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ALBERTO JUNQUEIRA PINTO NETO

FREQUÊNCIA DA VARIANTE GENÉTICA VAL66MET EM PACIENTES PORTADORES DE GLAUCOMA EM SALVADOR

Salvador BA
2022

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Orientadora: Prof.ª Dr.ª Camila A. V. de Figueiredo

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ATA DA SESSÃO PÚBLICA DO COLEGIADO DO PROGRAMA DE PÓS-GRADUAÇÃO EM IMUNOLOGIA PARA JULGAMENTO DO TRABALHO DE DISSERTAÇÃO INTITULADO: **"FREQUÊNCIA DA VARIANTE GENÉTICA VAL66MET EM PACIENTES PORTADORES DE GLAUCOMA EM SALVADOR"** DO MESTRANDO ALBERTO JUNQUEIRA PINTO NETO

Aos dezenove dias do mês de dezembro do ano de dois mil e vinte e dois, na sala de reunião vinculada ao Programa de Pós-graduação em Imunologia da Universidade Federal da Bahia (PPGIm -UFBA) a Banca Examinadora composta pelos Professores: Dra. Camila Alexandrina Viana Figueiredo Orientadora, Dr. Bruno Castelo Branco; Dra. Verônica Franco de Castro Lima, se reúne com a finalidade de discutir, avaliar e julgar o trabalho de dissertação intitulado "Frequência da variante genética val66met em pacientes portadores de glaucoma em salvador" do mestrando Alberto Junqueira Pinto Neto. Após a apresentação e comentários dos membros da Banca Examinadora fica determinado que as sugestões discutidas sejam implementadas na versão final da dissertação. Havendo cumprido as exigências do Programa quanto à defesa do trabalho final, a Banca Examinadora conclui que mediante a entrega do exemplar final pelo aluno com as devidas modificações no prazo de 60 dias, o Pós-Graduando está habilitado à obtenção do título de Mestre em Imunologia. Adicionalmente, os pareceres individuais dos membros da Banca Examinadora serão anexados à ata. Nada mais havendo a tratar se encerra a sessão da qual é lavrada a presente ata que após lida e aprovada vai assinada pelos componentes da Banca examinadora, pelo mestrando e pela Coordenadora do Programa de Pós-Graduação. Salvador, dezenove de dezembro do ano de dois mil e vinte e dois.

Dra. Camila Alexandrina V. Figueiredo
Orientadora

Dr. Bruno Castelo Branco
Banca Examinadora

Dra.Verônica Franco de Castro Lima
Banca Examinadora

Alberto Junqueira Pinto Neto
Mestrando

Dra. Silvia Lima Costa
Coordenadora do PPGIm
ICS-UFBA

RESUMO

O glaucoma é uma condição bastante frequente e tem o diagnóstico muitas vezes difícil e tardio. O BDNF é um neuropeptídeo, cuja função trófica ajuda na proteção das células ganglionares da retina. A diminuição dos níveis do BDNF no glaucoma tem sido tema de vários estudos. Na procura por elucidação da fisiopatologia glaucomatosa, neste trabalho, pretendemos descrever a frequência de uma variante genética, val66met (rs6265), numa população de portadores de glaucoma e associar a presença desta variante aos diferentes fenótipos da doença. Para isso, um total de 200 pacientes foram recrutados na clínica Oftalmodiagnose Os pacientes são acompanhados nesta clínica pelo Protocolo Clínico e Diretrizes Terapêuticas do Glaucoma, no qual os pacientes recebem acompanhamento regular da doença e colírios para o seu tratamento. O diagnóstico de glaucoma primário de ângulo aberto é feito com pressão intraocular acima de 21mmHg, escavação papilar acima de 0,5 DP e/ou defeito de visão no campo visual. Foram obtidos 8ml de sangue de cada paciente, separado plasma para dosagem de BDNF via Luminex e o *buffy coat* foi utilizado para extração de DNA genômico. A genotipagem foi realizada através de PCR em tempo real usando a tecnologia Taqman em equipamento QuantStudio 12K. A população teve uma média de idade de 63,19 (11,18) anos. 46,8% dos pacientes tinham doença avançada/grave e 53,2% foram classificados como tendo doença precoce/moderada. Encontramos uma frequência genotípica baixa (8,6%) do alelo variante (T) em relação ao mais comum (C). Não encontramos diferenças significantes entre a gravidade do glaucoma e as frequências alélicas. Testamos os níveis plasmáticos de BDNF entre os casos e controles. A média de valores foi de 17,01 (DP 3,92) pg/ml para casos e 365,46 (DP 111,59) pg/ml para controles. Essa diferença foi sugere uma significância estatística ($p<0,01$), apesar do pouco número de amostras já examinadas. Nosso estudo sugere que o BDNF pode ser um biomarcador para o diagnóstico do glaucoma.

Palavras-chave: Glaucoma; BDNF; Val66met; rs6265; Salvador; Brasil; Imunopatologia.

ABSTRACT

Glaucoma is a very common condition and its diagnosis is often difficult and late. BDNF is a neuropeptide whose trophic function helps protect retinal ganglion cells. Low levels of BDNF have been associated with glaucoma in several studies. In the search for elucidation of the pathophysiology of glaucoma, in this work, we described the frequency of a genetic variant, val66met (rs6265), in a population of glaucoma patients and associated the presence of this variant with the different phenotypes of the disease. To this end, a total of 200 patients were recruited at the Oftalmodiagnose clinic. Patients are followed up in this clinic by the Clinical Protocol and Therapeutic Guidelines for Glaucoma, in which patients receive regular monitoring of the disease and eye drops for its treatment. The diagnosis of primary open-angle glaucoma is made with intraocular pressure above 21 mmHg, papillary cupping above 0.5, and/or visual field defect. 8 ml of venous blood was obtained from each patient, plasma was separated for BDNF measurement via Luminex and the buffy coat was used to extract genomic DNA. Genotyping was performed through real-time PCR using Taqman technology in QuantStudio 12K equipment. The population had a mean age of 63.19 (11.18) years. 46.8% of patients had advanced / severe disease and 53.2% were classified as having an early / moderate disease. We found a low genotypic frequency (8.6%) of the variant allele (T) in relation to the most common one (C). We found no significant differences between glaucoma severity and allele frequencies. We tested BDNF plasma levels among cases and controls. Mean values were 17.01 (SD 3.92) pg/ml for cases and 365.46 (SD 111.59) pg/ml for controls. This difference was suggestive of statistical significance ($p<0.01$), despite the small number of samples already examined. Our study suggests that BDNF may be a biomarker for the diagnosis of glaucoma.

Keywords: Glaucoma; BDNF; val66met; Rs6265; Salvador; Brazil; Immunophysiology.

SUMÁRIO

1 INTRODUÇÃO GERAL

O glaucoma é uma neuropatia ótica progressiva e a segunda maior causa de cegueira irreversível do mundo afetando em torno de 3% da população mundial. Todo o tratamento do glaucoma é direcionado para a redução da pressão intraocular (PIO). Atualmente é o único fator modificável para a progressão do dano glaucomatoso. A redução da PIO é eficaz para o controle do glaucoma, embora, a margem dessa redução ainda seja questão de controvérsia.

Estudos recentes demonstraram uma redução dos níveis de *brain derived neutrotrophic factor* (BDNF) no glaucoma, correlacionando estágios da doença com flutuações no nível deste peptídeo e a progressão da lesão neuronal. Estudos laboratoriais em ratos demonstraram que, com o aumento do BDNF mediante aplicação exógena, há proliferação das fibras nervosas da camada de células ganglionares da retina, inclusive havendo sinais de recuperação da função visual.

Neste contexto, o presente estudo se propõe a avaliar a frequência da mutação no gene do BDNF, a val66met (rs6265), e verificar se há associação desta variante com fenótipos de glaucoma em uma amostra de indivíduos de Salvador, Bahia.

2 REVISÃO DA LITERATURA

O olho é uma estrutura bastante complexa e tem situação imunológica privilegiada. A luz chega ao olho pela córnea, sofre refração nesta estrutura e no cristalino para formar uma imagem na retina, de cabeça para baixo e espelhada, de forma bastante semelhante a uma câmera fotográfica. Esta imagem é captada pelos cones e bastonetes, através de despolarização destas células e este impulso elétrico é levado pelo nervo óptico até o lobo occipital no cérebro. Neste local é feita a decodificação e desinversão da imagem (1).

