positive regulation of metabolic process	08	*ABCG1*, *PKNOX1*, *TIAM1*, *DYRK1A*, *ITGB2*, *KCNE2*, *RUNX1*, *SOD1*	0.016
Response to lipoprotein particle	02	*ITGB2*, *ABCG1*	0.017
Cell adhesion	06	*IGSF5*, *PKNOX1*, *TIAM1*, *COL6A1*, *ITGB2*, *SOD1*	0.017
Biological adhesion	06	*IGSF5*, *PKNOX1*, *TIAM1*, *COL6A1*, *ITGB2*, *SOD1*	0.018
Anatomical structure formation involved in morphogenesis	05	*PKNOX1*, *PCNT*, *COL6A1*, *ITGB2*, *RUNX1*	0.020
Regulation of cholesterol metabolic process	02	*SOD1*, *ABCG1*	0.020
Response to chemical	09	*TIAM1*, *KCNE2*, *COL6A1*, *ITGB2*, *MX1*, *SOD1*, *SLC19A1*, *IFNGR2*, *ABCG1*	0.024
Single-organism process	17	*ABCG1*, *MX1*, *PKNOX1*, *TIAM1*, *COL6A1*, *CBS*, *DIP2A*, *DYRK1A*, *IGSF5*, *ITGB2*, *IFNGR2*, *PCNT*, *KCNE2*, *KCNJ6*, *RUNX1*, *SLC19A1*, *SOD1*	0.026
Aging	03	*KCNE2*, *ITGB2*, *SOD1*	0.028
Anatomical structure morphogenesis	07	*PKNOX1*, *TIAM1*, *PCNT*, *COL6A1*, *ITGB2*, *RUNX1*, *SOD1*	0.028
Potassium ion import	02	*KCNJ6*, *KCNE2*	0.029
Cellular response to chemical stimulus	07	*TIAM1*, *KCNE2*, *COL6A1*, *ITGB2*, *MX1*, *SOD1*, *IFNGR2*	0.029
Single organismal cell–cell adhesion	04	*IGSF5*, *PKNOX1*, *ITGB2*, *SOD1*	0.029
Regulation of response to stress	05	*TIAM1*, *DYRK1A*, *ITGB2*, *SOD1*, *IFNGR2*	0.029
Regulation of alcohol biosynthetic process	02	*SOD1*, *ABCG1*	0.031
Single-organism cell adhesion	04	*IGSF5*, *PKNOX1*, *ITGB2*, *SOD1*	0.035
Response to external stimulus	06	*TIAM1*, *ITGB2*, *MX1*, *SOD1*, *IFNGR2*, *CBS*	0.038
Response to organic substance	07	*TIAM1*, *COL6A1*, *ITGB2*, *MX1*, *SOD1*, *IFNGR2*, *ABCG1*	0.039
Positive regulation of cellular metabolic process	07	*PKNOX1*, *TIAM1*, *DYRK1A*, *KCNE2*, *ITGB2*, *RUNX1*, *SOD1*	0.040
Endodermal cell differentiation	02	*COL6A1*, *ITGB2*	0.042

Table 2 continuation.

Function	Count	Genes	*P*-value*
Anatomical structure development	10	*DIP2A*, *PKNOX1*, *TIAM1*, *PCNT*, *DYRK1A*, *KCNE2*, *COL6A1*, *ITGB2*, *RUNX1*, *SOD1*	0.043
Single-organism developmental process	10	*DIP2A*, *PKNOX1*, *TIAM1*, *PCNT*, *DYRK1A*, *KCNE2*, *COL6A1*, *ITGB2*, *RUNX1*, *SOD1*	0.043
Cholesterol biosynthetic process	02	*SOD1*, *ABCG1*	0.044
Single-organism cellular process	16	*PCNT*, *ITGB2*, *SOD1*, *SLC19A1*, *ABCG1*, *DIP2A*, *PKNOX1*, *KCNJ6*, *TIAM1*, *DYRK1A*, *COL6A1*, *KCNE2*, *MX1*, *RUNX1*, *IFNGR2*, *CBS*	0.044
Secondary alcohol biosynthetic process	02	*SOD1*, *ABCG1*	0.045
Regulation of steroid biosynthetic process	02	*SOD1*, *ABCG1*	0.047
Endoderm formation	02	*COL6A1*, *ITGB2*	0.050
Sterol biosynthetic process	02	*SOD1*, *ABCG1*	0.050

**P*< 0.05, multiple tests of Database for Annotation Visualization and Integrated Discovery (DAVID) version 6.

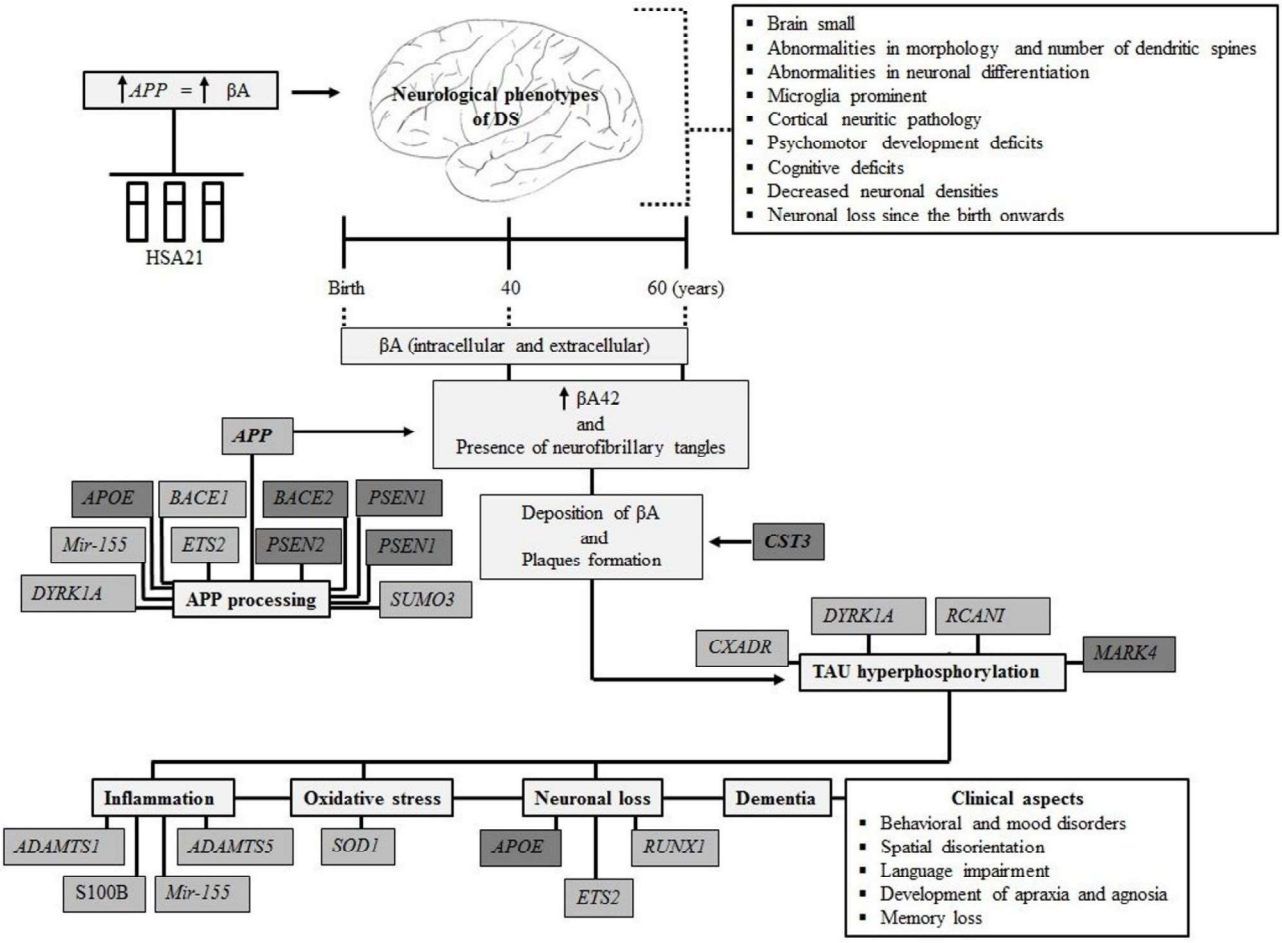

Figure 1: Schematic of genetic factors involved in clinical manifestations of AD-DS in which are described the events of pathogenesis AD-DS (light gray box) and the interaction of genes inside (medium-dark gray box) and outside (dark gray box) of chromosome 21 with the neurologic phenotypes and clinical aspects (white box): human chromosome 21 (HSA21); Down syndrome (DS); beta amyloid protein (Aβ); amyloid precursor protein (*APP*); apolipoprotein E (*APOE*); lipid transporter ATP-binding cassette G1 (*ABCG1*); beta-secretase 1 (*BACE1*) beta-secretase 2 (*BACE2*); microRNA 155 (*Mir-155*); ETS proto-oncogene 2, transcription factor (*ETS2*); Presenilin 1 (*PSEN* 1); Presenilin 2 (*PSEN 2*); dual-specificity tyrosine-phosphorylation-regulated kinase 1A (*Dyrk1A*); small ubiquitin-related modifier 3 (SUMO3); cystatin C (*CST3*); coxsackie virus and adenovirus receptor (CXADR); regulator of regulate calcineurin 1 (*RCAN1*); microtubule affinity-regulating kinase 4 (*MARK4*); ADAM metalloproteinase with thrombospondin type 1 motif, 1 (*ADAMTS1*); ADAM metalloproteinase with thrombospondin type 1 motif, 5 (*ADAMTS5*); S100 calcium binding protein astrocyte-derived (*S100B*); superoxide-dismutase type 1 (*SOD-1*); runt-related transcription factor 1 (*RUNX1*) genes.

Table 3: Main cytokines altered in Down syndrome.

Cytokines	Sample types	Result	References
IL-1	Serum	↑ level of IL-1α in DS vs control	Śmigielska-Kuzia *et al.*[81] †,‖
	Brain	↑ gene expression of IL-1Ra in DS <40 years old vs control	Wilcock *et al.*[68]‡‡
		↑ gene expression of IL-1Ra and IL-1β in DS with AD >40 years old vs control	Wilcock *et al.*[68]‡‡
		↑ immunoreactivity in astrocytes of IL-1 in postnatal DS vs control	Griffin *et al.*[74]‡‡
		↓ level of IL-1β Ts65Dn mouse model for DS vs control (wild-type)	Roberson *et al.*[85]‡‡
	Plasma	↑ level of IL-1β in DS vs control, both after stimulation with *influenza A*	Broers *et al.*[78]†
	GCF	↑ level of IL-1β in DS vs control	Tsilingaridis *et al.*[77]§,‖
	PBMCs	↓ secretion of IL-1β in aged DS vs control both after stimulation with LPS	Park *et al.*[87]‖
IL-2	Serum	↑ level in DS vs control†	Śmigielska-Kuzia *et al.*[81]†,‖
		↓ production in aged DS vs control both after stimulation with PHA	Park *et al.*[87]‖
	PBMCs	↓ in DS vs control when stimulated with $A\beta_{1-42}$	Loewenbrueck *et al.*[88]‖
IL-4	Serum	↑ level in DS vs control	Cetiner *et al.*[76]†
	GCF	↑ level in DS vs control	Tsilingaridis *et al.*[77]§,‖
IL-6	Brain	↑ gene expression in DS <40 years old vs control	Wilcock DM *et al.*[68]‡‡
		↑ gene expression in DS with AD >40 years old vs control	Wilcock DM *et al.*[68]‡‡
	Plasma or Serum	↑ level in DS with AD vs control	Iulita *et al.* [71] ‖
		↑ level in DS with severe AD vs DS without AD	Kálmán *et al.* [73]
		↑ level in DS vs control	Iulita *et al.*[71]‖, Zaki *et al.*[72]†, Broers *et al.*[78]†, Śmigielska-Kuzia *et al.*[81]†,‖
		↓ gene expression DS vs control	Zampieri *et al.* [86]†
	PBMCs	↓ level in DS vs control	Cetiner *et al.*[76]†
	GCF	↑ level in DS vs control	Tsilingaridis *et al.*[77]§,‖

Table 3 continuation.

IL-7	Plasma or serum	↑ level in DS vs control	Roat *et al*.[80]†, Guazzaroti *et al.*[89]§
IL-8	Plasma or serum	↑ level in DS with AD vs DS without AD	Iulita *et al*.[71]‖
		↑ level in DS vs control	Broers *et al*.[78]†, Nelson, *et al*.[83]†
IL-10	Brain	↑ gene expression in DS <40 years old vs control	Wilcock DM *et al.* [68]§,‡‡
		↑ gene expression in DS with AD >40 years old vs control	Wilcock DM *et al*. [68]§,‡‡
	Plasma or serum	↑ level in DS vs control	Iulita *et al*.,[71]‖ Cetiner *et al*.[76]†
		↑ level in DS with AD vs control	Iulita *et al*.[71]‖
		↓ level in DS vs ID	Natheghi Rostami *et al*.[70]†,§
		↑ level in DS vs control, both after stimulation with *Streptococcus pneumonia*	Broers *et al*.[78]†
	Gingival	↓ gene expression in DS vs control, both with periodontal disease	
	GCF	↑ level in DS vs control	
	PBMCs	↑ level in DS vs control, both after stimulation with Phytohemagglutinin	Cavalcante *et al*.[75]‖
		↑ level in DS vs ID	Tsilingaridis *et al*.[77]§,‖
		↑ gene expression in DS vs control	Guazzaroti *et al.*[89]§
			Trotta *et al.*[90] ‖
			Zampieri *et al*.[86]†
IL-12	Brain	↑ gene expression in DS <40 years old vs control	Wilcock *et al*.[68]‡‡
	GCF	↑ level in DS vs control	Tsilingaridis *et al*.[77]§,‖
IL-15	Plasma	↑ level in DS vs control	Roat *et al*.[80]†
IL-17A	PBMCs	↓ gene expression *IL-17A* in DS vs control	Jakubiuk-Tomaszuk *et al*.[82]†§
IFN- α	Plasma	↑ level in DS vs control	Broers *et al*.[79]†

Table 3 continuation.

IFN- γ	Plasma or serum	↑ level in DS vs control	Nateghi Rostami *et al*.[70]†,§
		↑ level in DS vs ID	Nateghi Rostami *et al*.[70]†,§
		↑ level in DS with AD vs control	Iulita *et al*.[71]‖
		↑ intracellular production in DS vs MR and HC	Franciotta *et al*.[84]‖
	GCF	↑ level in DS vs control	Tsilingaridis *et al*.[77]§,‖
	PBMCs	↑ level in DS vs control, both after stimulation with Phytohemagglutinin or Cytomegalovirus	Guazzaroti *et al*.[89]§
		↑ level in DS vs ID	Trotta *et al*.[90] ‖
		↓ in DS vs control when stimulated with Aβ1-42	Loewenbrueck *et al*.[88]‖
TGF-β	Brain	↑ gene expression in DS <40 years old vs control	Wilcock *et al*.[68]§,‡‡
		↑ gene expression DS with AD >40 years old vs control	Wilcock *et al*.[68]§,‡‡
TNF-α	Brain	↑ gene expression DS with AD >40 years old vs control	Wilcock *et al*.[68]‡‡
	Plasma	↑ level in DS with AD vs control	Iulita *et al*.[71]‖
		↑ level in DS vs control	Iulita *et al*.,[71]‖ Zaki *et al*.,[72]† Nateghi Rostami *et al*.,[70]†,§ Broers *et al*.,[78]†
		↓ level in DS vs control	Śmigielska-Kuzia *et al*.[81]† Cetiner *et al*.[76]†
	GCF	↑ level in DS vs control	Tsilingaridis *et al*.[77]§,‖
	PBMCs	↑ level in DS vs ID	Trotta *et al*.[90] ‖

AMHC: age-matched healthy control; HC: healthy control; DS: Down syndrome; AD: Alzheimer disease; ID: intellectual deficient; MR: mental retardation; GCF: gingival crevicular fluid; PBMCs: peripheral blood mononuclear cells; PHA: phytohemagglutinin.

†Children, §Adolescent, ‖Adult, ‡‡Postmortem, ↑: Increased, ↓: Decreased.

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ARTIGO 3

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Autores: Fabiana de Campos Gomes, João Simão de Melo-Neto, Merari de Fátima Ramires Ferrari, Carla Patrícia Carlos, Eny Maria Goloni-Bertollo, Érika Cristina Pavarino.

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Vitamin D_3 increases the Caspase-3 p12, MTHFR, and P-glycoprotein reducing amyloid-β_{42} in the kidney of a mouse model for Down syndrome

Fabiana de Campos Gomes[a], João Simão de Melo-Neto[b], Merari de Fátima Ramires Ferrari[c], Carla Patrícia Carlos[d], Eny Maria Goloni-Bertollo[a], Érika Cristina Pavarino[a,*]

[a] Genetics and Molecular Biology Research Unit (UPGEM), São José do Rio Preto Medical School (FAMERP), São José do Rio Preto, SP, Brazil
[b] Institute of Health Sciences, Federal University of Pará (UFPA), Belém, PA, Brazil
[c] Department of Genetics and Evolutionary Biology, Biosciences Institute, University of São Paulo (USP), São Paulo, SP, Brazil
[d] Faceres School of Medicine (FACERES), São José do Rio Preto, SP, Brazil

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ABSTRACT

Aims: Renal dysfunction has been reported in individuals with Down syndrome (DS); however, the causes and mechanisms involved remain unknown. Here, we present a proposal for how the triplication of the amyloid beta precursor protein (*APP*) and, mainly the amyloid β peptide 1–42 ($A\beta_{42}$) can favor the development of renal abnormalities in DS. We evaluated the effects of vitamin D_3 (VD_3) supplementation on morphofunctional aspects and the repercussions on the presence and localization of $A\beta_{42}$, methylenetetrahydrofolate reductase (MTHFR), caspase-3 p12, and P-glycoprotein (Pgp) in the renal tissue of DS mouse model.

