VNIVERSITAT DE VALÈNCIA

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Facultat de Medicina i Odontologia

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Neurodegeneration in diabetic retinopathy; an insight on its signalling profile

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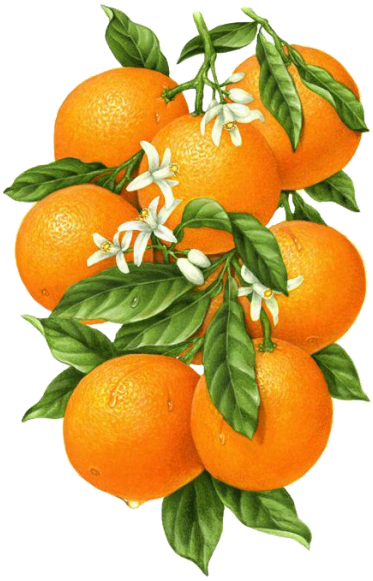
"And before I am gone, in gratitude and love I end, you have truly been a friend" — Audrey Hepburn



Per a la mare i la iaia

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Resum ampliat

Introducció

a. La retina: estructura i funció

El sistema visual és el principal encarregat de percebre els canvis que ocorren al nostre voltant, tenint l'ull un paper fonamental en el procés de transducció de la informació lluminosa i la seua conducció cap als elements funcionals superiors del sistema nerviós central (SNC). Anatòmicament, l'ull està dividit en tres compartiments principals: i) la càmera anterior, que conté l'humor aquós (HA), ii) la càmera posterior, localitzada entre l'iris i el cristal·lí, i iii) la càmera vítria, que conté l'humor vitri (HV). Localitzada en la part posterior de la càmera vítria es troba la retina, un teixit molt especialitzat de naturalesa neuronal encarregat de dur a terme el procés de transducció dels senyals lumínics en impulsos elèctrics que viatjaran pel nervi òptic (NO) fins al còrtex visual del cervell.

A nivell anatòmic i de superfície, la retina compta amb quatre elements diferencials: i) el disc òptic, coincident amb l'inici del NO, ii) la màcula, que conté al seu interior la fòvea, iii) la fòvea, caracteritzada per ser la zona amb major agudesa visual de la retina conseqüència del seu gruix reduït, i iv) els vasos sanguinis que conformen el sistema vascular retinal. D'altra banda, a nivell cel·lular, la retina es caracteritza per presentar una gran complexitat estructural, estant constituïda per una diversa quantitat de tipus cel·lulars que s'encarreguen de dur a terme i regular el procés de transducció

anteriorment esmentat. En aquest sentit, la retina presenta vuit capes ben diferenciades, dintre de les quals estan localitzats diferents elements cel·lulars: i) capa de segments externs (CSE), que conté els segments més externs dels fotoreceptors (cons i bastons), ii) membrana limitant externa (MLE), iii) capa nuclear externa (CNE), formada pels nuclis cel·lulars dels fotoreceptors, iv) capa plexiforme externa (CPE), que conté les connexions sinàptiques entre els fotoreceptors i les cèl·lules bipolars, v) capa nuclear interna (CNI), formada pels nuclis de les cèl·lules bipolars, horitzontals i amacrines, vi) capa plexiforme interna (CPI), corresponent a la regió de connexió sinàptica entre cèl·lules bipolars, amacrines i ganglionars, vii) capa de cèl·lules ganglionars (CCG), formada pels somes de les cèl·lules ganglionars, i viii) capa de fibres del nervi òptic (CFNO), formada pels axons de les cèl·lules ganglionars, la confluència dels quals acaba formant el NO. Al seu torn, aquestes capes descansen sobre una capa externa formada per cèl·lules amb un alt contingut en melanina anomenada epiteli pigmentari de la retina (EPR), en contacte directe amb la coroides.

A més d'aquests tipus neuronals, la retina compta amb un grup de cèl·lules conjuntament anomenades cèl·lules de la glia que presenten un paper important en el manteniment de la correcta funció tissular de la retina. Dintre de la glia destaquen les cèl·lules de la macròglia (cèl·lules de Müller i astròcits), encarregades de modular les connexions neuronals en totes les capes de la retina i mantenir l'homeòstasi tissular a través de la secreció de factors neurotròfics

(FNT), i les cèl·lules de la micròglia, principals agents del sistema immunitari de la retina encarregades de generar respostes front a antígens desconeguts i d'organitzar la resposta inflamatòria.

El manteniment de l'homeòstasi de la retina depèn del correcte funcionament i comunicació entre tots aquests tipus cel·lulars. No obstant, donada la seua elevada taxa d'activitat, la retina també és altament dependent del sistema vascular que la irriga, existint dos estructures fonamentals per al seu correcte funcionament: i) la barrera hemato-retiniana, encarregada de regular el flux de substàncies des del torrent sanguini a l'interior de la retina, i ii) la unitat neurovascular (UNV), una estreta comunicació entre les cèl·lules neuronals de la retina i les cèl·lules endotelials que conformen els vasos sanguinis encarregada de controlar la irrigació sanguínia en resposta a les demandes metabòliques tissulars.

Tenint açò en compte, qualsevol alteració tant en el component neuronal com vascular de la retina pot provocar disrupcions funcionals en la totalitat del teixit conduint a l'aparició de malalties.

b. Diabetis mellitus i retinopatia diabètica

La diabetis mellitus (DM) és una complexa patologia metabòlica caracteritzada per un estat hiperglucèmic crònic resultat d'alguna alteració en el metabolisme de la glucosa. Tot i que presenta diferents manifestacions etiològiques, les principals son la DM tipus 1

(DMT1), d'origen autoimmunitari, i la DM tipus 2 (DMT2), resultat d'una alteració en els processos de senyalització per insulina. Independentment del seu origen, els nivells elevats de glucosa induïts per la DM provoquen un estrès metabòlic a llarg termini a nivell sistèmic.

Donada la seua dependència per la glucosa i la seua elevada taxa metabòlica, la retina es veu altament afectada per aquest increment patològic derivat de la DM, el qual provoca una sèrie d'alteracions cel·lulars que en conjunt s'engloben dintre del terme retinopatia diabètica (RD), una de les principals causes de pèrdua de visió en la població adulta. A nivell clínic, la RD presenta dos estadis ben diferenciats: i) RD no proliferant (RDNP), sent l'estadi inicial de la patologia caracteritzat per la formació de microaneurismes i petites hemorràgies i dipòsits sòlids, i ii) RD proliferant (RDP), caracteritzada per un procés patològic de neovascularització mediat pel factor de creixement endotelial (VEGF, de l'anglès *vascular endothelial growth factor*) que deriva en una pèrdua de visió perifèrica i central. Addicionalment, la RD està associada a una altra condició patològica derivada de l'alteració en la permeabilitat del sistema vascular de la retina denominada edema macular diabètic (EMD), conseqüència de la sortida de plasma circulant a la regió macular a través dels vasos danyats, incrementant el seu gruix i alterant la seua estructura, conduint a una pèrdua de la visió central.

Etiològicament, el principal focus de la patologia ha estat orientat al seu component vascular, anant la majoria de les estratègies terapèutiques enfocades a revertir o frenar el fenomen de neovascularització (teràpia anti-VEGF). No obstant, recentment s'ha observat que la pèrdua de funció de les cèl·lules neuronals de la retina juga un paper fonamental en l'inici i progressió de la RD, sent la neurodegeneració un factor clau en l'estudi de la malaltia.

c. Neurodegeneració en la retinopatia diabètica

El concepte de neurodegeneració engloba tots aquells processos patològics que resulten en una pèrdua estructural o funcional de les cèl·lules neuronals. En aquest sentit, l'alteració del metabolisme de la glucosa conseqüència de la DM indueix una sèrie d'alteracions en les cèl·lules de la retina que contribueixen al desenvolupament i progressió de la RD, sent la neurodegeneració un factor clau en la seua patogènesi.

Com ocorre en altres patologies neurodegeneratives, existeixen múltiples vies de senyalització implicades tant en l'inici com en la progressió del dany neuronal, estant sovint directament associades les unes amb les altres. Així, la hiperglucèmia indueix l'activació d'una bateria de processos metabòlics en les cèl·lules de la retina que conformen la base de la resta d'alteracions associades a la neurodegeneració, entre les que destaquen: i) ruta poliol, ii) ruta de les hexosamines, iii) formació dels productes finals de glicació avançats

(AGEs, de l'anglès *advanced glycation end products*) i iv) sobre activació de la proteïna kinasa C (PKC). Aquests processos condueixen d'una banda a un increment en la producció d'espècies reactives d'oxigen (ROS, de l'anglès *reactive oxygen species*) i l'establiment de condicions d'estrès oxidatiu (EO), i d'altra banda a un increment en l'emissió de senyals proinflamatòries que, en conjunt, condueixen a l'activació de rutes més específicament implicades en l'alteració funcional de les cèl·lules neuronals.

Dintre de les diferents alteracions cel·lulars derivades d'aquests processos, la gliosi reactiva destaca com al procés de pèrdua de la funcionalitat de les cèl·lules de la glia. En aquest sentit, es diferencia una desregulació tant de la macròglia com de la micròglia que, en últim terme, condueixen a una alteració de l'homeòstasi neuronal de la retina. L'alteració de la macròglia té, entre altres, dues conseqüències funcionals implicades en el fenomen neurodegeneratiu de la RD: i) una alteració en la síntesi i alliberament de diversos FNT, com el factor neurotròfic derivat del cervell (BDNF, de l'anglès *Brain Derived Neurotrophic Factor*) i el factor de creixement transformant β (TGF- β, de l'anglès *Transformant Growth Factor β*), que té com a conseqüència una disminució de la capacitat de supervivència de les cèl·lules neuronals de la retina, i ii) una reducció en la seua capacitat de reciclatge de neurotransmissors, principalment el glutamat, que té com a conseqüència la seua acumulació i la inducció de fenòmens d'excitotoxicitat, especialment perjudicials per a les neurones de la

retina. D'altra banda, l'activació patològica de les cèl·lules de la micròglia conseqüència de l'alteració en el metabolisme de la glucosa condueix a l'establiment d'un estadi proinflamatori caracteritzat per l'alliberació incontrolada de molècules com les interleuquines (IL)-1β, IL-6, IL-18 i el factor de necrosi tumoral α (TNF-α, de l'anglès *Tumor Necrosis Factor* α).

De forma col·lectiva, tots els mecanismes descrits tenen com a resultat primari el dany a les cèl·lules de la retina, sent les neurones les principals en ser afectades donada la seua elevada taxa metabòlica. Entre els diferents processos cel·lulars activats com a conseqüència de l'establiment d'aquests processos, l'apoptosi destaca com el principal responsable de la inducció de la mort cel·lular, conduint a una reducció tant en la densitat com en la funcionalitat del component neuronal de la retina. L'apoptosi es tracta d'un procés complex, en el qual participen tota una bateria de molècules amb diferents funcions, sent les caspases les proteïnes característicament associades al procés. D'entre elles, la caspasa 3 (CAS3) destaca per ser el principal efector de la resposta apoptòtica. Tenint açò en compte, l'activació dels mecanismes prèviament descrits condueix a una activació patològica de l'apoptosi, existint una taxa apoptòtica més elevada que la observada sota condicions fisiològiques.

Amb això, el procés neurodegeneratiu que caracteritza la RD suposa una complexa matriu d'interaccions cel·lulars altament

dinàmic i dependent de l'estadi de la patologia. En aquest context, la identificació de molècules implicades en el procés és fonamental per entendre millor les bases del fenomen neurodegeneratiu i per millorar la monitorització i capacitat diagnòstica de la malaltia.

Hipòtesis

La hipòtesi central d'aquesta tesi doctoral està basada en la importància del procés neurodegeneratiu en l'inici i progressió de la RD com a complicació de la DM. Donada la natura neurodegenerativa de la patologia, son diferents els mecanismes que s'han proposat, de forma separada, per explicar el dany neuronal observat en els pacients amb RD. Entre aquests, destaquen l'excitotoxicitat, la desregulació dels FNT, la neuroinflamació i la inducció de fenòmens apoptòtics. Tenint això present, la hipòtesi del treball planteja que aquests mecanismes no operen de forma independent, sinó que existeix una intercomunicació entre les diferents rutes esmentades, contribuint de forma col·lectiva a l'alteració del component neuronal de la retina.

Objectius

L'objectiu principal d'aquesta tesi es incrementar el nostre coneixement sobre el paper que les rutes associades amb la neurodegeneració tenen en la patogènesi de la RD, donant una perspectiva holística d'aquest fenomen en el context de la patologia. D'aquest objectiu primari surten diversos objectius secundaris:

1. Determinar la concentració de glutamat en HV i plasma d'individus amb i sense RD per estudiar el paper de l'excitotoxicitat en el context neurodegeneratiu de la patologia.
2. Determinar la concentració de BDNF i TGF-β en HV i plasma d'individus amb i sense RD per estudiar el paper de la desregulació dels FNT en el context neurodegeneratiu de la patologia.
3. Determinar la concentració de IL-6, TNF-α, IL-1β i IL-18 en HV i plasma d'individus amb i sense RD per estudiar el paper de la neuroinflamació en el context neurodegeneratiu de la patologia.
4. Determinar la concentració de VEGF en HV i plasma d'individus amb i sense RD per estudiar el paper de la disfunció vascular en el context neurodegeneratiu de la patologia.
5. Determinar la concentració de CAS3 en HV i plasma d'individus amb i sense RD per estudiar el paper de l'apoptosi en el context neurodegeneratiu de la patologia.

Material i mètodes

a. Disseny de l'estudi i població

Aquest estudi es pot classificar com a descriptiu, transversal, observacional, cas-control i retrospectiu, en el que les mostres de plasma i HV foren recollides en múltiples moments dintre del període comprès entre setembre del 2022 a setembre del 2024.

La població d'estudi consisteix en pacients d'ambdós sexes, amb edats compreses entre els 40 i els 80 anys, seleccionats en diversos hospitals col·laboradors i classificats de forma individual per professionals oftalmòlegs. En base al seu diagnòstic, els participants foren classificats en dos grups principals: a) grup control comparatiu (G-CC), consistent en pacients que requerien una intervenció intravítria per a tractar una patologia no associada etiològicament amb la RD o la patogènesi de la malaltia, i b) un grup amb RD (G-RD), incloent pacients amb DMT2 i RDP que requerien una intervenció intravítria per al tractament de la seua patologia. La selecció dels participants en funció dels criteris d'inclusió i exclusió prèviament definits va conduir al reclutament de 75 pacients per a G-CC i 75 per al G-RD, donant un total de 150 participants en l'estudi els quals signaren el consentiment informat.

b. Variables de l'estudi

Les variables de l'estudi es classifiquen en sis categories principals segons la seua naturalesa:

i) Variables sociodemogràfiques, incloent sexe i edat.

ii) Variables antropomètriques, incloent l'índex de massa corporal (IMC).

iii) Variables clíniques oftalmològiques de funció, incloent l'agudesa visual millor corregida (AVMC) i la pressió intraocular (IOP).

iv) Variables oftalmològiques clíniques d'imatge, incloent el gruix del subcamp central (GSC) i el volum macular central (VMC).

v) Variables bioquímiques, incloent els nivells plasmàtics de glucosa (Gluc), els nivells d'hemoglobina glicosilada (HbA1c), els nivells de colesterol total (ColT), els nivells de colesterol associat a lipoproteïnes d'elevada densitat (HDL-Col), els nivells de colesterol associat a lipoproteïnes de baixa densitat (LDL-Col), els nivells de colesterol associat a lipoproteïnes de molt baixa densitat (VLDL-Col) i els nivells de triglicèrids (Trg).

vi) Variables moleculars, incloent la concentració en HV de glutamat (Glut-Vit), BDNF (BDNF-Vit), TGF-β (TGF-β-Vit), IL-6 (IL-6-Vit), TNF-α (TNF-α-Vit), IL-1β (IL-1β-Vit), IL-18 (IL-18-Vit), VEGF (VEGF-Vit) i CAS3 (CAS3-Vit), i la concentració plasmàtica de glutamat (Glut-Pls), BDNF (BDNF-Pls), TGF-β (TGF-β-Pls), IL-6 (IL-6-Pls), TNF-α (TNF-α-Pls), IL-1β (IL-1β-Pls), IL-18 (IL-18-Pls), VEGF (VEGF-Pls) i CAS3 (CAS3-Pls).

c. Obtenció de dades clíniques i mostres biològiques

Una vegada seleccionats, els participants foren sotmesos a una exploració clínica i oftalmològica per determinar l'edat, sexe, IMC, AVMC, IOP, GSC i VMC. La mesura d'AVMC es va fer amb el test de Snellen, mentre que la mesura de la IOP es va dur a terme mitjançant

un tonòmetre de Goldmann. Les variables d'imatge GSC i VMC es van obtenir per tomografia de coherència òptica (OCT, de l'anglès *Optical Coherence Tomography*). A més, l'anàlisi bioquímic rutinari de sang completa va registrar les variables Gluc, ColT, HDL-Col, LDL-Col i VLDL-Col.

Les mostres de sang per a l'estudi molecular foren recollides en tubs amb àcid etilendiaminotetraacètic (EDTA) i posteriorment centrifugades a 2000 x g durant 10 minuts a 4 ºC per a l'obtenció de plasma. D'altra banda, les mostres d'HV foren obtingudes per vitrectomia, un procediment quirúrgic consistent en l'extracció d'HV de la càmera vítria utiltizat per al tractament de les condicions incloses en els criteris d'inclusió per a ambdós grups. Una vegada recollides, ambdós tipus de mostres foren correctament etiquetades, distribuïdes en alíquotes i conservades en ultracongeladors a -80 ºC dins el seu posterior ús en els procediments experimentals.

d. Obtenció de dades experimentals

La quantificació de les diferents variables moleculars incloses en l'estudi es va dur a terme utilitzant dos aproximacions diferents: assaig d'immunoabsorció lligat a enzim (ELISA, de l'anglès *Enzyme-Linked ImmunoSorbent Assay*) o fluorimetria.

Els ELISA es realitzaren seguint les recomanacions del fabricant, contemplant una sèrie de passos clau: i) preparació de les

solucions amb els estàndards, ii) addició dels estàndards i les mostres als pouets, iii) llavat dels pouets amb un buffer de llavat per eliminar les substàncies no adherides, iv) addició de l'anticòs de detecció conjugat amb l'enzim peroxidasa, v) llavat dels pouets amb un buffer de llavat, vi) addició del substrat enzimàtic, vii) terminació de la reacció afegint una solució acídica, i viii) mesurament dels nivells d'absorbància a 450 nm en un espectrofotòmetre (Molecular Devices SPECTRAmax Plus 384) i transposició dels resultats segons la corba patró obtinguda de les lectures dels estàndards.

El procés per a l'assaig fluorimètric és semblant: i) preparació de les solucions estàndard, ii) addició de les solucions estàndard i les mostres a una placa opaca, iii) addició del mix de reacció, i iv) mesura de l'emissió de fluorescència amb un fluorímetre (Molecular Devices SPECTRAmax Gemini XPS), amb la posterior transposició dels resultats segons la corba patró obtinguda de les lectures dels estàndards.

e. Anàlisi de dades

Les dades foren recollides i registrades correctament en una base de dades Excel. Els càlculs estadístics foren realitzats mitjançant el programari R i es va calcular la mitjana ± la desviació estàndard per a totes les variables continues o els percentatges respecte al total per a les discretes. La normalitat de les variables continues va ser calculada mitjançant el test de Shapiro-Wilk. La comparació de les variables

discretes es va realitzar mitjançant la prova de Chi-quadrat. Per a les comparacions entre grups, el test U de Mann-Whitney o t de Student fou calculat segons la normalitat de les variables. A més, la correlació entre les variables continues fou analitzada mitjançant el càlcul del coeficient de Pearson o Spearman, segons la normalitat de les variables. Finalment, per assegurar la qualitat de les dades, es va realitzar una detecció dels valors outlier prèviament a la realització de les proves estadístiques mitjançant mètodes visuals (boxplots) i tècniques estadístiques. En tots els casos, un p-valor menor a 0.05 va ser considerat estadísticament significatiu.

f. Aspectes ètics

Aquest estudi va ser realitzat segons els protocols establerts i els principis acordats en la Declaració de Helsinki per als principis ètics de la investigació mèdica implicant a pacients i els protocols de bona pràctica clínica. A més, el protocol de l'estudi va ser presentat i avaluat pel Comitè Ètic d'Investigació Clínica de l'hospital Doctor Peset, rebent la seua aprovació amb el codi número 67.2.

Resultats

a. Dades clíniques i sociodemogràfiques

La comparació de les variables clíniques mostra que els pacients del G-RD presenten diferencies en algunes de les

característiques analitzades quan es comparen amb el G-CC. D'aquesta manera, G-RD presenta major percentatge d'homes (52.30% vs. 25.00%, p-valor = 0.00), major IMC (26.40 ± 2.80 vs. 23.20 ± 3.30 kg/m^2, p-valor = 0.02), major GSC (285.10 ± 45.17 vs. 258.26 ± 31.23 mm, p-valor = 0.00), major VMC (0.24 ± 0.01 vs. 0.21 ± 0.02 mm^3, p-valor = 0.00), major Gluc (102.20 ± 18.30 vs. 91.50 ± 14.20 mg/dL, p-valor = 0.01), major HbA1c (7.50 ± 1.90 vs. 5.30 ± 1.20 %, p-valor = 0.00), major LDL-Col (109.50 ± 38.50 vs. 96.00 ± 28.80 mg/dL, p-valor = 0.02), major VLDL-Col (38.10 ± 6.90 vs. 18.10 ± 5.80 mg/dL, p-valor = 0.00).

b. Perfil molecular

L'anàlisi de les mostres de plasma mostra diferències significatives en algunes de les molècules implicades en els processos de senyalització cel·lular d'interès en el context del present projecte doctoral. D'aquesta manera el G-RD presenta majors nivells plasmàtics de IL-1β (5.32 ± 3.09 vs. 2.03 ± 2.53 pg/mL, p-valor = 0.01), IL-6 (7.31 ± 3.80 vs. 4.63 ± 1.24 pg/mL, p-valor = 0.03), TNF-α (17.35 ± 2.57 vs. 15.60 ± 1.25 pg/mL, p-valor = 0.03), VEGF (152.10 ± 35.29 vs. 122.97 ± 24.60 pg/mL, p-valor = 0.00), glutamat (1.19 ± 0.53 vs. 0.69 ± 0.27 μM, p-valor = 0.03) i CAS3 (0.09 ± 0.04 vs. 0.06 ± 0.04 ng/mL, p-valor = 0.04).