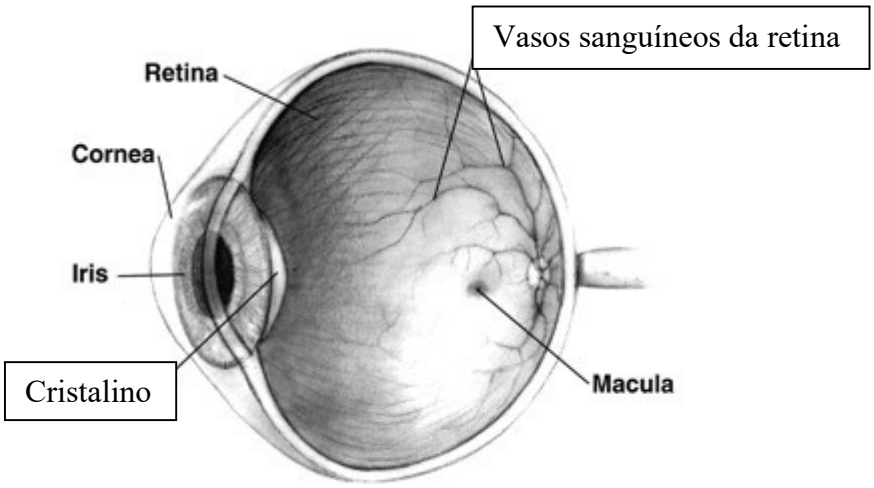

Fig 1 Anatomia do globo ocular. (Rehman I, Hazhirkarzar B, Patel BC. Anatomy, Head and Neck, Eye. [Updated 2021 Jul 31]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK48(adaptada)

Dada a sua função, o olho tem que ser preenchido com estruturas transparentes. Existem duas câmaras principais no olho. A anterior que é preenchida com humor aquoso e a posterior preenchida com humor vítreo. O humor aquoso sofre renovação constante e um desequilíbrio entre sua produção e drenagem acarreta aumento da pressão intraocular (PIO) causando o glaucoma. O aumento da PIO comprime todas as estruturas intraoculares e a mais susceptível ao dano é a camada de células ganglionares da retina, sendo elas as mais internas e que sofrem estreitamento ao atravessar a lâmina crivosa no fundo do olho formando o nervo óptico (1).

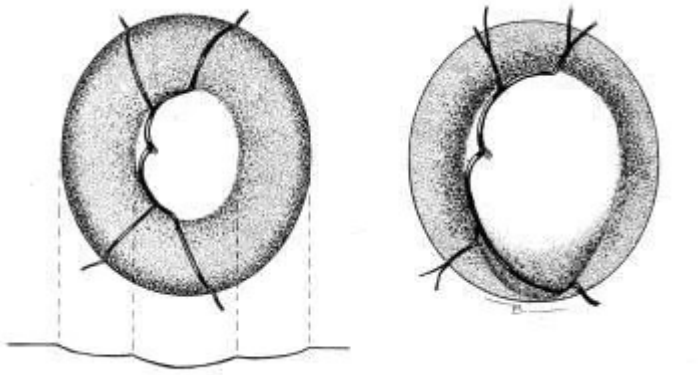

Fig 2 Aumento de escavação do nervo óptico com progressão do dano glaucomatoso. A perda de fibras nervosas causa diminuição da rima neural (fonte: https://emedicine.medscape.com/article/1206147-overview)

O glaucoma é responsável pelo maior número de pessoas portadoras de cegueira irreversível mundialmente e a segunda maior causa de cegueira no total (2). Em 2010, aproximadamente 66,8 milhões de pessoas no mundo padeciam da doença e, com o envelhecimento da população, a tendência é de aumento destes números (3).

O principal meio de tratamento do glaucoma consiste em redução da pressão intraocular (PIO), sendo o método mais embasado por estudos e comprovado e, até o momento, o único modificável (4) (5) (6). A doença se caracteriza por ser uma neuropatia óptica com perda progressiva e inicialmente periférica da visão, com lesão incialmente estrutural dos axônios retinianos com posterior perda funcional. O foco do tratamento do glaucoma desde a sua descoberta tem sido a redução da PIO (7).

O manejo do glaucoma consiste em medicações tópicas (colírios), procedimentos a laser com vistas a redução da PIO e a cirurgia incisional, classicamente a trabeculectomia. O avanço na produção de materiais cada vez menores e mais complexos permitiu o surgimento de cirurgias utilizando micro tubos de drenagem para reduzir a pressão intraocular. Essas cirurgias com próteses minimamente invasivas (MIGS) apresentam vantagem na uma redução na PIO independente da adesão ao tratamento tópico medicamentoso e sem os efeitos colaterais destas substâncias (8). Usualmente o tratamento inicial é realizado com medicações tópicas sendo a cirurgia reservada para casos refratários. O papel do laser como primeira linha é uma opção promissora por não depender da adesão ao tratamento e não ter efeitos colaterais das medicações utilizadas, embora seu uso ainda não seja amplamente utilizado entre os oftalmologistas (9). O tratamento com

colírios é, como com qualquer medicação, sujeito a efeitos colaterais. Efeitos locais como os das prostaglandinas (hiperemia, olheiras, escurecimento na cor de íris mais claras e aumento do tamanho dos cílios) (10) ou sistêmicos como os dos betabloqueadores (bradicardia, tonturas, deflagração de crises de asma e impotência) (11), assim como a baixa persistência e adesão dos pacientes ao tratamento, impõem um desafio enorme ao que é uma doença que acompanhará o paciente por toda vida e, na maior parte dos casos e da sua história natural, assintomática (5) (12) (13) (14). Desta forma, os pacientes não percebem melhora ao utilizar a medicação, sendo esta notada apenas pelo oftalmologista ao aferir a PIO, logo é sabido que entre 20 a 64% dos pacientes interrompem o tratamento ainda no seu primeiro ano, com consequências potencialmente devastadoras. Já no caso dos pacientes que aderem ao tratamento, 97% informam utilizar corretamente as medicações porém ao ser instalado um rastreador eletrônico no frasco das medicações apenas 77% realmente utilizam de forma adequada (15) (16). Uma alternativa terapêutica que minimize os problemas de adesão e tenha como alvo direto a fisiopatologia da lesão glaucomatosa ajudará sobremaneira a prática clínica.

O Glaucoma Primário de Ângulo Aberto (GPAA) é o tipo de glaucoma mais comum nas populações de origem africana e europeia, sendo a segunda causa, o glaucoma de ângulo fechado, representando apenas aproximadamente um terço dos casos de GPAA (3). O GPAA é caracterizado por um ângulo irido corneano da câmara anterior aberto e normal (17). Há um traço genético forte no GPAA já tendo sido identificados pelo menos 20 *locii* cromossômicos envolvidos na sua patogênese exibindo uma significante hereditariedade. Porém, os casos decorrentes de genes específicos respondem por menos de 10% dos casos totais de GPAA. É provável que o aspecto hereditário do restante dos casos do glaucoma seja poligênico e que interações entre os genes e o ambiente sejam importantes (18). Alterações genéticas consistentemente envolvidas na patogênese do GPAA estão localizados em genes que codificam as seguintes proteínas importantes em diversas rotas biológicas que vão desde enzimas metabolizadoras, moléculas pró-inflamatórias até neuropeptídios: miocilina (MYOC), optoneurina (OPTN), domínio de repetição WD36 (WDR36), repetição de anquirina e SOCS-box contendo 10 (ASB10), família 1 do citocromo P450, polipeptídio 1 subtipo B (CYP1B1), e neurotrofina 4 (NTF4) (19) (20). Além de alterações genéticas que afetam diretamente o surgimento do glaucoma e seu

fenótipo, alterações outras podem facilitar o seu surgimento, de forma indireta. No escopo deste estudo vamos nos deter numa variante genética que altera a síntese e metabolização de um neuropeptídeo trófico chamado fator fator neurotrófico derivado do cérebro (*brain derived neurotrophic factor*, BDNF) (21). Alterações genéticas na síntese do BDNF são bem estudadas no campo da neurologia e psiquiatria comumente facilitando o surgimento de doenças neurodegenerativas (22). Destas alterações a variante mais estudada a val66met. Esta variante é no nosso conhecimento a única estudada envolvendo glaucoma e alterações genéticas vinculadas ao BDNF (21). Evidências apontam que este polimorfismo está associado a diferenças na progressão do glaucoma, especialmente no sexo feminino (22).

A fisiopatologia do glaucoma envolve a existência de um estado de ativação da glia, isto sendo um marcador de neuro inflamação (23). Conforme pode ser visualizado na Figura 3, canais mecanosensíveis nas células ganglionares da retina (CGR) sofrem efeito do aumento da PIO causam influxo de íons de cálcio e esse fluxo libera ATP ativando células da glia. Estas células, em seu estado normal, ajudam na homeostase das CGR (24) e na sua proteção. As células de Müller, que fazem parte da glia, no início do dano celular secretam fatores neuro tróficos, tais como o BDNF (25). Porém, ao serem ativadas cronicamente, atuam participando da neuro degeneração por mecanismos imunomediados das CGR produzindo citocinas pró inflamatórias e fatores do complemento (26). As células da glia respondem ao estresse (27) e têm a habilidade de reconhecer padrões moleculares associados ao dano (DAMPs) através de receptores *toll like* (TLR) (28) (29). Em sequência ocorre a liberação de citocinas, sendo as mais importantes o TNF, IL-6 e IL-1 β (26), que culminam na ativação das vias apoptóticas das CGR resultando em morte celular (30) (31).