Main methods: Twenty female mice (14-week-old) belonging to the B6EiC3Sn-Rb(12.Ts171665Dn)2Cje/CjeDnJ lineage were divided into four experimental groups (n = 5/group): common diet; trisomy (Ts) and wild-type (Wt); and high doses VD_3, $Ts_{(VD3)}$, and $Wt_{(VD3)}$. All the groups were treated for 10 weeks. At 24 weeks, the protocol experimental was interrupted. The kidney was weighed, collected, and processed for immunochemical analysis for $A\beta_{42}$, Caspase-3 p12, MTHFR, and Pgp proteins. All data were analyzed statistically.

Key findings: Our results showed that VD_3 promoted an increase in caspase-3 p12, MTHFR, and Pgp, and consequently contributed to reduced $A\beta_{42}$ in the renal tissue of a mouse model of DS. Furthermore, VD_3 treatment affected the plasma creatinine and urea levels and contributed to the attenuation of the dilation of Bowman's space observed in trisomic mice.

Significance: Finally, the results showed that VD_3 may activate specific mechanisms involved in reduced $A\beta_{42}$ and tissue repair in the kidneys of a mouse model for Down syndrome.

1. Introduction

Down Syndrome (DS) is a chromosomal abnormality caused by alteration in number of genes on chromosome 21 [1]. Gene overexpression has implications throughout the genome, resulting in several abnormal phenotypes [2–4]. Among the phenotypes, a wide variety of urogenital abnormalities, such as renal dysfunction, have been described in adults with DS [4–6]. There are limited of studies that address the causes of nephropathy in DS [5,6]. However, the mechanisms involved in the occurrence of these abnormalities remain unknown. In the brain, the exacerbated production of amyloid β peptide 1–42 ($A\beta_{42}$) has been found to favor the activation of pro-apoptotic mechanisms, including the participation of caspase-3 [7]. After a cascade of events, in an attempt to maintain cellular homeostasis, other mediators induce the activation of pathways that may affect the morphological structure and/or cause programmed cell death [8–10]. In the kidneys, the mechanisms involved in the exacerbated production of $A\beta_{42}$ have not been investigated in experimental models, and humans with DS.

Among the nephropathies described in DS, the presence of chronic renal failure may represent a risk for the appearance of other renal diseases [5,6,11]. Kidney failure may progress to chronic kidney disease (CKD), favoring the appearance of complications and comorbidities [12,13]. People with CKD, and curiously in DS, have a deficiency of vitamin D_3 (VD_3). The causes are associated with reduced VD_3 levels in the CKD and DS that are multifactorial, including a poor diet quality, reduced sun exposure [5,14], and particularly described for CKD, a loss

* Corresponding author at: Genetics and Molecular Biology Research Unit (UPGEM), São José do Rio Preto Medical School (FAMERP), Av. Brigadeiro Faria Lima, 554, São José do Rio Preto, SP CEP: 15090-000, Brazil.
E-mail address: erika@famerp.br (É.C. Pavarino).

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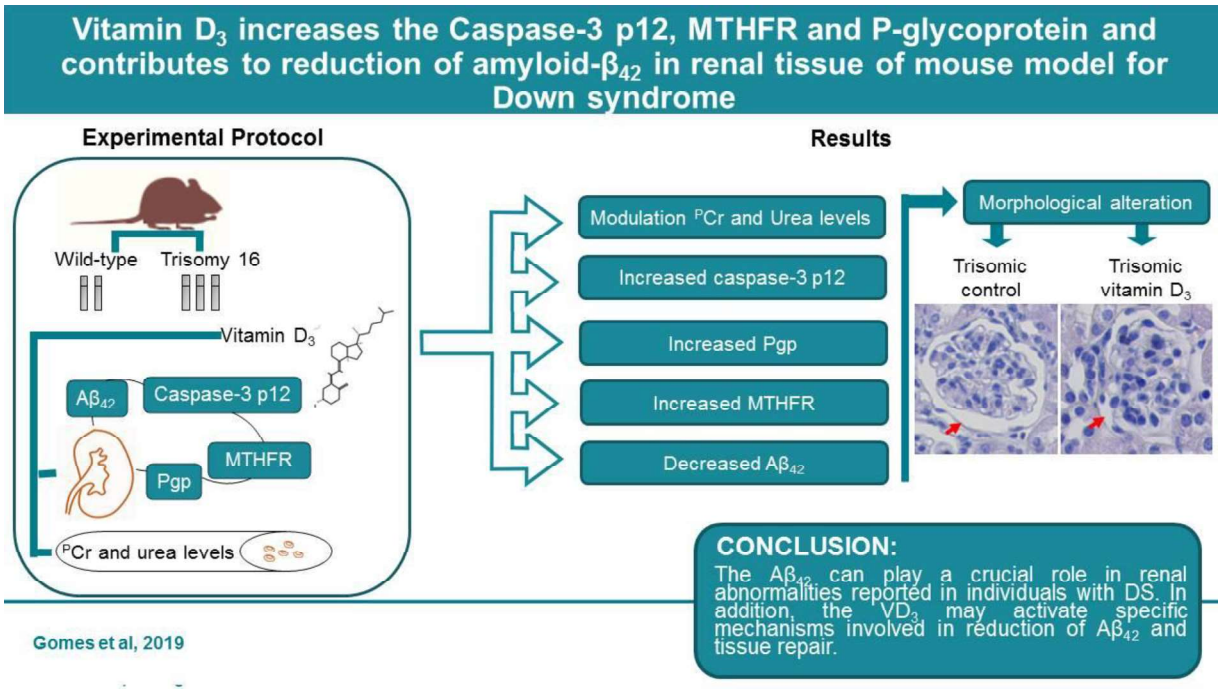
Graphical abstract



ABSTRACT

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Significance: Finally, the results showed that VD_3 may activate specific mechanisms involved in reduced $A\beta_{42}$ and tissue repair in the kidneys of a mouse model for Down syndrome.

***Keywords*:** Down syndrome; amyloid-β; vitamin D3; kidney.

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Among the nephropathies described in DS, the presence of chronic renal failure may represent a risk for the appearance of other renal diseases [5,6,11]. Kidney failure may progress to chronic kidney disease (CKD), favoring the appearance of complications and comorbidities [12,13]. People with CKD, and curiously in DS, have a deficiency of vitamin D_3 (VD_3). The causes are associated with reduced VD_3 levels in the CKD and DS that are multifactorial, including a poor diet quality, reduced sun exposure [5,14], and particularly described for CKD, a loss of 25-hydroxycholecalciferol in cases of proteinuria nephropathy [15].

Vitamin D [25(OH)D] is important for renal morphophysiology [16,17]. The kidney is also responsible for the synthesis of the active form of vitamin D (VD), 1,25-dihydroxyvitamin D3 [1,25 (OH)2D3] [18]. Through binding to the nuclear VD receptor (VDR), 1,25 (OH)2D3 activates transcriptional mediators and regulates genes responsive for

VD. The calcitriol-receptor complex can regulate important mechanisms, including the increase in transport mediated by P-glycoprotein (Pgp), a membrane protein important in the clearance de $A\beta_{42}$ in brain tissue [19]; and in regulating receptors involved in folate transport [20]. Folate has a key role in cell growth and proliferation [21], and the levels of this metabolite are regulated by the enzyme methylenetetrahydrofolate reductase (MTHFR) [22]. Genetic variations of *MTHFR* are associated with the presence of nephropathy [23,24]. In addition, VD_3 may influence folate levels [25]. Therefore, although still unknown, MTHFR can be influenced by VD_3, and play an important role in renal morphophysiology.

Given the importance of VD_3 in the physiological functions, prevention and/or treatment of renal diseases, and its role in $A\beta_{42}$ clearance [10,26,27], we analyzed the effects of VD_3 supplementation in the morphological/functional parameters and repercussions on the presence and localization $A\beta_{42}$, MTHFR, caspase-3 p12, and Pgp proteins in the kidney of a mouse model for DS. For the first time, we characterized, before and after VD_3 supplementation, a relationship between immunolocalization and expression of proteins involved in pathogenic processes, tissue repair, apoptosis and clearance of Aβ amyloid in the kidney of a mouse model for DS.

2. Methods

2.1. Animals and experimental environment

Female mice (*Mus musculus*) of the lineage B6EiC3Sn-Rb(12.Ts17^{16}65Dn)2Cje/CjeDnJ (#004850) carrying a Robertsonian translocation [28] (The Jackson Laboratory, Bar Harbor, ME, USA) were maintained with normal diploid B6EiC3SnF1/J male for mating (both with 3-month-old). The offspring of B6EiC3Sn-Rb(12.Ts17^{16}65Dn)2Cje/CjeDnJ and B6EiC3SnF1/J mice were karyotyped at 21 days of age, according to protocol of instructions provided by The Jackson Laboratory (USA) to determine the presence of the trisomy, and subsequently distributed into control and experimental

groups. The genotyping test was performed in the Biosciences Institute, University of São Paulo (USP), São Paulo, SP, Brazil. Throughout the experimental protocol, the mice were maintained in the Central Bioterium of the São José do Rio Preto Medical School (FAMERP) under adequate conditions of lighting (12-hour light-dark cycle) and temperature (23 ± 2ºC). The experiment protocol was approved by the Ethics Committee for Animal Use of the FAMERP, protocol n. 001-002447/2015. In the present study, only female offspring were used in the experimental protocol because the early pathophysiological mechanisms associated with Aβ have been reported more frequently in women with DS [29] and female Ts65Dn mice, a model for DS [30,31]; Therefore, knowledge about the role of peripheral clearance of Aβ in the kidney is extremely important.

2.2. Experimental groups

Initially all mice had access to a standard solid diet (Nuvilab®, Curitiba, PR, Brazil) and water *ad libitum*. With 14 weeks of age the animals were submitted to the experimental protocol. Female mice (14-week-old) were distributed in four experimental groups (n = 5/group) according to a presence (Ts) or absence of the trisomy (Wt) and supplemented with vitamin D_3 (VD_3) or control diet (CO) as follows: control diet with positive genotype ($Ts_{(CO)}$), control diet with negative genotype ($Wt_{(CO)}$), vitamin D_3 with positive genotype ($Ts_{(VD3)}$), vitamin D_3 with negative genotype ($Wt_{(VD3)}$).

2.3. Diet

The $Ts_{(CO)}$ and $Wt_{(CO)}$ groups were maintained with a standard diet throughout the experiment. Whereas, the mice of the $Wt_{(VD3)}$ and $Ts_{(VD3)}$ groups were fed a diet supplemented with high doses of VD_3 for 10 weeks (12,500 IU/kg, Domeneghetti & Corrêa Ltda®, Jaú, SP, Brazil), according to Wergeland et al. [32].

2.4. Euthanasia and tissue collection and processing

24-week-old mice were euthanized with high-dose 100 mg/kg sodium thiopental (Tiopental®), administered intraperitoneally according to animal weight. After total sedation, blood samples were collected. Posteriorly, transcardial perfusion was performed with phosphate-buffered saline solution (PBS) pH 7.4. Then, the kidneys (left antimer) were excised and weighed, fixed in 4% paraformaldehyde diluted in PBS, processed, and embedded in paraffin, cut into 5 μm thick sections. The slides followed for the morphometric and immunohistochemistry analyses.

2.5. Renal function analysis

The plasma samples were used to detect the following biochemical markers: plasma urea and creatinine (^{P}Cr). The samples of all groups were checked with colorimetric assay and analysis by spectrophotometry (BIO-200, Bioplus, São Paulo, SP, Brazil) using commercial kits (Biotécnica, Varginha, MG, Brazil).

2.6. Morphometric analysis

The slides were stained with hematoxylin-eosin (HE) and Bowman's space (μm), glomerulus diameter (μm), diameter of the renal corpuscles (μm), glomerular area (μm^2), and area of the renal corpuscles (μm^2) were analyzed. Fifteen microscopic fields were randomly selected (objective magnification 40 ×) per group. The analysis was performed in the Zeiss Primo Star microscope model coupled to a camera (Zeiss Axiocam 105 color model) and Zen Lite 2.3 software (Zeiss).

2.7. Immunohistochemistry analyses of Aβ$_{42}$, Pgp, MTHFR, and caspase-3 p12

The sections were deparaffinized, hydrated, and subjected to antigenic recovery. Posteriorly, submitted to endogenous peroxidase blocking and nonspecific proteins with skim milk (MOLICO®). The primary antibodies (abcam®, USA) used for incubation were: anti-beta Amyloid 1-42 (1:1000 concentration; ab201060), anti-P Glycoprotein (1:250 concentration; ab170904), anti-MTHFR (1:200 concentration; ab203789), and anti-caspase-3

p12 (1:500 concentration; ab179517). After overnight incubation with the primary antibodies, the sections were washed in PBS buffer and incubated with Goat antirabbit IgG H&L secondary antibodies (1:500 concentration, HRP, abcam®, USA, ab97051). Posteriorly, the slides were revealed with DAB chromogen and counterstained with hematoxylin. To confirm the specificity of the reaction, negative controls were used.

The analysis was performed in the renal cortex and medulla by photos acquired on the microscope Zeiss Primo Star model coupled to a camera (Zeiss Axiocam 105 color model) (objective magnification 20 × and 40 ×) and Zen Lite 2.3 software (Zeiss), according Fu et al. [33]. Tissue area fields per group were randomly used to evaluate the percentage (%) of immunoreactivity. These fields were analyzed using ImageJ 1.47 software, windows version (National Institutes of Health, USA), according to Ruifrok and Johnston's method [34]. During the immunohistochemistry analysis, the fixed threshold was established to obtain of the percentage of the immunostained tissue area to the proteins analyzed.

2.8. Statistical analysis

Data were analyzed by descriptive and inferential statistics. The data were initially subjected to the Shapiro-Wilk normality test. The results were presented as mean and standard deviation or median and 95% confidence interval as appropriate for parametric or non-parametric tests. The following between-groups factors were considered for statistical analyses: presence of trisomy, vitamin D_3, and their interaction (trisomy and vitamin D_3). Two-way analysis of variance (ANOVA) with the post hoc Bonferroni test (parametric) or Scheirer Ray Hare test with the post hoc Mann-Whitney U test (non-parametric) were applied to assess group heterogeneity in the biometric parameters and renal function, morphometry, and percentage of immunoreactive area. The F- (F) and H-statistic (H) were presented to analyze between-groups factors (trisomy, vitamin D3, and their interaction). The effect of size (low 0.01 to 0.33; moderate 0.34 to 0.66; high 0.66 to 0.99) was analyzed for parametric data

by eta partial squared analysis (ηp2). The probability of a Type I error for the statistical tests was evaluated at $p < 0.05$.

3. Results

3.1. Vitamin D3 supplementation reduces body and kidney weight in trisomic mice

Regardless of the positive or negative genotype for partial trisomy, the supplementation of VD_3 in high doses contributes to loss of body weight. The groups treated with VD_3 (trisomic or not) had a lower body weight than that of the controls. In addition, we observed that the relative weight of the kidney in ($Ts_{(CO)}$) mice is higher than that in ($Wt_{(CO)}$) mice, but not in control mice treated with VD3 ($Wt_{(VD3)}$) compared to that of control group ($Wt_{(CO)}$), suggesting the direct involvement of the trisomy factor in morphological alteration. After VD_3 treatment, the kidney absolute was reduced ($Ts_{(CO)}$ *versus* $Ts_{(VD3)}$ group), suggesting VD_3 is a protective factor contributing to this reduction (Table 1).

3.2. Supplementation of vitamin D3 reverts renal morphological parameters altered by trisomy

Morphometric analysis showed the presence of morphological alterations in the renal tissue of the trisomic mouse group. We identified a dilation of Bowman's space in the $Ts_{(CO)}$ group than that of the $Wt_{(CO)}$. Trisomy did not affect parameters, such as glomerular and renal corpuscle diameter/area ($Ts_{(CO)}$ vs $Wt_{(CO)}$). After VD_3 supplementation, it was observed the Bowman's space in $Ts_{(VD3)}$ group was reduced, an effect not observed in $Wt_{(VD3)}$ compared to that of the $Wt_{(CO)}$ group. In relation to the glomerular and renal corpuscle diameter/area, independent of genotype, VD_3 reduced these structures. For the glomerular area, a reduction was observed only in the $Wt_{(VD3)}$ group compared with that of the control ($Wt_{(CO)}$) (Table 1).

3.3. Vitamin D3 reduces plasma urea in trisomic mice

High-doses of VD_3 changed the ^{P}Cr and plasma urea levels in Ts and Wt groups. The urea level was reduced in $Ts_{(VD3)}$ and $Wt_{(VD3)}$ in comparison to that of their control groups.

However, a significant increase in ^{P}Cr was observed in the $Ts_{(VD3)}$ *versus* $Ts_{(CO)}$ group, being trisomy factor responsible by increase of ^{P}Cr (Table 1).

3.4. Immunohistochemistry

3.4.1. Vitamin D_3 reduces the expression of β-amyloid peptide ($Aβ_{42}$) induced by trisomy in the kidney

In all experimental groups, we verified the presence of immunoreactivity to $Aβ_{42}$ (Fig. 1.1A–D and 1.2E-H). However, the distribution pattern and immunoreactivity of this protein differs among control and experimental groups. In $Ts_{(CO)}$ (Fig. 1.1A and 1.2E) *versus* $Wt_{(CO)}$ (Fig. 1.1C and 1.2G), we observed a high immunostaining for $Aβ_{42}$ in the renal cortex and medulla. This protein in the $Ts_{(CO)}$ group was localized in the glomerular tuft (cell nucleus and cytoplasm; and between the glomerular cells), proximal and distal tubules and blood vessels (veins, arteries, glomerular capillary), and renal tubular cells and vessels in the inner and outer medulla (Fig. 1.1A and 1.2E). Interestingly, in the medulla an intense deposition of $Aβ_{42}$ similar to that of the amyloid plaques was observed. In addition, we observed the presence of inflammatory infiltrate in the renal cortical interstice (Fig. 1.1B). However, VD_3 decreased the immunoreactivity area percentage for $Aβ_{42}$ in $Ts_{(VD3)}$ *versus* $Ts_{(CO)}$ (Fig. 1.1A-B and 1.2 E-F) that was not observed in $Wt_{(VD3)}$ compared to $Wt_{(CO)}$. Unlike the control group, $Aβ_{42}$ in $Ts_{(VD3)}$ mice was visualized as small clusters in the glomerular tuft (extracellular and intracellular), interstitial space, and blood vessels; beyond the presence of inflammatory infiltrate (Fig. 1.1B). This result indicated that VD_3 is an important factor for reducing $Aβ_{42}$ accumulation (Fig. 1.2I-K).