D'altra banda, l'anàlisi de les mostres d'HV també mostra diferències significatives en algunes de les molècules analitzades. Així, G-RD presenta majors nivells vitris de IL-1β (10.40 ± 1.17 vs. 9.18 ± 1.26

pg/mL, p-valor = 0.04), IL-18 (112.43 ± 46.37 vs. 67.75 ± 12.62 pg/mL, p-valor = 0.04), TNF-α (13.75 ± 2.67 vs. 11.15 ± 2.30 pg/mL, p-valor = 0.03), TGF-β (86.15 ± 22.71 vs. 69.35 ± 18.52 pg/mL, p-valor = 0.04), VEGF (133.14 ± 199.60 vs. 49.43 ± 10.98 pg/mL, p-valor = 0.03), glutamat (0.19 ± 0.19 vs. 0.05 ± 0.08 μM, p-valor = 0.01) i CAS3 (0.44 ± 0.05 vs. 0.42 ± 0.02 ng/mL, p-valor = 0.04), i menors nivells de BDNF (7.41 ± 4.73 vs. 12.34 ± 5.11 pg/mL, p-valor = 0.03)

c. Estudi de correlacions

Es va realitzar una anàlisi de correlació per explorar possibles associacions entre les variables moleculars en plasma i humor vitri, paràmetres clínics oftalmològics i marcadors bioquímics. En aquest sentit, es van observar correlacions positives fortes entre GSC i VMC (r = 0,71), glucosa i HbA1c (r = 0,88), i glucosa i ColT (r = 0,68). També es va trobar una associació alta entre TGF-β-Vit i VMC (r = 0,74), així com entre VEGF-Vit i HbA1c (r = 0,65), i una correlació moderada entre TGF-β-Vit i GSC (r = 0,52). Altres correlacions positives destacades inclouen VEGF-Vit i TGF-β-Vit (r = 0,47), TNF-α-Vit i CAS3-Vit (r = 0,53), IL-18 en plasma i humor vitri (r = 0,61), i IL-1β en ambdós líquids (r = 0,67).

Pel que fa a correlacions negatives, la BCVA va mostrar associacions inverses marcades amb GSC (r = –0,65) i VMC (r = –0,73). També es van detectar correlacions negatives moderades entre BDNF-Vit i Gluc (r = –0,44), HbA1c (r = –0,46) i CAS3-Vit (r = –0,54).

Discussió

La RD continua sent una patologia oftalmològica amb una gran problemàtica associada donada la seua prevalença en la població i l'increment en el nombre d'individus amb DM. A més, les limitacions que presenten les principals opcions terapèutiques actuals fa necessari el desenvolupament de noves aproximacions per a un millor maneig dels pacients amb la patologia. En aquest sentit, la descripció de la neurodegeneració com a un mecanisme clau en la fisiopatologia de la RD obri les portes a una millor comprensió de la malaltia i al potencial descobriment de noves dianes terapèutiques més eficaces que les actuals. No obstant això, el fenomen neurodegeneratiu encara no es troba ben caracteritzat, i tot i que es coneixen diverses rutes implicades, pocs són els treballs que intenten donar una perspectiva holística del procés. És precisament amb el pretext de millorar la nostra comprensió sobre el fenomen neurodegeneratiu característic de la RD que naix el present treball d'investigació

Els participants del G-RD es caracteritzen per presentar diferencies en algunes de les variables clíniques analitzades. En el cas de l'IMC, els valors més elevats observats en el G-RD són d'especial interès donades les connexions existents entre l'obesitat i la neurodegeneració, sent un factor inductor de processos inflamatoris i alteracions metabòliques. Quan es comparen les variables oftalmològiques, destaquen les diferències entre els valors de GSC i

VMC, ambdós indicadors d'una pèrdua de l'estructura de la màcula especialment interessant si es considera el paper del EMD en el context de la patologia. Finalment, l'anàlisi de les variables bioquímiques mostra, en primera instància, un major perfil glucèmic en el G-RD, amb valors elevats i correlacionats tant de Gluc com de HbA1c, que està en completa concordança amb les alteracions associades amb la DM. D'altra banda, el perfil lipídic presenta diferències tant en els valors de LDL-Col com els de VLDL-Col, prèviament associats amb un major risc de comorbiditats en els pacients amb DM.

L'anàlisi del perfil molecular plasmàtic i de l'HV tant en el G-RD com en el G-CC mostra canvis significatius en els perfils de senyalització estudiats, donant una visió holística dels diferents missatgers moleculars implicats en el fenomen neurodegeneratiu associat a la RD.

L'alteració tant en HV com en plasma de les molècules IL-1β, IL-6, IL-18 i TNF-α reforça el paper de la neuroinflamació com a efector principal en la patogènesi de la RD. De fet, diversos treballs indiquen una associació directa entre la disfunció del metabolisme de la glucosa i la inducció de fenòmens proinflamatoris. En el cas de IL-1β, incrementat tant en HV com en plasma, son diversos els treballs que pareixen indicar el seu paper en la sobre activació del factor nuclear kappa B (NF-κB, de l'anglès *Nuclear factor kappa B*), però també contribuint en fenòmens de desregulació de la senyalització per

glutamat, alteració de la funció mitocondrial i increment de la taxa apoptòtica de diversos tipus cel·lulars. D'altra banda, IL-6 sols presenta un increment significatiu en mostres de plasma, tot i que s'observa una tendència a una major concentració vítria en les mostres del G-RD. Donat la seua importància com a molècula efectora de la resposta immunitària, es podria pensar que la seua expressió, tot i que reduïda, es un element addicional de la bateria de molècules proinflamatòries observades a l'estudi, tenint no obstant un efecte més subtil íntimament lligat, segons diversos estudis, amb l'alliberació de VEGF i la disfunció vascular. Pel que fa a IL-18, amb concentracions significativament elevades sols en mostres d'HV, son diferents els estudis que l'impliquen amb una activació tant del complex inflamasoma com de l'activació conjunta amb IL-1β de fenòmens de piroptosi, sent un vincle entre la neuroinflamacció i la mort de cèl·lules de la retina. A més, la seua elevació exclusiva en l'HV i no en plasma semblen indicar una especificitat en quan al seu mecanisme d'acció, semblant ser una molècula clau en la patogènesi neuroinflamatòria. Finalment, el TNF-α, amb valors incrementats tant a plasma com a HV, ha estat associat tant a l'activació de respostes proinflamatòries per activació del NF-κB com per l'activació de les cèl·lules de la glia, la síntesi de VEGF i l'inducció de fenòmens pro-apoptòtics com l'activació de la CAS3 i CAS8. D'aquesta manera, la sobreexpressió simultània de totes aquestes molècules indiquen l'existència d'una

sinèrgia que, en combinació amb altres rutes, amplifica el dany a les cèl·lules de la retina contribuint al procés de neurodegeneració.

En el cas dels FNT, la mesura de les concentracions de BDNF i TGF-β mostra una disminució i increment en les mostres d'HV, respectivament, però no en les de plasma. De nou, aquesta observació sembla indicar una contribució específica d'aquestes molècules en el context de la patologia, estant localitzades a nivell regional i no sistèmic. En qualsevol dels casos, la disminució de BDNF sembla estar associada a una disminució de la viabilitat neuronal conseqüència tant de la falta d'activació de senyals de supervivència cel·lular com de la incapacitat d'activació de mecanismes neuroprotectors com ara la mitigació de l'EO, la prevenció de l'activació de la micròglia o l'increment en la captació i reciclatge de glutamat per part de les cèl·lules de Müller. D'altra banda, el TGF-β ha estat descrit com un inductor del fenomen angiogènic, sent capaç d'alterar la UNV i alterar l'homeòstasi vascular de la retina. A més, són diversos els estudis que relacionen TGF-β amb el procés inflamatori, existint una associació entre ambdós fenòmens descrits i observats en aquest projecte.

La quantificació dels nivells de VEGF mostra un increment esperable tant en mostres de plasma com en mostres d'HV. Sent un dels principals agents etiològics de la patologia, el seu increment en HV ha estat àmpliament descrit en altres treballs. No obstant, no es molt el que es coneix d'aquesta molècula en el context de la

neurodegeneració, tot i que alguns estudis apunten a que podria tenir una activitat inductora de la supervivència i funcions neuronal. En aquest sentit, l'ús de teràpies anti-VEGF ha de ser correctament avaluat, ja que podria empitjorar l'agressió al teixit neuronal de la retina i per tant a la progressió de a patologia a llarg termini.

L'observació de nivells de glutamat significativament elevats en plasma però especialment en HV és especialment interessant donat l'impacte del fenomen excitotòxic en la pèrdua de viabilitat de les cèl·lules neuronals conseqüència directa de fenòmens de senyalització que indueixen alteracions en l'homeòstasi del calci intracel·lular. A més, diverses línies d'investigació han indicat una relació directa entre nivells elevats de glutamat en l'espai extracel·lular i un increment en la producció de VEGF, lligant aquest procés amb el fenomen angiogènic. No sols això, altres estudis han mostrat una relació entre l'excitotoxicitat induïda per glutamat i un increment en l'activitat de l'inflamasoma associada a una major producció de IL-1β tal i com s'observa en els resultats d'aquest estudi. D'aquesta manera, excitotoxicitat, disfunció vascular i inflamació semblen anar de la mà, existint una estreta comunicació entre tots els fenòmens que apareix representada en els resultats de l'estudi.

Finalment, l'anàlisi dels nivells de CAS3 mostren un increment en la seua concentració vítria i plasmàtica en el G-RD, remarcant la importància de l'apoptosi com a mecanisme clau en tota aquesta

constel·lació de processos de senyalització cel·lular. D'aquesta manera, l'observació simultània de nivells elevats de CAS3 i els altres marcadors moleculars analitzats dona una base ferma per a fer una associació entre aquests i l'increment de l'activitat apoptòtica, justificant d'aquesta manera el seu paper en el procés neurodegeneratiu associat a la RD.

Amb això, el present treball d'investigació té tota una sèrie de punts forts i limitacions que han de ser considerats a l'hora d'interpretar els resultats.

En quan als punts forts, en primer lloc destaca la realització d'una aproximació doble amb dos tipus de mostres diferents, la qual cosa és d'especial interès donats els estudis que indiquen que existeix una correlació entre la composició molecular entre HV i plasma. D'aquesta forma, aquesta aproximació metodològica dona una visió més ampla del procés neurodegeneratiu i valida el plasma com a element útil en la seua monitorització. A més, i en relació amb això, l'ús d'HV com a mostra en aquest estudi suposa una molt bona oportunitat per conèixer el medi directe en contacte amb les cèl·lules neuronals, oferint una perspectiva única per a l'estudi de la neurodegeneració en la retina diabètica. En últim lloc, l'anàlisi simultani d'un gran espectre de molècules implicades en el procés neurodegeneratiu dona una visió més completa dels mecanismes responsables de la disfunció de les cèl·lules neuronals de la retina,

contribuint a incrementar el coneixement actual d'aquest mecanisme patogènic.

Pel que fa a les limitacions de l'estudi, cal destacar en primer lloc la baixa mida mostral, sent aquesta la principal condicionant a l'hora de poder traslladar aquests resultats a l'àmbit clínic de forma directa. En aquest sentit, estudis futurs amb cohorts més grans serien beneficiosos per validar i expandir els nostres resultats. En addició, la dificultat pràctica i ètica per a obtenir mostres d'HV fa que l'aplicabilitat dels resultats obtinguts sols es pugui traduir en el cas dels anàlisis en mostres de plasma. Finalment, tot i que l'abast molecular i funcional d'aquest estudi es ample, altres autors han descrit altres molècules no incloses, per la qual cosa es requereix seguir incrementant el nombre d'agents moleculars analitzats per obtenir una visió cada vegada més completa de la totalitat del procés.

Finalment, són diverses les futures línies de recerca que deriven dels resultats obtinguts a l'estudi. Primerament, seria interessant incrementar la mida mostral per millorar el nivell de fortalesa dels resultats. A més, la inclusió d'anàlisis més holístics, com els que ofereixen les ciències òmiques, podrien ajudar a millorar la comprensió del procés neurodegeneratiu d'una forma més global. D'altra banda, l'ús de models experimentals controlats, tant *in vivo* com *in vitro*, podrien donar una perspectiva més lligada amb la funcionalitat de les rutes analitzades, poden ser interessant el

desenvolupament d'agents terapèutics amb dianes directament relacionades amb el procés neurodegeneratiu. En qualsevol dels casos, continuar elucidant les diferents vies implicades en el desenvolupament de la malaltia és necessari per tal de millorar la qualitat de vida dels pacients afectats per aquesta condició oftalmològica.

Conclusions

1. La neurodegeneració és un procés multifactorial que contribueix de forma significativa a la patogènesi de la RD.
2. El fenomen d'excitotoxicitat induït per concentracions elevades de glutamat apareix com un mecanisme fonamental en la neurodegeneració associada a la RD.
3. El desequilibri en la producció de FNT, específicament BDNF i TGF-β, juga un paper clau en la patofisiologia de la RD.
4. L'increment en la secreció de citocines proinflamatòries, incloent IL-6, TNF-α, IL-1β, i IL-18, remarca la importància de la neuroinflamació en l'establiment de dany en les cèl·lules de la retina i en la progressió de la RD.
5. L'observació de nivells elevats de VEGF en pacients amb DR indica el seu paper en el procés de desregulació de l'angiogènesi, i suggereix un potencial paper secundari contribuint al procés de neurodegeneració mitjançant la seua interacció amb les altres rutes moleculars analitzades.

6. L'increment de la taxa apoptòtica, evidenciat per nivells més alts de CAS en els pacients amb DR, sembla ser un agent clau en la neurodegeneració de la retina diabètica.
7. Els resultats d'aquesta tesi inicien el camí per a la detecció de nous biomarcadors orientats a l'avaluació de l'impacte de la neurodegeneració en el context de la RD.

Referències

Adamiec-Mroczek J, Zając-Pytrus H, Misiuk-Hojło M. Caspase-Dependent Apoptosis of Retinal Ganglion Cells During the Development of Diabetic Retinopathy. Adv Clin Exp Med. 2015;24(3):531-5

Bikbova G, Oshitari T, Baba T, Yamamoto S. Neurotrophic factors for retinal ganglion cell neuropathy – with a special reference to diabetic neuropathy in the retina. Curr Diab Rev. 2014; 10:166 176

Fragiotta, S., Pinazo-Durán, M.D., Scuderi, G. Understanding Neurodegeneration from a Clinical and Therapeutic Perspective in Early Diabetic Retinopathy. Nutrients. 2022 Feb 14;14(4):792. doi: 10.3390/nu14040792

Jindal V. Neurodegeneration as a primary change and role of neuroprotection in diabetic retinopathy. Mol Neurobiol. 2015; 51(3):878-84

Simó R, Hernández C. Neurodegeneration is an early event in diabetic retinopathy: therapeutic implications. Br J Ophthalmol. 2012; 96(10):1285–1290

Simó, R., Stitt, A. W., Gardner, T. W. (2018). Neurodegeneration in diabetic retinopathy: does it really matter? Diabetologia, 61(9), 1902 1912. https://doi.org/10.1007/s00125-018-4692- 1

Wang, W., Lo, A. C. Y. (2018). Diabetic retinopathy: Pathophysiology and treatments. International Journal of Molecular Sciences, 19(6). https://doi.org/10.3390/ijms19061816

Wong TY, Cheung CM, Larsen M, Sharma S, Simó R. Diabetic retinopathy. Nat Rev Dis Primers. 2016; 2:16012

Abbreviatures

AGEs: Advanced glycation end products

AH: Aqueous humour

BDNF: Brain-derived neurotrophic factor

BRB: Blood-Retinal Barrier

CAS3: Caspase 3

CAT: Catalase

CC-G: Comparative Control Group

CNS: Central Nervous System

DAG: Diacylglycerol

DM: Diabetes Mellitus

DMO: Diabetic macular oedema

DR: Diabetic retinopathy

DR-G: Diabetic retinopathy group

ELM: External limiting membrane

ERM: Epiretinal membrane

ERK: extracellular signal-regulated kinase

GCL: Ganglion Cell Layer

GFAT: Glutamine:fructose-6-phosphate amidotransferase

GLAST: L-glutamate/L-aspartate transporter

GPx: Glutathione peroxidase

GS: Glutamine synthetase

GSH: Glutathione

iBRB: inner Blood-Retinal Barrier

IL: Interleukin

ILM: Inner Limiting Membrane

INL: Inner Nuclear Layer

IPL: Inner Plexiform Layer

MH: Macular hole

NADPH: Nicotinamide adenine dinucleotide phosphate

NFL: Nerve Fibre Layer

NF-κB: nuclear factor kappa B

NGF: Nerve growth factor

NLRP3: Nod-Like Receptor Family Pyrin Domain Containing 3

NMDA: N-methyl-D-aspartate

NPDR: Non-proliferative diabetic retinopathy

NTF: Neurotrophic factor

NVU: Neurovascular Unit

oBRB: outer Blood-Retinal Barrier

OGT: O-linked N-acetylglucosamine transferase

ONL: Outer Nuclear Layer

OPL: Outer Plexiform Layer

OS: Oxidative stress

OSL: Outer Segment Layer

PDR: Proliferative diabetic retinopathy

PEDF: Pigment epithelial-derived factor

PI3K: phospharidylinositol-3 kinase

PKC: Protein kinase C

RAGEs: Advanced glycation end products receptors

RD: Retinal Detachment

RECs: Retinal Endothelial Cells

RGCs: Retinal Ganglion Cells

ROS: Reactive oxygen species

RPE: Retinal Pigment Epithelium

SOD: Superoxide dismutase

STAT3: signal transducer and activator of transcription 3

T1DM: Type 1 Diabetes Mellitus

T2DM: Type 2 Diabetes Mellitus

TGF-β: Transforming growth factor β

TNF-α: Tumour necrosis factor α

TrkB: tropomyosin receptor kinase B

UDP-GlcNAc: uridin-5-dihosphate-N-acetylglucosamine

VEGF: Vascular Endothelial Growth Factor

VB: vitreous body

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Research groups, Projects, Fellowships and Funding

During the course of this study, I have been actively involved in the following research groups:

1. The Cellular and Molecular Ophthalmo-biology Group at the University of Valencia (UOCEM-UV: GIUV2013-152),
2. The Ophthalmic Research Unit "Santiago Grisolía" at FISABIO, Valencia (UIO SG/FISABIO), and
3. The Glaucoma Unit at the Centre of Preclinical and Translational Pharmacology, University of Calabria, Italy.

Throughout this period, I had access to the infrastructure, funding, and human resources provided by these institutions. In addition, I contributed as a collaborating researcher in the following official research projects:

1. Spanish Research Network in Inflammation and Immunopathology of Organs and Systems (REI-RICORS), Institute of Health Carlos III, Madrid, Spain. Project codes: RD21/0002/0032 and RD24/0007/0004 (2021–present).
2. Strategic Action in Health (Acción Estratégica en Salud), FIS-FEDER Project, Institute of Health Carlos III, Madrid, Spain. Project code: PI24/01722 (2024–2027).
3. MACBIO Research Project, FISABIO–Generalitat Valenciana. Project code: UGP21-216 (2021–2023).

Additionally, I was selected through a public call to receive a fellowship under the “INVESTIGO” program (UGP-22-058), aimed at promoting research and innovation initiatives for young researchers in the Valencian Community. This fellowship covers the period from October 1, 2022, to October 31, 2024.

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I. Introduction

1. General structure of the visual system

Every living organism is characterized by their ability to interact with their environment, receiving stimuli and generating adequate adaptative responses to them. From an evolutionary perspective, mammals are characterized by the development of the so-called neurosensory organs, structures whose main function is to perceive changes in their surroundings and transmitting the information they detect to the central nervous system (CNS). Amongst these stimuli-sensible structures, the visual system establishes itself as the key element responsible of receiving and transducing changes in the intensity of light, being able to provide spatial information that can be processed and integrated to create images that can be interpreted by the superior functional elements of the CNS.

From an morphological perspective, the eye can be divided into three main compartments (Kels et al., 2015): i) the anterior chamber, containing the aqueous humour (AH) and delimited anteriorly by the cornea and posteriorly by the iris, pupil and lens; ii) the posterior chamber, located between the iris, the lens and the zonule fibres, and iii) the vitreous chamber, containing the vitreous body (VB) and delimited anteriorly by the back-side of the iris and the lens and posteriorly by the retina and its associated structures (Figure 1, Table 1). Functionally, and from a simplistic approach, the vision process

occurs when light goes across the cornea to all three spatial compartments of the eye until it reaches the retina. Here, it activates a plethora of cell responses that will ultimately lead to the transformation of light information by a series of biochemical processes to an electric nerve impulse, able to travel through the optic pathway to be interpreted in an adequate manner.

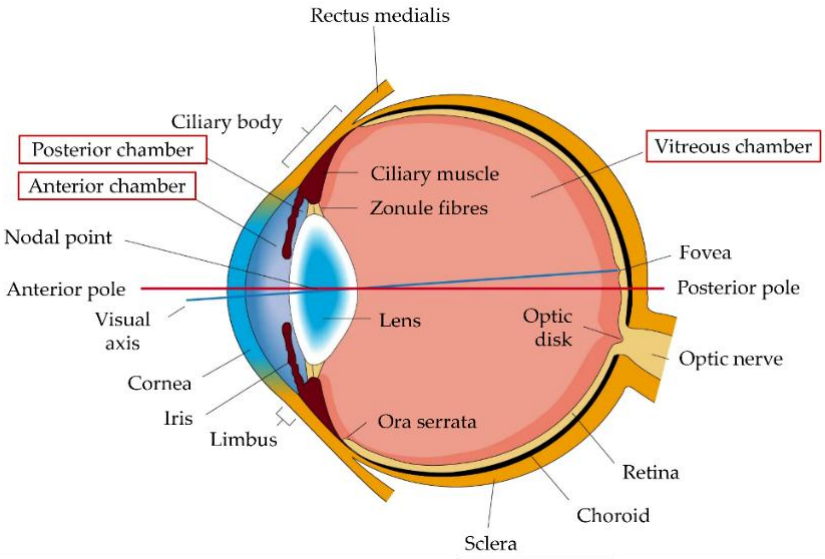


Figure 1. *Sagittal plane of the eyeball. The three main spatial divisions of the eyeball (anterior, posterior and vitreous chambers) are highlighted. Figure adapted from https://www.britannica.com/science/human-eye.*

Having a general idea of the normal morphology and physiology of the visual system may allow us to fully understand what occurs when it is altered. In this sense, the main anatomic, cellular and physiological aspects of the visual function and the retinal tissue are reviewed in the following sections.