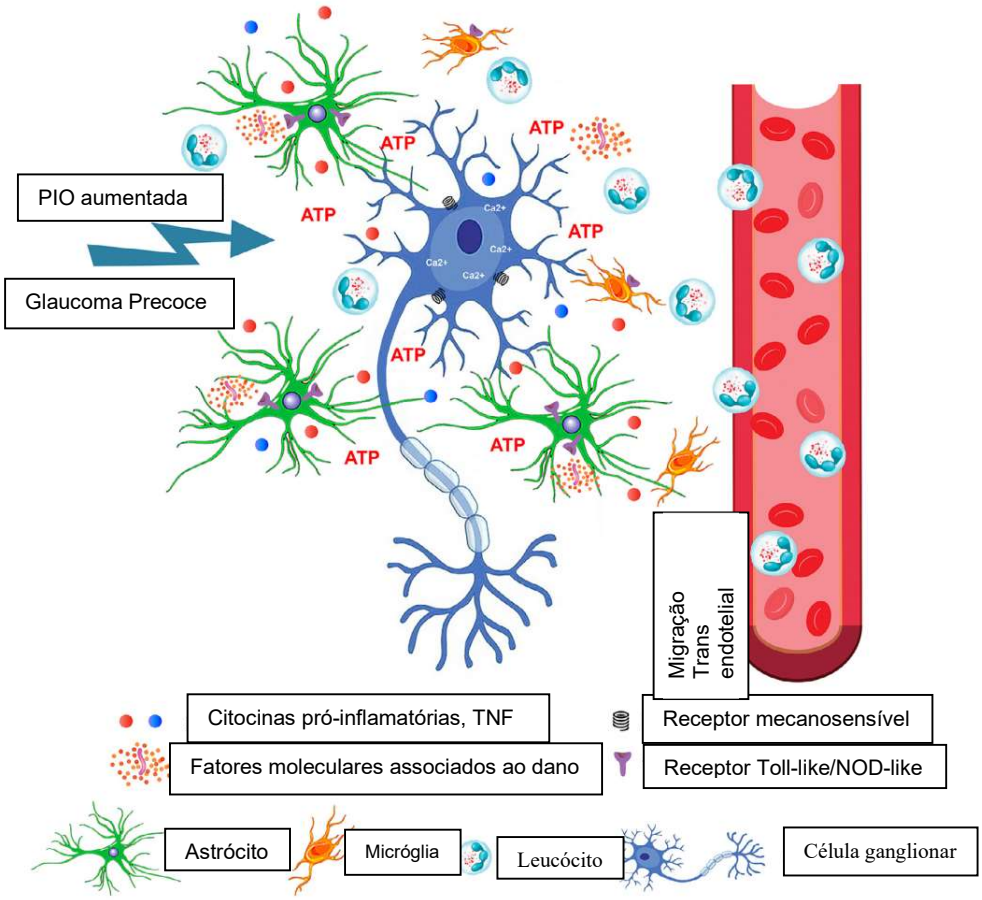

Fig 3 O aumento da PIO cronicamente ativa a micróglia causando lesão das CGR (31) –(ADAPTADA)

Neste contexto neuro inflamatório o BDNF ganha importância. Esta molécula é um fator de crescimento bastante estudado responsável por várias funções no sistema nervoso central (SNC) (32). O BDNF tem uma importante ação na sobrevida das células ganglionares da retina e na proteção ao dano (33) (34) (35). Estudos em modelos experimentais demonstraram correlação entre o aumento da PIO e o bloqueio do transporte retrógrado axonal do BDNF com provável aumento da suscetibilidade das células ganglionares a lesão oxidativa e apoptose (36) (34). Este pode ser um fator decisivo na progressão do dano glaucomatosos e explicar porque alguns pacientes continuam progredindo na doença a despeito do controle pressórico (36) (37) (38) (39). Demonstrando o efeito do BDNF, um estudo utilizando modelo animal injetou o neuropeptídeo dentro do olhos de ratos já cegos por glaucoma provocado experimentalmente e, após seis semanas com doses

programadas, demonstrou a regeneração axonal e recuperação função visual destes anumais (40).

Até o momento, em pacientes glaucomatosos, foram feitas dosagens do BDNF no soro e na lágrima, correlacionando os níveis do mesmo com as fases da progressão da lesão funcional do glaucoma. Esses estudos encontraram uma diminuição da síntese do peptídeo nas fases iniciais e moderadas da doença, com uma elevação desses níveis em fases avançadas (41) (42) (43) (44). Como dito anteriormente, correlações na síntese do BDNF com o glaucoma primário de ângulo aberto já foram demonstradas no estudo da variante val66met (rs6265). Essa variante resulta na substituição de uma valina por uma metionina no códon 66 na região promotora do gene do BDNF e está associada com alterações de processamento intracelular e secreção ativa dependente de BDNF (45). Os pacientes sem a alteração exibem duas citosinas (CC) na posição mencionada. Heterozigotos possuem uma timina e uma citosina (TC) e homozigotos para a variante duas timinas (TT) causando a variante com alteração na metabolização do BDNF. Essa variante não interfere na produção de BDNF, mas reduz sua secreção celular, reduzindo seus níveis extracelulares (46). Estes estudos foram realizados na Europa, Austrália e Irã. Não encontramos estudos sobre o tema conduzidos no Brasil. A natureza dessa influência necessita ser mais precisamente determinada (46) (47).

Assim, considerando o exposto, neste trabalho pretendemos descrever a associação da variante val66met (rs6265) no gene do BDNF em pacientes portadores de glaucoma e verificar se a presença desta mutação afeta os níveis de BDNF no plasma destes pacientes.

3 HIPÓTESE E OBJETIVOS

3.1 HIPÓTESE

A frequência da variante val66met (rs6265) na população glaucomatosa é diferente da frequência da população geral e varia de acordo com a gravidade da doença por afetar os níveis de BDNF no plasma.

3.2. OBJETIVO GERAL

Analisar a frequência da mutação val66met (rs6265) no gene do BDNF em pacientes portadores de glaucoma de acordo com sua gravidade e verificar se a presença desta mutação afeta os níveis de BDNF no plasma destes comparando com controles sem a doença.

3.3. OBJETIVOS ESPECÍFICOS

3.2.1 Descrever a frequência do rs6265 no gene do BDNF em amostra da população glaucomatosa soteropolitana;

3.2.2 Avaliar a associação de rs6265 com a progressão da doença glaucomatosa e sua gravidade;

3.2.3 Determinar os níveis de BDNF no plasma dos pacientes do estudo, bem como associar com gravidade da doença, comparando com controles sem a doença;

3.2.4 Avaliar a associação de rs6265 com os níveis de BDNF no plasma dos pacientes.

4 CAPÍTULO 1: ARTIGO: BDNF VAL66MET (RS6265) GENETIC VARIANT IN A GLAUCOMATOUS POPULATION FROM BRAZIL

Pinto-Neto, AJ; Santos, HS; Andrade, CM; Pena, LC; Figueiredo, CA

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4.1. ABSTRACT

Glaucoma is a very common condition and its diagnosis is often difficult and late. BDNF is a neuropeptide whose trophic function helps protect retinal ganglion cells. Low levels of BDNF have been associated with glaucoma in several studies. In the search for elucidation of the pathophysiology of glaucoma, in this work, we described the frequency of a genetic variant, val66met (rs6265), in a population of glaucoma patients and associated the presence of this variant with the different phenotypes of the disease. To this end, a total of 200 patients were recruited at the Oftalmodiagnose clinic. Patients are followed up in this clinic by the Clinical Protocol and Therapeutic Guidelines for Glaucoma, in which patients receive regular monitoring of the disease and eye drops for its treatment. The diagnosis of primary open-angle glaucoma is made with intraocular pressure above 21 mmHg, papillary cupping above 0.5, and/or visual field defect. 8 ml of venous blood was obtained from each patient, plasma was separated for BDNF measurement via Luminex and the buffy coat was used to extract genomic DNA. Genotyping was performed through real-time PCR using Taqman technology in QuantStudio 12K equipment. The population had a mean age of 65.43 (10.42) years. 46.8% of patients had advanced / severe disease and 53.2% were classified as having an early / moderate disease. We found a low genotypic frequency (8.6%) of the variant allele (T) in relation to the most common one (C). We found no significant differences between glaucoma severity and allele frequencies. We tested BDNF plasma levels among cases and controls. We have recruited fifty disease-free controls, of these 10 controls have their BDNF levels measured. Mean values were 17.01 (SD 3.92) pg/ml for cases and 365.46 (SD 111.59) pg/ml for controls. This difference was suggestive of statistical significance ($p<0.01$), despite the small number of samples already examined. Our study suggests that BDNF may be a biomarker for the diagnosis of glaucoma.

Keywords: Glaucoma; BDNF; val66met; rs6265; Salvador; Brazil; Immunophysiology

4.1 INTRODUCTION

Glaucoma is a frequent pathology affecting about 3% of world population which may increase up to 20% as individuals age. It is the second most frequent cause of blindness and first cause of irreversible blindness (3). There are several types of glaucoma. The most common is Primary Open Angle Glaucoma (POAG), and it is defined by elevation of intra ocular pressure (IOP) levels, increase in cup to disc ratio and/or visual field damage, with a normal open angle of the anterior chamber (17). This neurodegenerative disorder causes diminishing peripheral vision through retinal ganglion cells (RGCs) apoptosis, culminating with total vision loss (48).