3.4.2. Caspase-3 p12 is enhanced by vitamin D_3 in trisomic mice

We observed immunoreactivity to caspase-3 p12 intracellularly (cytoplasm and nucleus) in the tubular system, medulla (inner and outer zones) and blood vessels (endothelial cells) in the kidney of the control and experimental groups (Fig. 2.1A-D and 2.2E-H). For the

$Ts_{(VD3)}$ group, a discrete marking of caspase-3 p12 was observed in the glomerular tuft. Although the localization pattern for caspase-3 p12 was similar among groups, it was possible to observe perceptible differences in the percentage and intensity of the immunoreaction. The $Ts_{(CO)}$ group *versus* $Wt_{(CO)}$ has a lower immunoreaction for caspase-3 p12 in the renal medulla (Fig. 2.2K). However, the VD_3 induced an increase in caspase-3 p12 in the total kidney, cortex, and medulla in $Ts_{(VD3)}$ *versus* $Ts_{(CO)}$; this protein was visualized in the cytoplasm and nucleus of a large number of cells (Fig. 2.1B and 2.2F). Unlike, $Wt_{(VD3)}$ group presented a lower caspase-3 p12-immunoreactive percentage than that of $Wt_{(CO)}$ (Fig. 2.2I-K).

3.4.3. Vitamin D_3 enhanced P-glycoprotein (Pgp) in the renal cortex

In the kidney, a similar pattern of immunolocalization for Pgp was observed among the control and experimental groups (Fig. 3A-D). Pgp was localized in the cortical region, specifically in the proximal tubular cells (brush border membrane). Although the localization of Pgp was similar among groups, a significant increase in the percentage of the immunoreactive area was observed in $Ts_{(VD3)}$ *versus* $Ts_{(CO)}$ and $Wt_{(VD3\ )}$ *versus* $Wt_{(CO)}$ (Fig. 3B and D). There was no difference between $Ts_{(CO)}$ and $Wt_{(CO)}$ or between $Ts_{(VD3)}$ and $Wt_{(CO)}$ (Fig. 3E).

3.4.4. Methylenetetrahydrofolate reductase (MTHFR) is enhanced by vitamin D_3 in trisomic mice

In the renal tissue of trisomic and wild-type mice, MTHFR was immunolocalized in cytoplasm and/or cell membranes in the cortical region, including Bowman's capsule (membrane basement and parietal cells) and proximal convoluted tubule epithelial cells (cytoplasm and brush border membrane) near the renal corpuscle (Fig. 4A-D). A high intensity of MTHFR was observed in the parietal layer of the Bowman's capsule in $Wt_{(CO)}$. In renal tissue of the $Ts_{(VD3)}$ mice, it was possible to observe the intracellular presence of MTHFR in the parietal cells (Fig. 4B) at a higher magnification. Regarding immunoreactivity,

we observed an increase in MTHFR in $Ts_{(VD3)}$ *versus* $Ts_{(CO)}$ and $Ts_{(VD3)}$ *versus* $Wt_{(VD3)}$, but not in $Wt_{(VD3)}$ *versus* $Wt_{(CO)}$ (Fig. 4E).

4. Discussion

In DS, the presence of kidney abnormalities may compromise the renal function and favors the emergence of complications and comorbidities. Although it has been reported that congenital abnormalities may be related the malformations in kidney [35], the causes of these events remain unknown. Taking into consideration that adults individuals with DS present renal abnormalities [4-6,36] and develop early-onset Alzheimer's disease (EODA) associated, mainly, to the increased Aβ accumulation [37]; and that elevated serum Aβ levels may be associated with a renal failure [38, 39], being the kidney one of the main organs involved in peripheral clearance of Aβ peptides [40]; the knowledge about the presence and localization of the $A\beta_{42}$ protein in the kidney of a mouse model for DS is of great importance.

Several studies have shown that the VD_3 acts in the activation of mechanisms involved in the degradation and clearance Aβ peptides [19, 41], and that diets enriched with VD_3 may help in the prevention or treatment of kidney diseases [15,42,43]. In this study we analyzed the effect of high-doses of VD_3 in kidney of adult female Rb(12.Ts17^{16}65Dn)2Cje mice. Our findings showed an association between the localization and quantitative presence of Pgp, MTHFR, and Caspase-3 p12 proteins in response to VD_3 treatment and an increase in $A\beta_{42}$ peptides in the kidney.

The $A\beta_{42}$, Pgp, MTHFR, and Caspase-3 p12 proteins are modulated according VD_3 availability [7,19,25,26], and are involved in pathogenic processes; clearance; DNA repair and cell survival; and apoptosis, respectively [7,19,22]. The increased protein $A\beta_{42}$ may favor the activation of pathways that induce DNA damage and apoptosis leading to cell death [44]; therefore, it is fundamental to analyze these proteins in this context.

For the first time, our research showed the localization and accentuated presence of $A\beta_{42}$ in the kidney of a DS mouse model. These findings strengthen the supposition that increased $A\beta_{42}$ can be involved with the dilation of the Bowman's space (BS) in $Ts_{(CO)}$ mice, and it is possible a similar condition may occur in people with DS. Owing limited information about factors involved in the renal abnormalities in DS, and based on the literature [29,37,38,39,45-47], we hypothesized that the overexpression of *APP* and, mainly the $A\beta_{42}$ protein, may be involved with abnormalities reported in the renal tissue of individuals with DS and experimental models. This proposal, as well as the involved action mechanisms [1,19,45,46,48-52] are presented in Figure 5.

After treatment with high-doses of VD_3, the decrease in $A\beta_{42}$ was accompanied by a reduction in Bowman's space in $Ts_{(VD3)}$ similarly to parameters in Wt mice. In humans, the enlarged Bowman's space in the kidneys of DS individuals is associated with a nephropathology, known as cystic kidney disease [53]. There are also reports about a severe disease form, glomerulocystic kidney disease [63,64]. Therefore, in DS, considering cystic kidney disease and risk of complications, interventions that could attenuate the progression of the disease are of extreme importance.

In addition, the presence of $A\beta_{42}$ in the glomerular capillaries, veins and medulla, suggests that this protein comes from systemic circulation. Previously, it was described that in humans and mice, the blood Aβ levels are lower in the inferior vena cava than in the femoral artery, indicating Aβ effluxes of the brain to peripheral blood, which reinforces our hypothesis [40]. In addition, these authors [40] demonstrated that there is a peripheral clearance of brain-derived Aβ.

Although treatment with high-dose VD_3 caused some beneficial actions, such as reduced $A\beta_{42}$, adverse effects in the kidneys of $Ts_{(VD3)}$ and $Wt_{(VD3)}$ mice were observed. Among the adverse effects, a reduction in body weight was noted in groups treated with VD_3.

Experimental studies in humans and mice also observed a body weight loss after VD_3 supplementation [44,56]. The mechanism by which VD_3 can reduce body weight might be related to lipid metabolism [51,57]. These findings suggested the existence of feedback between the supplementation and need to store VD_3, and consequently, could affect lipolysis rates, resulting in a loss of body weight. The mechanisms [51,52,58] are presented in Figure 5.

Additionally, VD_3 promotes structural alterations in the kidneys. These morphological changes were accompanied by a reduction in caspase-3 p12 in $Wt_{(VD3)}$ and increase in $Ts_{(VD3)}$ groups. Caspase-3 is an important mediator for cellular apoptosis in the kidneys [10]. Here, we analyzed caspase-3 p12 to investigate alternative pathways, because depending on the stimulus, complexes involved in inflammation and apoptosis, such as p17/p12 or p19/p12, can be formed [8]. The activation of caspase 3 is a critical step in the pathways that lead to morphological and biochemical alterations [9]. Based on these studies, a reduction in caspase-3 p12 in $Wt_{(VD3)}$ could indicate the participation of other cellular mechanisms in an attempt to inhibit the apoptosis or pro-inflammatory pathway activation.

In $Ts_{(VD3)}$ mice in response to intrinsic increased $A\beta_{42}$, a high immunoreactivity of caspase-3 p12 may be related to the reversion of morphological changes, as Bowman's space dilation and enhanced relative kidney weight, as previously observed in the $Ts_{(CO)}$. Caspase 3 also has an important role in the cleavage APP protein [59], consequently this can affect $A\beta_{42}$ deposition and apoptosis. For $Ts_{(CO)}$ adult mice, we did not find a relationship between the increase in $A\beta_{42}$ and caspase-3 p12. However, the presence of small clusters of inflammatory cells in the interstitial space (Fig. 5B) may indicate an early-stage response, which over time may progress to complications and affect the kidney morphophysiology.

In parallel with the morphofunctional changes, the Ts factor and high-dose VD_3 also affect plasma creatinine and urea concentration. The alterations in urea and creatinine levels

in relation to the normal limit refers to variations in glomerular filtration rate and tubular function [60,61], and may be associated with renal dysfunction. Despite the VD_3 protective effect to reverse increased urea levels in Ts mice, an elevation in the creatinine level in the group treated with VD_3 was observed, the opposite effect when compared to urea in this group. It is possible that at the time of the animal sacrifice, 10 weeks after the initiation of experiments and treatments, VD_3 worsened the creatinine level, despite improvement in the urea excretion and simultaneous renal structure. Therefore, this effect could be improved with an extended VD_3 treatment. It is interesting to verify the kidney function by assessing glomerular filtration at different times of treatment with VD3. Therefore, although the VD_3 plays an important role in several mechanisms, such as the reduction in $A\beta_{42}$ in the cerebral tissue [19,50,62], more research is needed, especially to assess the adverse effects in peripheral organs, such as the kidneys.

In addition, the data in relation to urea levels showed an association between increased Pgp in the brush border of apical membrane of proximal tubular epithelial cells and reduction urea levels in trisomic and non-trisomic groups that received VD_3 (Fig. 6). Proximal tubules participate in the excretion and reabsorption mechanisms of urea [63]. Moreover, the kidney exerts a role in VD_3 metabolism and owing to the expression of VDR-VD3 receptors in various structures in the renal tissue [17,64,65], VD_3 can also regulate Pgp expression [19] in this tissue.

Moreover, the increase in Pgp contributed to reduced $A\beta_{42}$ in the kidneys of $Ts_{(VD3)}$ mice in the present study. Pgp plays an important role in $A\beta_{42}$ clearance in cerebral tissue [66]. Studies with mice show that VD_3 supplementation increases Pgp immunostaining and expression with reduced amounts of $A\beta_{42}$ in the brain [19]. This suggests that mechanisms similar to those observed in the brain [50,52,41], also occur in the kidney in response the increased levels of Aβ. Further, the presence and localization of small agglomerates of $A\beta_{42}$ in

the interstitial space, glomerulus, vessels, and medulla in the kidneys of $Ts_{(VD3)}$ mice (Fig. 5C), indicate the participation of other effective mechanisms responsive to the VD_3 that may favor $A\beta_{42}$ excretion (Fig. 5E).

Besides these mechanisms, increased MTHFR in $Ts_{(VD3)}$ the kidneys seems to further support the role of VD_3 in $A\beta_{42}$ degradation and morphological restoration (Figure 6). To the best of our knowledge, we are the first to show the immunolocalization of MTHFR in the renal tissue of trisomic and non-trisomic mouse models. The presence of MTHFR in the proximal tubule epithelial cells (cytoplasm and brush-border membrane) and in Bowman's capsules (basement membrane and parietal cells) indicate that this protein may be involved in mechanisms of cell proliferation and survival, such as podocytes and tissue restoration, contributing to a reduced Bowman's space in $TS_{(VD3)}$ mice, similarly to the standard parameters, as observed in wild-type mice. This assumption was strengthened by the fact that MTHFR is important in the process of folate metabolism regulation, which is involved in nucleotide synthesis, DNA replication, cell growth, and survival [22,67].

5. Conclusion

Supplementation with high-dose VD_3 affects renal morphophysiology and attenuates Bowman's space dilation in the kidney of adult female mice model for DS. Moreover, VD_3 supplementation influences the presence and localization of caspase-3 p12, Pgp, and MTHFR proteins and contributes to reduced $A\beta_{42}$.

Thus, this research can contribute to new studies and perspectives for a better understanding of the possible causes of nephropathies in individuals with DS and the role of kidney in the peripheral clearance of $A\beta_{42}$, as well as possible implications involved in the development of early-onset Alzheimer disease in DS. In addition, the use of high doses of VD_3 requires further investigation to determine if VD_3 supplementation could lead to adverse implications in renal morphophysiology.

Conflict of interest statement

The authors have declared that have no conflicts of interest.

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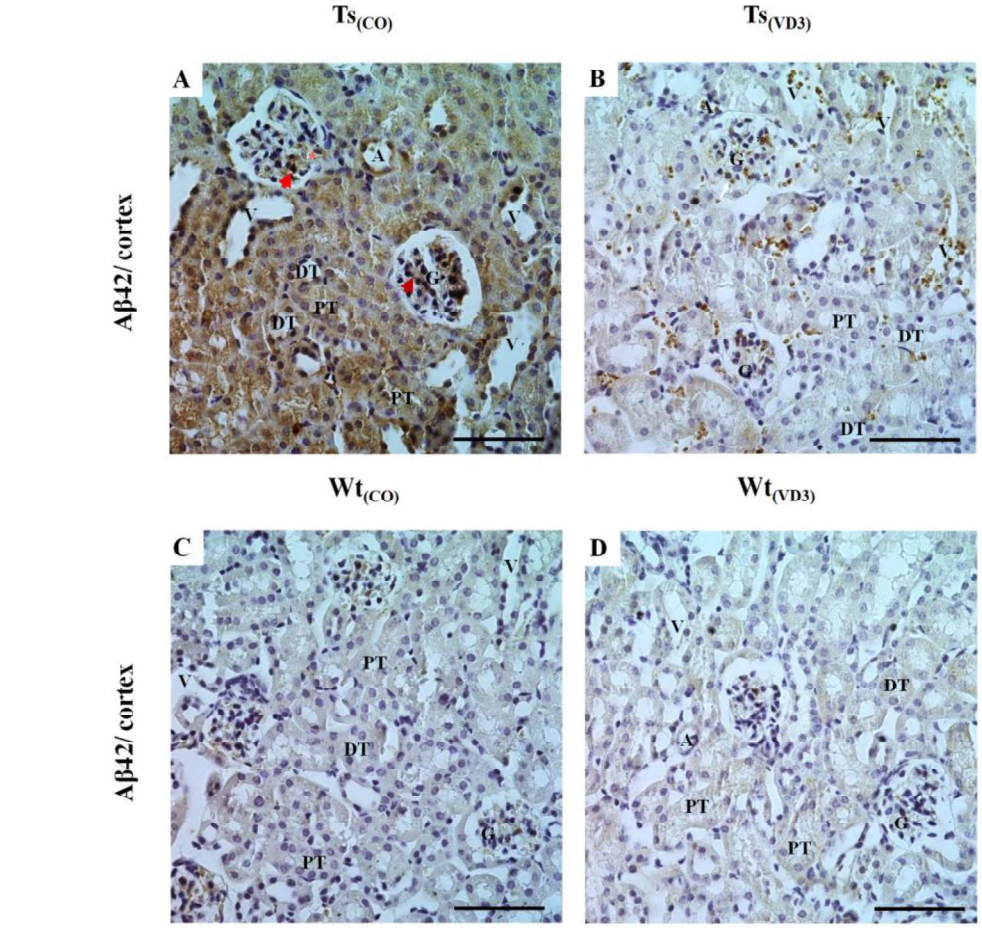
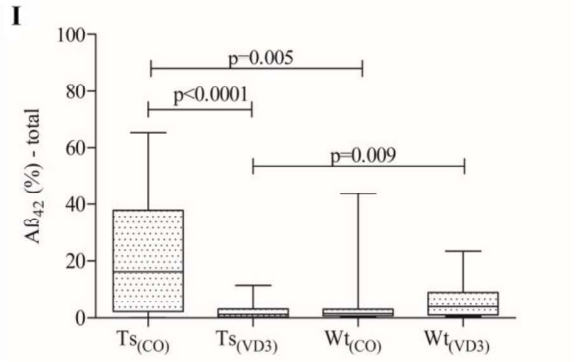
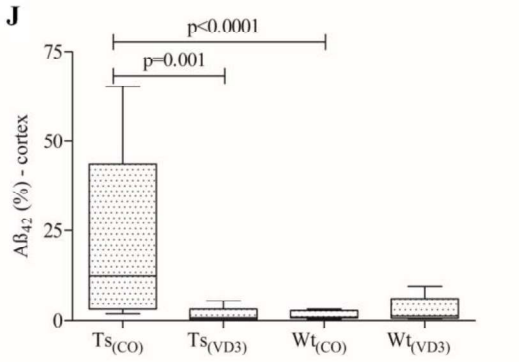
Table 1. Biometric parameters, renal functional, and morphometric analyses in the kidney of the different groups.