Table 1. Anatomical components of the eye

Cornea	Transparent protective layer located in the front part of the eye.
Iris	Pigmented structure located in front of the lens; it reflects the light due to its high melanocyte density.
Pupil	Central round opening in the centre of the iris whose diameter regulation controls the amount of light that passes through the lens.
Lens	Transparent biconvex structure able to correctly focus the incident light on the retina.
Zonule fibres	Group of connective fibres that maintain the shape and position of the lens.
Ciliary body	Structure located posteriorly respect to the iris; its main function is producing the aqueous humor and regulating the lens shape.
Retina	Innermost eyeball layer constituted by a neurosensorial layer and the retinal pigmentary epithelium. It is responsible of the transduction process of light stimuli into electric impulses that travel through the II cranial pair, the optic nerve, to the visual cortex located in the brain occipital lobe. Most relevant anatomical structures of the retina are the macula, the most central region of the retina which contains the fovea, characterized by being the area with the highest visual acuity of the retina. Additionally, the optic disk appears as the beginning of the optic nerve, being considered a blind spot in the retina due to its cellular composition.
Ora serrata	Most anterior and peripheric part of the retina, in contact with the ciliary body.
Sclera	External layer characterized by its resistance; it is anteriorly limited by the cornea (limbus) and posteriorly by the choroid.
Choroid	Vascularized layer adjacent to the retina and located under the sclera.
Limbus	Circular area located in the anterior part of the eyeball, between the cornea and the sclera.

2. The retina

The retina is a well-organized tissue located in the innermost surface of the eye globe, covering the whole posterior side of the eye. Anteriorly, it borders the ciliary body constituting the ora serrata or peripheric retina. Transversally, the retina is constituted by a series of different cellular layers (see subsection 2.1), being limited by the outermost retinal pigment epithelium (RPE) and by the inner limiting membrane (ILM) and the vitreous chamber on its internal surface (Frith and Mehta, 2021; Grossniklaus et al., 2015; Ramon y Cajal, 1893). In this sense, the retina's functionality is interrelated with its anatomical borders, specially the RPE and the vitreous chamber, being reciprocally influenced by their physiological status.

Anatomically, four main elements stand out on the retinal surface (Frith and Mehta, 2021; Grossniklaus et al., 2015; Kels et al., 2015; Ramon y Cajal, 1893), as depicted in Figure 2: i) the optic disc, which coincides with the beginning of the optic nerve, being the collection point of the retinal nerve fibres, ii) the macula, which appears as a dark central spot and contains the fovea, iii) the fovea, characterized by being the area with the highest visual acuity of the retina as a result of its reduced thickness, and iv) the blood vessels that form the retinal vascular system.

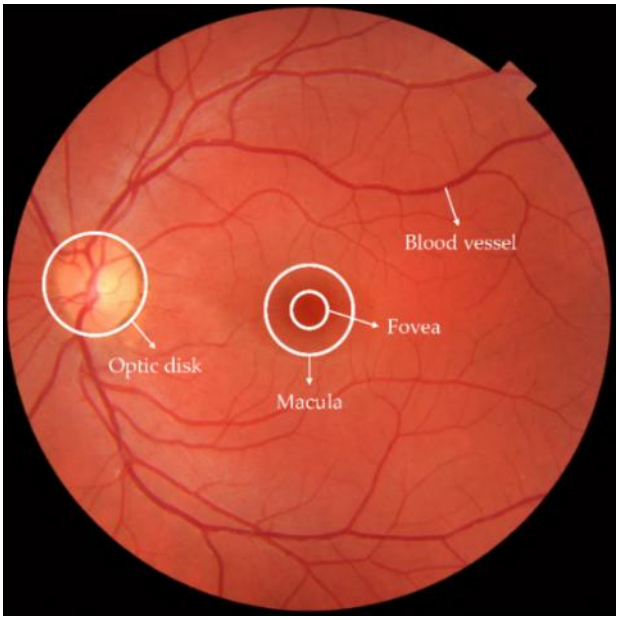


Figure 2. *Ocular fundus. Retinography image. The main elements of the normal retinal anatomy are depicted. Figure adapted from Häggström (2014).*

The retina is characterized by having an active metabolism due to its high physiological activity, consequently having an increased demand of oxygen and metabolic substrates when compared with other organs. In this sense, the retinal tissue counts with a highly developed vascular system that comprises a plethora of blood vessels distributed on its inner and outer layers (Grossniklaus et al., 2015). These retinal blood vessels, derived from the ophthalmic artery (and this, in turn, from the internal carotid), are part of two different vascular systems, depicted in Figure 3: i) the blood vessels that irrigate the innermost retinal layers, which are part of the retinal vascular system and are originated from the central retinal artery, presenting a constant and reduced blood flux, and ii) the blood vessels that irrigate

the external layers and the RPE, which come from the ciliary arteries, have an intense blood flux and are part of the choroid vascular system, responsible of providing most of the oxygen and nutrients suppliance in the retina (Grossniklaus et al., 2015).

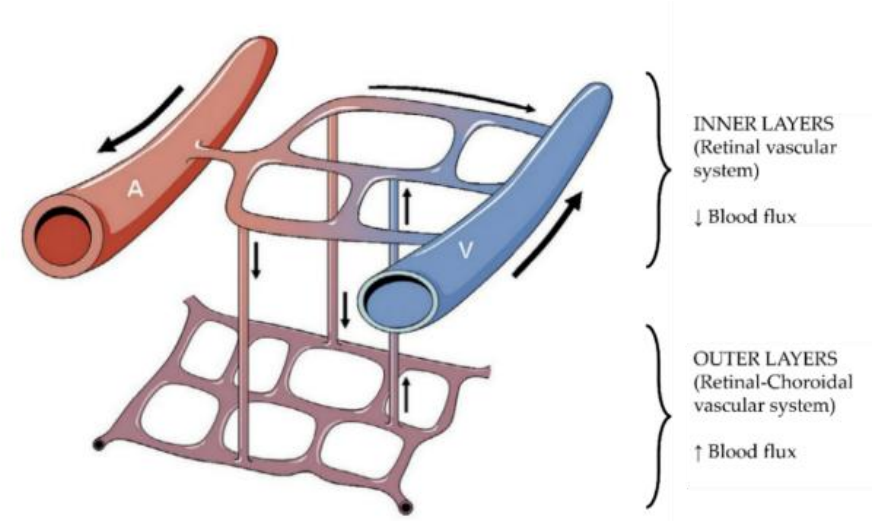


Figure 3. *Schematic representation of the retinal vascular system. The existence of communication vessels between both circulatory systems allows a better tissular irrigation. A: artery, V: vein. Figure adapted from Grossniklaus et al. (2015).*

The configuration and functionality of this complex circulatory system, of capital importance for the maintenance of the homeostasis of the retinal tissue, are adequately discussed in subsection 2.2.

2.1 Cellular organization and layering

From a structural perspective, the retina stands out as one of the most cellular-diverse and well-organized tissues of the human body. It is constituted by different cellular types whose main mission is to

transform the light stimuli into electrical impulses and sending them to the brain through the optic nerve, assuring an adequate maintenance of the tissular homeostasis during the whole process(Hoon et al., 2014). In this sense, the retina has eight well-differentiated layers (Hoon et al., 2014): as shown in Figure 4 i) the outer segment layer (OSL), ii) the external limiting membrane (ELM), iii) the outer nuclear layer (ONL), iv) the outer plexiform layer (OPL), v) the inner nuclear layer (INL), vi) the inner plexiform layer (IPL), vii) the ganglion cell layer (GCL) and viii) the nerve fibre layer (NFL), this latter also named retinal nerve fibre layer (RNFL).

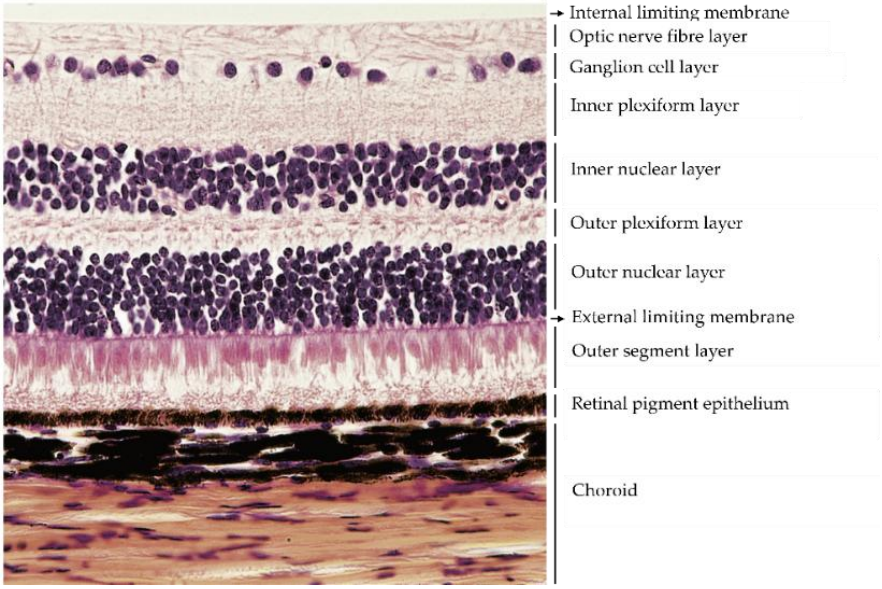


Figure 4. *Histological section of the retina. The figure transversally shows the different layers of the human retina. Adapted image licensed under Wikimedia Commons (CCO 1.0).*

Having this histological architecture in mind, and following an outer-inner axis, the first cellular type that one encounters are the photoreceptors, responsible for capturing the light stimuli and transforming them into nervous information using the so-called photopigments. There are two different types of photoreceptors with different pigment content and complementary functions in the visual context: i) rods, rich in opsin and responsible for vision at low light levels (scotopic vision), and ii) cones, rich in rhodopsin, active at higher light levels (photopic vision), responsible for high spatial acuity and capable of colour vision. Both types are formed by two segments: i) an external segment which contains the light-reactive pigments and form the OSL, and ii) an inner segment which includes the cellular nuclei and the organelles needed to maintain the energetic homeostasis, constituting the ONL (Hoon et al., 2014).

When light hits the photopigments, a myriad of biochemical events known as phototransduction cascade and visual cycle allows the generation of nerve impulses that are conducted to the next cellular element, the bipolar cells or intermediate neurons, whose nuclei are located in the INL. The electric impulse travels through the axons of the bipolar cells until it reaches the retinal ganglion cells (RGCs) in the GCL, whose axons are jointly directed to the optic disk jointly forming the optic nerve and sending the transduced visual information through the optic pathway to the superior cerebral areas (Hoon et al., 2014).

This photoreceptor-bipolar-ganglion cell axis is tightly modulated by two additional neuronal cell types whose nuclei are located into the INL: i) the horizontal cells, located in the synaptic areas between the bipolar cells and the photoreceptors, and ii) the amacrine cells, present in the synaptic processes between the bipolar and RGCs. In this sense, by secreting specific molecular modulators, these secondary neuronal cells are able to modulate the synaptic connections between neurons and control the process by which the visual information is transferred to the brain, being responsible of accentuating the borders of the images perceived by a mechanism of lateral cell inhibition, and modifying the signals that the RGCs receive, determining their sensibility to moving visual stimuli, respectively (Hoon et al., 2014).

In addition to these cellular actors, the retina counts with a group of cells, collectively named "glia" or "neuroglia" (Ramon y Cajal, 1896) that have a capital role maintaining the correct function of this tissue and whose functions commonly overlap with those that horizontal and amacrine cells are responsible for. The neuroglial cells can be divided in two groups, with different ontogenetic origins: i) the macroglial cells, which include astrocytes and Müller cells, and ii) the microglial cells. Both macroglial and microglial cells have crucial functions inside the retinal tissue. Astrocytes, located alongside the axonal terminations of the RGCs in the ILM, have an important role in modulating the electric signal transmission that will arrive to the optic

nerve (Peña and Vazquez, 2022; Rasmussen and Samson, 2017). On the other hand, Müller cells have a more holistic role, stabilising and physically maintaining the retinal architecture due to their location alongside the whole retinal width but also regulating the homeostasis of the retina through the secretion of multiple molecular modulators with several roles in terms of cell survival maintenance, immune response and cell migration, among others (Peña and Vazquez, 2022).

Finally, microglial cells stand out as the main immunologic actor on the retina, having an essential function on the defence against external and internal injuries (Peña and Vazquez, 2022; Peng et al., 2014). All these cellular types (Table 2), work synergically to maintain the homeostasis in the retina, having an effective communication rate by secreting a series of molecules that can modify each other's behaviours.

Having this in mind, different pathological conditions can cause a functional dysregulation on these cells, inducing the activation of different biochemical and cellular processes that, by extension, can alter the functionality of the retina, as further explained in following sections of this text.

Table 2. Cell types of the human retina.

Cell Group	Cell type	Location	Function
Visual Axis	*Photoreceptor*	OSL, OLM, ONL	Transduction of light information into electric impulses.
	Bipolar cell	OPL, INL, IPL	Transferring of electric impulses to the ganglion cells.
	Ganglion cell	IPL, GCL, NFL	Their axons collectively constitute the optic nerve, leading the visual information to the brain.
Complementary Neurons	*Horizontal cell*	OPL, INL	Accentuating the borders of the images perceived by a mechanism of lateral cell inhibition.
	Amacrine cell	IPL. INL	Modifying the signals that the ganglion cells receive, determining their sensibility to moving visual stimuli.
Glia	*Astrocyte*	ILM	Modulation of the electric signal transmission that will arrive to the optic nerve.
	Müller cell	From the NFL to the ELM	Structural and homeostatic role, participating in cell survival maintenance, immune response and cell migration processes, among others.
	Microglia	Through all retinal layers	Immune response.

2.2 Retinal vasculature

The retina is characterized by presenting a high demand of oxygen and nutrients to fulfil its energetic requirements, as previously commented in subsection 1.2. Consequently, the blood flux in the retinal tissue is constant, and the adequate functionality of the circulatory system is one of the key factors when studying retinal homeostasis (Grossniklaus et al., 2015). Having this important physiological role, the retinal circulatory system is characterized by being highly regulated and utterly controlled by the cellular elements that compose this tissue. With this in mind, the two main elements that characterize the retinal vasculature and whose related mechanisms are paramount to understanding any retinal pathology are discussed in subsections 2.2.1 and 2.2.2.

2.2.1 The blood-retinal barrier

The blood-retinal barrier (BRB) is a cellular formation whose main objective is protecting the retinal cells from any harmful substances that may be transported by the blood flow, tightly controlling the flux of substances into and out of the retina and preventing the infiltration of immune system-related elements, turning the retina into an immunoprivileged tissue (Rudraraju et al., 2020). In this sense, its main role is to preserve an adequate medium

in which the retinal cells can conduct its function without being perturbed by any harmful stimulus (Runkle and Antonetti 2011).

From a regional perspective, the BRB is divided into two parts (Patel et al., 2023) (Figure 5): i) the inner BRB (iBRB) and ii) the outer BRB (oBRB). The iBRB includes two main elements that guarantee the separation between the blood flux and the retina: i) the tight junctions of the retinal endothelial cells (RECs) that conform the blood vessels, and ii) the prolongation of different cell types that cover the walls of the blood vessels. The group of cells that are part of the iBRB include astrocytes, Müller cells and pericytes, being all of them characterized by recovering the external surface of the blood vessel and having the ability of modulating its functionality, as described in the next section. On the other hand, the oBRB is mainly composed of the tight junctions formed between the cells of the RPE, serving as a filter that regulates the exchange of solutes from the choroid into the retina.

Disruption of this structural and functional barrier may lead to the development of pathological alterations in the retinal tissue due to an imbalance in the exchange of substances with the circulatory system (O'Leary and Campbell, 2023; J. Zhou and Chen, 2023).

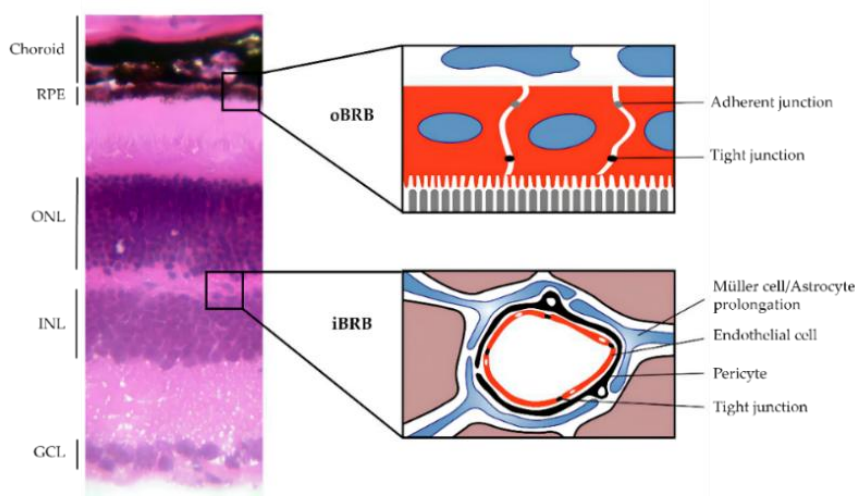


Figure 5. *Elements of the blood-retinal barrier (BRB). BRB is divided into an outer (oBRB) part, formed by the intricate communication between the RPE cells, and an inner (iBRB) part, including the tight junctions between the endothelial cells and the cellular terminations of Müller cells, astrocytes and pericytes. RPE: Retinal Pigment Epithelium; ONL: Outer Nuclear Layer; INL: Inner Nuclear Layer; GCL: Ganglion Cell Layer. Figure adapted from Pan et al. (2021).*

2.2.2 The neurovascular unit

Due to its importance for the correct functioning of the retina, the vascular system must be strictly regulated to fulfil any tissular requirement. In order to do so, there is a strong association between the neural and the vascular part of the retina in which several cell mediators are synthesized allowing to change the functionality of the vascular elements (Mrugacz et al., 2021; Nian et al., 2021). This cell-cooperative system receives the name of neurovascular unit (NVU), and it allows the retina to regulate the blood flow in response to its

metabolic demands, simultaneously maintaining the integrity of the iBRB (Grimes et al., 2024; Zafar et al., 2019). In this sense, any disturbance in either the vascular or the neural part of the NVU may lead to alterations in the whole tissue functioning, being a key factor in most retinal pathologies.

3. Diabetes mellitus and diabetic retinopathy

Diabetes mellitus (DM) refers to a collective group of complex metabolic pathologies characterized by a chronic hyperglycaemic state, direct result of an alteration in the metabolism of the glucose (American Diabetes Association Professional Practice Committee, 2022). Pathogenically, DM can be divided into 4 main groups, as depicted in table 3: i) type 1 DM (T1DM; DM1), of autoimmune origin, ii) type 2 DM (T2DM, DM2), result of an alteration in the signalling processes related with insulin, iii) gestational diabetes, consequence of the changes associated with the pregnancy, and iv) specific gene-related DM, which include various mutations in genes somehow associated with the metabolism of the glucose.

Independently of its origin, the increase of blood glucose levels leads to long-term detrimental effects in many organs as a result of an alteration in several cell signalling processes. Due to its high energetic demands and irrigation, the retina is keen to suffer the consequences

of persistent hyperglycaemic conditions. This situation causes functional alterations in the vascular and neuronal retinal cells that altogether lead to the establishment of a pathologic condition termed diabetic retinopathy (DR),(Fischer, 1989; Wong et al., 2016) whose main clinical aspects are discussed in section 3.1.

Table 3. Diabetes mellitus types and their main traits.

Diabetes type	Characteristics
Type 1	Autoimmune pathology commonly diagnosed in an early-age characterized by an insufficient insulin production as a consequence of the loss of β-pancreatic cells. Patients with this condition require the exogenic administration of insulin and a strict regulation of its glycaemic levels.
Type 2	Multifactorial metabolic disease result of an alteration in the signalling pathway of insulin often described as a "resistance" phenomenon. It is strongly associated to dietary patterns and unhealthy lifestyle, with a typical onset around 40-60 years old. It is treated with specific anti-diabetic pharmacotherapy.
Gestational	Pathology associated to the pregnant state and commonly diagnosed in the second or third trimester of pregnancy in women without a previous DM diagnosis. It requires a strict monitoring to preserve the health of the foetus.
Specific gene-related	Diabetic phenotypes whose causes are a direct consequence of a single mutation on a specific gene or a syndrome. This group includes the Maturity Onset Diabetes of the Young (MODY), cystic fibrosis-related diabetes and diabetes caused by rare syndromes (Wolfram syndrome, Alström syndrome).

3.1 Clinical aspects of diabetic retinopathy

DR can be defined as a microvascular and neuronal complication derived from the progression of DM that can induce a series of structural and functional vitreoretinal changes, ultimately leading to visual impairment and blindness if left untreated (Cheung et al., 2010; Wong et al., 2016). In this sense, DR is the first cause of vision disability in working aged people worldwide (Kropp et al., 2023), being associated with serious emotional conditions and a reduction in the life quality and autonomy of affected patients, their families and the society (Jampol et al., 2020).

From a clinical perspective, multiple population studies have emerged to better grading DR, including the Early Treatment Diabetic Retinopathy Study (ETDRS), classically considered the referential classification for multiple clinical trials (ETDRS, 1991), and the Global Diabetic Retinopathy Project Group (GDRPG), that launched the International Severity Scale for DR (Wilkinson et al., 2003). The DR classification modified from both the ETDRS and GDRPG study groups, widely used in clinical practice, divides the disease in two well-differentiated states (Figure 6): i) non-proliferative DR (NPDR) and ii) proliferative DR (PDR) (Wong et al., 2016). NPDR is considered the initial state of the pathology, characterized by the formation of isolated retinal microaneurysms without any additional lesions or

visible abnormalities on its mild/early state. As the disease progresses, microaneurysms and other vascular alterations-derived formations, such as haemorrhages, solid exudates and cotton-wool spots become more common, causing visual symptoms settling the moderate and subsequently severe NPDR stages (Danek et al., 2021).