Glaucoma is more common, appears earlier and has increased severity in African-decedent populations, which compose most of our city population (49). Central corneal thickness (CCT) is a predictor of glaucoma progression with thinner corneas increasing risk (7).

The pathophysiology of POAG has been subject of many studies (25) (19). Changes in the trabecular meshwork caused by aging impair aqueous humor drainage elevating IOP (48). Treatment focus on pressure management. Up to date, there are no other targets for glaucoma treatment. Lowering IOP can be achieved through eye drops, laser treatments and surgery (4) (9) (8). Patient adherence to treatment and unsatisfactory surgery results are commonplace, so early diagnosis and intervention are essential (10) (11) (14) (13).

There is a strong genetic factor in POAG and, therefore, significant inheritance. However, cases arising from specific genes alone account for less than 10% of total POAG cases. This suggests that the disease has a polygenic factor and, as such, gene-environment interactions are important (18) (20).

Several immune mechanisms are linked to glaucoma. Chronic high IOP leads to activation of RGCs mechanosensitive channels and calcium influx, in turn releasing ATP. This activates glia and microglia which produces cytokine, mainly TNF, interleukin 1β (IL-1β), and interleukin 6 (IL-6) (29) (25). Inflammation is established with increased vessel permeability and leucocyte trans endothelial migration (25) (27) (23) (37). The inflammatory state leads to irreversible vision damage with RGCs apoptosis (30). High pressure also contributes reducing blood flow at optic nerve head. Less blood means an increased risk for ischemic lesions. BDNF axonal transport is

also impaired by elevated IOP, decreasing RGCs damage threshold (39) (48) (17). (Figure 1)

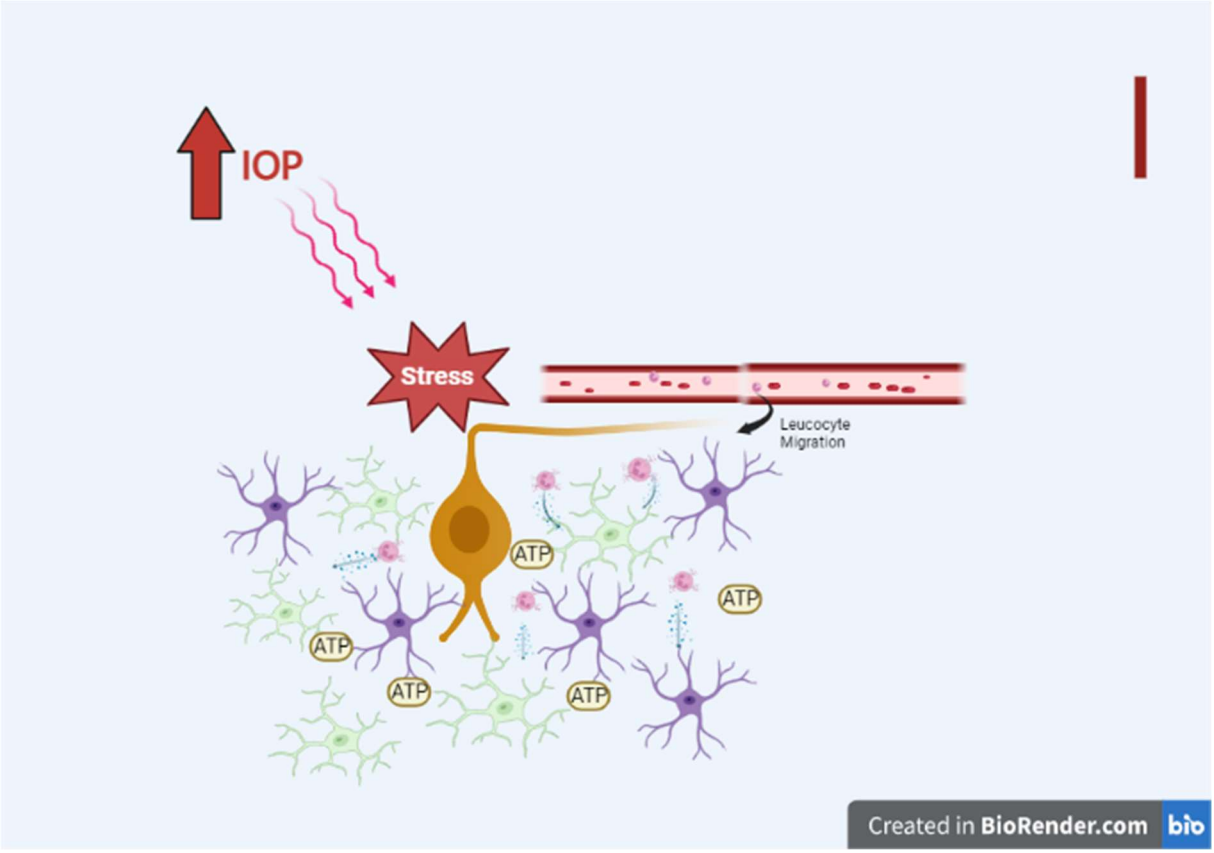


Figure 1 Immune mechanism in glaucoma

Fig1: Orange cell: Retinal ganglion cell; Purple cell: astrocytes; green cells: microglia; pink cells: leucocytes; IOP: intra ocular pressure; Blue arrow with dots: cytokine release.

BDNF is a neurotrophic peptide responsible for neuron growth, regenerating RGCs and can protect optic nerve axons and RGCs from damage (21). Thus, low BDNF levels facilitates cell damage. Low BDNF levels are linked with early glaucoma disease (46) (42) (43). Genetic polymorphisms may disturb BDNF secretion and the variant val66met, in which a valine is substituted by a methionine on codon 66 is the most studied variant that may led to alterations on BDNF route (47) (45). Patients without the alteration exhibit two cytosines (CC) in the mentioned position. Heterozygotes have one thymine and one cytosine (TC) and homozygotes for the variant have two thymines (TT) causing the alteration in BDNF metabolism. This variant does not interfere with BDNF production, but reduces its cellular secretion, reducing its extracellular levels (46). In this study, we aim to describe the frequency of this genetic variant in a glaucomatous population from Salvador, Bahia, Brazil. BDNF

serum levels will be measured and associated to glaucoma severity and val66met polymorphism and compare with disease free controls.

4.2 METHODS

4.2.1 STUDY POPULATION

In this study, a convenience sample of 200 individuals (calculated using a proportion confidence interval assuming IC 95%, expected proportion in population 50%, beta error 20%, design effect 1,5 and sample losses 20%, resulting in a total n=46 patients with a confirmed diagnosis of primary open-angle glaucoma) will be included, with eye pressure control using hypotensive eye drops, which are latanoprost, travoprost, bimatoprost, timolol, brimonidine and dorzolamide. Optic nerve cupping was also evaluated. Glaucoma occurs with augmented cupping but these may apperar in only one eye, so some patients with glaucoma may have normal cupping in one eye. These patients are being followed on Oftalmodiagnose ophthalmology clinic. All patients were of age 40 years and up. Inclusion criteria were not have a history of trauma to the eye, congenital eye diseases and not have other pathologies influenced by the synthesis of BDNF, such as neurodegenerative diseases or depression.

We have recruited 50 disease free controls. They follow the same inclusion and exclusion criteria of cases, except for previous diagnosis of glaucoma.

This study has been approved by Instituto de Ciências da Saúde Ethics Committee (**CAAE:** 42634821.4.0000.5662).

4.2.2 GENOTYPING

Genotyping was performed using TaqMan probe-based 5'-nuclease assays technology (Applied Biosystems, Foster City, CA, USA) in Applied Biosystem's QuantStudio 12K equipment. After performing the assays, the genotyping call rate of at least 93% and a $p>0.05$ in the Hardy–Weinberg equilibrium analysis using healthy individuals from the population were considered for analysis. As controls, we used DNA-free wells to evaluate nonspecific amplification.

4.2.3 BDNF levels in plasma

BDNF levels in plasma were measured using Luminex technology using a MagPiX device following the manufacturer standardized protocol BDNF HumanProcartaPlex™ Simplex Kit (Thermo Fisher Scientific, Waltham, MA, USA).

4.2.4. In silico analysis

In silico gene expression was assessed using an online browser of the Genotype Tissue expression Project (GTEx) (http://www.gtexportal.org). This project established a database which contains tissue gene expression according to the genetic variation. We examined whether genotypes of rs6265 were associated with differential expression of BDNF in peripheral blood.

In addition, to evaluate the frequency of this variant in comparison to ancestral populations we have used data from 1000 Genomes Project. This project is a catalogue of common human genetics variations, using openly consented samples from people who declared themselves to be healthy. The reference data resources generated by the project remain heavily used by the biomedical science community (https://www.internationalgenome.org/). Also, as reference, we used another Brazilian dataset, the ProAR .

The ProAR Foundation is the union of health professionals, patients and enthusiasts, which aims to expand access to the diagnosis and treatment of chronic respiratory diseases such as asthma, COPD, lung cancer, cystic fibrosis and tuberculosis. (https://www.fundacaoproar.org.br/)

4.2.5 STATISTICAL ANALYSIS

Initially, a simple chi-square test was performed to see the distribution of allele frequencies to obtain nominal, unadjusted values of p. For adjusted association tests, we used logistic regression corrected for age, and sex.