	$Ts_{(CO)}$	$Ts_{(VD3)}$	$Wt_{(CO)}$	$Wt_{(VD3)}$	**p (Trisomy)**	**p (Vitamin D3)**	**p (Interaction)**
Biometric parameters							
Body weight (Kg)	0.024 (0.020 – 0.027)	0.020 (0.017 – 0.024)[a]	0.026 (0.022 – 0.030)	0.022 (0.019 – 0.025)[b]	0.102	0.027[*]	0.970
Kidney weight (g): absolute[**]	0.173 ± 0.020	0.126 ± 0.018[a]	0.143 ± 0.025	0.134 ± 0.022	0.290	0.010[§]	0.064
Kidney weight (OW/BW): relative[**]	7.360 ± 0.509[d]	6.227 ± 0.585	5.567 ± 0.754	6.062 ± 0.939	0.008[§]	0.334	0.022[§]
Functional analysis							
Plasma urea (mg/dL)	40.43 ± 4.293	21.24 ± 3.853[a]	50.65 ± 10.74	24.64 ± 2.799[b]	0.018[§]	<0.0001[§]	0.181
Plasma creatinine (mg/dL)	0.359 (0.254-0.444)	0.410 (0.392-0.449)[a,c]	0.308 (0.191–0.384)	0.346 (0.294–0.399)	0.028[*]	0.059	0.488
Morphometry							
Bowman's space (µm)	12.72 (10.95 – 22.62)[d]	6.52 (5.72 – 9.31)[a]	5.89 (4.91 – 12.42)	7.00 (6.49 – 11.03)	0.072	0.078	0.004[*]
Glomerulus diameter (µm)	102.66 ± 14.40	81.63 ± 16.41[a]	104.82 ± 23.75	77.79 ± 16.66[b]	0.857	<0.0001[§]	0.525
Diameter of the renal corpuscles (µm)	122.57 ± 17.47	93.81 ± 20.84[a]	118.27 ± 22.63	88.36 ± 19.36[b]	0.353	<0.0001[§]	0.912
Glomerular area (µm²)	8465 ± 1795	6446 ± 2113	8526 ± 2800	5020 ± 1871[b]	0.231	<0.0001[§]	0.192
Area of the renal corpuscles (µm²)	11401 (10152 – 12257)	7468.5 (6627.7 – 9916.8)[a]	10129 (8705.9 – 12232)	6807.6 (5148.5 – 7793)[b]	0.151	<0.0001[*]	0.813

OW: Organ weight. BW: Body weight. Ts: presence of trisomy. Wt: absence of trisomy. CO: control diet. VD_3: vitamin D_3 diet. Interaction (trisomy and vitaminD_3) [*] $p<0.05$, Scheirer–Ray–Hare test + Mann-Whitney post-test or [§] $p<0.05$, two-way Anova + Bonferroni post-test as follows: [a] $Ts_{(VD3)}$ *vs* $Ts_{(CO)}$; [b] $Wt_{(VD3)}$ *vs* $Wt_{(CO)}$; [c] $Ts_{(VD3)}$ *vs* $Wt_{(VD3)}$; [d] $Ts_{(CO)}$ *vs* $Wt_{(CO)}$.



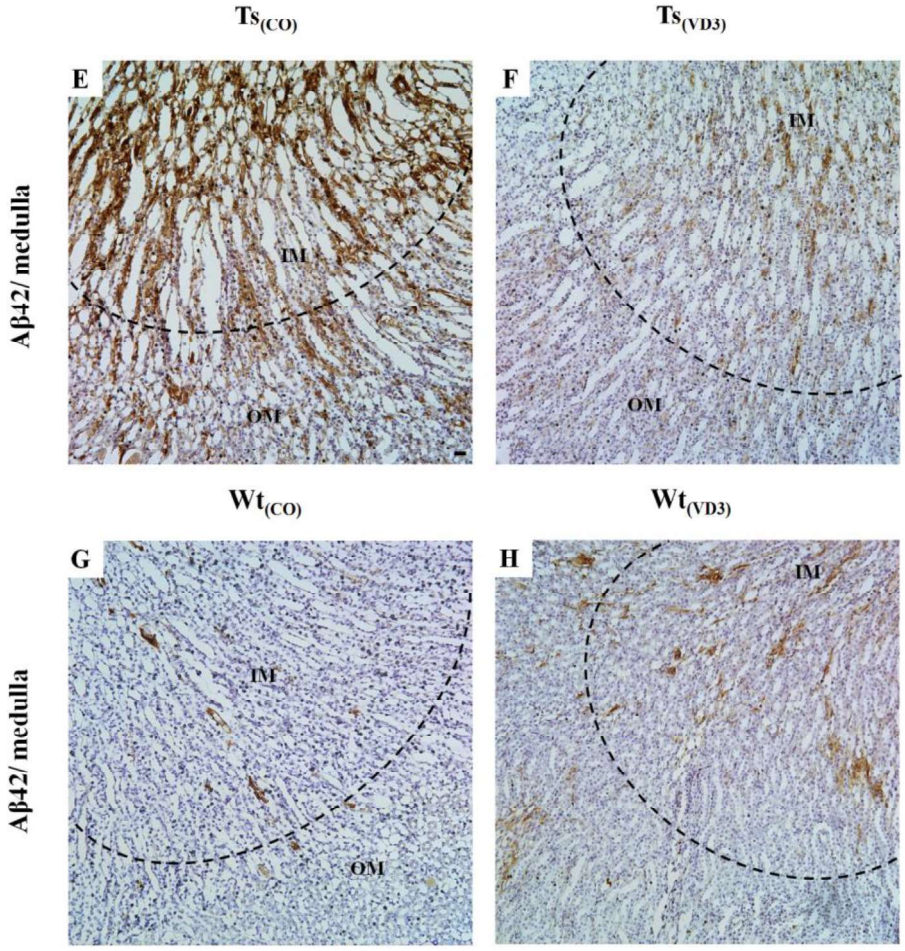


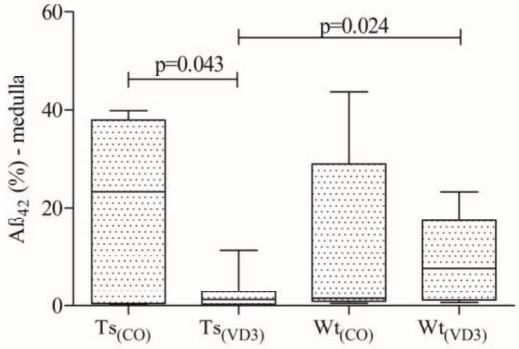


	Ts	VD_3	Interaction
H	0.181	4.610	13.478
p	0.671	0.032	<0.001

	Ts	VD_3	Interaction
H	2.392	4.337	11.158
p	0.122	0.037	<0.001

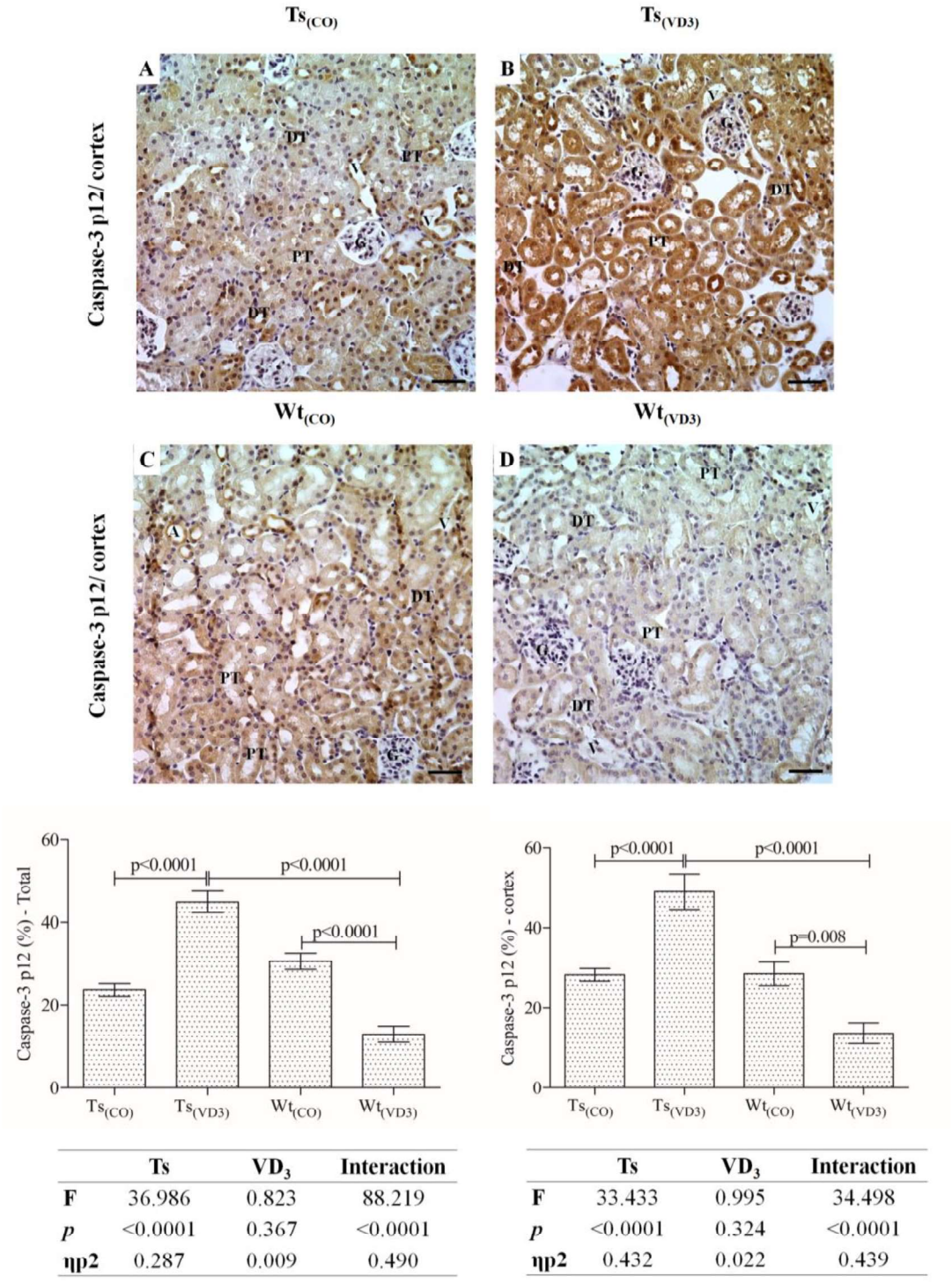
Figure 1.1. Aβ42 immunolocalization. $A\beta_{42}$ protein was identified in renal cortex of the control and experimental groups [$Ts_{(CO)}$ (A), $Ts_{(VD3)}$ (B), $Wt_{(CO)}$ (C), $Wt_{(VD3)}$ (D)]. Kidney structures indicated as proximal tubule (PT), distal tubule (DT), glomerulus (G), veins (V), arteries (A). Yellow arrow indicates the presence of $A\beta_{42}$ in the cell nucleus and among glomerular cells; yellow arrowhead refers to the glomerular capillary. Scale-bars: 60 μm (A-D) objective magnification 60 ×.





	Ts	VD_3	Interaction
H	0.491	1.634	3.599
p	0.483	0.201	0.058

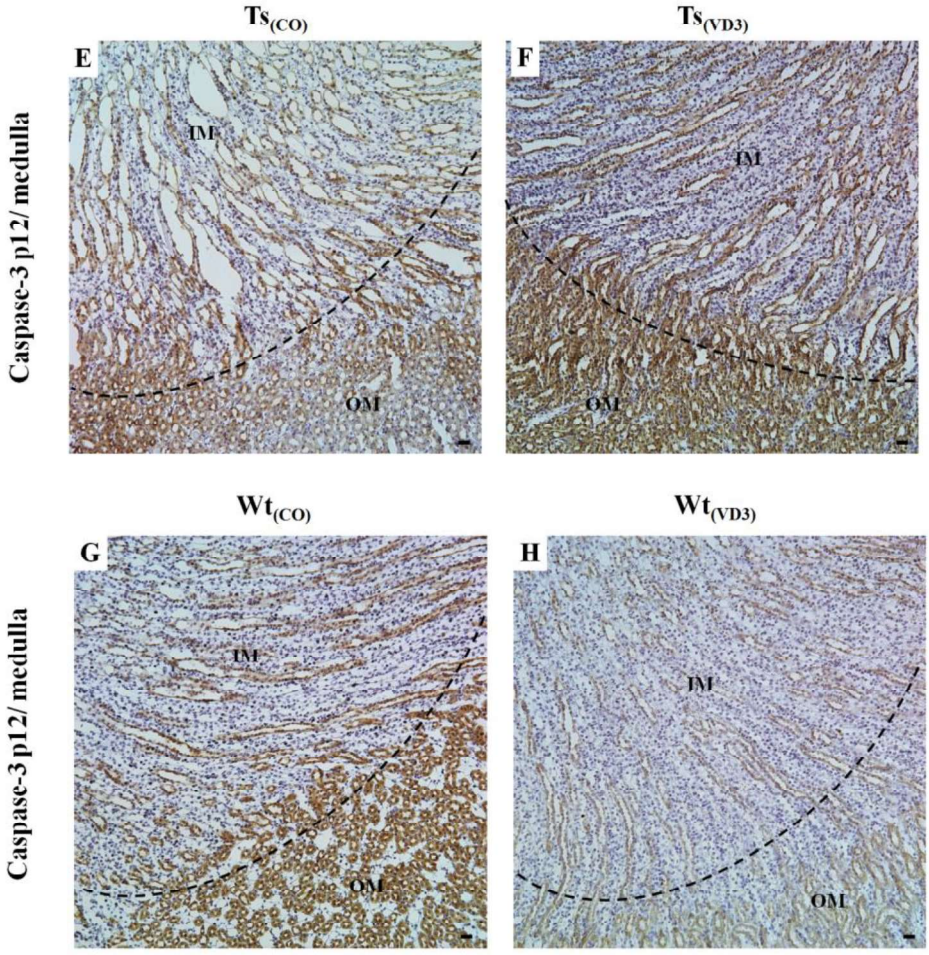
Figure 1.2 Aβ42 immunolocalization. $A\beta_{42}$ protein was identified in medulla of the control and experimental groups [$Ts_{(CO)}$ (E), $Ts_{(VD3)}$ (F), $Wt_{(CO)}$ (G), $Wt_{(VD3)}$ (H)]. Kidney structures indicated as inner medulla (IM), outer medulla (OM). Black dashed circles delimitate medulla IM and OM. Scale-bars: 20 μm (E-H) objective magnification 20 × respectively.

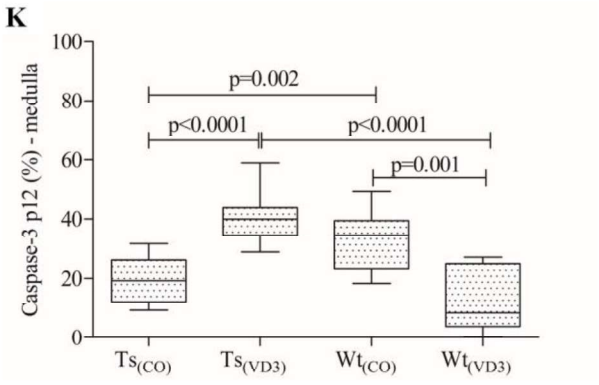


	Ts	VD_3	Interaction
F	36.986	0.823	88.219
p	<0.0001	0.367	<0.0001
ηp2	0.287	0.009	0.490

	Ts	VD_3	Interaction
F	33.433	0.995	34.498
p	<0.0001	0.324	<0.0001
ηp2	0.432	0.022	0.439

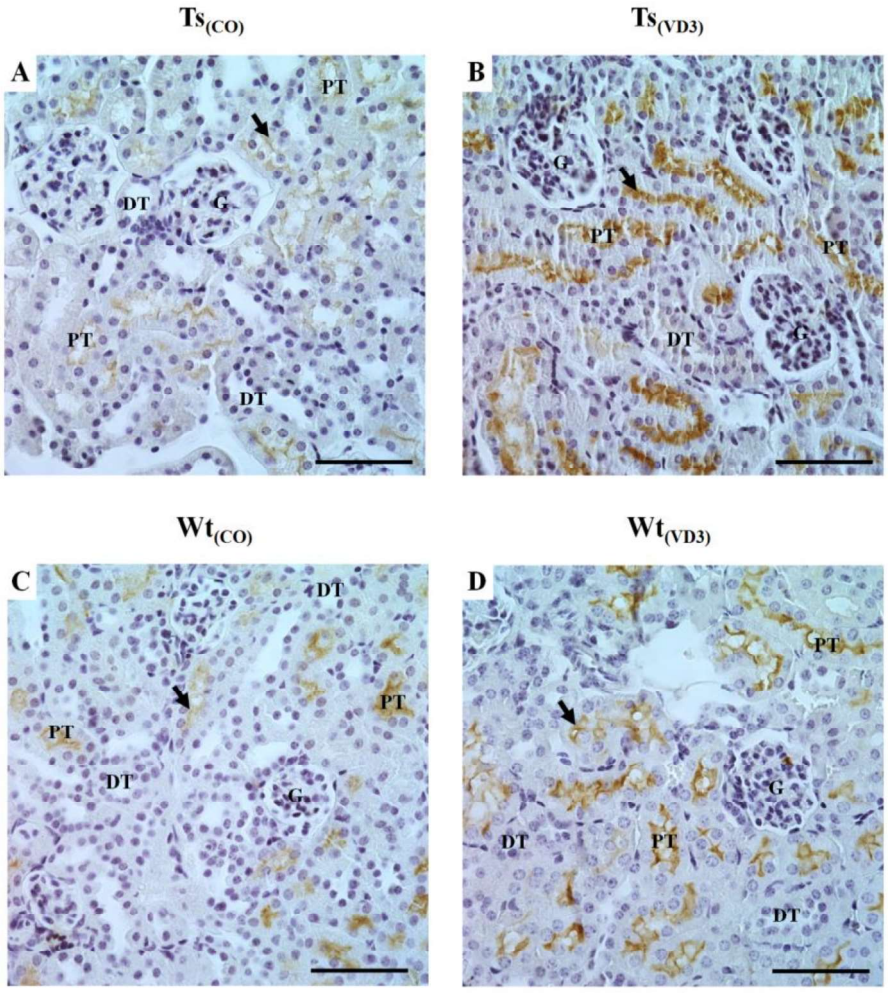
Figure 2.1. Caspase-3 p12 immunolocalization. Renal cortex of the control and experimental groups [$Ts_{(CO)}$ (A), $Ts_{(VD3)}$ (B), $Wt_{(CO)}$ (C), $Wt_{(VD3)}$ (D)] have caspase-3 p12 expression. Kidney structures indicated as proximal tubule (PT), distal tubule (DT), glomerulus (G), veins (V), arteries (A). Scale-bars: 40 µm (E-H), objective 40 ×.