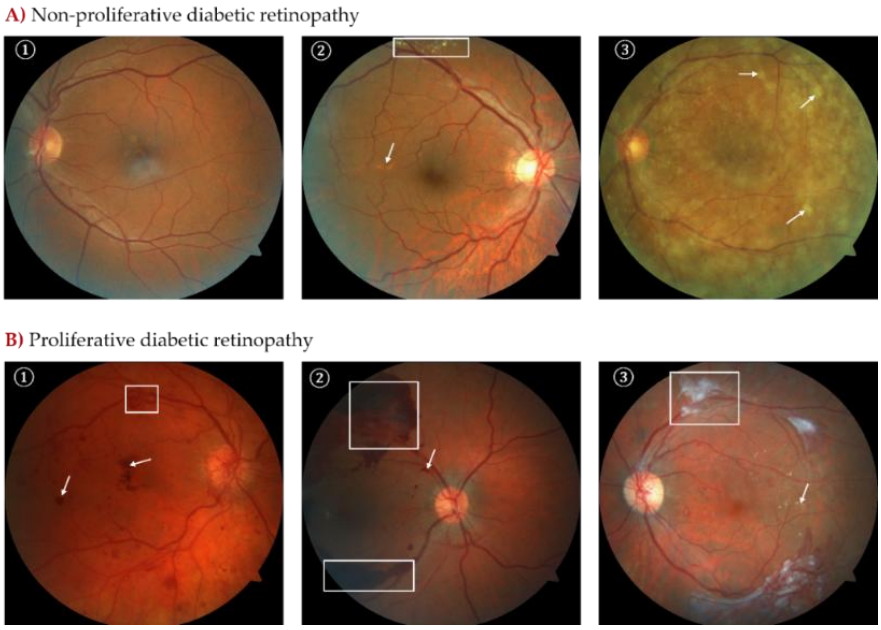


Figure 6. *Clinical DR staging. A) Non-proliferative diabetic retinopathy has 3 different stages: 1) a mild/early state, 2) moderate state, characterized by the presence of some exudates (arrow) and solid deposits (squared), and 3) a severe state, characterized by an increased number of deposits and exudates (arrows). B) Proliferative diabetic retinopathy has 3 different stages: 1) moderate state, characterized by the presence of intravitreal haemorrhages (arrows) and blood vessels alterations (squared), 2) severe state, characterized by a worsening of the intravitreal haemorrhages (squared) and the formation of new ones (arrow), and 3) fibrotic state, characterized by the formation of fibrotic tissue around the newly grown blood vessels (squared). Retinal fundus images extracted from Castillo Benítez et al. (2021).*

Overtime, hyperglycaemic conditions induce a series of retinal vascular alterations that ultimately lead to their occlusion and loose of permeability, causing a hypoxic stimulus that induces the releasing of growth factors, mainly vascular endothelial growth factor (VEGF) (W. Wang and Lo, 2018), to generate new blood vessels. This neovascularization process fails to solve the hypoxic situation and results in the synthesis of week and unstable blood vessels, prone to bleed into the vitreous cavity worsening the situation and settling the PDR stage, which ultimately leads to loss of both central and peripheral vision (Chaudhary et al., 2021). In most severe cases, the neovascularization response leads to a fibrotic phenomenon that may induce the physical separation of the nervous retina and the RPE, known as retinal detachment (W. Wang and Lo, 2018).

Additionally, changes in permeability of retinal vasculature, leaking fluid into the macula, causes the diabetic macular oedema (DMO), being the main cause of vision loss in diabetics. This situation occurs when the circulating plasma infiltrates into the macular region through the damaged blood vessels, increasing its thickness and altering its structure, which ultimately leads to a central vision loss. Interestingly, DMO can occur in any RD state, although it is usually more frequent on the more advanced ones (Bandello et al., 2017).

Up today, the vitreoretinal complication of DR has no definitive cure. Classically, clinical practice mainly considers DR as a

microvascular condition that primarily affects the retinal blood vessels, being most of its treatment strategies directed to the neovascularization phenomena (W. Wang and Lo, 2018), as depicted in Table 4.

Table 4. Therapeutic approaches for diabetic retinopathy.

Treatment	Description	Examples
Laser photocoagulation	Approach based on the generation of microscopic lesions using laser technology. Its main purpose is to lower down the oxygen demand, reducing the neovascularization process.	PASCAL, D-MPL, NAVILAS
Anti-VEGF agents	Intravitreal administration of antibodies that target and blocks VEGF's function, preventing the associated neovascularization process.	Ranibizumab, aflibercept, bevacizumab
Corticosteroids	Drugs that, when injected intravitreally, reduce the vascular permeability, help restoring the BRB and minimize the inflammatory process associated with DR. They are usually applied in form of ocular implants, which release the drug in a constant way for a longer time period without having to reintervene.	Triamcinolone, dexamethasone, fluocinolone

However, its aetiology is way more complex and involves multiple cellular processes that affect both the neural and vascular parts of the retina. Amongst these, the neurodegeneration of retinal

nervous cells has recently arisen as a key mechanism to consider when analysing the onset and evolution of DR (Simó et al., 2022). With this in mind, the pathogenic mechanisms of DR viewed under the prism of the neurodegenerative process are discussed in section 4 of this text.

4. Neurodegeneration in diabetic retinopathy

The concept of neurodegeneration englobes every pathological process in which neuronal cells lose their structure and/or functionality. In this sense, neurodegenerative diseases can potentially affect any tissue that contains neurons as a cellular component, such as the retina. Based on this, several studies have found that during the progression of DM, hyperglycaemia causes metabolic disturbances that progressively damage the retinal nervous cells, contributing to the development and progression of DR (Ciprés et al., 2022; Pillar et al., 2020). Furthermore, this neurodegenerative process seems to be an early phenomenon in the course of the pathology, and it may occur even before the vascular alterations settle in (Soni et al., 2021).

Neurodegeneration of the retinal neuron cells has recently become an important aspect to consider when studying and clinically managing DR. The mechanism, role and implications of this process in this pathology are properly discussed in the following subsections.

4.1 Neurocentrism vs. vasculocentrism

Currently, the different hypothesis that try to explain the pathogenic origin of DR could be divided into two groups: i) a neurocentric approach, based on the idea that the damage to the neural cells of the retina precedes every subsequent clinical manifestation of the pathology, and ii) a vasculocentric approach, according to which the vascular alterations would precede the neural damage. In this sense, a series of studies have systematically shown that animal models and patients with DM but without any visual manifestations present active signalling routes that directly target retinal neurons, accompanied by a thinning of the nervous layers of the retina, such as NFL and GCL (Gasecka et al., 2020; Soni et al., 2021). However, the improvement on the resolution in vascular imaging has also demonstrated preclinical retinal vascular and choroidal changes (Sachdeva, 2021).

Both realities seem to fade in when the NVU concept is considered. On this basis, and as commented in subsection 2.2.2, the tight communication between the neural and vascular parts of the retina makes it difficult to perceive both pathogenic possibilities separately. Thereby, whether a specific process derived from a dysregulation of glucose metabolism occurs in a neural or vascular cell

is not as important for the global outcome of the pathology, given that both cellular types will be mutually affected by it.

The following subsections analyse the signalling processes that directly affect the retinal neural cells and that can be englobed in the neurodegenerative-associated processes group. However, as mentioned in this subsection, all of them are tightly associated with the vascular dysfunction processes due to an impairment of the neurovascular unit, being this concept crucial to fully understand the nature of DR as a visual pathology.

4.2 Neurodegeneration signalling in diabetic retinopathy

Such as in any other neurodegenerative pathology, there are multiple cell-signalling pathways involved in the onset and evolution of the neuronal damage, regularly interrelated with each other. However, all these processes share a common origin: the metabolic dysregulations caused by hyperglycaemia. The following subsections collect the main molecular mechanisms that intervene in the neurodegenerative process associated with DR, whose aetiological sequence is compiled in Figure 7.

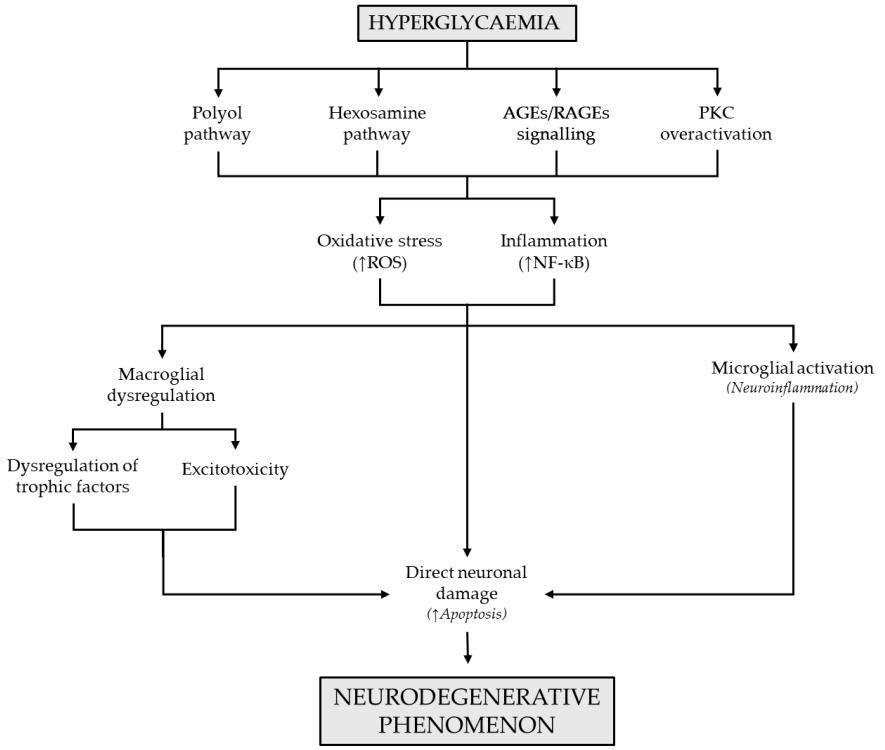


Figure 7. *Signalling mechanisms associated with neurodegeneration in DR. Hyperglycaemia alters several biochemical pathways linked with the metabolism of glucose (aldose reductase overactivation, hexosamine pathway, AGEs/RAGEs signalling and PKC overactivation). In turn, this settles a pro-oxidant (↑ROS) and pro-inflammatory (↑NF-κB) state, leading to a series of modifications in the retinal neural cells, both glial (microglial activation) and neuronal (direct neuronal damage). This ultimately determines the neurodegenerative phenomenon characteristic of DR. AGEs: advanced glycation end products; RAGEs: receptor for advanced glycation end products; PKC: protein kinase C; ROS: reactive oxygen species; NF-κB: nuclear factor kappa B.*

4.2.1 Hyperglycaemia-related routes

Diabetes-induced hyperglycaemia involves two situations in the retina: i) an intracellular glucose accumulation due to the exceed on the demand of this carbohydrate by the retinal cells, and ii) a glucose extracellular accumulation direct consequence of the rising on blood glucose levels. Conjunctively, both situations cause an imbalance in metabolic pathways responsible for transforming glucose into energy or other organic molecules, as well as other mechanisms related to the cellular ability of interacting with their environment.

Under euglycemic conditions, glucose is metabolized in various steps, involving the glycolysis, citric acid cycle and oxidative phosphorylation biochemical pathways, to obtain energy and maintaining the homeostasis of this sugar. However, under diabetic conditions there is an increased flux of substrates to these routes, particularly to the glycolytic pathway, leading to an abnormal increase in the concentrations of its intermediates (Aldosari et al., 2022). This accumulation causes the overactivation of three main pathways with harmful effects, as depicted in Figure 8: i) the polyol pathway, ii) the hexosamine pathway and iii) the overactivation of protein kinase C (PKC).

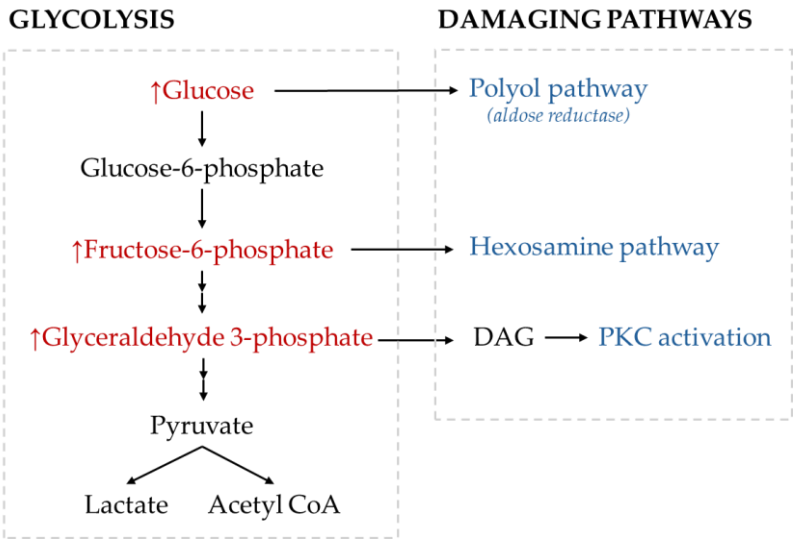


Figure 8. *Hyperglycaemia-related pathways. The accumulation of glucose and its glycolytic intermediates leads to the activation of different deleterious pathways. DAG: Diacylglycerol; PKC: Protein kinase C.*

The saturation of the glycolytic pathway causes the metabolization of the glucose excess by the aldose reductase, which synthesizes sorbitol; in turn, sorbitol is transformed into fructose by the enzyme sorbitol dehydrogenase (Lorenzi, 2007). Both reactions, englobed in the so-named polyol pathway, cause a depletion of nicotinamide-adenine dinucleotide phosphate (NADPH), cofactor needed to maintain the cellular antioxidant capacity. In this sense, the overactivation of this pathway leads to a diminished intracellular concentration of nitric oxide (NO) and reduced glutathione (GSH), establishing a pro-oxidant milieu in the affected cells (dos Santos et al., 2023; Lorenzi, 2007).

The glucose molecules that enter the glycolytic route are metabolized into glucose-6-phosphate by the enzyme glucokinase. In turn, glucose-6-phosphate is reversibly metabolized into fructose-6-phosphate by the enzyme glucose-6-phosphate isomerase. At this point, fructose-6-phosphate molecules can follow the glycolytic path getting turned into 1,3-diphosphoglycerate, or they can be metabolized by the enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT) to be transformed into glucosamine-6-phosphate and enter the hexosamine pathway (Paneque et al., 2023). The activity of several enzymes of this route, such as O-linked N-acetylglucosamine transferase (OGT), results in the transformation of the glucosamine-6-phosphate molecules into uridine-5-dihosphate-N-acetylglucosamine (UDP-GlcNAc), precursor of several important molecules such as glycoproteins, glycolipids proteoglycans and glycosaminoglycans (Paneque et al., 2023). Diabetic conditions induce the activity of GFAT, favouring the functioning of the hexosamine pathway (Srinivasan et al., 2007). In turn, this route contributes to the establishment of a pro-oxidant and pro-inflammatory state as a consequence of the post-translational O-GlcNAcylation of key molecules capital for the correct functioning of the retinal cells, triggering their death by apoptosis (Semba et al., 2014).

Lastly, high intracellular glucose concentration leads to an accumulation of glyceraldehyde-3-phosphate which can be

enzymatically transformed to diacylglycerol (DAG). This buildup of DAG can over-activate several PKC isoforms within the cell, inducing an alteration in several signal transduction cascades (Panda et al., 2023). In this sense, PKC triggers the production of reactive oxygen species (ROS), activates the synthesis of proinflammatory mediators in several retinal cells, may induce an alteration of vascular permeability and causes a general dysfunction of the neuronal activity (Aldosari et al., 2022; dos Santos et al., 2023).

In addition to this, the hyperglycaemic milieu caused by the diabetic condition induces the formation of the so-called advanced glycation end products (AGEs), a heterogeneous group of molecules produced from the non-enzymatic glycation of other macromolecules such as proteins, lipids or nucleic acids (Qingzheng et al., 2022). The accumulation of these glucose-modified products may trigger several signalling pathways, often through the direct activation of specific receptors [AGEs receptors (RAGEs)], inducing inflammatory processes, reactive oxygen species (ROS) formation and an increased apoptosis rate of the retinal cells, amongst other processes (Mrugacz et al., 2021; Sachdeva, 2021)n increased apoptosis rate of the retinal cells, amongst other processes (Mrugacz et al., 2021; Sachdeva, 2021).

All these described metabolic alterations, directly related with glucose homeostasis, constitute the first pathological mechanisms that will ultimately lead to the activation of subsequential signalling routes

involved in the loss of retinal cells' functionality under diabetic conditions. Altogether, hyperglycaemia-induced signalling pathways lead to a prooxidant and proinflammatory condition that constitute the basis of the neurodegenerative phenomenon and general pathophysiology of DR, as described in the following subsections. However, it is important to note that these pathways will continue to occur in the course of the disease, being key players in all DR stages.

4.2.2 Oxidative stress and inflammation

Under physiological conditions, cell metabolism naturally induces the synthesis of respiratory chain-derived products known as reactive oxygen species (ROS), including the superoxide (O_2^-), oxygen peroxide (H_2O_2) and hydroxyl radical ($HO\cdot$) (Augustine et al., 2021; Sies, 2015). These molecules have detrimental effects and must be neutralised in order to keep the homeostasis. To do so, cells count with a plethora of enzymatic [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), etc.] and non-enzymatic [glutathione (GSH), vitamin C, vitamin E, etc.] antioxidant elements that allow them to keep an adequate redox balance (Mendonca et al., 2020). However, under diabetic conditions the alteration of glucose metabolism increases the production of cytosolic ROS establishing a situation of oxidative stress (OS) that directly affects retinal functionality, as described in subsection 4.2.1.

Amongst the multiple alterations induced by OS in the retinal cells, a crosslink between ROS accumulation and the inducement of an inflammatory response mainly mediated by the transcription factor nuclear factor kappa B (NF-κB) has been widely described (Park, 2023; Soni et al., 2021; Z. Xu et al., 2023). In this sense, ROS facilitates the nuclear translocation of NF-κB inducing the synthesis of proinflammatory mediators, such as interleukin (IL)-1β, IL-6, IL-8, IL-18 and tumour necrosis factor α (TNF-α) (Nebbioso et al., 2022). In turn, this proinflammatory response alters the antioxidant capacity and functionality of retinal cells, increasing ROS production and creating a feedback loop that leads to cell dysfunction and contributes to the neurodegenerative phenomenon of DR (Wei et al., 2022).

Having this in mind, OS and proinflammatory cell signals can be considered as the central actors in the pathophysiology of DR, having a connective role between the initial glucose-related metabolic disturbances and the posterior cellular functional alterations that characterize the more advanced stages of the disease.

4.2.3 Reactive gliosis

The proinflammatory and prooxidant conditions triggered by the diabetic metabolic changes lead to the disruption of retinal homeostasis due to cellular dysregulation. Although OS and inflammation initially appear as a general phenomenon that involves

every cellular type, they activate a range of cell-specific responses. Due to their plethora of functions, glial cells are key actors in this process, and their functionality loss has been named reactive gliosis (Pekny and Pekna, 2016).

4.2.3.1 Macroglia dysregulation

Macroglia ensures the sustainment of the extracellular retinal environment and guarantees a correct regulation of neuronal function (Ghaseminejad et al., 2020). However, reactive gliosis induces a series of changes that constitute the basis of two key mechanisms involved in the neurodegenerative process associated with DR: i) dysregulation of trophic factors signalling and ii) excitotoxicity.

Under physiological conditions, macroglia cells are involved in the synthesis and secretion of neurotrophic factors (NTF), molecules that regulate the physiological growth, maturation and functionality of retinal cells (Jindal, 2015; Vecino et al., 2016). In the first stages of the disease, NFTs are secreted as a response to the initial cell damage. However, long-term hyperglycaemia and its metabolic alterations alter macroglia cells' capacity to synthesize these products. Consequently, this causes an abnormal expression of some of these molecular messengers, including brain-derived neurotrophic factor (BDNF), transforming growth factor β (TGF-β), pigment epithelial-derived factor (PEDF), somatostatin, erythropoietin and VEGF,

amongst several others (Bikbova et al., 2014; Gasecka et al., 2020). This situation, alongside with the increased synthesis of proinflammatory mediators, ultimately leads to an imbalance in retinal homeostasis, contributing to both neovascularization and neurodegeneration phenomena (Simó et al., 2021; Soni et al., 2021).

Macroglial cells also have a capital function maintaining retinal homeostasis by participating in the recycling of neurotransmitters, the molecules responsible for transmitting information throughout the retinal neuronal axis. Amongst the different neurotransmitter types found within retinal cells, glutamate outstands for being the main excitatory neurotransmitter, responsible of transmitting the visual information to the superior cerebral nuclei (Thoreson, 1999). Physiologically, the normal activity of the retinal cells causes the release of these messengers to the so-called synaptic spaces, located between two communicating neurons. Although most of these molecules meet their target receptors in the post synaptic membrane, some escape to the extracellular space, having potentially deleterious effects when accumulated due to the overactivation of their receptors (Thoreson, 1999). In this context, macroglial cells function by inactivating the excess of glutamate molecules capturing them through specific receptors, such as L-glutamate/L-aspartate transporter (GLAST). Once in the intracellular space, glutamate can then be transformed into less harmful molecules such as glutamine by

the ATP-dependent enzymatic action of glutamine synthetase (GS) (Aldosari et al., 2022; Thoreson, 1999; Verma et al., 2022).

Hyperglycaemia and its metabolic consequences cause the dysfunction of macroglial cells, directly affecting their capacity to regulate neurotransmitter signalling as a result of a downregulation in the expression of GLAST and GS, amongst other mechanisms (Mrugacz et al., 2021; Verma et al., 2022). This favours the accumulation of glutamate in the extracellular space, inducing a deleterious signalling cascade directly related to the activation of specific glutamate receptors and inducing an excitotoxic phenomenon (Figure 9) (Boccuni and Fairless, 2022). Amongst them, N-methyl-D-aspartate (NMDA) receptors family appear as the key responsible for the toxic effects of glutamate, functioning as cation channels of high conductance permeable to calcium (Ca^{2+}), amongst other cations (Bai et al., 2013; Smith, 2002). In this sense, the activation of these receptors induces a plethora of mechanisms that end up promoting neuronal degeneration (Figure 9) (Bading, 2017; Pichavaram et al., 2019). Additionally, glutamate accumulation may lead to a depletion of GSH as a consequence of its inhibitory effect on glutamate/cystine antiporters, responsible of importing cystine to the intracellular space. Being an essential element for the synthesis of GSH, the consequences of a glutamate excess result in the worsening of OS (Aldosari et al., 2022; Boccuni and Fairless, 2022; Lau and Tymianski, 2010).