Quantitative data was presented as mean and standard deviation, when applicable. Statistical differences between groups were verified using the ANOVA test, non-parametric Kruskal Wallis test and Mann Whitney U test, as applicable.

The tests were be two-tailed and the statistical significance was established for the confidence interval of 95%. The genetic associations were performed in the PLINK program, normality and comparison tests and the graphics were produced in SPSS Statistics for Windows, Version 25.0 (IBM Corp. Released 2017 Armonk, NY).

4.4. RESULTS

4.4.1 POPULATION CARACTERISTICS

All patients from the case group have been sampled. Mean age was 65.43 (SD10.42) years. Most of the individuals were from female sex, 63%, with the remaining 37% male.

These patients have been followed up in our service for a mean 8.3 (SD 2.45) years. The number of drugs used per patient is 2.19 (SD 1.01) drugs, usually starting with prostaglandin eye drops (travoprost, latonoprost or bimatoprost). Patients also were treated with dorzolamide, timolol and brimonidine, when needed. Mean IOP levels were 13.63 (SD 2.67) mmHg for OD and 13.74 (SD 2.79) mmHg for OS. We also measured central corneal thickness (CCT) and mean deviation (MD) (Table 1). MD is a score that increases with glaucoma severity and is used to glaucoma classification. In this study we used Anderson's score (figure 2).

Figure 2 Anderson glaucoma classification

Minimum criteria for diagnosing acquired glaucomatous damage
- A Glaucoma Hemifield Test outside normal limits on at least two fields; OR
- A cluster of three or more non-edge points in a location typical for glaucoma, all of which are depressed on the pattern deviation plot at a p<5% level and one of which is depressed at a p<1% level on two consecutive fields; OR
- A corrected pattern standard deviation that occurs in less than 5% of normal fields on two consecutive fields

Classification of defects

Early defect:
- MD less than -6 dB
- Less than 25% of the points (18) are depressed below the 5% level and less than 10 points are depressed below the 1% level on the pattern deviation plot
- All point in the central 5° must have a sensitivity of at least 15 dB

Moderate defect:
- MD less than -12 dB
- Less than 50% of the points (37) are depressed below the 5% level and less than 20 points are depressed below the 1% level on the pattern deviation plot,
- No points in the central 5° can have a sensitivity of 0 dB
- Only one hemifield may have a point with sensitivity of <15 dB within 5° of fixation

Severe defect (any of the following results):
- MD greater than -12 dB
- More than 50% of the points (37) are depressed below the 5% level or more than 20 points are depressed below the 1% level on the pattern deviation plot
- At least one point in the central 5° has a sensitivity of 0 dB
- Points within the central 5° with sensitivity <15 dB in both hemifields

Fig 2 adapted from Susanna R Jr, Vessani RM. Staging glaucoma patient: why and how?

Table 1 Population characteristics

		Early / Moderate				Advanced / Severe			
		Mean	Standard deviation	N	% Total N	Mean	Standard deviation	N	% Total N
Age (years)		63,19	11,18			66,81	9,57		
Sex	Female			36	20,00			75	41,67
	Male			42	23,33			27	15,00
Time of follow-up (years)		8,41	2,30			8,22	2,43		
Number of drugs		2,05	0,98			2,29	1,05		
IOP OD (mmHg)		13,31	2,22			13,65	2,78		
IOP OS (mmHg)		13,42	2,17			13,75	2,99		
CUP OD		0,75	0,10			0,79	0,13		
CUP OS		0,75	0,11			0,76	0,15		
CCT OD (µm)		522,91	36,86			531,41	36,71		
CCT OS (µm)		524,38	38,86			531,05	39,59		
Genotype	CC			61	36,75			80	48,19
	TC+TT			10	6,02			15	9,04

Table 1 CUP = Optic nerve head cup to disc radio; IOP = intraocular pressure, CCT= central corneal thickness, MD = mean deviation

All stages of optic nerve head cupping were displayed. Round numbers are more common than its respective fractions.

As can be seen in Table 2, most of our population (88.5%) did not require a glaucoma surgery (trabeculectomy or tube) with 4% needing in one eye and 7.5% in both. Cataracts surgery was more frequent, with 63.5% having no surgery, but 36.5% having in at least in one eye.

Cup size was significantly higher in patients who have undergone glaucoma surgery ($p<0.01$). Cataract surgeries were more common with age ($p<0.01$).

Table 2 Surgery history

GLAUCOMA SURGERY	Frequency	Percent	CATARACTS SURGERY	Frequency	Percent
No	177	88.5		127	63.5
OD	6	3.0		3	1.5
OS	2	1.0		5	2.5
OU	15	7.5		65	32.5
Total	200	100.0		200	100.0

Table 2 OD = right eye, OS = left eye, OU = both eyes

Our controls had a mean age of 61,3 (SD 9,14) years. IOP and sex frequencies can be seen in table 3.

Table 3 Control population caracteristics

		Mean	N	Standard deviation	% N total
Age (Years)		54.61	49	11.21	
Sex	Female		36		73.47
	Male		13		26.53
IOP OD (mmHg)		15.80	49	2.86	
IOP OS (mmHg)		15.57	49	2.99	

Table 3 IOP = intraocular pressure; OD = right eye; OS = left eye

4.4.2 DNA and Genotypes

Table 4 presents the mean values for 460/480, parameter related to purity of the samples, where a mean of 1.77 (SD 0.09) was obtained. DNA concentration was in average 305.42ng/mL (SD 319.68).

Table 4 DNA samples concentration and quality

DNA Samples	**A260/280**	**Concentration (ng/ml)**
N	250	250
Mean	1.77	305.42
Standard deviation	0.09	319.68

Table 4 A260/280 = DNA purity, ideal ratio ~1,8;

Regarding genotyping (Table 5), 83.25% of the participants were CC, 16.75% of TC heterozygotes, and only 0,01% for the minor allele TT genotype. Allelic frequency was 91.39% for C and 8.6% for T. In controls, similar distributions were found. CC homozygotes were 82.22%, heterozygotes TC were 17.78% and no individuals TT homozygotes were found in controls. Genotype frequencies between cases and controls were not significant ($p>0.05$)

In comparison with ancestral populations from 1000 Genomes Project, as seen in figure 3, we can note that in African decent population such as AFR the genotype TT

was not found. CC frequency was 97.9% and TC 2.1%, the variant allele appearing even less than in our population. On the other hand, in EUR the frequency of CC is 64.2% and TT is 3.6%. Heterozygotes TC account for 32.2%.

In East Asian population the frequency is 27% for CC, 48.4% TC and 24.6% TT. South Asian population has frequencies of 64.2% for CC, 31.1% TC and 4.7% TT.

In another Brazilian dataset, the ProAR (https://www.fundacaoproar.org.br/) the frequency of CC was 81.6%, TC 17.7% and TT was 0,8%, the latter very similar to ours (Salvador).

Table 5 Frequency of genotypes and allele frequency in our case-control study.

	Genotypes	Sample Frequency	Percent	Allele	Sample Frequency	Percent
Cases	CC	164	83.25	C	361	91.39
	TC+TT	33	16.75	T	34	8.60
	Total	197	100			
Controls	CC	37	82.22	C	82	91,11
	TC + TT	8	17.78	T	8	8,88
	Total	45	100			

Figure 3 Genotype in ancestral populations

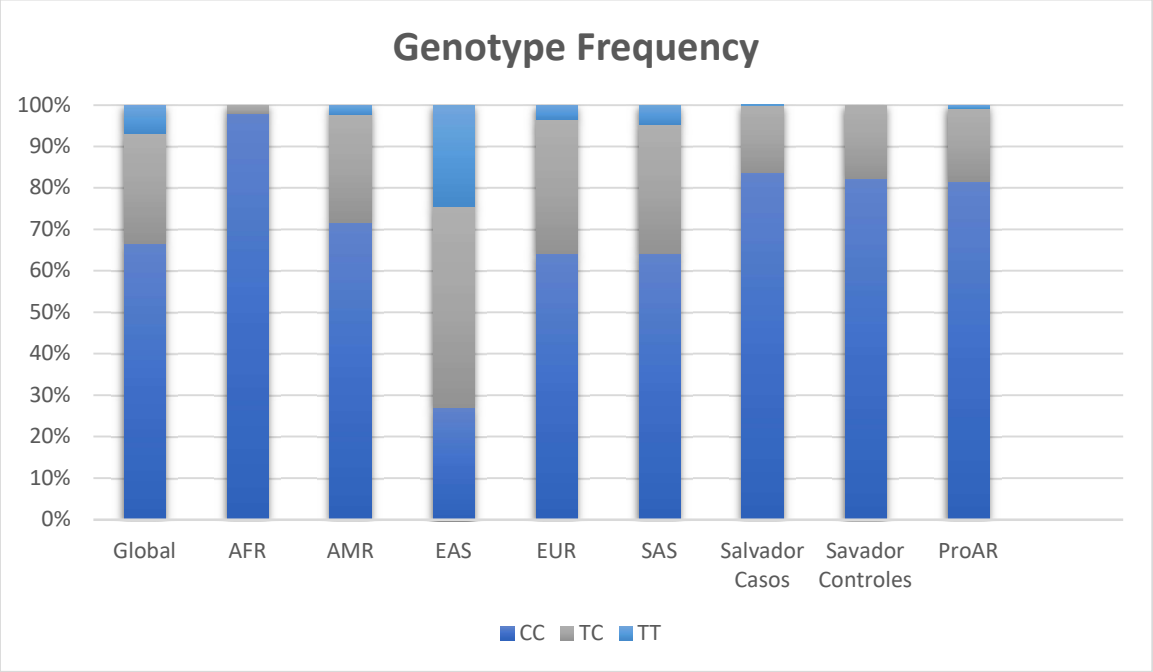


Fig 2 Genotypes frequencies in ancestral populations. AFR = African, AMR = American, EAS = East Asian, SAS = south Asian.