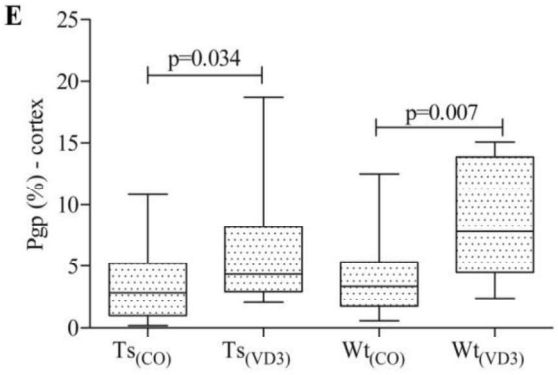




	Ts	VD_3	Interaction
H	3.368	0.083	27.215
p	0.066	0.773	<0.0001

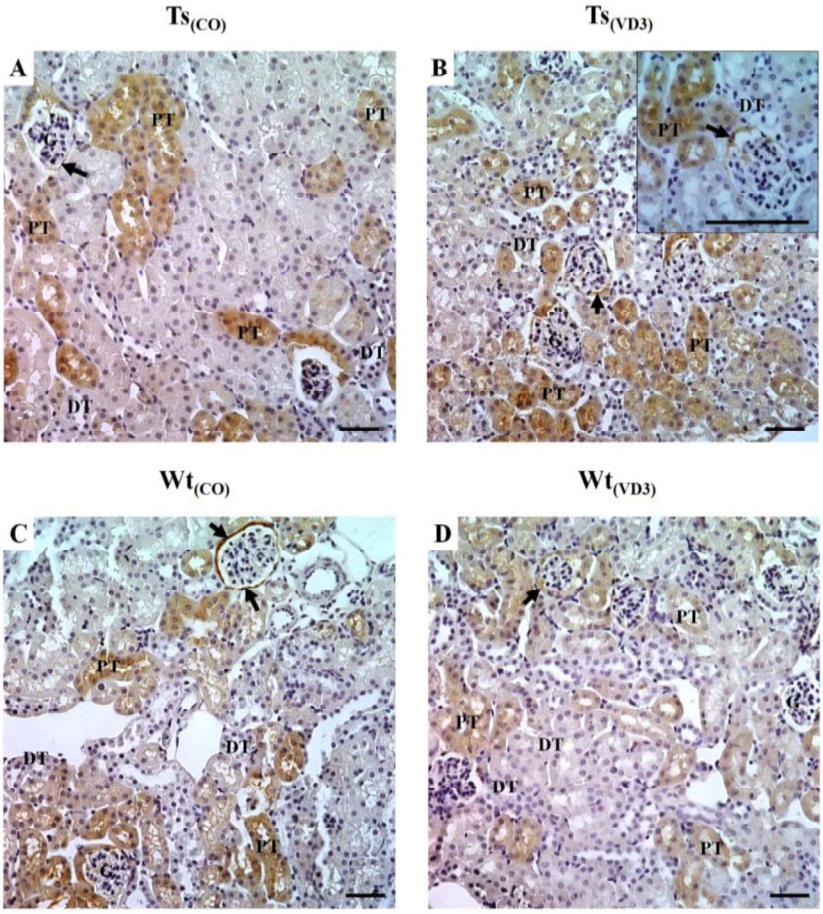
Figure 2.2. Caspase-3 p12 immunolocalization. Renal medulla of the control and experimental groups [$Ts_{(CO)}$ (E), $Ts_{(VD3)}$ (F), $Wt_{(CO)}$ (G), $Wt_{(VD3)}$ (H)] have caspase-3 p12 expression. Kidney structures indicated as inner medulla (IM), outer medulla (OM). Black dashed circles delimitate medulla IM and OM. 20 μm (E-H), objective magnification 20 ×.

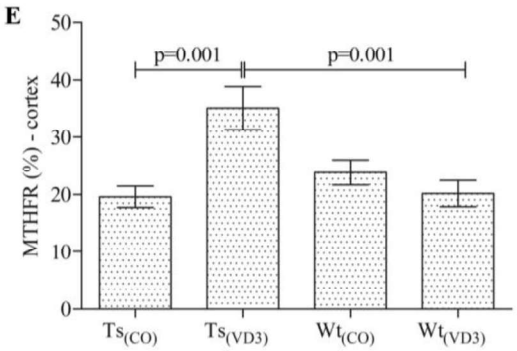




	Ts	VD_3	Interaction
H	1.430	8.696	0.225
p	0.232	0.003	0.635

Figure 3. Pgp immunolocalization. (A-D, scale-bars 60 µm) identified in the renal cortex of the control and experimental groups [$Ts_{(CO)}$ (A), $Ts_{(VD3)}$ (B,), $Wt_{(CO)}$ (C), $Wt_{(VD3)}$ (D)]. Kidney structures indicated as proximal tubule (PT), distal tubule (DT), glomerulus (G). Black arrow indicates brush borders of proximal tubular cells. Objective magnification 40 ×, 60 × and 100 ×. Scale-bars: 60 µm, objective 60 ×.





	Ts	VD_3	Interaction
F	4.091	4.997	13.215
p	0.049	0.031	0.001
ηp2	0.085	0.102	0.231

Figure 4. MTHFR immunolocalization. Renal cortex of the control and experimental groups [$Ts_{(CO)}$ (A), $Ts_{(VD3)}$ (B), $Wt_{(CO)}$ (C), $Wt_{(VD3)}$ (D)] presents expression of MTHFR. Kidney structures indicated as proximal tubule (PT), distal tubule (DT), glomerulus (G). Black arrow indicates the presence of MTHFR in the Bowman's capsule (parietal layer). In higher magnification (detail in B), it is possible to observe the intracellular presence of MTHFR in parietal cells. Scale-bars: 40 µm (A-D) and 100 µm (B), objective magnification 40 × and 100 ×, respectively.

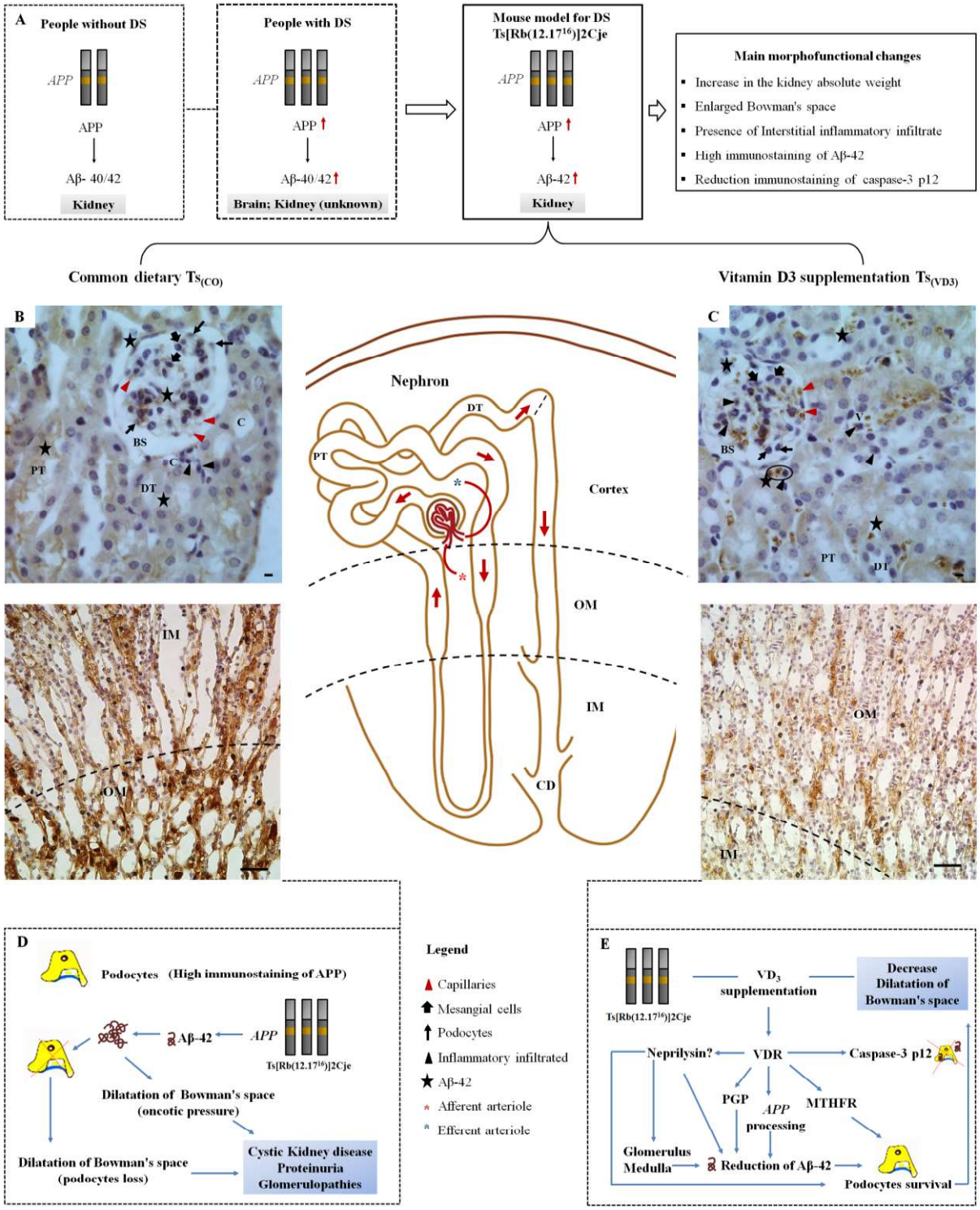


Figure 5. Proposed model for how the overexpression of *APP* and its product, Aβ$_{42}$, may favor the appearance of renal tissue abnormalities in DS mouse model and human trisomy 21. A. Presence of APP protein and Aβ- 40/42 peptides (immunostained) (white box) has been observed in kidneys of people without Down syndrome (DS) [45]. In DS individuals, it has been well-described that *APP* overexpression results in increased levels of Aβ- 40/42 (red arrow) in the brain. However, its expression pattern remains unknown in the kidney. In our study with DS mouse model Ts[Rb(12.17^{16})]2Cje, the overexpression of *APP* and increased levels

of $A\beta_{42}$ are associated with morphofunctional changes. **B and C.** Presence and location of $A\beta_{42}$ in the kidney of $Ts_{(CO)}$ and $Ts_{(VD3)}$ mice. The renal cortex and medulla of both groups (between C and D) when compared to the nephron scheme show structural delimitations. This provides an indication of how $A\beta_{42}$ protein can affect the functional unit of the kidney. The red arrows in the figure (nephron) indicate the blood flow in the vessels from the afferent and efferent arteriole. Kidney structures are indicated as Bowman's space (BS), proximal tubule (PT), distal tubule (DT), glomerulus (G), outer medulla (OM), inner medulla (IM), and collecting duct (CD). Black dashed circles delimitate IM and OM. Other symbols used are explained in the legend box. B and C scale-bars are 10 and 40 μm, respectively (objective magnification 40 and 100 ×). **D**. Mechanism proposed by which *APP* overexpression and increase in $A\beta_{42}$ may be involved in renal abnormalities. In rats, APP is immunolocalized in podocytes with an important role in the glomerular filtration [49]. In our experimental mouse model ($Ts_{(CO)}$ group), *APP* triplication is associated with an increase in levels of $A\beta_{42}$. The agglomeration and nuclear localization of $A\beta_{42}$ in podocytes suggest a crucial point to initiate events that can induce cell death, contributing to the dilation of Bowman's space. $A\beta_{42}$ can also accumulate in the Bowman space and increase the oncotic pressure. Possibly a deregulation caused by increased $a\beta_{42}$ may be involved with some nephropathies reported in individuals with DS (blue box). **E.** Proposed mechanism by which VD_3 can act on clearance of $A\beta_{42}$ in the kidney of Ts mice. The active form of VD_3 binds to the VDR receptor and can induce the expression of genes that regulate or are involved in the expression of proteins, such as Pgp and possibly neprilysin enzymes that participate clearance of $A\beta_{42}$. VD3 may also induce the increase of caspase-3 p12, favoring cellular apoptosis and/or promote the increase of MTHFR contributing to cell proliferation and survival (blue arrows). After the activation of specific mechanisms [19,41,52] several events occur favoring the reduction of $A\beta_{42}$ and cell survival (blue arrow).

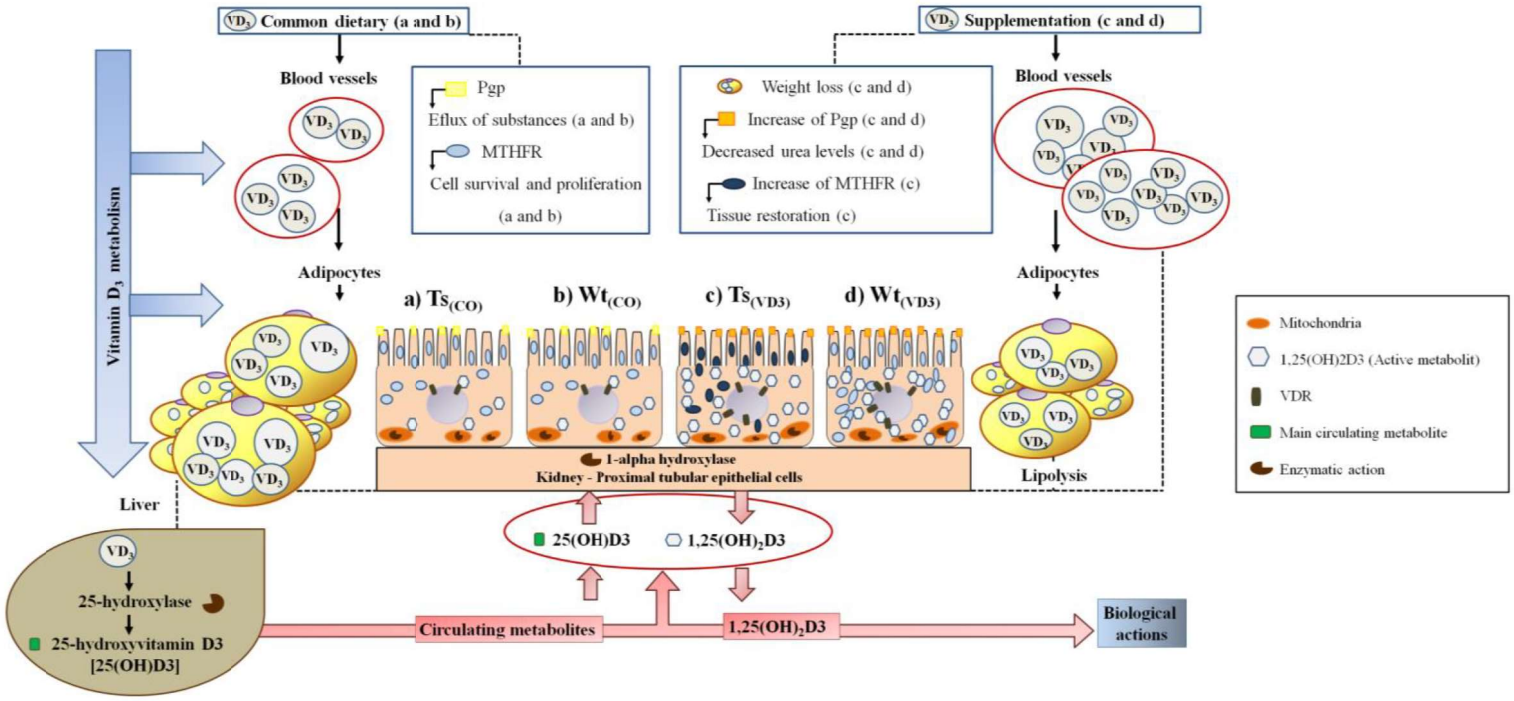


Figure 6. Scheme proposed for the interactions between Pgp and MTHFR proteins in kidney of Ts and Wild-type mice. The VD_3 from a standard diet or supplementation are incorporated into cytochrome and transported through the blood vessels. VD_3 can be stored and released by adipocytes. Circulating VD_3 is transported to the liver and converted into 25-hydroxyvitamin D3 [25(OH)D3] by the enzyme, 25-hydroxylase. 25(OH)D3 is the primary circulating metabolite. In kidneys, specifically in the mitochondria of proximal tubule epithelial cells, 25(OH)D3 is converted by the enzyme 1-alpha hydroxylase into its active form 1,25(OH)2D3, that has biological action [18, 58]. Our findings suggest that VD_3 supplementation causes a high availability of VD_3 and reduces the need for storage, leading to increased lipolysis and thus resulting in body weight loss (c-d). VD_3 supplementation may result in increased levels of 1,25(OH)2D3 that can bind to the nuclear receptor VDR, favoring the increase in Pgp (c-d), and that are associated with the reduction of urea levels. The presence of MTHFR, induced by VD_3, suggests a role for this protein in proliferation and survival cell and with potential contributions to homeostasis in Ts mice renal tissue (d). Notably, the increase in MTHFR is observed only in Ts treated with VD_3. Possibly, this protective role may be due to increased intrinsic $A\beta_{42}$, and therefore, there is a greater need for VD_3-responsive mechanisms [19, 52].

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ARTIGO 4

Título: *Vitamin D_3 supplementation affects the morphology and MTHFR and caspase-3 p12 expression in the olfactory bulb of a mouse model for Down syndrome*

Autores: Fabiana de Campos Gomes[1], João Simão de Melo-Neto[2], Merari de Fátima Ramires Ferrari[3], Orfa Yineth Galvis-Alonso[4], Eny Maria Goloni-Bertollo[1], Érika Cristina Pavarino[1]

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Title page

Vitamin D_3 supplementation affects the morphology and MTHFR and caspase-3 p12 expression in the olfactory bulb of a mouse model for Down syndrome

Fabiana de Campos Gomes[1], João Simão de Melo-Neto[2], Merari de Fátima Ramires Ferrari[3], Orfa Yineth Galvis-Alonso[4], Eny Maria Goloni-Bertollo[1], Érika Cristina Pavarino[1]

Running headline: Vitamin D_3, olfactory bulb & Down syndrome.

[1]Genetics and Molecular Biology Research Unit (UPGEM), São José do Rio Preto Medical School (FAMERP), São José do Rio Preto, SP, Brazil;

[2]Institute of Health Sciences, Federal University of Pará (UFPA), Belém, PA, Brazil;

[3]Department of Genetics and Evolutionary Biology, Biosciences Institute, University of São Paulo (USP), São Paulo, SP, Brazil.

[4]Department of Molecular Biology, São José do Rio Preto Medical School (FAMERP), São José do Rio Preto, SP, Brazil.

Corresponding author:

Dr. Érika C. Pavarino: Genetics and Molecular Biology Research Unit (UPGEM), São José do Rio Preto Medical School (FAMERP). Av. Brigadeiro Faria Lima, 554, São José do Rio Preto, SP, Brazil. CEP: 15090-000. *E-mail*: erika@famerp.br.