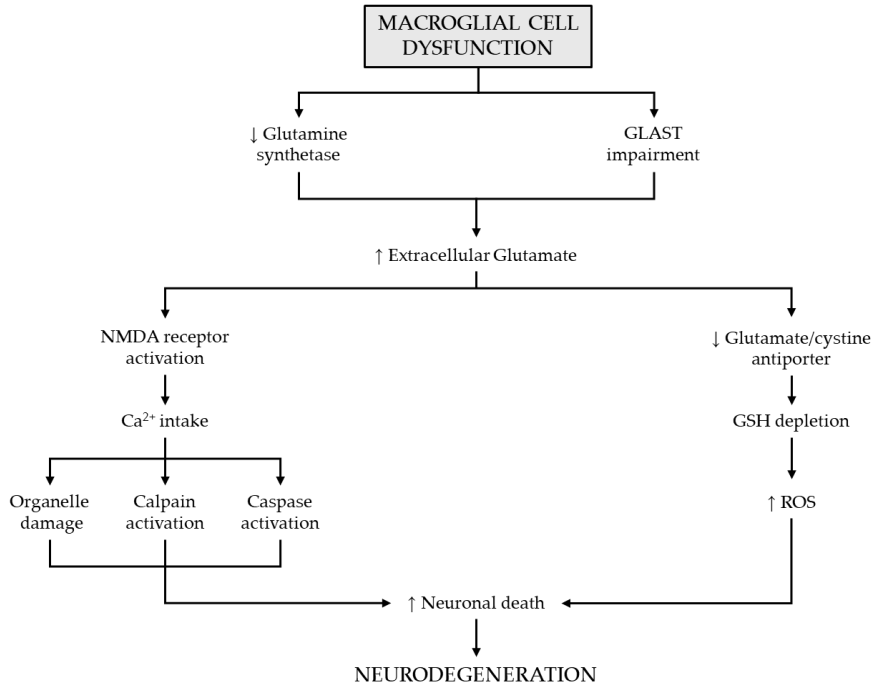


Figure 9. *Glutamate excitotoxicity mechanism. Under hyperglycaemic conditions macroglial cells suffer a series of alterations that significantly reduce their capacity to capture and recycle glutamate, including a reduction of GS and GLAST activities. Accumulated extracellular glutamate can induce neuronal damage through two main mechanisms: a) activation of NMDA receptors, which causes an increased intake of* Ca^{2+} *responsible of activating different harmful signals (organelle damage, calpain and caspase activation); and b) the inhibition of the glutamate/cystine antiporter, which causes a depletion of GSH consequence of a cystine deficit worsening the prooxidant milieu. These pathways ultimately lead to neuronal death and contribute to the neurodegenerative phenomenon. GLAST: L-glutamate/L-aspartate transporter; NMDA: N-methyl-D-aspartate; GSH: glutathione; ROS: reactive oxygen species.*

4.2.3.2 Microglial overactivation

Under physiological conditions, microglial cells are located in the plexiform layers of the retina, emitting ramifications that surround the retinal cells and constantly receptive to any potential signal that might activate an immune response (Karlstetter et al., 2015). Whenever they detect a harmful stimulus, microglial cells get activated and develop a specific phenotype characterized by a change in their morphology, mobility and secretion capacity (Kovács-Valasek et al., 2023). More specifically, they adopt an amoeboid structure by retracting their cellular processes, becoming more mobile and therefore increasing their ability to displace to the damaged areas (Karlstetter et al., 2015; Lim et al., 2019). Additionally, they increase their ability to release inflammatory mediators and organize the activity of other cellular elements from the immune system (Ibrahim et al., 2011).

According to the nature and functionality of the released inflammatory mediators, activated microglia can be divided into two different phenotypes named M1 and M2, according to their macrophage nature (Wu et al., 2021). M1-like microglial cells play a proinflammatory role by producing and releasing proinflammatory cytokines such as TNF-α, IL-1α, IL-1β, IL-6, IL-12 and IL-23, mostly depending on NF-κB nuclear translocation, as well as chemoattractant molecular messengers to attract other cells that will participate in the

immune response (Wu et al., 2021). On the other hand, M2-like microglial cells produce anti-inflammatory molecules such as IL-4, IL-10 and IL-13 which help regulating the immune response and maintaining retinal homeostasis (Wu et al., 2021). In this sense, the distinction between both phenotypes can vary according to the retinal demands and microglia adapts to maintain a balance in the immune response (Graeber et al., 2011). Under normal physiological conditions, the activated microglia-initiated inflammatory response initiated by the M1 microglial cells is countered by a number of immune-suppressive mechanisms conducted by M2 microglial cells in order to re-establish equilibrium in the retina (Pan et al., 2021).

The harmful and chronic metabolic effects derived from the diabetic condition cause the dysfunction of this phenomenon, inducing and overactivation of the M1 phenotype and a diminishing of the M2 functions, causing a polarization towards a proinflammatory response (Kovács-Valasek et al., 2023; Pillar et al., 2020). This imbalance has holistic consequences in the physiology of the retina, potentially damaging every cell type. However, there is a direct deleterious effect of the activated microglia on retinal neurons, being a key contributor to the neurodegenerative phenomenon and generally to the evolution of DR (Pan et al., 2021). In this sense, the overexpression of molecules such as IL-1β and TNFα by the microglial cells, although beneficial in short-term responses, ends up getting

associated with cytotoxicity and cell death as a consequence of an alteration of the normal immune response (Pan et al., 2021). Figure 10 illustrates the dynamism of the macroglia activity in physiological and pathological conditions.

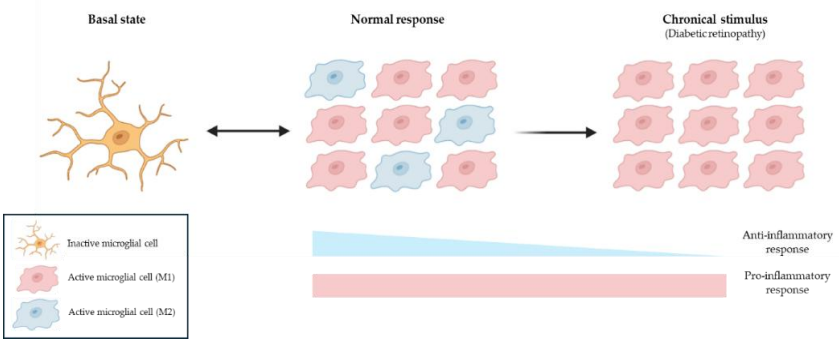


Figure 10. *Microglia overactivation in diabetic retinopathy. Under physiological conditions and without the presence of a specific harmful stimulus, microglial cells remain quiescent in a ramified form englobing the surrounding retinal cells. When a retinal insult occurs, microglial cells experience a phenotypic change and get activated, creating a refined equilibrium between a proinflammatory (M1) and anti-inflammatory (M2) response that allows an adequate resolution of the initial offence. However, the diabetic conditions, mainly characterised by the releasing of proinflammatory molecules and the establishment of an oxidative stress situation, the microglial activity switches towards a proinflammatory response, losing the previously mentioned equilibrium and playing an important role in the neurodegenerative process by over-secreting molecules with cytotoxicity activity.*

4.2.4 Neuronal death

The main signalling alterations directly related to the neurodegenerative process have been sequentially reviewed throughout subsection 4.2. Collectively, all these lead to a primary

outcome: retinal cell damage. In the context of this research, this phenomenon affects all types of neuronal retinal cells, inducing the neurodegenerative-associated events that characterize DR. Amongst the different routes activated under these conditions, apoptosis stands out as the main process responsible for the induction of cell death, which leads to a reduction of the density and functionality of the neuronal component of the retina (N. Zhang et al., 2023).

Apoptosis can be defined as a programmed process whereby cells undergo a series of morphological and biochemical changes that ultimately lead to a complete loss of functionality or cell death (Elmore, 2007). This cellular response involves a complex interaction between cell signals and effector proteins, and it can be initiated through two different pathways depending on the detonating stimulus (Kashyap et al., 2021; Savitskaya and Onishchenko, 2015) (Figure 11): i) the extrinsic pathway, initiated as a result of an extracellular signal; and ii) the intrinsic pathway, direct consequence of an intracellular harmful element. Both pathways depend on the activity of caspases, proteolytic enzymes that have the ability to exert the functional changes associated with enzymes (M. L. G. Ramirez and Salvesen, 2018). Both extrinsic and intrinsic pathways activate specific families of caspases that end up converging into the execution pathway, characterized by the activation of caspase 3 (CAS3), the main effector of the apoptotic response (T. A. Chen et al., 2001; D'Amelio et

al., 2010). Proinflammatory molecules such as TNF-α can initiate the extrinsic pathway, whereas the increased production of ROS and the consequent alteration of mitochondrial function can activate the intrinsic pathway (Savitskaya and Onishchenko, 2015).

Physiologically, apoptosis is a natural-occurring phenomenon necessary for the correct development and regeneration of tissues (Kuranaga, 2012; Rai et al., 2005). However, the increase in pro-apoptotic signalling that characterizes the diabetic condition induces an imbalance between cell death and renovation, leading to the loss of cell density and tissular functionality (Adamiec-Mroczek et al., 2016; Krijnen et al., 2009). In the case of DR, the increased production of ROS, the uncontrolled secretion of proinflammatory molecules, the decline in trophic factor's production and the effect of other harmful signalling processes such as glutamate's accumulation cause cells to undergo apoptosis at a higher rate than they would normally do (Barber et al., 2011; Soni et al., 2021). Importantly, this increased apoptotic rate has a greater impact on neuronal cells, characterized by presenting a higher metabolic rate and being extremely dependent on their microenvironment composition (Rossino et al., 2019).

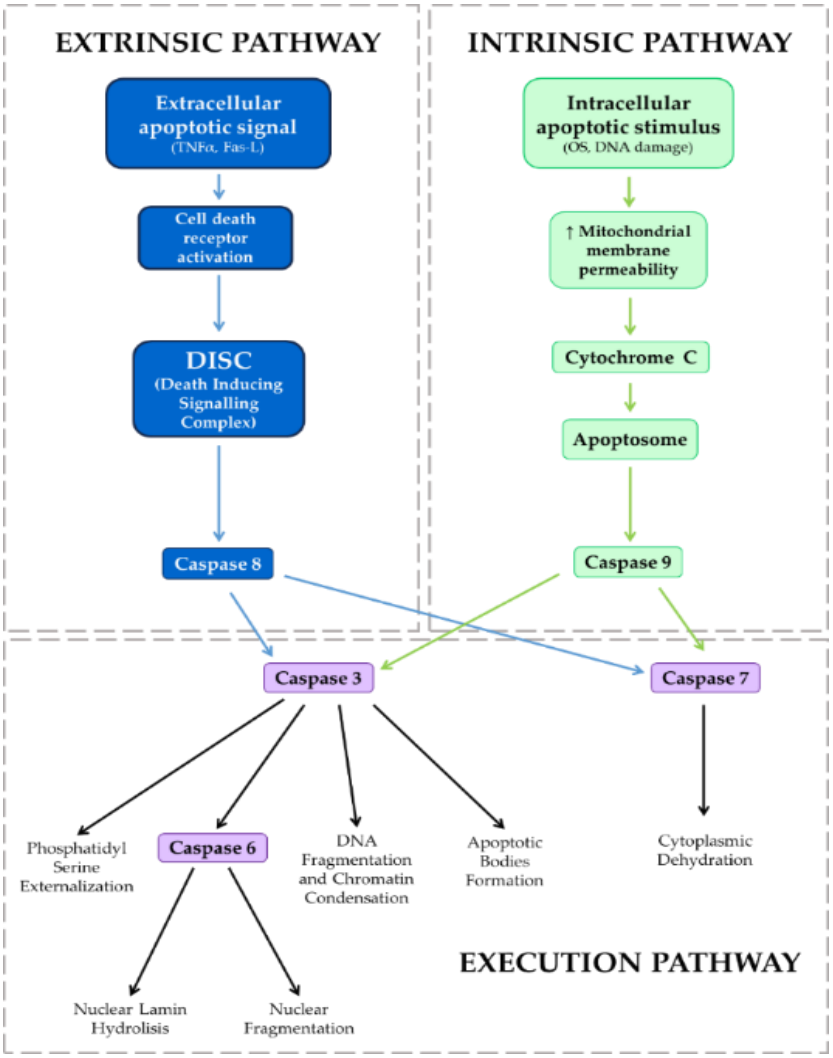


Figure 11. *Apoptotic pathways. The extrinsic pathway begins with the recognition of some extracellular apoptotic signal, such as TNF-α, by specific cell membrane receptors. This molecule-receptor interaction leads to the recruitment of the Death-Inducing Signalling Complex (DISC), which activates caspase 8. Intracellular proapoptotic stimuli, such as oxidative stress, alter mitochondrial physiology leading to cytochrome C's exit to the cytoplasm. This induces the assembling of the so-called apoptosome, a multiproteic element that activates caspase 9. Both apoptotic pathways converge in the execution pathway, where the activated effector caspases 3 and 7 are responsible for inducing all cellular changes that define the apoptotic process.*

Having all of this in mind, the neurodegenerative process that characterizes DR onset and progression appears as a complex signalling matrix that is highly dynamic and depends on the stage of the pathology. In this context, the identification of molecules that may play a role in the evolution of DR, also called biomarkers, is capital to fully understand the signalling profile and molecular basis of the pathology, and it could play a key role in the diagnosis and monitoring of the disease. Throughout the present research work, a holistic molecular characterization of the main important signalling routes in patients with DR has been conducted, with a special emphasis on those molecular elements directly related to the neurodegenerative phenomenon.

II. Study justification, hypothesis and objectives

Study Justification

The DM pandemic has deleterious effects for the patients, the families and the society, creating major challenges for patient care and clinical and pre-clinical research worldwide. The International Diabetes Federation (IDF) Congress (Stockholm, Sweden, 2024) stated that up to 550 million adults aged 20–80 are currently suffering DM.

The impact of the disease in the eyes and vision is inexorable, further exacerbating the burden of the pathology (Cheung et al., 2010; ETDRS, 1991; Wilkinson et al., 2003; Wong et al., 2016). The DR has no definitive cure, and the medical-laser-surgical treatment is directed to the ocular fundus signs. A 5-year cumulative rate of severe visual impairment has been described in NPDR patients without treatment, that rises to 50% in PDR cases (Das et al., 2021; Owsley et al., 2015; Shyam et al., 2025).

The DR is a major vision-threatening complication, that demands to take proper measures for screening, early diagnosis and timely therapeutic interventions.

Hypothesis

The present research work focuses on the importance that the neurodegenerative process has in the onset and progression of DR as a complication of DM. Given the neurodegenerative nature of DR, different processes have been suggested, separately, to explain the

observed death of neuronal cells in the retina of diabetic patients. Amongst these mechanisms, excitotoxicity, NTF dysregulation, neuroinflammation and apoptosis induction have been suggested as key actors in the thinning of neuronal retinal cell layers. Having this into account, this study hypothesizes that these mechanisms do not operate independently; instead, there is likely a significant crosstalk between these signalling pathways, which collectively drive the neurodegenerative process characteristic of DR.

Objectives

The **primary objective** of this research work is to increase our understanding of the role that neurodegeneration-associated signalling pathways have on DR's pathological evolution, giving a holistic view of neurodegeneration in the context of the disease. With this, the main goal is to improve our clinical ability to make a proper diagnosis and have a better tracking of the pathology. From this main objective, several **secondary objectives** arise:

1. Determine and quantify the concentration of glutamate in VB and plasma samples in individuals with and without DR with the goal of studying the role of excitotoxicity as an actor in the neurodegenerative process.

2. Determine and quantify the concentration of BDNF and TGF-β in VB and plasma samples in individuals with and without a DR in order to determine the role of NTFs' dysregulation in the pathology.
3. Determine and quantify the concentration of several pro-inflammatory molecules, including IL-6, TNF-α, IL-1β and IL-18 in VB and plasma samples of individuals with and without DR to analyse the role of neuroinflammation in the neurodegenerative process.
4. Determine and quantify the levels of VEGF in VB and plasma samples of individuals with and without DR to elucidate the role of angiogenesis in the context of neurodegeneration in DR.
5. Determine and quantify the levels of CAS3 in VB and plasma samples of individuals with and without DR to assess the impact of apoptosis in the context of the neurodegeneration process associated with DR.
6. Selection of biomarkers with potential applicability in the study and translation to the clinical DR diagnosis/prognosis and managing.

III. Materials and methods

1. Study type

The design of this study is descriptive, cross-sectional, observational, case-control, and retrospective. This multifaceted approach enables a comprehensive analysis, allowing for both the identification of patterns within the data and the comparison between groups of interest. Samples for the study were collected at multiple intervals, ensuring a consistent and representative dataset across the entire research period, which spanned from September 2022 to September 2024.

The present study was conducted according to the stablished protocol and following the principles collected in accordance with the Declaration of Helsinki for the ethical principles of medical research involving human participants (World Medical Association, 2013), the European Research Council (ERC) and the Association for Vision and Eye Research (ARVO) guidelines, and the good clinical practice guidelines (Guerra-Farfan et al., 2023). Accordingly, a protocol of the study was presented to the Clinical Research Ethics Committee (CEIm) of the Hospital Doctor Peset, receiving the corresponding approbation with the CEIm code 67.2 (Annexe I).

Regarding the ethical concerns, the risk-benefit assessment concluded that the procedures involved in the study posed no additional risk to the participants' health or interfered with their

disease diagnosis and treatment. The primary invasive procedure, vitrectomy, was deemed safe as it is the standard technique used for treating the complications specified in the inclusion criteria. Furthermore, none of the experimental techniques employed in the study compromised the physical or psychological well-being of the participants. Anonymity of the data was strictly maintained in all cases, ensuring ethical compliance and the protection of participant integrity.

The processing, communication, and transfer of personal data for all study participants complied with the provisions of the Spanish Organic Law 3/2018, of December 5, on Personal Data Protection and the Guarantee of Digital Rights, as well as Regulation (EU) 2016/679 of the European Parliament and Council, of April 27, 2016, on Data Protection. In accordance with data protection legislation, participants may exercise their rights to access, modify, oppose, or delete their data by contacting the study physician. Additionally, participants may request to restrict the processing of incorrect data, obtain a copy of their data, or request the transfer (portability) of the data provided for the study to a third party. However, if a participant withdraws from the study, the data already collected cannot be deleted to ensure the validity of the research. Participants also have the right to file a complaint with the Data Protection Agency if they are not satisfied.

2. Study population

The study population consists of Spanish nationals of both sexes, aged between 40 and 80 years old. All participants were individually selected by ophthalmology specialists from various collaborative medical centres, including Hospital Clínico (Barcelona, Spain), Clínica Innova Ocular (Valencia, Spain), Hospital Clínico Universitario (Valladolid, Spain) and Hospital Punta de Europa (Algeciras, Spain). All initial volunteers were informed and documented about the study characteristics and firmed the consent for participation.

Based on the diagnoses, participants were classified into two main groups: a) surrogative comparative control group (CC-G), comprising patients requiring intravitreal interventions for ophthalmological complications unrelated to DR and not associated with the pathogenesis of the disease [rhegmatogenous retinal detachment (RD), epiretinal membrane (ERM), or macular hole (MH)], and b) diabetic retinopathy group (DR-G), including patients with PDR that required an intravitreal intervention for the management of their pathology. A total of 150 participants were included in the study, with 75 in the CC-G and 75 in the DR-G. This sample size was determined using calculations performed with G*Power software (version 3.2.9.7) to ensure adequate statistical power (Faul et al., 2009).

3. Inclusion and exclusion criteria

Participants recruitment for the groups included in this study considered a series of inclusion/exclusion criteria, collected in Table 5, to ensure the sample was as uniform as possible.

Patients included in the DR-G had a confirmed T2DM/DM2 diagnosis accompanied by a PDR diagnosis according to the criteria collected by the International Council of Ophthalmology (ICO, Guidelines for Diabetic Eye Care). DR-G included patients of both sexes and ages comprised between 40 and 80 years of age that agreed to participate in the study by signing the informed consent. Patients younger than 40 years of age and older than 80 years of age were excluded, and so were patients with NPDR or patients with some other retinal pathology different to PDR that could interfere with the ophthalmological tests conducted. Patients with other severe systemic/neurodegenerative pathologies and treatments that could interfere with the result were discarded, as well as those patients with cognitive deficit or severe mobility problems. Conversely, the CC-G comprised patients of both sexes, aged between 40 and 80 years, who did not have DM or any sign of DR and agreed to participate in the study by signing the informed consent. These participants required intravitreal surgery for unrelated ophthalmological alterations that do not interfere with the DR pathogenesis.

Table 5. Inclusion and exclusion criteria for the study participants.

Inclusion criteria	Exclusion criteria
Comparative control group (CC-G): • 40-80 years old. • No clinical signs of DM. Glucose blood levels between 70-100 mg/dL. • No clinical signs of DR. • Presence of non-DM related ophthalmological complications, including retinal detachment (RD), macular perforations (MP) or epiretinal membranes (EM), that requires a vitrectomy. • Absence of a severe systemic or neurodegenerative disease. • Not taking any treatment that could interfere with the results. • Having signed the informed consent. • Having psychological and physical autonomy.	**Comparative control group (CC-G)**: • Age < 40 or > 80 years old. • Clinical signs of DM and/or glucose blood levels > 100 mg/dL • Detection of any clinical sign of DR that justifies conducting a vitrectomy. • Presence of any systemic or neurodegenerative disease. • Subjected to a specific treatment that could interfere with the results. • Not having signed the informed consent or revoking it. • Having cognitive deficit or severe mobility problems.
Diabetic retinopathy group (DR-G): • 40-80 years old. • DM diagnosis Glucose blood levels > 100 mg/dL. • Clinical diagnosis of PDR according to the ICO guidelines for Diabetic Eye Care. • Absence of any other severe systemic or neurodegenerative disease. • Absence of any treatment that could interfere with the results of the study. • Having signed the informed consent. • Having psychological and physical autonomy.	**Diabetic retinopathy group (DR-G)**: • Age < 40 or > 80 years old. • No signs of DM nor PDR. • Presence of advanced stages of DR. • Presence of DMO. • Presence of any severe systemic or neurodegenerative disease. • Subjected to a specific treatment that could interfere with the results. • Not having signed the informed consent or revoking it. • Having cognitive deficit or severe mobility problems.

4. Variables

The analysed variables in the study can be classified into six main categories depending on their nature:

i) Sociodemographic variables, including sex and age.

ii) Anthropometric variables, including body-mass index (BMI)

iii) Clinical ophthalmological function variables, including best corrected visual acuity (BCVA) and intraocular pressure (IOP).

iv) Clinical ophthalmological image variables, including central subfield thickness (CST) and central macular volume (CMV).

v) Biochemical variables, including the plasmatic glucose levels (Gluc), the glycosylated haemoglobin levels (HbA1c), the total cholesterol (CholT), the high-density lipoprotein cholesterol (HDL-Chol), the low-density lipoprotein cholesterol (LDL-Chol), the very low-density lipoprotein cholesterol (VLDL-Chol) and the triglyceride levels (Trg).

vi) Molecular variables, including vitreous glutamate concentration (Glut-Vit), plasmatic glutamate concentration (Glut-Pls), vitreous BDNF concentration (BDNF-Vit), plasmatic BDNF concentration (BDNF-Pls), vitreous IL-6 concentration (IL-6-Vit), plasmatic IL-6 concentration (IL-6-Pls), vitreous TGF-β concentration (TGF-β-Vit), plasmatic TGF-β concentration (TGF-β-Pls), vitreous VEGF concentration (VEGF-Vit), plasmatic VEGF concentration

(VEGF-Pls), vitreous CAS3 concentration (CAS3-Vit), plasmatic CAS3 concentration (CAS3-Pls), vitreous TNF-α concentration (TNF-α-Vit), plasmatic TNF-α concentration (TNF-α-Pls), vitreous IL-18 concentration (IL-18-Vit), plasmatic IL-18 concentration (IL-18-Pls), vitreous IL-1β concentration (IL-1β-Vit), and plasmatic IL-1β concentration (IL-1β-Pls).