Allelic frequencies are described in figure 4.

Figure 4 Allelic frequencies in ancestral populations

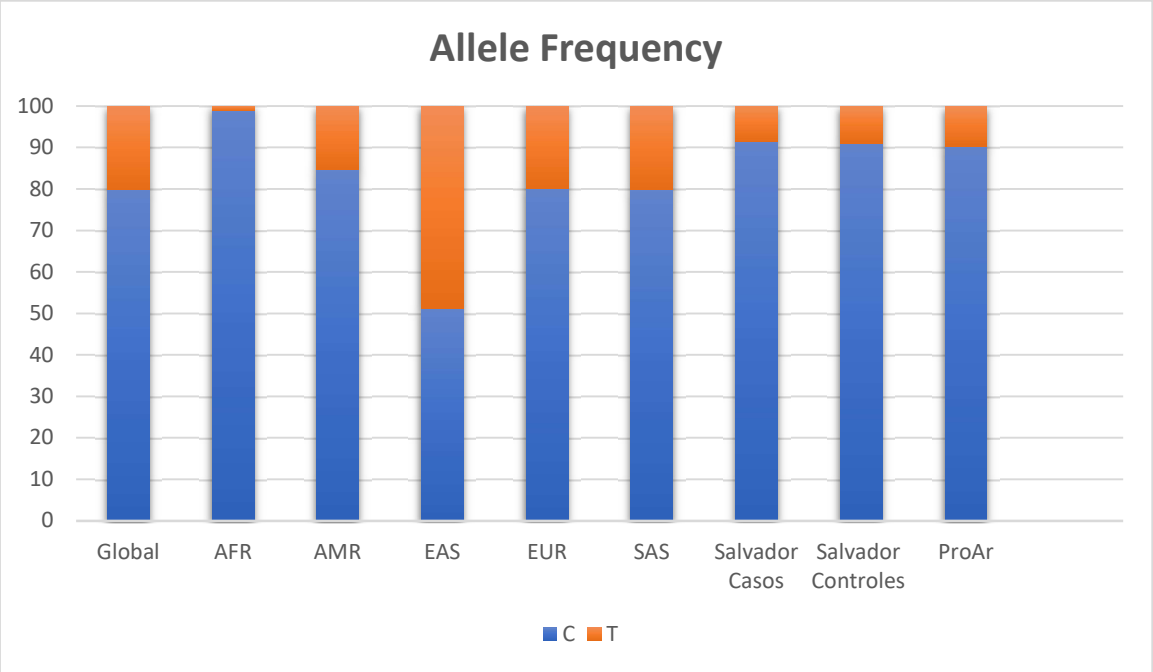


Fig 3. AFR = African, AMR = American, EAS = East Asian, SAS = south Asian.

Hardy-Weinberg equilibrium was performed to verify expected allelic frequencies. The sample had a balanced distribution as shown in table 6.

Table 6 Hardy-Weinberg equilibrium test

CHR	SNP	A1	A2	GENO	O(HET)	E(HET)	P
11	rs6265	T	C	1/27/157	0.1459	0.1445	1

Table 6 CHR = chromosome; SNP = single nucleotide variant; A1 = first allele; A2= second allele; GENO = genotype, O(HET)= frequency of heterozygotes found; E(HET)= expected frequency of heterozygotes; P = p value

4.4.3 Glaucoma versus Genotypes

To test whether genotypes influenced in glaucoma severity we did regression, linear or logistic, when applicable. IOP, MD, cup to disc ratio, CCT and number of anti-glaucomatous drugs in use were compared with different genotypes. Figure 5 displays values between traits with mean values and standard deviation. These data were adjusted for age and sex. No significant differences were found ($p>0.05$). Next, we again analyzed the same traits with exception of CCT. Data this time were also

adjusted for time of follow-up and CCT. No significant differences were found once more (p>0.05).

Figure 5 Glaucoma severity in different genotypes

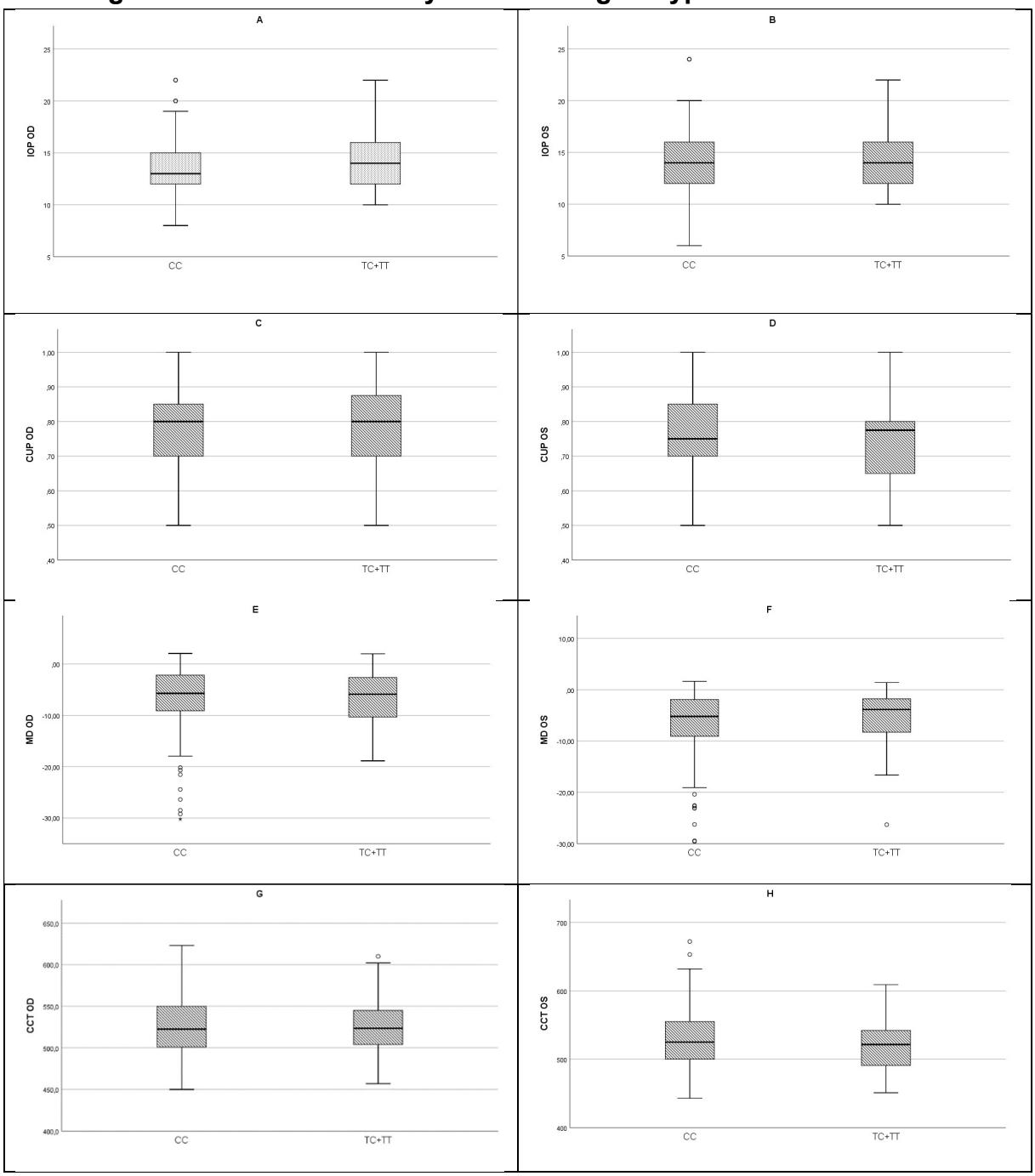
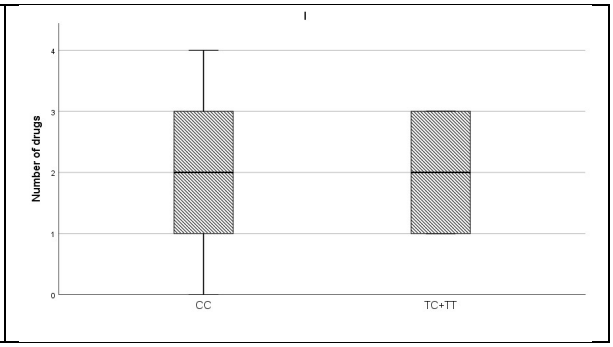