Abstract

Individuals with Down syndrome (DS) have olfactory function impairment and are likely to develop Alzheimer's disease (AD). Olfactory dysfunction may be an early clinical symptom of AD. Recent studies show that vitamin D_3 (VD_3) has neuroprotective effects in mouse models of AD. In this study, we evaluated the effects of VD_3 on the morphology, immunolocalization, and expression of markes involved in neuropathogenic processes, apoptosis, proliferation and cell survival, clearance of amyloid peptides and neuron detection in the olfactory bulb (OB) of an adult female mouse model for DS. Morphologic and molecular analysis showed that trisomic mice had a volume reduction in the external plexiform layer, decrease in the number of mitral and granule cells; increase in expression of $A\beta_{42}$, caspase-3 p12 and Pgp. VD_3 may reverse some morphological abnormalities in the OB in $TS_{(VD3)}$ mice; and reduced levels of caspase-3 p12 and MTHFR in the treated groups. The findings showed that the trisomy factor cause morphofunctional abnormalities in the OB of $TS_{(CO)}$ mice. In addition, VD_3 may be a therapeutic target to attenuate morphological and molecular alterations in OB.

***Keywords*:** Down syndrome; trisomic mice; vitamin D_3; olfactory bulb; amyloid beta.

Abbreviations:

Aβ - amyloid beta

AD - Alzheimer disease

APP - beta-amyloid precursor protein

DS - Down syndrome

EOAD - early-onset Alzheimer's disease

EPL - external plexiform

GCL - granule cell layer

GL - Glomerular layer

HE - hematoxylin-eosin

IPL - internal plexiform layer

MCL - mitral cell layer

MTHFR - methylenetetrahydrofolate reductase

NeuN - nuclear protein

Pgp - P-glycoprotein

OB - olfactory bulb

Vitamin D - VD

Vitamin D3 - VD_3

1. Introduction

Down syndrome (DS) is a genetic abnormalities caused by trisomy of chromosome 21 (Antonarakis et al., 2004). The trisomy 21 affect the physiological and morphological development of the brain, resulting in impairment of important functions, such as olfactory processing (Murphy and Jinich, 1996; Chen et al. 2006; Cecchini et al., 2016). The olfactory dysfunction in individuals with or without DS has been associated with Alzheimer disease (AD) (Laak et al., 1994; Cecchini et al., 2016), and can indicate an early clinical sign of dementia (Roberts et al., 2016; Zou et al., 2016; Silva et al., 2018).

In DS, the olfactory deficit and early-onset Alzheimer's disease (EOAD) are observed mainly in adult women, and occasionally in men (Hartley et al., 2014; Cecchini et al., 2016; Schupf et al., 2017). The triplication of beta-amyloid precursor protein (*APP*) located on chromosome 21 is a crucial point in development of EOAD in DS (Hartley et al., 2014). The increase of APP and its derivatives contribute to abnormal production amyloid-β peptides (Aβ), mainly $A\beta_{40}$ and $A\beta_{42}$ peptides (Wiseman et al., 2018). An increased production of peptides $A\beta_{42}$ favors the deposition and formation of amyloid plaques in various brain regions (Head et al., 2012; Head et al., 2016), including the entorhinal cortex, which is involved in olfactory processing (Hof et al., 1995). In Ts65Dn mice, a reduction in olfactory bulb (OB) neurogenesis and impairment of olfactory function has been reported (Bianchi et al., 2014), but the causes of these abnormalities and a potential relationship between morphological and molecular alterations in response to Aβ peptides in the OB remain unknown.

The accumulation of Aβ may compromise the processing and propagation of signals via olfactory glomeruli and among the cells located in different layers of the OB (Laak et al., 1994). Moreover, the increase or deposition of $A\beta_{42}$ peptides can activate pro-apoptotic mechanisms mediated by caspase-3, induce inflammation, and consequently, lead to neuronal death. (Kavanagh et al., 2014; Han et al., 2017). Under pathological conditions, there is a

reduction of nuclear protein (NeuN) positive cells, indicating a possible neuronal loss (Gusel'nikova & Korzhevskiy, 2015).

On the other hand, the 1,25-dihydroxyvitamin D_3 [1,25 (OH)2D3] or VD_3 has shown neuroprotective effects, including neurogenesis and clearance of Aβ peptides (Anjum et al., 2018). VD_3 regulates the expression of mediators involved in the efflux of Aβ, such as P-glycoprotein (Pgp) (Durk et al., 2012; Durk et al., 2014; Landel et al., 2016). In addition, a possible association between VD_3 and folate levels has been described (Lucoc et al., 2018). The folate metabolism is regulated by methylenetetrahydrofolate reductase (MTHFR) that is essential for converts folate into metabolites that can be used in various cellular processes including methylation mechanisms of gene promoter enhancers and proteins, amino acids, DNA, RNA (Spellicy et al., 2012), and process of repair and cell proliferation (Salbaum and Kappen, 2012; Leclerc et al., 2013).

Based on these findings, we investigated the effects of high doses of VD_3 on morphology, immunolocalization and expression of $A\beta_{42}$, caspase-3 p12, MTHFR, Pgp and NeuN of which are involved in the neuropathogenic processes, apoptosis, proliferation and cell survival, clearance and marker of postmitotic neurons in the OB of an adult female mouse model for DS.

2. Materials and methods

2.1. Experimental groups and treatment

Female mice (*Mus musculus,* lineage B6EiC3Sn-Rb(12.Ts17^{16}65Dn)2Cje/CjeDnJ (#004850)) from Jackson Laboratory (Bar Harbor, ME, USA) were kept with the normal diploid male mice of the lineage B6EiC3SnF1/J for mating. The offspring were karyotyped at 21 days of age to determine the presence of the trisomy. Genotyping was performed in the Biosciences Institute, University of São Paulo (USP), São Paulo, SP, Brazil. Animals were maintained in the Bioterium of the São José do Rio Preto Medical School (FAMERP) under

appropriate conditions of lighting (12-hour light-dark cycle) and temperature (23º ± 2ºC). The research was approved by the Ethics Committee for Animal Use of the FAMERP, protocol n. 001-002447/2015.

Twenty female mice (14 weeks old) were distributed into four experimental groups (n = 5 per group): genotype positive (trisomic mice) and negative (wild-type mice) for partial trisomy of chromosome 16, and diet without (control) or with VD_3 supplementation. The distribution of the experimental groups and treatment period are shown in Figure 1. That is, mice were distributed as follows: $Ts_{(CO)}$ (standard diet with positive genotype), $Wt_{(CO)}$ (standard diet with negative genotype), $Ts_{(VD3)}$ (high-dose VD_3 with positive genotype), $Wt_{(VD3)}$ (high-dose VD_3 with negative genotype).

During the first 14 weeks of life, all mice were fed with a standard diet (Nuvilab®, Curitiba, PR, Brazil) and *ad libitum* water. After this period, the $Wt_{(VD3)}$ and $Ts_{(VD3)}$ groups were subjected to a high-dose VD_3 diet for 10 weeks (12,500 IU/kg; Domeneghetti & Corrêa Ltda®, Jaú, SP, Brazil), as proposed by Wergeland et al. (2011).

2.2. Euthanasia, tissue collection, and processing

The mice (24 weeks of age) were anesthetized with high doses (100 mg/kg) of intraperitoneal injection of sodium thiopental (Thiopental®). After total sedation, transcardial perfusion was performed with phosphate-buffered saline solution (PBS). Posteriorly, brains were collected and the left OB was dissected and fixed by immersion in 4% paraformaldehyde diluted in PBS for 24 hours at 4ºC. After fixation, the tissue was embedded in paraffin, and cut with microtome in 6-µm-thick serial coronal sections, and attached to a gelatinized slide. The right OB was immersed in liquid nitrogen and stored at -80° C for Western blotting.

2.3. Stereology and morphology

For stereology and morphological analysis, the slides were stained with Hematoxylin-eosin (HE). Three microscopic fields from each group (three animals group) were captured using a Zeiss Primo Star microscope model coupled to a camera (Zeiss Axiocam 105 color model) and Zen Lite 2.3 software (Zeiss) at magnification 400×. The fields were photographed when all regions evaluated were visible (Tsutiya et al., 2016). Each field was photographed at intervals of 90 μm. As proposed by Weibel et al. (1996), the relative volume (%) of the following layers was analyzed: glomerular (GL), external plexiform (EPL), mitral cell (MCL), internal plexiform (IPL) and granule cell layer (GCL). The method of counting by stereology points has been applied to estimate the volume fraction in the brain (Zhuang et al., 1999). For morphological analyze, the procedures for counting of the number of mitral and granule cells were performed according to the histological procedures described to Zhang et al. (2018). However, the criteria for mitral and granular cell count were performed according to the cells morphological characteristics, being considering 3 fields per animal / at magnification 400×. The images were analyzed by the program ImageJ 1.47, Windows version (National Institute of Health, United States Code, USA).

2.4. Immunohistochemistry and immunolocalization

Sections were deparaffinized and dehydrated with xylene/ethanol and submitted to antigenic recovering with citrate buffer (pH 6.0). After sequential wash stages with PBS, the samples were subjected to a blockade of endogenous peroxidase with 3% hydrogen peroxide, and blockage of non-specific proteins with nonfat milk (MOLICO®) for 1 hour at room temperature. The sections were incubated at 4ºC overnight with the following primary antibodies (abcam®, USA): anti-beta Amyloid 1-42 (1:1000; ab201060; incubation: 2 hours at room temperature), anti-caspase-3 p12 (1:500; ab179517), MTHFR (1:200; ab203789), anti-P Glycoprotein (1:250; ab170904) and anti-NeuN (1:2000; ab177487). On the next day, the sections were washed with PBS and incubated with the secondary antibody (Goat Anti-

Rabbit IgG H &L; 1:500, HRP, abcam®, USA, ab97051) for 1 hour at room temperature. For negative control, seriated sections were incubated only with the secondary antibody. Posteriorly, the sections were washed with PBS, revealed with diaminobenzidine (DAB) chromogen, and counterstained with hematoxylin. The sections were visualized and photographed with a microscope (Camera Zeiss Axiocam 105 color model coupled to the Zeiss Primo Star microscope model at objective magnification 40x) and Zen Lite 2.3 software (Zeiss). Subsequently the images were analyzed using software ImageJ 1.47, Windows version (National Institute of Health, United States Code, USA) for description of immunolocalization.

2.5. Western blotting

OB samples of control and treated groups (five samples/ group) were lysed in PBS (pH 7.4) 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, and 1 mM EGTA, 1% SDS, supplemented with a 1% protease inhibitor cocktail (Sigma-Aldrich; St. Louis, MO). After incubation on ice (30 minutes) the sample were centrifuged and protein fractions were obtained and quantified using the Pierce™ BCA Protein Assay Kit. The protein pools (25 μg of total protein) were applied to 10% or 12% SDS-polyacrylamide gels, according to the molecular weight of each protein. After electrophoresis, proteins were transferred to a nitrocellulose membrane. The membrane was blocked for 1 hour in room temperature with 5% nonfat milk diluted in TBS-T and incubated overnight at 4ºC with: β-actin (1:500; ab8227; abcam®, USA), anti-beta Amyloid 1-42 (1:1000; ab201060; abcam®, USA), anti-caspase-3 p12 (1:500; ab179517; abcam®, USA), MTHFR (1:200; ab203789; abcam®, USA), anti-P Glycoprotein (1:250; ab170904; abcam®, USA) and anti-distribution NeuN (1:2000; ab177487; abcam®, USA). The membrane was washed in a TBS-T buffer and incubated for 2 hours with a secondary antibody (Goat Anti-Rabbit IgG H&L; 1:20.000; HRP, abcam®, USA, ab97051). The reaction was revealed using a chemiluminescent

substrate for the detection of protein bands (Novex™ ECL Chemiluminescent Substrate Reagent Kit). Semi-quantitative densitometry analyses of the bands were performed in ImageJ 1.47, Windows version (National Institute of Health, United States Code, USA). β-actin was used for endogenous control. Results of immunoblot (%) are presented as optical densitometry values (positive band intensity/β-actin ratio).

2.6. Statistical analysis

Data were subjected to the Shapiro–Wilk test of normality. Parametric data were analyzed using a two-way ANOVA (two factors: treatment and trisomy) with a post-hoc Bonferroni test. The independent variables were trisomy (Ts), vitamin D_3 (VD_3), and their interaction (Ts and VD_3). Effect sizes were assessed as follows: low (0.01 to 0.33), moderate (0.34 to 0.66), or high (0.66 to 0.99) as analyzed by partial eta squared (ηp^2). Non-parametric data were analyzed by Scheirer–Ray–Hare test and Dunn's test. Statistical significance was determined at $p < 0.05$.

3. Results

3.1. Stereology and Morphology

Mice trisomic showed a reduction of the EPL volume (Fig. 2.1., 2.2. A; E). In the GL (DS: H = 1.251, p = 0.263; VD_3: H = 1.550, p = 0.213; interaction: H = 1.292, p = 0.256), IPL, and GCL layers did not show significant differences (Fig. 2.1., 2.2. B-E). For morphological analyze, trisomic mice have a lower number of mitral cells (F = 9.941, p = 0.006; η2 0.383) and granule cells (F = 6,343, p = 0.023; η2 0.284). Regarding the effects of treatment, the VD_3 is a determinant factor for the increase of the MCL (Fig. 2.1. B) and number granule cells in $Ts_{(VD3)}$ mice (p = 0.049) compared to the $Ts_{(CO)}$ group (Fig. 2.2. G).

3.2. Immunostaining and Immunoblotting

3.2.1. Trisomic mice showed present of diffuse amyloid deposition and increased expression of $A\beta_{42}$ and caspase-3 p12 in the OB

To investigate a possible neuropathology in the OB, we analyzed the immunolocalization and expression of $A\beta_{42}$ peptides and caspase-3 p12 in tissue (Fig. 3). An immunoreactivity of $A\beta_{42}$ and caspase-3 p12 was detected in all layers of the OB into trisomic mice. In the $Ts_{(CO)}$ group, the presence of diffuse plaques of $A\beta_{42}$, was observed in the EPL (Fig. 3.1. A-B). In the other OB layers, deposits of $A\beta_{42}$ was noted in the nucleus and cytoplasm of neurons, neuroglia cells, mitral and granule cells, and around the blood vessels (Fig. 3.1.B). In $Ts_{(VD3)}$, it was not observed the presence of diffuse $A\beta_{42}$ plaques in the OB after the VD_3 supplementation (Fig. 3.1. C). For the Wt(CO) group, we did not detect a positive immunoreactivity for $A\beta_{42}$ (Fig. 3D). In relation the caspase-3 p12, an immunolocalization was observed in the nucleus and/or cytoplasm of cells located in the GL, EPL, MCL, IPL, and GCL layers, including neurons and interneurons (Fig. 3.1. E-F).

Regard to the expression of $A\beta_{42}$ and caspase-3 p12, the $TS_{(CO)}$ group showed an increase in the expression of $A\beta_{42}$ and caspase-3 p12 as compared to the $WT_{(CO)}$ group (Fig. 3G-I). After VD_3 supplementation for expression of $A\beta_{42}$ we did not find significant differences between $Ts_{(VD3)}$ and $Ts_{(CO)}$ group (Fig. 3.2. H). However, there was a reduction of caspase-3 p12, in the treated groups, being more accentuated in $Ts_{(VD3)}$ mice (Fig. 3.2. I).

3.2.2. VD_3 reduces MTHFR expression, but does not affect Pgp and NeuN expression

To analyze the repercussion of the VD_3 treatment in response to the presence of $A\beta_{42}$ peptides we analyzed the immunolocalization and expression of MTHFR, Pgp and NeuN (Fig. 4). MTHFR was detected intracellularly in almost all layers, and mainly in the GLC, IPL, MCL, and EPL (Fig.4.1. -B). For Pgp an immunostaining was detected in the astrocytes

and endothelial cells of vessels in the layers GL, MCL, IPL, GCL, and mainly in the EPL (Fig. 3C-D). Regard to NeuN, nuclear immunoreactivity was observed in almost all cells of the OB layers (GL, EPL, MCL, IPL, and GCL. (Fig. 4.1. E-F).

In the analysis of protein expression (Fig. 4.2. G-J), VD_3 factor affects the expression of MTHFR causing the reduction in the $Ts_{(VD3)}$ and $Wt_{(VD3)}$ groups (Fig. 4.2. H). For Pgp we detected that trisomic factor contributes to the increase of Pgp in $Ts_{(CO)}$ group, however, the Pgp expression in the OB has not been altered after treatment with VD_3 (Fig. 4.2. I). Regarding to NeuN, the expression were not altered among the experimental groups (Fig. 4.2. J).

3. Discussion

During adulthood, individuals with DS show a severe impairment of olfactory function related to odor discrimination, identification, and threshold (Cecchini et al., 2016). In the mouse model Ts65Dn, there is an impairment of OB neurogenesis and olfactory function in mid-age mice (Bianchi et al., 2014); however, the mechanisms involved in theses change are not well understood. Given that individuals with DS develop early AD (Head et al., 2004) and that olfactory dysfunction is one of the first clinical symptoms of AD (Zou et al., 2016; Roberts et al., 2016; Silva et al., 2018), knowledge about the effects of $A\beta_{42}$ peptides on morphological structure and other cell mechanisms in the OB is critically important.

Morphologically, was observed in trisomic mice an intrinsic reduction in the volume of EPL and number of mitral and granule cells. The EPL is largely neuropil and has an important role in the processing of the olfactory information (Hamilton et al., 2005). This layer is composed of different cell types, including tufted cells and intrinsic interneurons (Hamilton et al., 2005; Nagayama et al., 2014). The signs of odors are processed within the glomerulus, and subsequently transmitted to tufted cells and dendrites of mitral cells that extend into the EPL, where other events occur so that the information can be processed in the

OB (Nagayama et al., 2014). Recently, a study showed that the OB of Ts65Dn mice present impaired neurogenesis (Bianchi et al., 2014), possibly this impairment, may affect the morphologic structure of the tissue at the cellular level, affecting the morphology and number of some cells types, and concurrently the volume of the OB layers as observed in this study.