5. Procedures

5.1 Patient selection

The study participants were selected based on the inclusion and exclusion criteria outlined in subsection 3 of this section. Clinical ophthalmologists from the collaborating hospitals evaluated each participant's eligibility by reviewing their medical history and conducting detailed fundus examinations using biomicroscopic techniques. Before enrolment, all participants were provided with an informed consent document that clearly explained the study's procedures, objectives, and potential risks. Participants were included in the study only after they fully understood and voluntarily agreed to the terms outlined in the consent form.

Figure 12 shows a flowchart reflecting the study participants and study groups at the onset of this work.

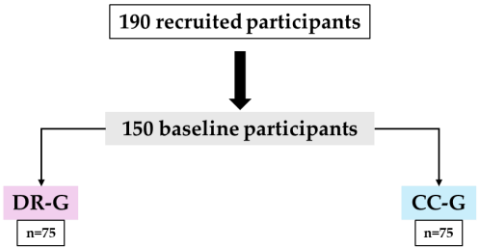


***Figure 12**. Participants selection flowchart. It displays the selection of participants and the distribution according to the main groups and subgroups of the study.*

5.2 Clinical data collection

Once selected, all participants were subjected to an ophthalmological examination to collect the variables included in subsection 4. BCVA measurement was conducted using the Snellen chart (Tsui and Patel, 2020), whereas IOP was determined through Goldmann applanation tonometry approaches (Zeppieri and Gurnani, 2024) by means of the Applanation Tonometer 900 (Haag Streit, Switzerland) mounted in a SL-D2 slit lamp with IMAGEnet 6 software (Topcon Healthcare, Barcelona, Spain). Retinal imaging variables were obtained by spectral domain optical coherence tomography (SD-OCT) (Spectralis, Heidelberg engineering, Germany) as the CST and CMV parameters, according to the stablished procedures used in conventional clinical practice (Podolenau, 2012; Waheed et al., 2023).

A classic biochemical analysis of the whole blood was performed by automatic procedures at the clinical laboratories of the collaborative hospitals to determine Gluc, HbA1c, CholT, HDL-Chol, LDL-Chol and Trg levels.

5.3 Sample collection

Two distinct sample types were obtained from the study participants to analyse the molecular variables: plasma and VB.

Whole blood was drawn following the stablished clinical protocol in fasten conditions, from the antecubital vein by using Becton Dickinson Vacutainer® Safety-Lok™ blood collection set. Ethylene-diamine-tetra-acetic acid (EDTA)-treated tubes were used. Blood cells were removed from plasma by centrifuging the blood at 2000 x g for 10 minutes at 4 ºC using a refrigerated centrifuge. The resulting supernatant was promptly transferred into clean 1.5 mL cryogenic tubes and stored at -80 ºC in ultra-low-temperature freezers.

The VB samples were obtained at the onset of the programmed vitrectomy in the corresponding study participants, being this a surgical procedure that involves the pathologic vitreous removal that is typically replaced with a similar product to maintain the eye structure, such as inert gas, silicone/heavy silicone oil, hydrogels, etc, being the most commonly used the octafluoropropane (C3F8) and sulfur hexafluoride (SF6), n-perfluorpropane gas (C3F8), and polydimetylsiloxane. Said procedure is used for a variety of ophthalmological disorders, including DR/DMO as well as in the cases of the pathologies included in the CC-G (Diaz and Arroyo, 2020). The extracted VB was collected at the onset of the vitrectomy, prior to the

opening of infusion, into clean 1.5 mL cryogenic tubes and immediately stored at -80 ºC in ultra-low temperature freezers.

Both sample types were carefully labelled, sealed and transported to the to the recipient research laboratories in Valencia. During transport, the samples were immersed in dry ice to maintain the cold chain and ensure their stability. Upon arrival, they were aliquoted and stored at -80 ºC in ultra-low-temperature freezers until further use in experimental procedures.

5.4 Experimental procedures

Once obtained, samples were processed in order to analyse the molecular variables included in the study. More specifically, the quantification of the different signalling molecules potentially involved in the neurodegenerative phenomena of DR was conducted using two main approaches: Enzyme-linked immunosorbent assay (ELISA) and fluorimetry.

The ELISA is a widely used method for quantifying proteins and other antibody-binding molecules present in various biological fluids, including plasma and VB. This assay is based on the high specificity of antibodies for their target proteins, known as antigens. Among the different ELISA modalities, the direct sandwich ELISA (Figure 13) was selected for the quantification of the molecules of interest, being the most common approach to detect soluble proteins.

Table 6 indicates the commercial kits used for each molecule included in the study.

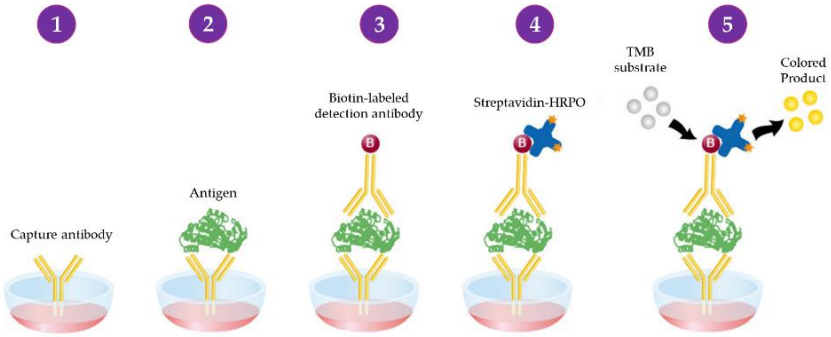


Figure 13. *Principles of the direct sandwich ELISA. Plate wells are coated with a primary capture antibody that specifically binds the target antigen present in the sample. After antigen binding, a biotin-labelled detection antibody is introduced, forming a sandwich complex. Subsequently, a streptavidin–horseradish peroxidase (HRP) conjugate binds to the biotin. This enzyme complex catalyses the conversion of the chromogenic substrate 3,3′,5,5′-Tetramethylbenzidine (TMB) into a coloured product, allowing for quantitative detection of the antigen.*

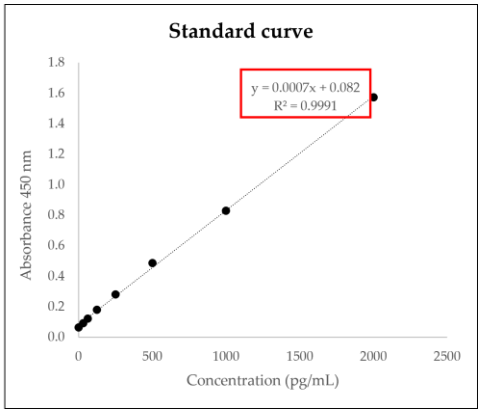
Table 6. Commercial ELISA kits used in the study.

Molecule	ELISA Kit	Reference	Brand
BDNF	Human BDNF ELISA kit	CEK1017	Cohesion Biosciences
CAS3	Human Caspase-3 (active) ELISA kit	KHO1091	Invitrogen
IL-1β	Human IL-1 beta ELISA kit	KHC0011	Invitrogen
IL-18	Human IL-18 ELISA Kit	BMS267-2	Invitrogen
IL-6	Human IL-6 ELISA Kit	EH2IL6	Invitrogen
TGF-β	Human TGF beta-1 ELISA Kit	BMS249-4	Invitrogen
TNF-α	Human TNF-alpha ELISA Kit	ab285312	Abcam
VEGF	Human VEGF ELISA kit	KHG0111	Invitrogen

In general lines, and as reported elsewhere (Aydin, 2015), all ELISAs performed followed these key steps: i) preparation of standard solutions with known concentrations to generate a standard curve; ii) addition of standards and samples to the wells pre-coated with the primary antibody; iii) washing of the wells with a wash buffer to remove unbound substances; iv) addition of a biotin-conjugated detection antibody; v) repeated washing of the wells with a wash buffer; vi) addition of an streptavidin-HRP conjugate, vii) washing of the wells with a wash buffer viii) addition of an enzymatic substrate specific to the detection antibody; ix) termination of the enzymatic reaction by adding an acidic stop solution; x) measurement of absorbance at 450 nm using a spectrophotometer (Molecular Devices SPECTRAmax Plus 384). The absorbance values obtained were directly proportional to the antigen concentration. Using the known concentration values from the standard curve, the sample wells' absorbance readings were converted into quantitative concentration values through a linear regression analysis (Figure 14).

Although the specific steps could vary slightly depending on the commercial kit used, all procedures strictly adhered to the manufacturer's instructions to ensure optimal assay performance and reproducibility of the results, including standards and reagents preparation, sample pre-processing (if needed), incubation conditions and spectrophotometry data acquisition.

Standard	Absorbance 450 nm	Concentration (pg/mL)
1	1.573	2000
2	0.830	1000
3	0.488	500
4	0.281	250
5	0.181	125
6	0.122	63
7	0.093	31
Blank	0.064	0



Absorbance

0.081	0.075	0.106	0.072	0.065	0.066	0.067	0.070
0.030	0.137	0.054	0.061	0.040	0.047	0.038	0.042
0.065	0.078	0.065	0.063	0.065	0.069	0.068	0.071
0.078	0.060	0.080	0.078	0.061	0.077	0.062	0.070
0.066	0.068	0.096	0.069	0.065	0.070	0.067	0.067
0.091	0.064	0.085	0.072	0.068	0.066	0.068	0.089
0.104	0.061	0.066	0.122	0.066	0.066	0.072	0.091
0.085	0.079	0.065	0.075	0.064	0.069	0.067	0.070

Linear regression

Concentration (pg/mL)

344.0	313.1	489.1	292.6	68.0	71.7	74.9	81.7
54.3	665.1	193.7	232.0	-	16.3	-	1.4
251.4	328.0	253.7	243.4	67.7	80.6	77.7	84.9
325.7	224.6	339.4	330.3	56.3	102.0	59.4	83.4
261.1	270.9	433.7	274.3	69.4	83.1	73.4	74.6
401.1	248.6	366.9	296.6	77.4	71.1	76.0	137.1
475.4	230.9	259.4	582.3	70.6	70.3	87.7	142.0
369.7	332.0	252.6	310.3	66.3	80.6	75.1	83.7

Figure 14. *Quantitative analysis of ELISA's results using a linear regression approach. Known standard concentrations and their corresponding absorbance readings are plotted to generate a standard curve. A linear regression analysis is performed, obtaining the equation for the line (squared in red), which is then used to calculate the concentrations of the samples based on their absorbance values.*

On the other hand, fluorimetry relies on the enzymatic generation of fluorescent compounds and the subsequent quantification of the fluorescent signal (Auld, 1999). In the context of this study, fluorimetry was employed to quantify the concentration of glutamate in both sample types, using a specific commercial kit (Glutamate Assay Kit, ref: STA-674, Cell Biolabs). The assay involved four main steps: i) preparation of standard solutions, ii) addition of the standard solutions and samples to an opaque black plate; iii) addition of the reaction mix containing glutamine oxidase and the horseradish peroxidase (HRP) enzymes; iv) measurement of fluorescence emission using a fluorometer (Molecular Devices SPECTRAmax Gemini XPS). Similarly to the ELISA technique, higher initial concentrations of glutamate correspond to greater fluorescence emission, enabling the quantification of glutamate based on the standard curve and a linear regression analysis.

6. Data analysis

All data were recorded in a Microsoft Excel spreadsheet, specifically designed for this work. Statistical analysis for this study was performed using the R programming environment (R Core Team, R: A Language and Environment for Statistical Computing, version 4.2.1, R Foundation for Statistical Computing, Vienna, Austria). The mean ± standard deviation was calculated for the continuous variables in each group. For the discrete variables, a percentage out of the total

was calculated. Chi-squared test was conducted to compare the distribution of discrete variables. The Shapiro-Wilk test was employed to evaluate the normality of continuous variables. Group comparisons were conducted using Student's t-test for normally distributed variables, while the Mann-Whitney U test was applied to non-normally distributed continuous variables. For the correlation analysis, Pearson or Spearman correlation coefficients were obtained according to the normality of the analysed variables.

To ensure data quality, an outlier detection process was implemented prior to statistical comparisons. Outliers were identified using both visual inspection methods, such as boxplots, and statistical techniques, including the interquartile range (IQR) method and z-score analysis. Observations with z-scores exceeding ±3 or values falling outside 1.5 times the IQR were flagged as potential outliers. When outliers were detected, their impact on the results was assessed, and those attributed to measurement errors or biological anomalies were excluded from further analysis. Sensitivity analyses were conducted to verify that outlier removal did not significantly alter the overall findings.

A p-value of less than 0.05 was considered statistically significant for all analyses.

IV. Results

1. Sociodemographics

From the 150 participants at baseline, 150 volunteers constituted the final sample of our study population, concluding the statistical processing.

Mean age of the whole study participants was 55.25 ± 16.40 years. The healthy subjects displayed 52.00 ± 11.50 years, whereas participants classified in the DR-G showed 56.50 ± 21.00 years. The age of the participants lacked statistical significance between groups and subgroups (p-value = 0.23).

Gender distribution was 52.3% men vs 47.7% women in the DR-G versus 25% men vs 75% women in the CC-G. Statistical significance in gender distribution was observed between groups and subgroups (p-value = 0.00).

The analysis of the anthropometric variable BMI also showed significant differences between the CC-G, with a BMI average of 23.20 ± 3.30 kg/m2, and the DR-G, with an average of 26.40 ± 2.80 kg/m2 (p-value = 0.02).

2. Clinical data

The clinical data analysis showed significant differences in some of the measured variables between both study groups (Table 7).

Table 7. Clinical data of the study participants.

	CC-G (n = 75)	DR-G (n = 75)	p-value
Demographic variables			
Gender male (%)	25.00	52.30	**0.00**
Age (years)	52.00 ± 11.50	56.50 ± 21.00	0.23
Anthropometric variables			
BMI (kg/m^2)	23.20 ± 3.30	26.40 ± 2.80	**0.02**
Ophthalmological variables			
BCVA	0.93 ± 0.14	0.95 ± 0.16	0.21
IOP (mmHg)	15.70 ± 2.40	14.90 ± 1.90	0.81
CST (mm)	258.26 ± 31.23	285.10 ± 45.17	**0.00**
CMV (mm^3)	0.21 ± 0.02	0.24 ± 0.01	**0.00**
Biochemical variables			
Gluc (mg/dL)	91.50 ± 14.20	102.20 ± 18.30	**0.01**
HbA1c (%)	5.30 ± 1.20	7.50 ± 1.90	**0.00**
CholT (mg/dL)	170.10 ± 40.50	172.50 ± 53.40	0.14
HDL-Chol (mg/dL)	62.00 ± 15.20	59.00 ± 18.00	0.51
LDL-Chol (mg/dL)	96.00 ± 28.80	109.50 ± 38.50	**0.02**
VLDL-Chol (mg/dL)	18.10 ± 5.80	38.10 ± 6.90	**0.00**
Trg (mg/dL)	102.70 ± 14.50	125.00 ± 38.40	0.20

BMI: body-mass index; BCVA: best corrected visual acuity; IOP: intraocular pressure; CST: central subfield thickness; CMV: central macular volume; Gluc: plasmatic glucose; HbA1c: glycosylated haemoglobin; CholT: total cholesterol; HDL-Chol: high-density lipoprotein cholesterol; LDL-Chol: very low-density lipoprotein cholesterol; VLDL-Chol: very low-density lipoprotein cholesterol; Trg: triglycerides.

The ophthalmological examination did not show any significant differences in BCVA (p-value = 0.21) or PIO (p-value = 0.81) values when comparing both groups. However, DR-G showed higher CST (285.1 ± 45.17 mm, p-value = 0.00) and CMV (0.24 ± 0.01 mm^3, p-value = 0.00) values when compared to CC-G.

Finally, the analysis of biochemical variables showed no statistically significant differences between groups in CholT (p-value = 0.14), HDL-Chol (p-value = 0.51) and Trg (p-value = 0.2). However, DR-G showed increased levels of Gluc (102.20 ± 18.30 mg/dL, p-value = 0.01), HbA1c (7.50 ± 1.90 %, p-value = 0.00), LDL-Chol (109.50 ± 38.50 mg/dL, p-value = 0.02) and VLDL-Chol (38.10 ± 6.90 mg/dL, p-value = 0.00) when compared to CC-G.

3. Molecular profiling

3.1 Neuroinflammation

For this section, VB and plasma levels of IL-1β, IL-6, IL-18 and TNF-α were measured. As shown in Figure 15, the CC-G showed IL-1β-Pls values of 2.03 ± 2.53 pg/mL, IL-6-Pls values of 4.63 ± 1.24 pg/mL, IL-18-Pls values of 217.23 ± 27.53 pg/mL and TNF-α-Pls values of 15.60 ± 1.25 pg/mL, whereas the DR-G showed IL-1β-Pls values of 5.32 ± 3.09 pg/mL, IL-6-Pls values of 7.31 ± 3.98 pg/mL, IL-18-Pls values of 237.84 ± 37.68 pg/mL and TNF-α-Pls values of 17.37 ± 2.57 pg/mL.

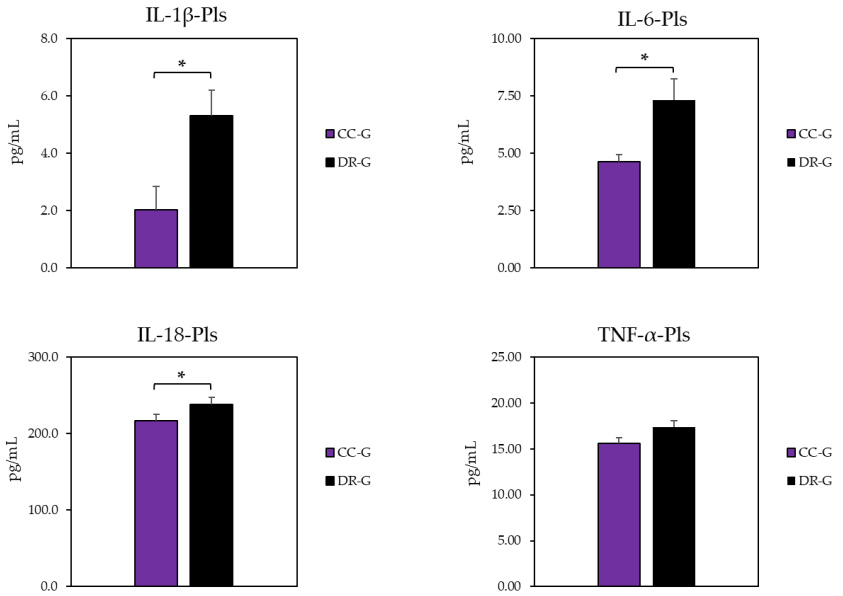


***Figure 15**. Plasmatic levels of the proinflammatory molecules IL-1β, IL-6, IL-18 and TNF-α. The (*) symbol indicates the presence of statistically significative differences when comparing the mean of both groups (p-value < 0.05).*

In this sense, statistically significant differences were observed when comparing the plasmatic levels of IL-1β (p-value = 0.01), IL-6 (p-value = 0.03) and TNF-α (p-value = 0.03) between both groups, but not when comparing the plasmatic levels of IL-18 (p-value = 0.18).

On the other hand, when comparing the VB levels of these molecules (Figure 16), CC-G showed IL-1β-Vit values of 9.18 ± 1.26 pg/mL, IL-6-Vit values of 60.02 ± 24.03 pg/mL, IL-18-Vit values of 72.11 ± 16.30 pg/mL and TNF-α-Vit values of 11.14 ± 2.30 pg/mL, whereas the DR-G showed IL-1β-Vit values of 10.40 ± 1.17 pg/mL, IL-6-Vit

values of 56.41 ± 13.67 pg/mL, IL-18-Vit values of 112.43 ± 46.37 pg/mL and TNF-α-Vit values of 13.75 ± 2.67 pg/mL.

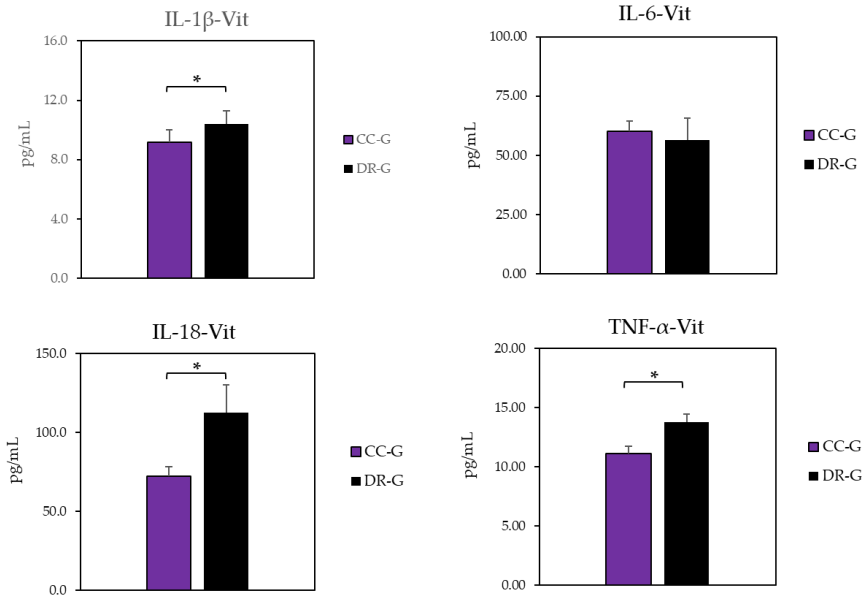


***Figure 16**. Vitreous body levels of the proinflammatory molecules IL-1β, IL-6, IL-18 and TNF-α. The (*) symbol indicates the presence of statistically significative differences when comparing the mean of both groups (p-value < 0.05).*

When comparing both groups, statistically significant differences were observed when comparing the VB levels of IL-1β (p-value = 0.04), IL-18 (p-value = 0.04) and TNF-α (p-value = 0.01), whereas IL-6 lacked statistical significance between groups (p-value = 0.74).

3.2 Neurotrophic factors

For this section, VB and plasma levels of BDNF and TGF-β were measured. In the case of plasma samples (Figure 17), CC-G showed BDNF-Pls values of 272.90 ± 141.98 pg/mL and TGF-β-Pls values of 297.54 ± 98.80 pg/mL, whereas DR-G presented BDNF-Pls values of 256.05 ± 177.75 pg/mL and TGF-β-Pls values of 322.49 ± 99.59 pg/mL. No statistically significant differences were found when comparing the group's mean values of BDNF-Pls (p-value = 0.78) nor TGF-β-Pls (p-value = 0.41).