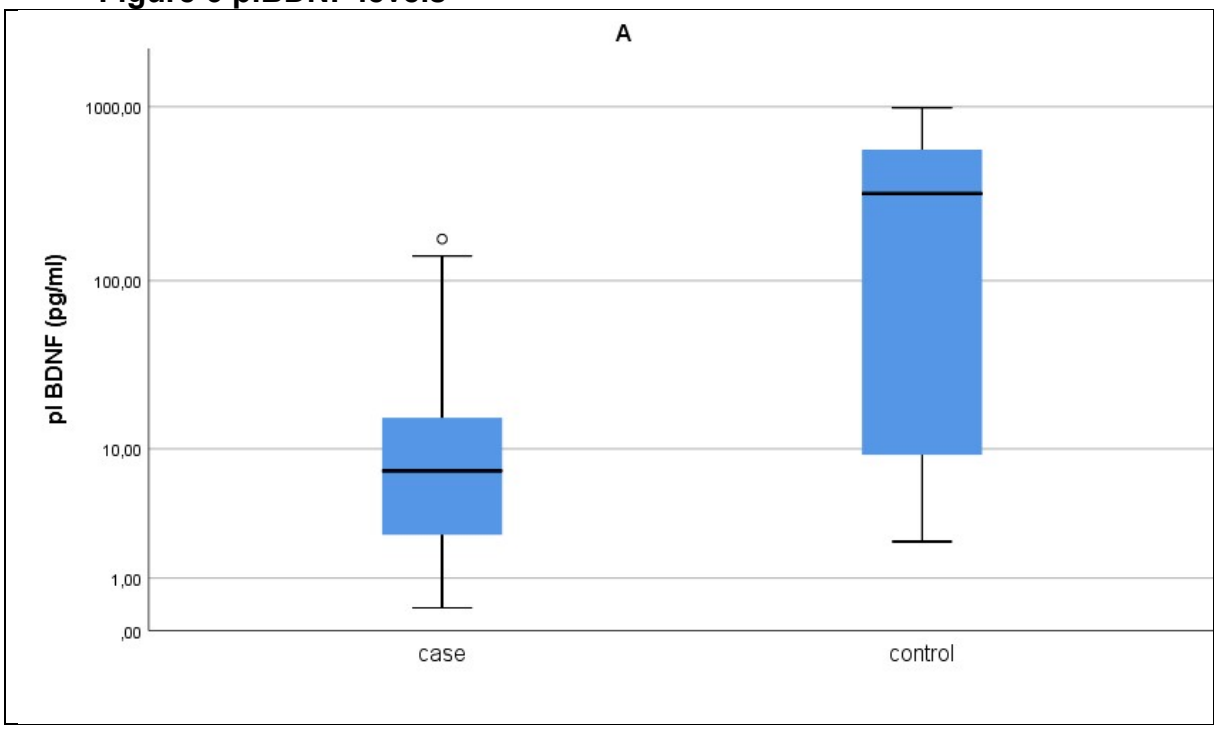
Fig 4 Comparison between genotypes and glaucoma traits = right eye; OS = left eye A/B- IOP = intraocular pressure, C/D - CUP = optic nerve head cup to disc ratio; E/F - MD = visual field mean deviation. G/H - PAC = pachymetry, I – DRUG NUMBER = number of drugs used



4.4.4 Plasmatic BDNF levels

Plasmatic BDNF (plBDNF) levels were measured in glaucoma patients and disease-free controls. Mean levels were 17.01 (SD 3.92) pg/ml for cases and 365.46 (SD 111.59) pg/ml for controls (figure 6). Genotypes were compared for these cases and no difference was found between groups CC and TC+TT (p>0,05).

Figure 6 plBDNF levels



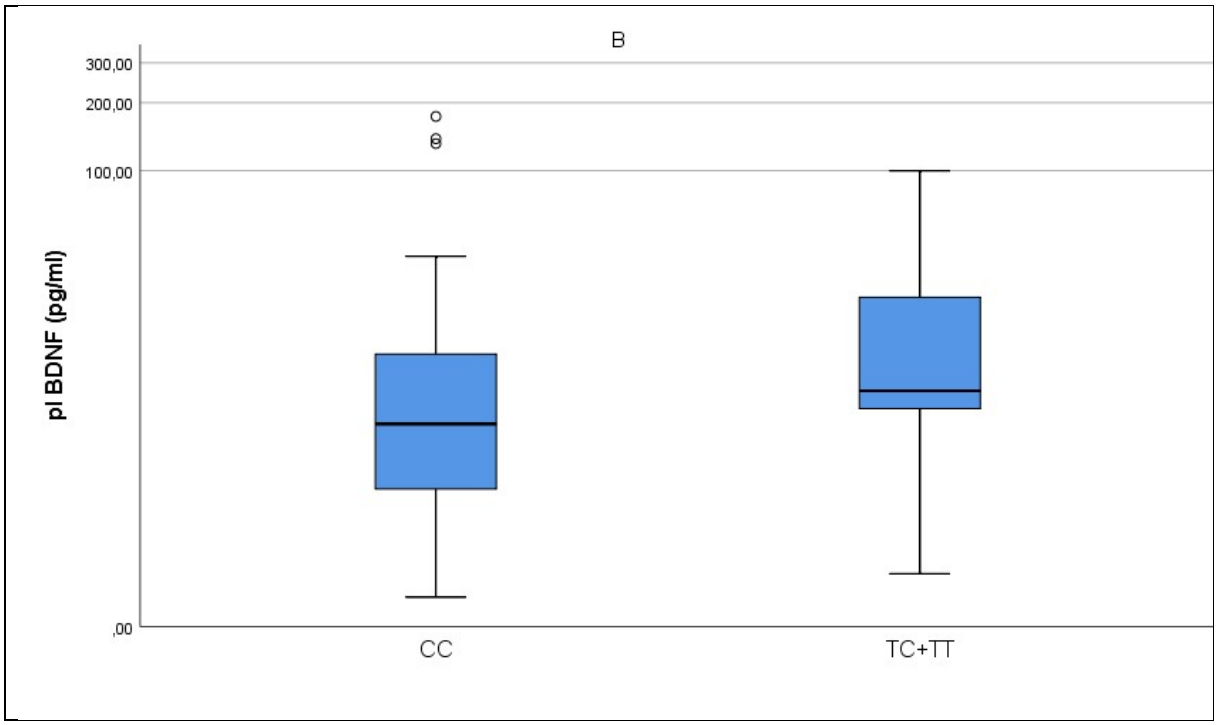


Fig 5 Means and SD plasmatic BDNF levels. A = cases versus controls; B = genotype groups. Data is presented in logarithmic scale.

Only in controls plBDNF levels followed a normal curve. 70 cases and 10 controls were tested. Soon a more complete analysis with total numbers will be performed. So far comparison between groups suggest a significant difference ($p<0,01$), with cases having lower plBDNF levels than controls.
In figure 7 we can see the contrast in individual plBDNF levels, cases on the left and controls on right of the image.