Nevertheless, beneficial effects on MCL was observed after the VD_3 supplementation. The VD_3 factor contribute to increases in the volume of the MCL and number of granule cells. The MCL is composed of mitral cells, which cells receive olfactory stimuli, and through the axonal projections out of the OB, participate in the regulation of information directed to the olfactory cortex (Nagayama et al., 2014). The mitral cells represent a small percentage of cells of the MCL, other cell types, including local interneurons such as granule cells, are observed in this layer (Panhuber et al., 1985; Nagayama et al., 2014).

Granular cells are a type of abundant inhibitory interneuron in the OB, play an important role in the processing of the olfactory information including the inhibition of tufted and mitral cells via dendrodendritic synapses (Nunes and Kuner, 2015). In addition, most newly generated cells in OB become granules cells, a small percentage of cells become periglomerular cells or astrocytes (Pignatelli and Belluzzi, 2010; Li et al., 2015; Lledo and Valley, 2016). Therefore, taking into account that VD_3 acts in the regulation of a variety of neurotrophic factors, of which influence the process of differentiation, survival, growth, neuronal proliferation and neurogenesis (Groves and Burne, 2017), VD_3 supplementation had a positive effect on number of granule cells and in the volume of the MCL, as observed in the treated mice.

In parallel the morphological changes, in the $Ts_{(CO)}$ group, we found $A\beta_{42}$ positive staining in the cytoplasm and intercellular space in all OB layers. The increased $A\beta_{42}$ expression and $A\beta_{42}$ staining with or without plaque formation in the different OB layers indicates a possible relationship with morphological and molecular alterations observed in

trisomic mice. Accumulation and deposition of amyloid plaques has been detected through immunohistochemistry in the OB and other brain regions of individuals with AD and in the Tg2576 AD mouse model of AD (Zhang et al., 2010, Kenney et al., 2018). In APP/PS1 transgenic mouse models of AD, plaque deposition of $A\beta_{42}$ compromises olfactory function and odor behavior (Yao et al., 2016). Therefore, according to these previous findings, in this study it is probable that morphofunctional alterations in the OB in $Ts_{(CO)}$ may occur in response to increase of $A\beta_{42}$.

Although VD_3 supplementation has not significantly reduced $A\beta_{42}$ expression in OB of $Ts_{(VD3)}$ group, it is important to highlight that was not observed the presence of deposits in the OB after VD_3 supplementation, perhaps a longer treatment time could result in a significant reduction of $A\beta_{42}$ expression in trisomic mice. The B6EiC3Sn-Rb(12.Ts17^{16}65Dn)2Cje model used in this study is genetically identical to the Ts65Dn model, both have elevated *APP* expression (Villar et al., 2005). In normal physiological conditions the *APP* gene is expressed in the brain of humans and mice (Puig and Combs, 2012). Although not entirely clear, APP and its products play an important role in synaptic function and plasticity in the mouse brain (Nalivaeva and Turner, 2013). However, alterations in the processing and cleavage of APP induces the amyloidogenic pathway causing an increase in the long fragments of Aβ, resulting in the development of neuropathologies as AD (Ludewig and Korte, 2017).

During the development of neuropathology several markers and pathways are altered including caspase-3 p12 (Shen et al., 2017). In the OB of $Ts_{(CO)}$ mice exist an increase in the expression of caspase-3 p12. However, the VD_3 supplementation reduced the expression of caspase-3 p12 in the $Ts_{(VD3)}$ and $Wt_{(VD3)}$ group. In the brain, caspase-3 p12 is involved in the activation of apoptotic and pro-inflammatory pathways (Kavanagh et al., 2014). Moreover, caspase 3 has an important role in degradation of APP (Rohn and Head, 2008). The increase

in caspase 3 indicates greater proteolytic APP processing, production of Aβ peptides, synaptic loss, neuronal death, and development of AD (Gervais et al., 1999; Rohn and Head, 2008). On the other hand, vitamin D reduces activation of apoptotic mechanisms and markers, including caspase-3 (Yuan et al., 2018). In this context, the reduction of caspase-3 p12 in treated groups indicates a positive effect of the VD_3 on OB.

Still considering others effects of the VD_3 supplementation, the reduction of MTHFR in treated groups suggests that the VD_3 may affect the expression of this protein. Alterations in the expression and production of metabolites involved in the folate metabolism regulated by MTHFR induce demethylation of DNA and may result in increased expression of some genes associated with pathophysiology of AD, that encode enzymes involved in the cleavage of APP, and consequently contributes for increase and deposition de $A\beta_{42}$ (Román et al., 2019). However, further studies are needed to investigate the impact of MTHFR reduction on the morphophysiology of the OB.

For Pgp protein, although some studies have shown an increase this protein after treatment with VD_3 (Chow et al., 2011; Durk et al., 2014), in this study, we did not find similar results. Interestingly, the trisomy factor contributes to the increased expression of Pgp in the OB. In the brains of mouse models of DS the enzymes expression involved in the clearance of Aβ, such as insulin-degrading enzyme and neprilysin are not changed in hippocampus (Wiseman et al., 2018), however, the Pgp expression has not been analyzed in the brain in DS mouse models. Pgp is a protein expressed in the brain, mainly in vessels and astrocytes (Aryal et al., 2017). This protein and others transporters of the ATP-binding cassette (ABC) are important elements of the blood-brain barrier (BBB) for avoiding or minimizing the effects of toxic substances or components that may penetrate or accumulate in brain (Löscher and Potschka, 2005), such as $A\beta_{42}$ (Durk et al., 2014). In this study, the

increased expression of Pgp in the OB of the $Ts_{(CO)}$ may indicate the need of the increase of efflux mediated by this protein, probably, to optimize the clearance of $A\beta_{42}$.

In our previous study (Gomes et al., 2019), we found increased Pgp in the renal tissue of trisomic mice treated with high doses of VD_3. This finding indicates two important events: VD_3 can act primarily by stimulating the increase of peripheral clearance of $A\beta_{42}$, and secondarily, the increase in peripheral clearance of $A\beta_{42}$ in the kidney helps in optimizing the efflux of this peptide, reducing the necessity of the activation of Pgp-mediated clearance mechanisms in the OB, as reported in this study.

Despite new findings on morphological and molecular aspects in the OB in DS mouse model, it is important to consider that our study present some limitations regarding the low sample size for group used in histological and morphological analyzes. However, we emphasize that all analyzes were performed as described in other studies, which does not make the relevance and new contributions on the subject unfeasible.

4. Conclusion

In sum, our findings show the intrinsic presence of some morphological and molecular abnormalities in the OB of Ts mice, suggesting that $A\beta_{42}$ may play a crucial role in morphofunctional abnormalities, and consequently, can lead to functional impairment of the OB. However, treatment with high-dose diets of VD_3 can attenuate and reverse some morphofunctional and molecular alterations in OB of Ts mice, indicating its neuroprotective function to minimize some morphofunctional alterations in the OB, probably, in response to increase of $A\beta_{42}$.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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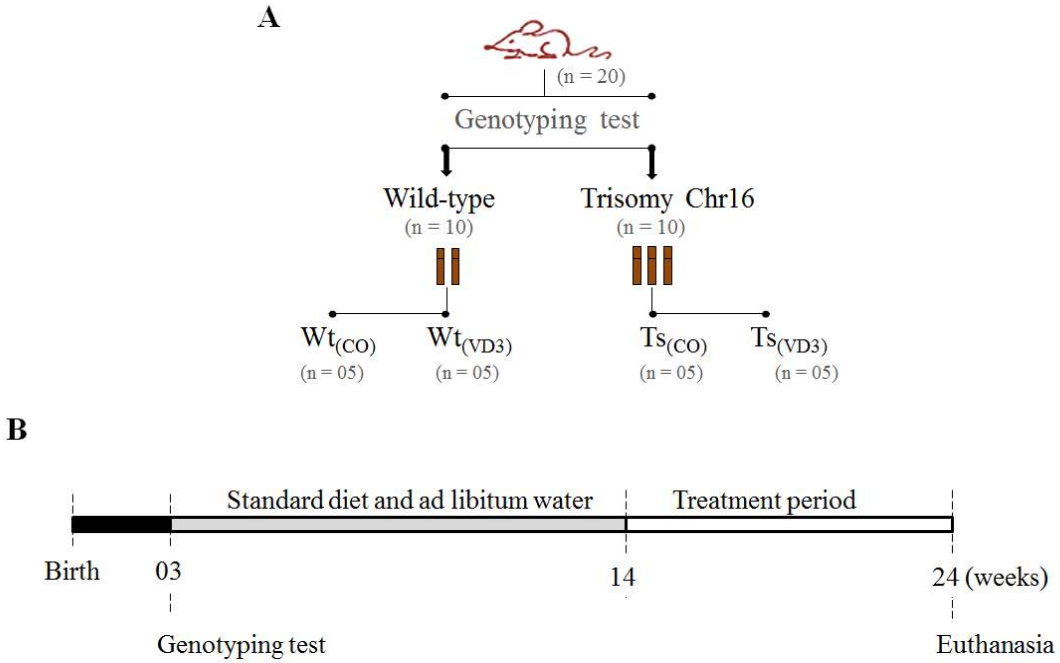
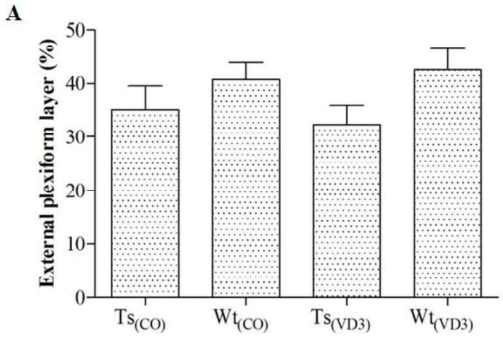
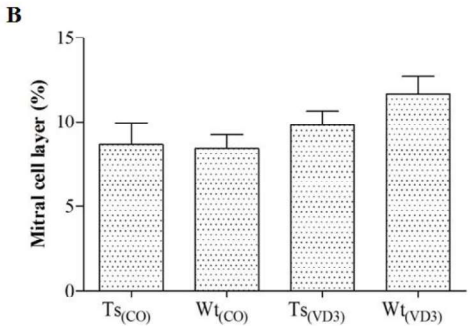


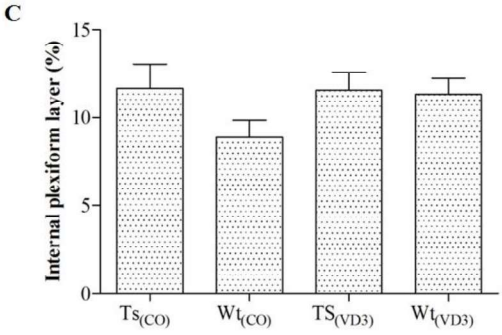
Figure 1. Experimental groups (A) and timeline of experimental procedures (B).



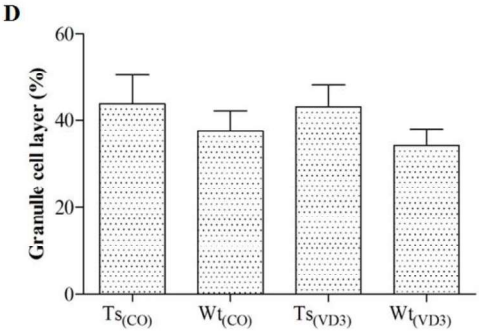
	Ts	VD_3	Interaction
F	4.313	0.017	0.355
p	0.046	0.897	0.556
ηp^2	0.119	0.001	0.011



	Ts	VD_3	Interaction
F	0.681	4.602	0.021
p	0.415	0.040	0.126
ηp^2	0.021	0.304	0.033



	Ts	VD_3	Interaction
F	1.934	1.170	1.403
p	0.174	0.288	0.245
ηp^2	0.057	0.035	0.042



	Ts	VD_3	Interaction
F	2.141	0.161	0.062
p	0.153	0.691	0.805
ηp^2	0.063	0.005	0.002

Figure 2.1 Stereological analysis in different layers of the OB in adult female mouse Ts and Wt of control and experimental groups. OB: olfactory bulb; Ts: trissomic; Wt: wild-type.

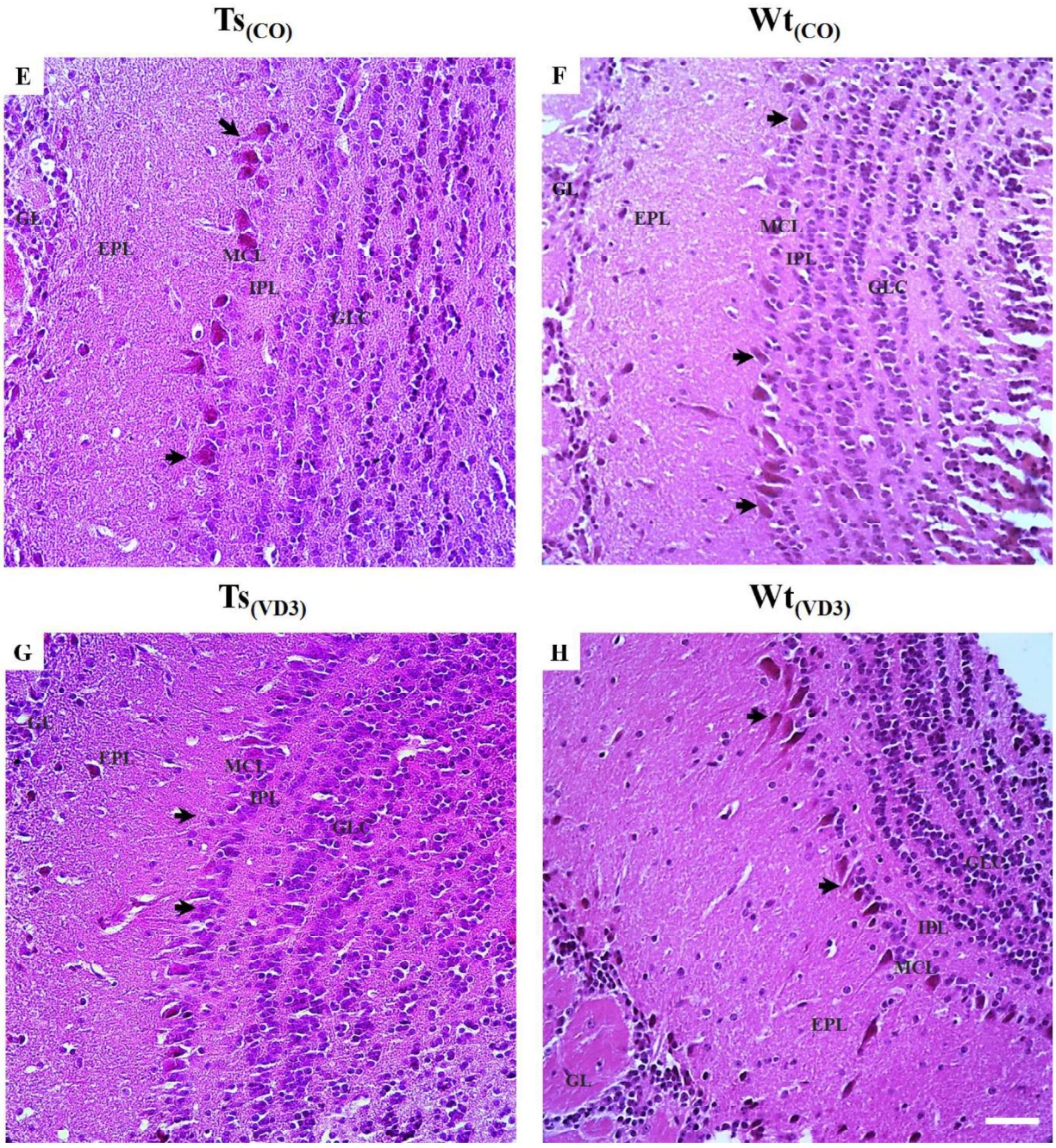


Figure 2.2. Representative images of HE-stained showing morphological aspects of olfactory bulb in groups $Ts_{(CO)}$ (E), $Wt_{(CO)}$ (F), $Ts_{(VD3)}$ (G), and $Wt_{(VD3)}$ (H). Glomerular layer (GL); external plexiform layer (EPL); mitral cell layer (MCL); internal plexiform layer (IPL); granule cell layer (GCL). Mitral cells (arrows). Objective magnification: 40x (E–H). Scale bar: 40 μm (E–H).