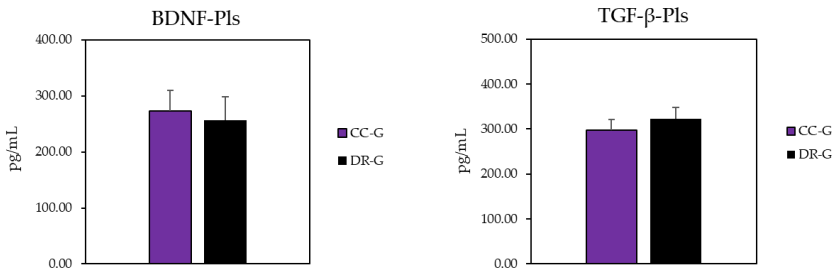


***Figure 17**. Plasmatic levels of the neurotrophic factors BDNF and TGF-β.*

In the case of the VB samples (Figure 18), the CC-G showed BDNF-Vit values of 12.34 ± 5.11 pg/mL and TGF-β-Vit values of 69.35 ± 18.52 pg/mL, whereas DR-G presented BDNF-Vit values of 7.41 ± 4.73 pg/mL and TGF-β-Vit values of 86.15 ± 22.71 pg/mL. Both BDNF-Vit (p-value = 0.03) and TGF-β-Vit (p-value = 0.04) showed statistically significant differences when comparing CC-G and DR-G.

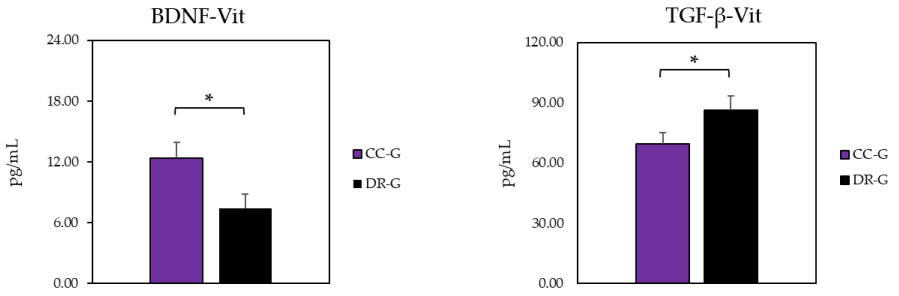


Figure 18. *Vitreous body levels of the neurotrophic factors BDNF and TGF-β. The (*) symbol indicates the presence of statistically significative differences when comparing the mean of both groups (p-value < 0.05).*

3.3 Vascular dysfunction and angiogenesis

In order to assess the importance of pathogenic angiogenesis in DR progression, the VEGF levels were measured both in VB and plasma samples (Figure 19). In the case of plasma assays, the CC-G showed VEGF-Pls levels of 122.97 ± 24.60 pg/mL, whereas the DR-G presented VEGF-Pls levels of 152.10 ± 35.29 pg/mL, existing significant differences between both groups (p-value = 0.001). When analysing the VB samples, the CC-G showed VEGF-Vit levels of 49.43 ± 10.98 pg/mL, whereas the DR-G presented VEGF-Vit concentration of 133.14 ± 199.60 pg/mL. When comparing both groups, statistics showed significant differences between them (p-value = 0.03).

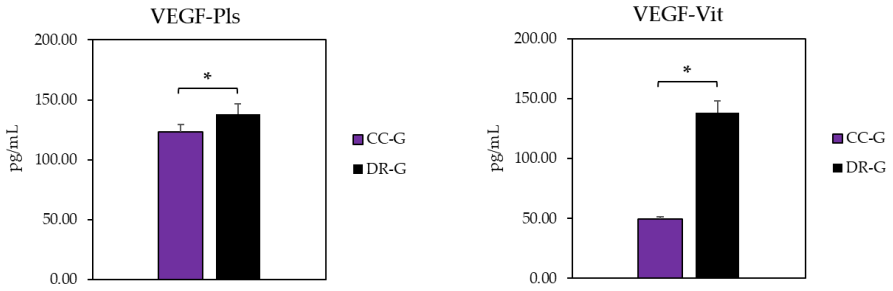


Figure 19. *Plasmatic and vitreous body levels of VEGF. The (*) symbol indicates the presence of statistically significative differences when comparing the mean of both groups (p-value < 0.05).*

3.4 Excitotoxicity

The study of the excitotoxicity phenomenon was assessed through the quantification of the glutamate levels both in plasma and VB samples (Figure 20). Analysing the plasma samples, the CC-G showed Glut-Pls levels of 0.70 ± 0.27 μM, whereas the DR-G presented Glut-Pls levels of 1.19 ± 0.53 μM, existing statistically significant differences between both groups (p-value = 0.03). In the case of the VB samples, the CC-G showed Glut-Vit levels of 0.05 ± 0.08 μM whereas the DR-G presented Glut-Vit levels of 0.20 ± 0.19 μM, existing statistically significative differences between both groups (p-value = 0.008).

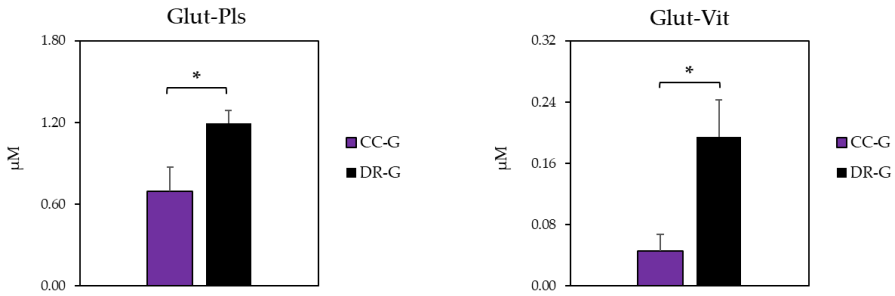


Figure 20. *Plasmatic and vitreous body levels of Glutamate. The (*) symbol indicates the presence of statistically significative differences when comparing the mean of both groups (p-value < 0.05).*

3.5 Apoptosis

The apoptotic process was analysed through the quantification in plasma and VB samples of CAS3 (Figure 21). In the case of the plasma samples, CC-G showed CAS3-Pls levels of 0.06 ± 0.04 ng/mL, whereas DR-G presented CAS3-Pls levels of 0.09 ± 0.04 ng/mL, existing statistically significant differences between both groups (p-value = 0.041). In the case of VB samples, the CC-G showed CAS3-Vit levels of 0.42 ± 0.02 ng/mL, whereas DR-G presented CAS3-Vit levels of 0.44 ± 0.05 ng/mL, existing statistically significant differences between both groups (p-value = 0.046).

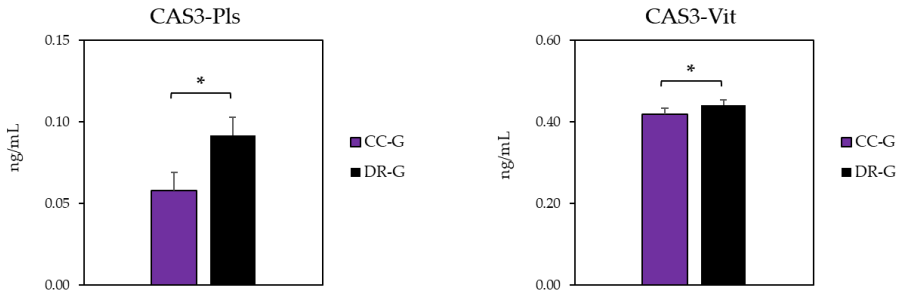


Figure 21. *Plasmatic and vitreous body levels of CAS3. The (*) symbol indicates the presence of statistically significative differences when comparing the mean of both groups (p-value<0.05).*

In order to get a better visualization of all the data, Table 8 compiles all the molecular data results obtained from the study with its corresponding significance values.

Table 8. Result summary for the analysed molecular variables.

	Plasma			Vitreous Humour		
Molecule	**CC-G**	**DR-G**	**p-value**	**CC-G**	**DR-G**	**p-value**
IL-1β (pg/mL)	2.03 ± 2.53	5.32 ± 3.09	**0.01**	9.18 ± 1.26	10.40 ± 1.17	**0.04**
IL-6 (pg/mL)	4.63 ± 1.24	7.31 ± 3.80	**0.03**	56.41 ± 13.67	60.02 ± 29.03	0.74
IL-18 (pg/mL)	217.23 ± 27.53	237.84 ± 37.68	0.18	67.75 ± 12.62	112.43 ± 46.37	**0.04**
TNF-α (pg/mL)	15.60 ±1.25	17.37 ± 2.57	**0.03**	11.15 ± 2.30	13.75 ± 2.67	**0.01**
BDNF (pg/mL)	272.90 ± 141.97	256.05 ± 177.75	0.78	12.34 ± 5.11	7.41 ± 4.73	**0.03**
TGF-β (pg/mL)	297.54 ± 98.79	322.49 ± 99.59	0.41	69.35 ± 18.52	86.15 ± 22.71	**0.04**
VEGF (pg/mL)	122.97 ± 24.60	152.10 ± 35.29	**0.00**	49.43 ± 10.98	133.14 ± 199.60	**0.03**
Glutamate (μM)	0.69 ± 0.27	1.19 ± 0.53	**0.03**	0.05 ± 0.08	0.19 ± 0.19	**0.01**
CAS3 (ng/mL)	0.06 ± 0.04	0.09 ± 0.04	**0.04**	0.42 ± 0.02	0.44 ± 0.05	**0.04**

4. Correlation analysis

A correlation analysis was performed to explore potential associations between molecular variables measured in plasma and vitreous humor, clinical ophthalmological parameters, and biochemical markers. For simplicity, Figure 21 collects the correlation matrix including the variables with some significant result.

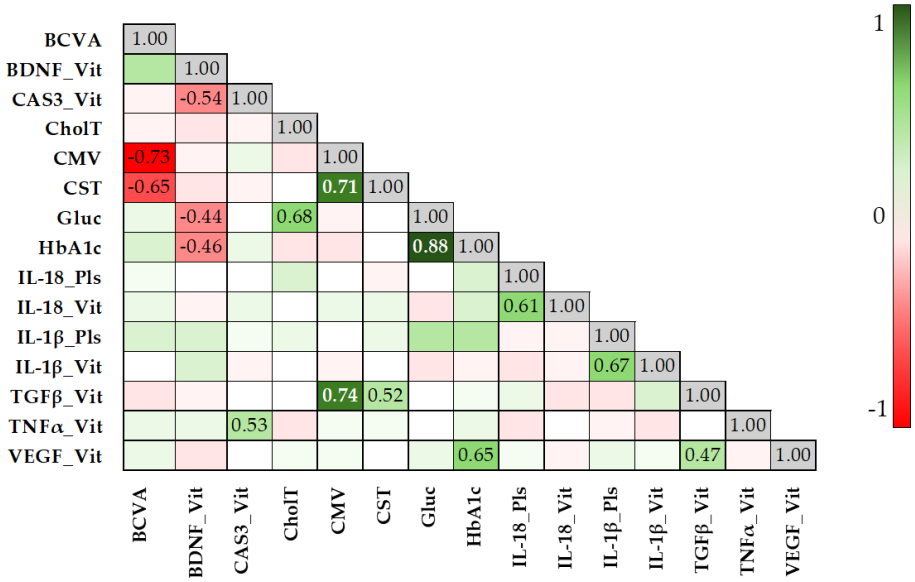


***Figure 22**. Correlation matrix for the clinical and molecular variables. The colour scheme corresponds to the Pearson coefficient value, in which 1 indicates a positive correlation whereas -1 indicates a negative correlation.*

Notably, strong positive correlations were observed between CST and central macular volume CMV (r = 0.71), as well as between Gluc and HbA1c (r = 0.88), and Gluc and Chol (r = 0.68) reflecting consistent clinical and metabolic relationships. A strong association

was also found between TGF-β in the vitreous and CMV (r = 0.74), and between VEGF-Vit and HbA1c (r = 0.65). and a moderate correlation between TGF-β-Vit and CST (r = 0.52). Moderate positive correlations were found between VEGF-Vit and TGF-β-Vit (r = 0.47), TNF-α-Vit and CAS3-Vit (r = 0.53), between IL-18 in both plasma and VB (r = 0.61) and between IL-1β in both plasma and VB (r = 0.67).

Among negative correlations, BCVA showed strong inverse associations with CST (r = –0.65) and CMV (r = –0.73). Additionally, a modest negative correlation was found between Gluc and BDNF-Vit (r = –0.44), HbA1c and BDNF-Vit (r = –0.46), and CAS3-Vit and BDNF-Vit (r = –0.54).

V. Discussion

DR is a major public health concern from both sociological and economic perspectives, ranking among the leading causes of blindness in modern societies (Flaxman et al., 2017; G. Li et al., 2025; T. Xu et al., 2020). Given its clinical significance and the alarming rise in DM's prevalence (Lovic et al., 2020), understanding the disease's etiological mechanisms has become more important than ever. In this sense, the current clinical tendency is to consider DR as a vascular disorder, leading to therapeutic strategies focused on its hallmark angiogenic processes—most notably, anti-VEGF therapy, which remains the cornerstone of treatment (Chong et al., 2024). However, the significant limitations of anti-VEGF therapy (Smit-McBride and Morse, 2021) have highlighted the urgent need for alternative therapeutic approaches. Because of this, our work focused on the role of neurodegeneration in DR, and an extensive search for molecular biomarkers that may help ophthalmologists to preclinically identifying people at high risk of diabetic blindness.

In this context, multiple studies have underscored the role of neurodegeneration in shaping the DR phenotype, demonstrating the involvement of various neurodegenerative mechanisms in DR's onset and progression (Barber and Baccouche, 2017; Soni et al., 2021), as reviewed in Section 4 of the Introduction. Nevertheless, the interplay between these pathways in the induction of retinal neuronal damage remains poorly understood.

This knowledge gap served as the primary motivation for the present research project, which aims to provide a comprehensive understanding of neurodegeneration in the context of DR. To address this, the molecular analyses of plasma and VB samples have uncovered concurrent dysregulation across multiple molecular pathways, underscoring the interconnected nature of the disease's pathogenic mechanisms. Specifically, the molecular profiling of these samples has shown significant dysregulation in neuroinflammatory (IL-1β, IL-6, IL-18, TNF-α), neurotrophic (BDNF, TGF-β), angiogenic (VEGF), excitotoxic (glutamate) and apoptotic (CAS3) mediators, suggesting their potential pathogenic roles in DR. These findings collectively indicate that these molecular processes do not act in isolation but rather interact in a highly integrated manner, driving disease progression through complex, interdependent mechanisms.

Our findings are discussed below.

Clinical data collection is an important aspect of any study with a translational focus. In this case, it is a key tool to appropriately characterise the patient from which samples come from, in order to determine whether other possible variables could be playing a role in the observed processes.

Age distribution between both groups showed no statistical differences between CC-G (52.00 ± 11.50) and DR-G (56.50 ± 21.00 years). These data support the strength of the patient selection

methodology, having achieved sufficiently similar populations to be able to conduct proper comparisons between groups. Other researchers reported similar ages in their participants to ours (Bianco et al., 2022; Roig-Revert et al., 2015).

The analysis of gender distribution showed that there were significant differences in the male percentage when comparing CC-G and DR-G, pointing out a gender imbalance between both groups. The comparison of gender distribution in the DR-G, however, showed no significant differences, existing a similar DR distribution between women and men. Similarly, the age distribution between both groups showed no statistical differences between CC-G (52.00 ± 11.50) and DR-G (56.50 ± 21.00 years). This data supports the strength of the patient selection methodology, having achieved sufficiently similar populations to be able to conduct proper comparisons between groups.

When analysing the anthropometric variable BMI, the CC-G group showed values of 23.20 ± 3.30 kg/m^2, whereas the DR-G showed values of 26.40 ± 2.80 kg/m^2, existing significant differences between both groups (p-value = 0.02). These results are in accordance with other studies that point out that obesity is a risk factor for DR, existing an association between higher BMI and the likelihood to have DR (Dirani et al., 2011). This observation is especially interesting in the context of this research, considering the linkage between obesity and

neurodegeneration, as recently reported (Kueck et al., 2024). In this sense, being a contributor of inflammatory and metabolic alterations, a higher BMI could be a contributing factor to the development of retinal neurodegeneration

The comparison of ophthalmological variables revealed no significant differences in either BCVA or IOP values when comparing both study groups. In this sense, although BCVA is a standard visual function measurement in clinical practice, some studies have pointed out that it is affected late in the disease process of DR (Glassman et al., 2024), which could explain the observed similarities between both groups. Further analyses using different DR stages could specifically assess the impact of the disease progression in BCVA. Alternatively, the study conducted by Jung et al. in a rat DM model points out how fluctuations in IOP are linked with neurodegeneration mechanisms in an animal model (Jung et al., 2020). In this sense, although no differences were observed between our groups, a periodic and constat measurement of IOP values could be an additional strategy to assess the neurodegenerative impact of DR.

Oppositely, the OCT image analysis revealed higher CST and CMV values when compared to CC-G, indicating an uptrend in the macular thickness volume in our DR patients. These measurements, with a significant correlation between both variables ($r = 0.70$), indicate the vulnerability of these patients to develop DME and visual loss, the

major complication of this disease (Torjani et al., 2023), which also appears reflected by the negative correlation of both CMV (r = -0.73) and CST (r = -0.65) with BCVA. Further studies should be conducted to evaluate the RNFL thickness in both groups, since previous research has pointed out that there is a reduction in RNFL that could be a direct result of neuronal loss, consequence of the neurodegenerative process that affects DR patients (Bhaskaran et al., 2023).

Regarding the biochemical analyses, quantification and comparison of these parameters revealed two different observations. Firstly, the DR-G group exhibited significantly higher glycaemic profile values compared to the CC-G group, both in Gluc and HbA1c values, which appeared to be highly correlated (r = 0.88). Secondly, when analysing the lipidic profile, only LDL-Chol and VLDL-Chol values showed statistically significant differences between groups, whereas CholT, HDL-Chol and Trg remained similar between groups. Several studies have pointed out that high levels of HbA1c play an important role in the progression of DR, being a key element to control for improving the management of DR patients (Poshtchaman et al., 2023). Furthermore, some authors have described a direct association between high HbA1c and neuronal dysfunction (A. Ramirez et al., 2015; Uyar et al., 2022), which could be a potential contributing factor to the neurodegenerative processes that affect the diabetic retina and,

therefore, a therapeutic target to further explore. In this sense, a negative correlation between HbA1c and VB BDNF levels (r = -0.46) and a positive correlation with vitreous VEGF levels (r = 0.65) seems to point towards a key role of this molecule in determining the phenotype of DR. Similarly, Gluc levels appear negatively correlated with vitreous BDNF levels (r = -0.44), highlighting the importance of glycaemic profile in the context of the pathology through altering the NTF balance.

In the case of the lipidic profile, the observation of higher concentrations of LDL-Chol is of special relevance to the disease pathogenesis, since oxidized and glycated LDL can induce inflammation and damage retinal cells, further worsening the degeneration of the retinal tissue (Busik, 2021). In addition, the characteristic breakdown of the BRB in DR patients, reflected by the increased values of CST and CMV, can lead to increased retinal levels of this lipidic product, leading to an increased retinal damage through OS-related processes (Busik, 2021). Similarly, higher VLDL-Chol levels have been defined as risk factors in the development of DR (Bryl et al., 2022), contributing to the aforementioned pathogenic processes and worsening the retinal function.

Next, the molecular profile in the neurodegeneration event that affects out study population will be discussed.

The analysis of the proinflammatory molecules IL-1β, IL-6, IL-18 and TNF-α in both plasma and VB samples reinforces the central role of neuroinflammation in DR pathogenesis. In this sense, it has previously been demonstrated that disruptions in glucose metabolism as a consequence of the DM condition directly activate NF-κB, triggering ROS production and inducing key signalling pathways such as extracellular signal-regulated kinase (ERK) activation. This cascade ultimately leads to microglial activation and the release of proinflammatory mediators (Z. Xu et al., 2023). Furthermore, beyond microglia, RPE cells, endothelial cells, glial cells and neurons have also been implicated in dysregulated cytokine secretion, serving as additional sources of proinflammatory mediators and further amplifying the inflammatory response in DR (Simó et al., 2022). The significance of these proinflammatory pathways is underscored by the findings of Ding et al., who demonstrated that inhibiting NF-κB with the specific blocker IMD-0354 effectively protects the retina by suppressing the inflammatory response in an experimental animal model (Ding et al., 2023).

In this context, the quantification of IL-1β showed a significant increase of its concentration in both plasma and VB samples of patients with DR, consistent with previous studies in humans (Feng et al., 2018; Mason et al., 2022; Qian and Huang, 2022) and animal models (Ge et al., 2022), and showing a significant positive correlation

between sample types (r = 0.67). Functionally, and under physiological conditions, IL-1β plays a crucial role in orchestrating a coordinated inflammatory response (Mendonca et al., 2020). However, in the context of DR, its chronically elevated secretion leads to an overactivation of NF-κB (Simó et al., 2022), mitochondrial dysfunction (C. Liu et al., 2022), and pericyte apoptosis (Yun, 2021), while also contributing to glutamate dysregulation (C. Chen et al., 2014)—all key drivers of neurodegeneration. These diverse pathogenic roles align with the observed alteration of other key molecules analysed, such as glutamate and CAS3, suggesting a potential crosstalk between inflammation, excitotoxicity and apoptotic dysregulation. Furthermore, the role of IL-1β in driving the proinflammatory response is reinforced by the increased concentrations of other proinflammatory molecules identified in this study.

Similarly, the analysis of IL-6, IL-18, and TNF-α revealed altered expression levels in both plasma and VB samples, though with some discrepancies depending on the sample type. Notably, IL-6 exhibited higher concentration in plasma samples, but no statistically significant changes were observed in VB samples, contrasting with previous findings (Yao et al., 2019). However, our DR patients displayed a trend toward higher vitreal IL-6 levels, suggesting a potential but more subtle role of this cytokine in DR pathogenesis. In this sense, beyond its well-established proinflammatory effects,

similar to those of IL-1β, IL-6 has been shown to activate the signal transducer and activator of transcription 3 (STAT3), promoting VEGF production and contributing to the vascular pathology of DR (Jha et al., 2013). This underscores IL-6 as a key mediator linking inflammation to vascular dysfunction in DR.