Figure 7 plBDNF Levels in case and controls

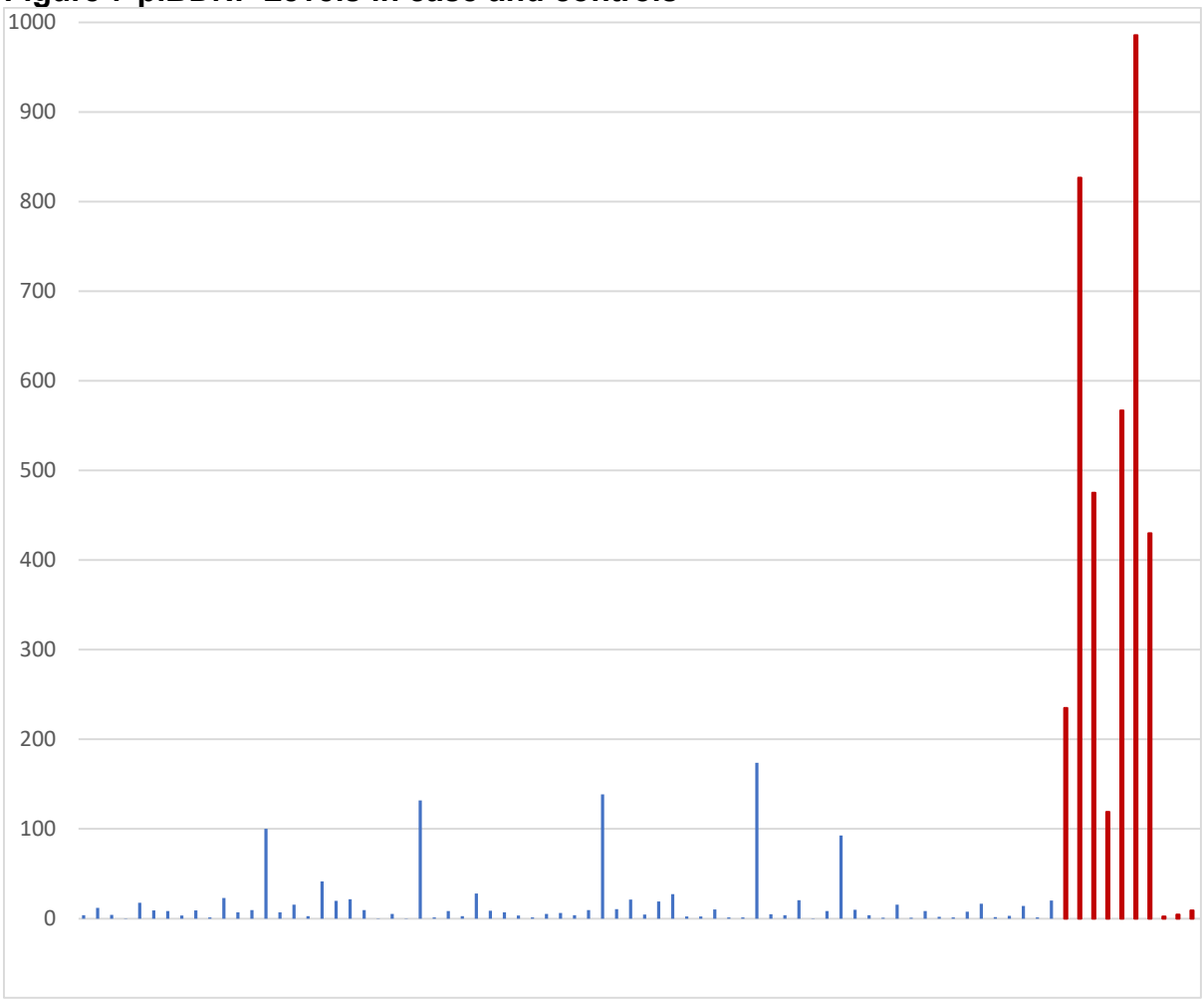


Figure 7 plBDNF levels. Blue bar= cases; Red bar= controls. Levels measured in pg/ml

4.5 DISCUSSION

Glaucoma is a particularly difficult disease to diagnose because of few or no symptoms (7). Patient adherence is essential to progression control. In this study, we explored whether variant rs6265 snp, also known as val66met, could be correlated with glaucoma severity and if it would interfere with plBDNF levels. This variant impairs BDNF release in neuron synapsis and its metabolization (45).

Animal models demonstrate alterations BDNF axonal transport and in its receptor trkB (36) (39), sometimes causing an up regulation in BDNF synthesis in eye cells (37). The rationale is that BDNF is essential to RGC survival, and so when transport from the brain is impaired, the eye must compensate production. The next step was to evaluate if BDNF not only could prevent cell death (34) (33) but also promote recovery of

damage RGCs. An experimental model in rats administered human recombinant BDNF and managed to rescue visual acuity in rats (40).

Trials in different human populations have consistently implicated low plBDNF levels with glaucoma (43) (42) (41). Measures were taken from aqueous humor, lacrimal fluid, and blood serum. This correlates to our data with mean plBDNF levels in controls significantly higher than in cases ($p<0,01$). We analyzed whether plBDNF levels changed with cup to disc ratio, MD values and Anderson categories but no differences were detected ($p>0,05$).

Our city, Salvador, has a mixed ancestral line. It´s supposed to approximate a mix of Yoruba (YRI), an African subpopulation and north and western European ancestry (CEU) (https://epigen.grude.ufmg.br/). The frequency of T allele in YRI population is around 0.5% and in CEU 19.7%, while our sample 7.8%.

As seen in figure 8, if we take a mean allelic frequency from our ancestral populations, it can be correlated to our numbers. This can be proof that we have a hybrid ancestral line combining European and African descendances.

Figure 8 Mean allelic distribution

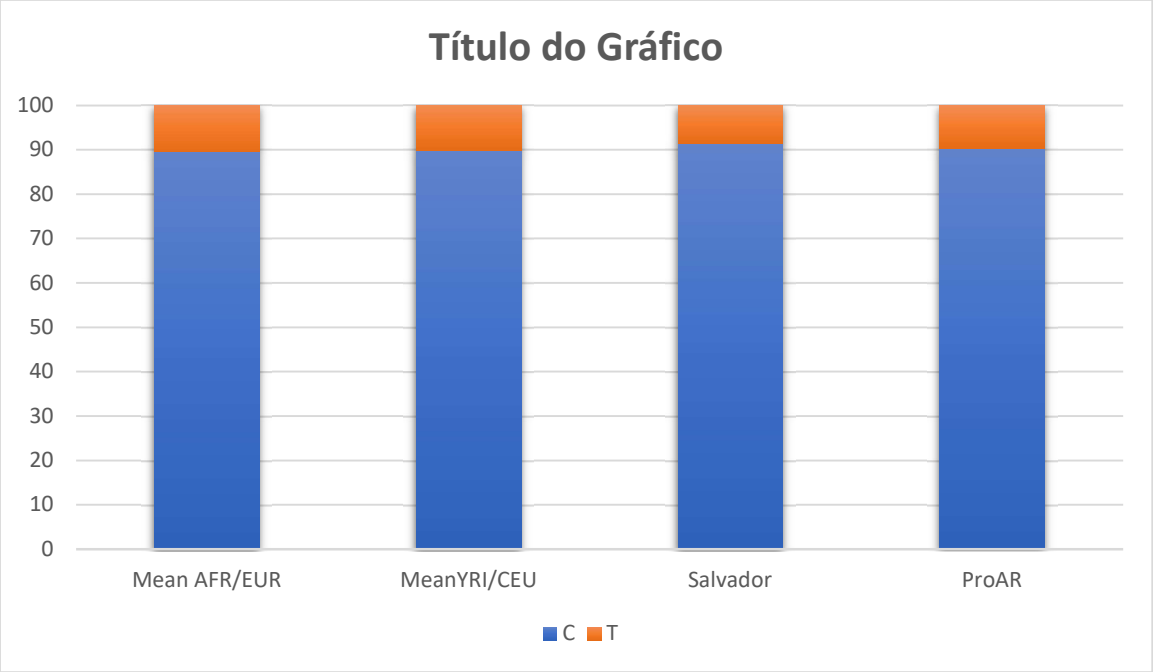


Fig 7 AFR = African, EUR = European; YRI = Yoruba; CEU = north and western eupropean

The minor allele frequency in our population was low. The exceptionally small number certainly is a limitation of our study. However, this information will be particularly

important to future gene studies in our city. This African heritage in out majority of our Black population must be accounted for in health policies. Glaucoma in African nations and in islands as Barbados can achieve a prevalence of 7.7% (50); more than double the 3.5% expected prevalence around the world. The disease in this populations have an earlier onset and is more aggressive. This can be correlated to the number of drug usage in our study. As such we aim to low IOP levels, like those shown in our sample. A Chinese survey showed 60% of population using only one drug (51). In our case, 30.5% uses one drug, but 30% uses two and 39% use three or more.

Eye drops remain the primary treatment option, especially in public health. Recent papers promote the efficiency of primary laser treatment (52), but that remains to be evaluated in the public health service.

The relationship between val66met polymorphism and glaucoma is still uncertain. A polish paper found differences in nerve fiber layer analyzer (GDx) and rim area, but not on cup-disk ratio or retinal nerve fiber layer (47) and another study found slower open angle glaucoma progression but only in female patients (46). In our data, glaucoma severity showed no difference between different genotypes. We tested cup to disc ratio, MD, IOP, pachymetry and number of drugs used, adjusted for age and sex. Since pachymetry showed no differences between groups, we moved this trait as a covariable, because pachymetry influences glaucoma severity with lower thicknesses increasing risk (53). Next, we also adjusted for follow-up times. Same results were found. As we follow these cases we will see if the alleles influence disease progression. Also, retrospective data is already being collected.

Val66met mutation influences BDNF production and intracellular transport. In met allele carriers BDNF depolarization dependent secretion and liberation at synapses were impaired (45). More research has been done correlating low BDNF levels with glaucoma (43) (42). More recently increase in BDNF levels with prostaglandin glaucoma medications may prove a neuroprotective effect (54). Soon we will have the neuropeptide plasma levels to compare with the literature.

Visual field index MD means were -6.98 for OD and -6.42 for OS. After analysis with overall MD, patients were categorized according to Anderson classification, that determines glaucoma severity according to MD values (fig 2). No significant difference between genotypes and glaucoma severity was found in both cases.

The mean MD found represents a moderate glaucoma in Anderson glaucoma classification system (55). This score was created based on the Humphrey perimeter.

This device is the gold standard, but, in our service, we use PcLab ocular perimeter which is a limitation of our work. Our non-gold standard device includes a confusion factor. This brand is the most widespread device. It´s critical to devise a way to create a classification using PcLab taking in account how important is to our city and even our country, due to its price and availability.

Although we haven't identified differences between genotypes and glaucoma severity, this is a important step to define our population genetics so we can aim the treatment more precisely towards individual patient needs. BNDF is a promising biomarker for glaucoma diagnostics, especially in early cases and in a population with increased risk for glaucoma. Soon all cases and controls will be fully screened generating a detailed report.

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