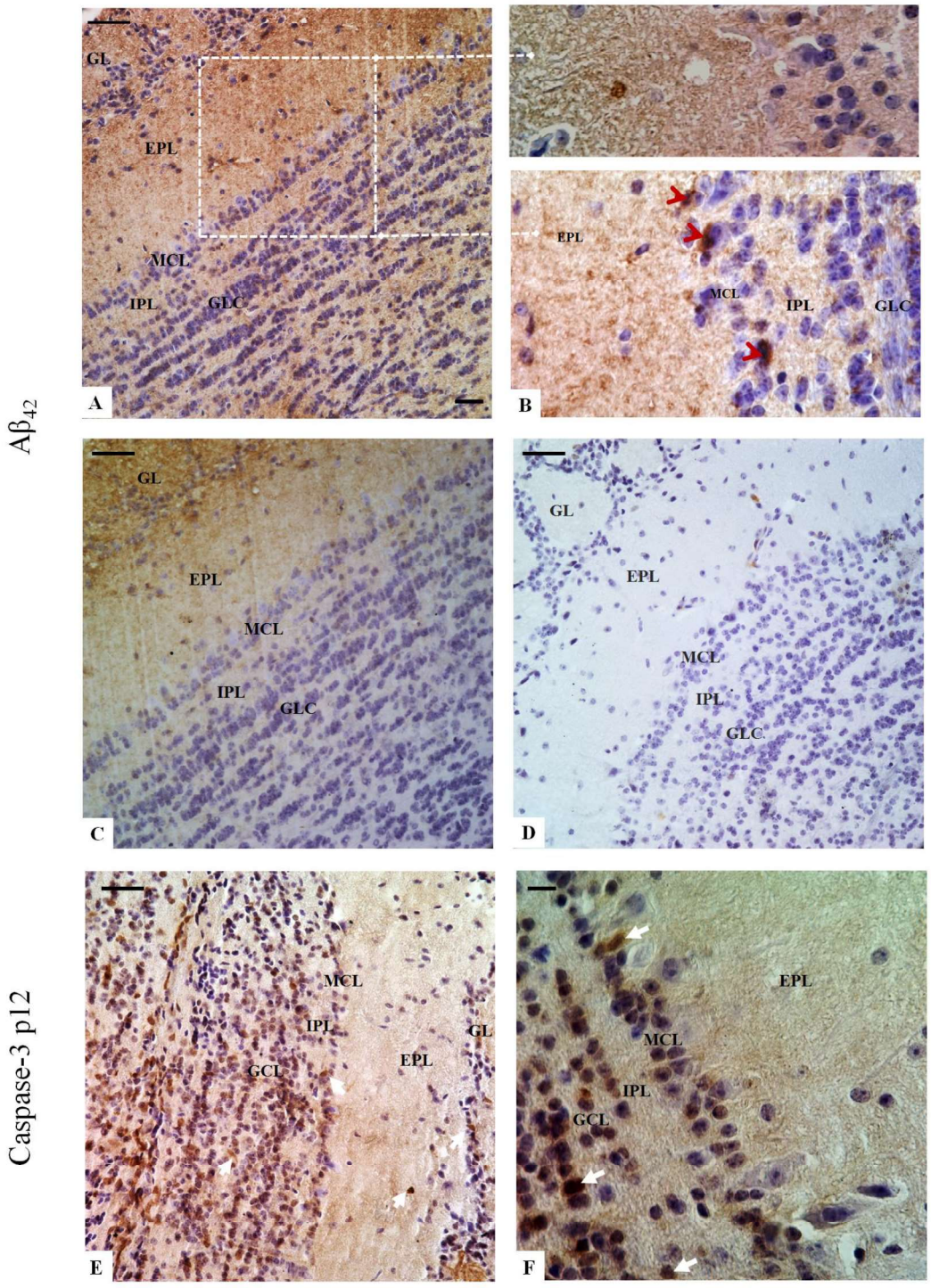
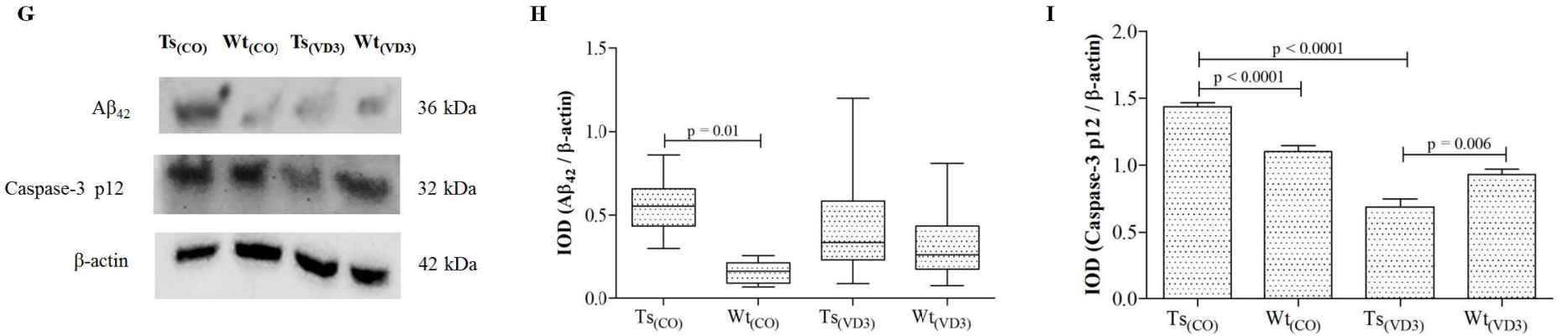


Figure 3.1. Immunohistochemical localization and expression of $A\beta_{42}$ and caspase-3 p12. Immunolocalization of $A\beta_{42}$ and presence of plaques (arrow) in the OB for the $Ts_{(CO)}$ (A-B). $Ts_{(VD3)}$ group is possible to observe ausence of plaques of $A\beta_{42}$ (C). $Wt_{(CO)}$ group (D). Immunolocalization for caspase- 3 p12 (white arrows) in the OB for the $Wt_{(CO)}$ group (E-F). External plexiform layer (EPL); mitral cell layer (MCL); internal plexiform layer (IPL); granule cell layer (GCL). $A\beta_{42}$ deposition in the layers and cells (arrow); Amyloid plaques (dotted frame); Presence of $A\beta_{42}$ deposition in the MCL (arrow head). Objective magnification: 40× and 100×. Scale bar: 10 µm.



	Ts	VD_3	Interaction
H	8.674	0.041	3.102
p	0.003	0.840	0.078

	Ts	VD_3	Interaction
F	1.153	107.729	42.725
p	0.296	<0.0001	<0.0001
ηp^2	0.055	0.843	0.681

Figure 3.2. Representation and analyse of the immunoblotting for Aβ42, caspase-3 p12 and β-actin (G) and analyze of the expression for Aβ42 (H) and caspase-3 p12 (I). Aβ42: amyloid beta-42.

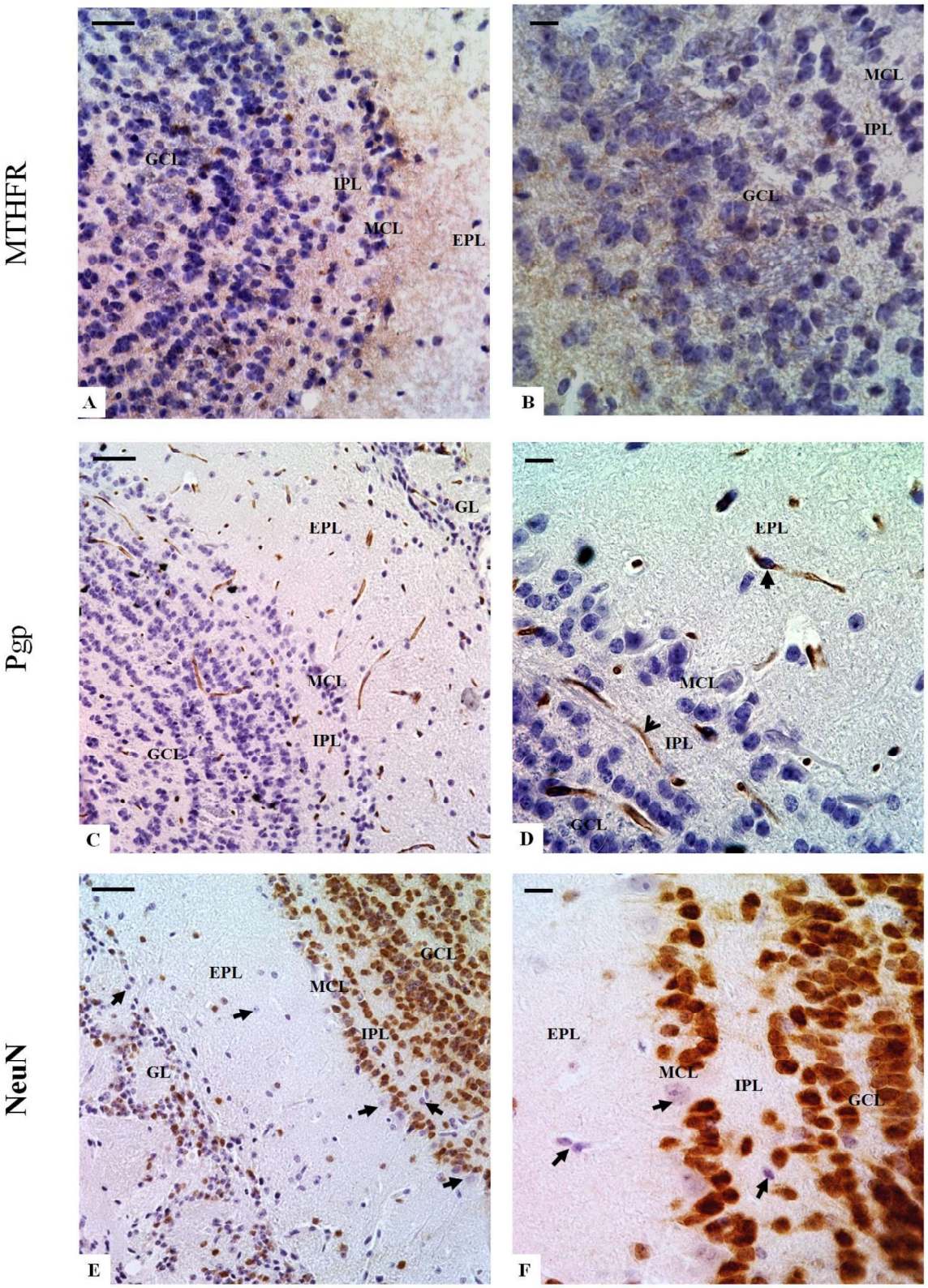
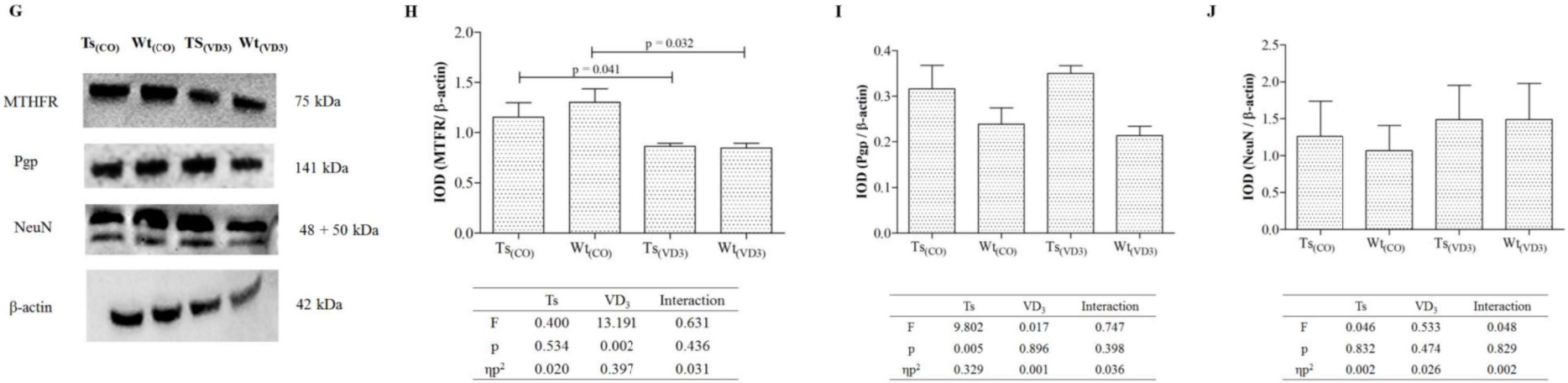


Figure 4.1. Immunohistochemical localization MTHFR (A-B), Pgp (C-D) and NeuN (E-F). Black arrows for indicate immunolocalization of the proteins in the OB. External plexiform layer (EPL); mitral cell layer (MCL); internal plexiform layer (IPL); granule cell layer (GCL). Objective magnification: 40× and 100×. Scale bar: 10 µm.



	Ts	VD_3	Interaction
F	0.400	13.191	0.631
p	0.534	0.002	0.436
ηp^2	0.020	0.397	0.031

	Ts	VD_3	Interaction
F	9.802	0.017	0.747
p	0.005	0.896	0.398
ηp^2	0.329	0.001	0.036

	Ts	VD_3	Interaction
F	0.046	0.533	0.048
p	0.832	0.474	0.829
ηp^2	0.002	0.026	0.002

Figure 4.2. Representation and analyse of the immunoblotting for MTHFR, Pgp, NeuN and β-actin (G) and analyze of the expression for MTHFR (H), Pgp (I) and NeuN (J). MTHFR: Methylenetetrahydrofolate reductase; Pgp: P-glycoprotein; NeuN: neuronal nuclei.

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3. CONCLUSÕES

Conclusões

- Os dados sobre a mortalidade e sobrevida, disponibilizados pelo DATASUS, mostram que existe diferença no número de óbitos entre as regiões administrativas, afetando principalmente crianças com idade inferior a dois anos de idade. Etnia e nível de educação estão associados com a mortalidade de indivíduos com SD nas regiões administrativas do Brasil.
- Alguns genes, localizados na região considerada como crítica para muitos fenótipos da SD, estão envolvidos com disfunções e doenças neurológicas, porém as informações sobre um número representativo destes genes permanecem desconhecidas.
- A suplementação com VD_3 atenuou as anormalidades morfofuncionais observadas no rim e BO de camundongos trissômicos.
- O tratamento com VD3 aumentou a imunomarcação das proteínas metilenotetrahidrofolato redudase (MTHFR), caspase-3 p12 e glicoproteína-P (Pgp); e reduziu a βA42 no rim. No BO a VD3 foi um fator importante para redução de caspase-3 p12 e MTHFR.

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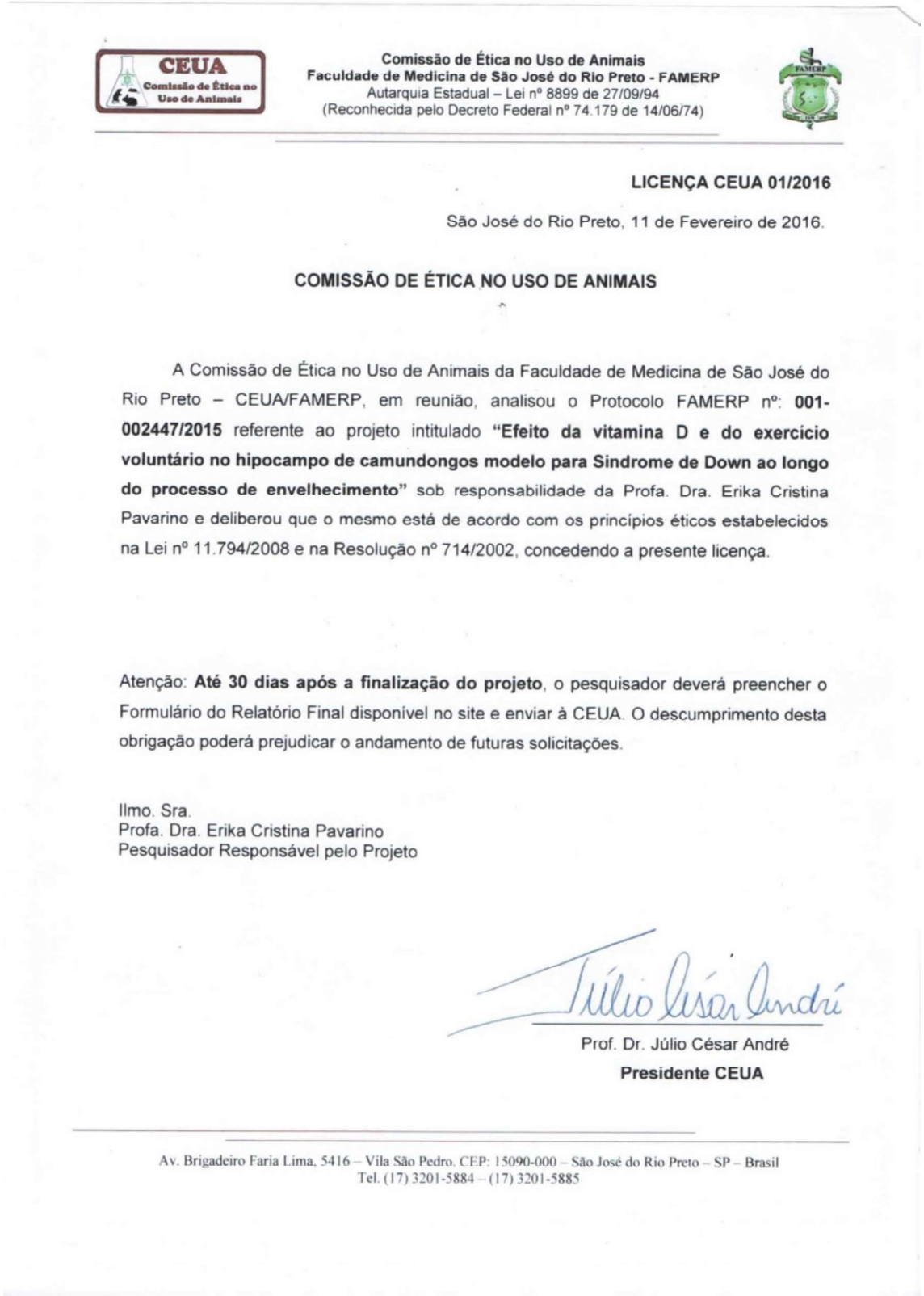
ANEXOS

Aprovação na comissão de ética no uso de animais



Comissão de Ética no Uso de Animais
Faculdade de Medicina de São José do Rio Preto - FAMERP
Autarquia Estadual – Lei nº 8899 de 27/09/94
(Reconhecida pelo Decreto Federal nº 74.179 de 14/06/74)

LICENÇA CEUA 01/2016

São José do Rio Preto, 11 de Fevereiro de 2016.

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

A Comissão de Ética no Uso de Animais da Faculdade de Medicina de São José do Rio Preto – CEUA/FAMERP, em reunião, analisou o Protocolo FAMERP nº: **001-002447/2015** referente ao projeto intitulado **"Efeito da vitamina D e do exercício voluntário no hipocampo de camundongos modelo para Sindrome de Down ao longo do processo de envelhecimento"** sob responsabilidade da Profa. Dra. Erika Cristina Pavarino e deliberou que o mesmo está de acordo com os princípios éticos estabelecidos na Lei nº 11.794/2008 e na Resolução nº 714/2002, concedendo a presente licença.

Atenção: **Até 30 dias após a finalização do projeto**, o pesquisador deverá preencher o Formulário do Relatório Final disponível no site e enviar à CEUA. O descumprimento desta obrigação poderá prejudicar o andamento de futuras solicitações.

Ilmo. Sra.
Profa. Dra. Erika Cristina Pavarino
Pesquisador Responsável pelo Projeto

Prof. Dr. Júlio César André
Presidente CEUA

Av. Brigadeiro Faria Lima, 5416 – Vila São Pedro. CEP: 15090-000 – São José do Rio Preto – SP – Brasil
Tel. (17) 3201-5884 – (17) 3201-5885