In contrast, IL-18 showed significantly higher concentrations in VB samples of DR patients, while no significant differences were observed in plasma samples, although a similar trend to IL-6 levels in VB towards increased plasma IL-18 concentration was noted and a significant correlation between plasmatic and vitreous samples was observed ($r = 0.61$). These findings align with previous studies reporting elevated IL-18 levels in both plasma (Jiang et al., 2023) and VB (Song et al., 2014) samples of DR patients. In this case, the observed discrepancies between sample types suggest that IL-18 may may play a more localized role in DR's pathogenesis, potentially contributing to retinal-specific processes. On these lines, its overproduction has previously been correlated to an increased secretion of IL-1β—consistent with the findings of this study—due to the activation of the Nod-Like Receptor Family Pyrin Domain Containing 3 (NLRP3) inflammasome in retinal cells (Zheng et al., 2023). Chronic inflammasome activation has, in turn, been associated with pyroptosis, a specific form of lytic programmed cell death (Al Mamun et al., 2021), establishing a crucial link between neuroinflammation

and retinal cell death in DR pathogenesis that is reflected by the obtained results.

TNF-α also displayed altered expression patterns in both sample types, reinforcing its role as a key inflammatory mediator in the context of DR (Yao et al., 2018). This proinflammatory cytokine is well known for its ability to activate NF-κB signalling, leading to the transcription of multiple proinflammatory genes (Wajant and Scheurich, 2011). In this sense, TNF-α contributes to retinal inflammation and plays a pivotal role in DR pathogenesis. Its involvement is multifaceted, as several studies have reported that it not only exacerbates endothelial dysfunction by disrupting the BRB but also promotes glial activation and upregulates VEGF synthesis (Forrester et al., 2020). Additionally, TNF-α has been linked to CAS3 and CAS8 activation (Joussen et al., 2009; Y. Liu et al., 2021), establishing a direct connection between chronic inflammation and dysregulated apoptosis in DR—both central to its neurodegenerative progression and the loss of neuronal cells. Interestingly, the correlation analysis shows a positive correlation between TNF-α and CAS3 ($r = 0.53$), in accordance with these studies.

The simultaneous upregulation of TNF-α, IL-1β, IL-6 and IL-18 observed in this study suggests a synergistic effect, where chronic inflammation amplifies retinal damage through interconnected

pathways and contributes to other pathogenic mechanisms, further driving the neurodegenerative processes characteristic of DR.

Regarding the neurotrophins, it is well known that under physiological conditions, NTF signalling plays a crucial role in preserving neuronal cell homeostasis. Disruptions in the balance of these molecules can lead to tissue alterations and loss of cellular function, ultimately contributing to detrimental mechanisms and neurodegeneration processes (Chiarelli et al., 2000). When evaluating the impact of NTF imbalance in DR, a significant decrease in BDNF and increase in TGF-β vitreous levels was observed, while plasma levels remained unchanged. This finding is particularly noteworthy, as it suggests that the imbalance is restricted to the retinal environment rather than being a systemic phenomenon, indicating a functional alteration in retinal cells. In this context, our results further support the role of this mechanism in DR pathogenesis, consistent with previous findings (Hernández and Simó, 2012).

Physiologically, BDNF has a neuroprotective effect by binding to its receptor tropomyosin receptor kinase B (TrkB), which is expressed by both amacrine and retinal ganglion cells. This activation triggers the extracellular signal-regulated kinase (ERK) and phospharidylinositol-3 kinase (PI3K) pathways, both known for their neuroprotective roles (Almeida et al., 2005; Cusato et al., 2002; Y. Liu et al., 2013). Additionally, BDNF mitigates OS by preventing

microglial activation, thereby preserving antioxidant enzyme regulation (Behl and Kotwani, 2017). Moreover, BDNF enhances glutamate uptake by stimulating the upregulation of GLAST and GS in Müller glia, particularly under hypoxic conditions (Dai et al., 2012). Through these mechanisms, BDNF serves as a key neuroprotective molecule, safeguarding against OS and excitotoxicity while promoting cell survival and synaptic plasticity (Eyileten et al., 2017).

Previous studies have reported a decrease in mature BDNF levels similar to those observed in this study, particularly in the AH (Jun and Kim, 2021) and VB (Kaviarasan et al., 2015) of DR patients, as well as in their plasma (Ram et al., 2024; Rashid et al., 2020) and in the retinas of diabetic rats (Kim et al., 2021; Ola et al., 2013). Given BDNF's critical role in promoting cell survival, synaptic plasticity, and neuronal homeostasis (Gasecka et al., 2020), its reduction is likely to contribute to a range of detrimental effects in the retina, ultimately exacerbating the neurodegeneration phenomenon. Amongst these effects, Boutahar et al. demonstrated that a decrease in BDNF-mediated intracellular cAMP signalling is associated with increased apoptotic activity in neuronal cells exposed to NMDA or H_2O_2 toxicity (Boutahar et al., 2010). In this sense, a negative correlation between BDNF and CAS3 ($r = -0.54$) was found in the correlation analysis, suggesting an inverse relationship between both of these molecular actors. These findings highlight the importance of maintaining BDNF

levels and suggest that its depletion may exacerbate excitotoxicity and OS vulnerability in DR patients.

Moreover, similar findings have been reported for other neurotrophins, such as nerve growth factor (NGF), which has previously shown reduced retinal levels alongside an accumulation of its precursor, pro-NGF (Ali et al., 2011). This is particularly significant, as neurotrophin precursors are not neuroprotective and have been shown to exert pro-inflammatory effects (Barcelona et al., 2016), further exacerbating the neurodegeneration and angiogenic processes characteristic of DR (Elshaer et al., 2013). Taking this into account, a similar mechanism may contribute to BDNF dysregulation, underscoring the need for further studies to quantify its proform and explore its potential role in shaping the neuroinflammatory signalling profile of retinal cells.

The quantification of TGF-β and the observed increase in its VB levels align with findings from various studies across different sample types, including AH, VB, and plasma (Bonfiglio et al., 2020; Mason et al., 2025; H. Zhang et al., 2020). Functionally, TGF-β is a multifaceted cytokine that regulates cell growth and differentiation by binding to specific transmembrane receptors, facilitating the attachment of intracellular proteins such as SMADs, which mediate signal transduction and govern cell development and growth (Massagué and Sheppard, 2023). As a growth factor, several studies

have highlighted its role in neuronal survival and development (Meyers and Kessler, 2017). However, in the context of DR, TGF-β also promotes angiogenesis and basal lamina thickening in retinal capillaries, disrupting the NVU and impairing retinal vascular homeostasis (Gasecka et al., 2020). Notably, TGF-β induces VEGF expression (Nagineni et al., 2003) and can alter the extracellular matrix composition, leading to fibrosis (Ong et al., 2021), further contributing to retinal vascular remodeling. Given the close functional interplay within the NVU, these vascular alterations inevitably affect the neuronal compartment, exacerbating neurodegeneration. These observations gain relevance when analysing the results of the correlation study, which shows a direct positive correlation between the vitreous levels of this molecule and CMV ($r = 0.74$), CST ($r = 0.52$) and vitreous VEGF ($r = 0.47$).

In addition to this, several studies have related TGF-β's recruitment of SMAD proteins with inflammatory processes (Yoshimura et al., 2010). While a direct inflammatory pathway has not been identified in retinal tissue, TGF-β's proinflammatory activity suggests a potential role in the neuroinflammatory processes discussed earlier in this section.

The analysis of molecules involved in angiogenesis has been assayed by quantifying VEGF levels in the VB and plasma samples of our study participants. As expected, VEGF was significantly elevated

in both sample types, consistent with numerous previous reports (Mason et al., 2022; Z. Zhou et al., 2019).

The pathological role of VEGF in relation to the vascular dysfunction observed in DR has been extensively reviewed (Behl and Kotwani, 2015). In response to hypoxia and inflammatory signals, various retinal cell phenotypes release VEGF, which binds to its receptors on endothelial cells, triggering the formation of fragile, leaky blood vessels that contribute to microvascular damage, a hallmark of DR (Rossino et al., 2019). Additionally, VEGF upregulates intercellular adhesion molecule 1 (ICAM-1), disrupts endothelial tight junctions, and increases vascular permeability, ultimately compromising the integrity of the BRB (J. Wang et al., 2010). This BRB breakdown further alters the NVU, exacerbating neuronal dysfunction in the diabetic retina. Nevertheless, the role of VEGF in neuronal survival remains less understood. Some studies have shown that VEGF exerts a direct protective effect on cultured neurons exposed to excitotoxic conditions (Matsuzaki et al., 2001), and an increased VEGF production can activate survival signalling pathways in neurons, promoting neuroprotection (Nishijima et al., 2007). However, this potential neuroprotective function has often been overlooked due to the widespread accepted use of anti-VEGF agents as a primary therapeutic strategy for DR (Saint-Geniez et al., 2008). Furthermore, the studies of Hombrebueno et al. have demonstrated that VEGF plays

a crucial role in neurogenesis and synaptogenesis (Hombrebueno et al., 2015), suggesting that a prolonged VEGF depletion resulting from anti-VEGF therapy could have unintended adverse effects on the human retina.

Considering all these factors, VEGF emerges as a multifaceted molecule at the intersection of multiple pathogenic processes in DR. It serves as a critical link between vascular dysfunction and inflammatory responses—both hallmarks of the disease—while also playing a neuroprotective role in the context of retinal neurodegeneration. This duality highlights the complexity of targeting VEGF therapeutically, as anti-VEGF strategies, while effective in managing vascular complications, may inadvertently impair its neuroprotective functions. Understanding VEGF's diverse roles is therefore essential for refining treatment approaches and minimizing potential adverse effects on retinal health.

As outlined in the introduction section, excitotoxicity is a key pathological process driving neurodegeneration. It directly damages neuronal cells due to excessive extracellular glutamate accumulation (Choi, 2020; Ishikawa, 2013) and the sustained activation of NMDA receptors on post-synaptic membranes disrupts calcium homeostasis by depleting endoplasmic reticulum calcium stores, triggering neurotoxic cascades and promoting apoptotic pathways (Ruiz et al., 2009). This sustained dysregulation of calcium signalling exacerbates

neuronal vulnerability, further contributing to the progression of neurodegeneration.

The analysis conducted in this study revealed a significant increase in glutamate levels in both study samples from our participants. This finding highlights the potential role of excitotoxicity in DR pathogenesis, directly contributing to its neurodegenerative processes through the previously described mechanisms.Although previous studies have reported a significant increase in glutamate concentrations in VB (Ambati, 1997; Deng et al., 2000; Diederen et al., 2006) and plasma samples (Y. Sun et al., 2021) of DR patients, research efforts have predominantly focused on other pathogenic factors, such as VEGF and proinflammatory cytokines. However, accumulating evidence suggests that glutamate excitotoxicity is not an isolated phenomenon but is intricately linked to other key mechanisms driving DR progression. On these lines, some authors have observed that excessive retinal glutamate contributes to the upregulation of VEGF expression (Cervantes-Villagrana et al., 2010) and increase OS levels (Ishikawa, 2013; Narayanan et al., 2019), both of which contribute to neuronal damage in DR. In support of this interplay, NMDA receptor inhibition has been demonstrated to reduce vitreoretinal VEGF levels in diabetic rat models (Rossino et al., 2019), further emphasizing the crosstalk between excitotoxicity, angiogenesis, and oxidative stress in DR pathology.

Furthermore, in their study, Zhou et al. demonstrated that diabetic conditions upregulate the expression of thioredoxin-interacting protein (TXNIP), a key regulator of oxidative stress that directly activates the ROS system, while simultaneously downregulating GS, an essential enzyme for glutamate clearance. This dysregulation not only exacerbates oxidative stress but also impairs the removal of excess extracellular glutamate, further promoting excitotoxicity. Additionally, their study showed that IL-1β reduces GS levels, highlighting a direct interplay between inflammatory processes and the worsening of excitotoxic damage (J. Zhou et al., 2016). This connection is particularly relevant considering the findings by Tsoka et al., who demonstrated that NMDA-induced excitotoxicity can activate the NLRP3 inflammasome complex, amplifying the neuroinflammatory response and further contributing to the neurodegenerative phenomenon. This activation establishes a self-perpetuating loop in which excitotoxicity triggers IL-1β production, leading to a reduction in GS levels and impairing the cell's ability to clear excess glutamate. The resulting glutamate accumulation further stimulates NLRP3 inflammasome activation, exacerbating neuroinflammation and neuronal damage (Tsoka et al., 2019). This intricate molecular interplay underscores the importance of developing therapeutic strategies that simultaneously target excitotoxicity, inflammation, and oxidative stress to mitigate the neurodegenerative progression of DR.

The combined effect of all the aforementioned mechanisms in the context of retinal neurodegeneration ultimately drives apoptosis, a process described in the introduction section as a progressive loss of cellular functionality and structure, culminating in cell death. In this context, the analysis of CAS3 levels revealed a significant upregulation of this apoptotic marker in both VB and plasma samples from patients with DR, highlighting apoptosis dysregulation as a key pathological mechanism in the context of the disease. These findings are consistent with previous studies reporting elevated CAS3 concentrations and other pathogenesis-related molecules in the VB (Andrés-Blasco et al., 2023; Tian et al., 2019). Furthermore, the overexpression of CAS3 and other proapoptotic molecules has been observed in post-mortem human diabetic retinas, particularly in RGCs, emphasizing the direct impact of apoptotic dysregulation on neuronal integrity (El-Asrar et al., 2004; Oshitari et al., 2008; Podestà et al., 2000).

This imbalance in apoptotic homeostasis not only jeopardizes neuronal survival, but also disrupts the retinal microenvironment, amplifying inflammation and OS disruption—two key drivers of retinal dysfunction in DR. This aligns with the general signalling landscape observed in the present study, where inflammatory and apoptotic factors coexist, reinforcing their interplay in disease progression. Furthermore, given that apoptosis is a primary consequence of glutamate excitotoxicity (Leist and Nicotera, 1998), the

simultaneous presence of elevated CAS3 and glutamate levels in the VB strongly suggests a direct association between these processes. This connection likely plays a critical role in shaping the retinal phenotype observed in diabetic patients, highlighting excitotoxicity-driven apoptosis as a key mechanism in DR pathogenesis.

The current research work has a series of strengths and limitations, which should be considered when interpreting the findings. The strengths of this study enhance the reliability and applicability of the results, while the limitations provide context for potential constraints and future research directions. By acknowledging both, we ensure a balanced and transparent evaluation of our study's contributions to the field.

One of the major strengths of this study is the dual-sample approach, in which molecules were measured in both plasma and VB. Plasma proteins have been shown to be transported to the VB, and a correlation exists between molecular compositions in these two biological fluids (Beebe et al., 1986). This methodological approach enhances our understanding of the specificity of these molecules and their potential as biomarkers. Importantly, plasma is far more accessible to clinicians than VB, making it a more practical medium for biomarker identification in clinical settings.

Another key strength of this study is the use of VB as a biological fluid for analysis. The vitreous is in direct contact with the retina, making it a highly accurate indicator of retinal pathology. As a result, our findings provide valuable insights into the molecular changes occurring within the retina, offering a unique window into the neurodegenerative processes associated with DR. The use of VB samples thus ensures that the study results are not only relevant but also highly informative for understanding disease mechanisms.

Furthermore, our study's comprehensive molecular analysis allows for a more holistic view of the signalling landscape characterizing DR. By simultaneously analysing a wide range of molecules, we can construct a more complete picture of the mechanisms underlying the neurodegenerative processes associated with this pathology. This multi-faceted approach strengthens the reliability and depth of our findings, contributing significantly to the existing body of knowledge on DR and retinal neurodegeneration.

Despite its strengths, this study also has certain limitations that should be considered. One of the primary limitations is the relatively small sample size, which may impact the generalizability of the findings, and the specific inclusion of only patients with PDR, and not on other stages of the disease. Future studies with larger cohorts that include patients with different stages of DR would be beneficial in validating and expanding upon these results.

Additionally, another practical limitation is the inherent difficulty of obtaining VB samples. While VB provides valuable insight into retinal pathology, its collection is invasive and not feasible for routine clinical use, which restricts its applicability in biomarker identification, as plasma remains the more accessible and practical option for clinicians. In addition, it is ethically impossible to collect vitreous from healthy eyes, except in programmed surgeries, such as in our surrogate comparative-control group of participants.

Finally, while this study employs a broad molecular analysis, it does not encompass all possible molecules involved in DR pathogenesis. Previous studies utilizing proteomics (Valdivia et al., 2023; Xiao et al., 2021), metabolomics (He et al., 2023; Zeng et al., 2024), and lipidomics (Fang et al., 2023; W. Sun et al., 2024) have identified a wide range of differentially expressed molecules in eye-derived samples within the context of DR. However, our study does not include the full spectrum of potential contributors to the disease. Further research is necessary to identify additional key molecules and better understand their roles in DR pathophysiology.

Based on the findings of this study, several promising directions emerge for future research. First, increasing the sample size would help validate the current results, thereby strengthening their reliability and laying a more robust foundation for subsequent investigations. Additionally, the integration of multi-omics

approaches, including proteomics, metabolomics, lipidomics, and transcriptomics, could greatly enhance our understanding of the molecular variability observed and help elucidate interaction networks and pathways involved in diabetic retinal changes at a systems level. In this context, growing attention has been directed toward the role of microRNAs and other non-coding RNAs as key regulatory molecules in DR (Friedrich et al., 2020; Santovito et al., 2021; H. Zhou and Kuang, 2021). Investigating their expression could provide deeper insights into disease pathogenesis, particularly in relation to retinal neurodegeneration, an area that remains relatively underexplored. Furthermore, the identification of new molecular agents could lead to the identification of new diagnostic and prognostic biomarkers that could improve the clinical management of the patients with DR (Sanz-González et al., 2020).

This approach could be extended to both in vitro and in vivo models, including cell and organoid cultures as well as animal models, to deepen our mechanistic understanding of retinal neurodegeneration (Garcia-Medina et al., 2020). In this sense, current retinal organoid cultivation techniques hold significant promise for retinal research, having well-established protocols and representing a valuable tool for conducting both mechanistic and therapeutic studies (X. Li et al., 2021). Taking this into account, designing targeted experimental procedures to evaluate the effects of neurodegeneration-

inducing stimuli within these models could provide an interesting approach for elucidating the pathogenic mechanisms driving retinal neurodegeneration. More specifically, given its importance in other neurodegenerative disorders, these in vitro approaches could be used to better characterize the excitotoxic response of retinal cells in hyperglutamatergic conditions, which seems to be a crucial and less explored mechanism participating in the disease's outcome. Alternatively, the development of several animal models that compile the phenotypic changes observed in patients with DR opens a window to explore all the aforementioned mechanisms in an in vivo model (Quiroz and Yazdanyar, 2021).

The use of all these approaches should be directed to better understand the neurodegenerative processes occurring in the diabetic eyes, and identify potential mechanistic targets involved in it to preserve the retinal function in diabetic patients (Barber and Baccouche, 2017). In this sense, although some nutraceutical approaches have been explored (Ye et al., 2024), the scarce pathophysiological knowledge of the neuronal loss mechanisms in the context of DR makes it difficult to establish effective and safe therapeutic agents. Continuing with the elucidation of the disease's pathogenic routes will improve our capacity to provide better therapeutic approaches and improve the quality of life of patients with this ophthalmological condition.

VI. Conclusions

In the light of the research presented in this dissertation, the following conclusions can be drawn:

1. Neurodegeneration is a multifactorial process that significantly contributes to the pathogenesis of DR.
2. Excitotoxicity induced by elevated glutamate levels emerges as a key mechanism driving retinal neurodegeneration in DR.
3. An imbalance in the production of neurotrophic factors, particularly BDNF and TGF-β, plays a critical role in DR pathophysiology.
4. The upregulation of pro-inflammatory cytokines, including IL-6, TNF-α, IL-1β, and IL-18, underscores the involvement of neuroinflammation in retinal cell damage and the progression of DR.
5. Increased VEGF levels observed in DR patients highlight the pivotal role of angiogenesis dysregulation in disease development and points out a potential role contributing to the neurodegenerative phenomenon through an interaction with the other analysed molecular pathways.

6. An elevated apoptosis rate, evidenced by higher levels of CAS3 in DR retinas, is a major contributor to the neurodegenerative process associated with the disease.
7. The findings from this thesis pave the way for the detection of new biomarkers to assess the impact of neurodegeneration in the context of DR.

VII. Bibliography

Adamiec-Mroczek, J., Zając-Pytrus, H., and Misiuk-Hojło, M. (2016). Caspase-Dependent Apoptosis of Retinal Ganglion Cells During the Development of Diabetic Retinopathy. *Advances in Clinical and Experimental Medicine*, *24*(3), 531–535. https://doi.org/10.17219/acem/31805

Al Mamun, A., Mimi, A. A., Zaeem, M., Wu, Y., Monalisa, I., Akter, A., Munir, F., and Xiao, J. (2021). Role of pyroptosis in diabetic retinopathy and its therapeutic implications. *European Journal of Pharmacology*, *904*, 174166. https://doi.org/10.1016/j.ejphar.2021.174166

Aldosari, D. I., Malik, A., Alhomida, A. S., and Ola, M. S. (2022). Implications of Diabetes-Induced Altered Metabolites on Retinal Neurodegeneration. In *Frontiers in Neuroscience* (Vol. 16). Frontiers Media S.A. https://doi.org/10.3389/fnins.2022.938029

Ali, T. K., Al-Gayyar, M. M. H., Matragoon, S., Pillai, B. A., Abdelsaid, M. A., Nussbaum, J. J., and El-Remessy, A. B. (2011). Diabetes-induced peroxynitrite impairs the balance of pro-nerve growth factor and nerve growth factor, and causes neurovascular injury. *Diabetologia*, *54*(3), 657–668. https://doi.org/10.1007/s00125-010-1935-1

Almeida, R. D., Manadas, B. J., Melo, C. V, Gomes, J. R., Mendes, C. S., Grãos, M. M., Carvalho, R. F., Carvalho, A. P., and Duarte, C. B. (2005). Neuroprotection by BDNF against glutamate-induced apoptotic cell death is mediated by ERK and PI3-kinase pathways. *Cell Death and Differentiation*, *12*(10), 1329–1343. https://doi.org/10.1038/sj.cdd.4401662

Ambati, J. (1997). Elevated γ-Aminobutyric Acid, Glutamate, and Vascular Endothelial Growth Factor Levels in the Vitreous of Patients With Proliferative Diabetic Retinopathy. *Archives of Ophthalmology*, *115*(9), 1161. https://doi.org/10.1001/archopht.1997.01100160331011

American Diabetes Association Professional Practice Committee. (2022). 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes—2022. *Diabetes Care*, *45*, S17–S38. https://doi.org/10.2337/dc22-S002

Andrés-Blasco, I., Gallego-Martínez, A., Machado, X., Cruz-Espinosa, J., Di Lauro, S., Casaroli-Marano, R., Alegre-Ituarte, V., Arévalo, J. F., and Pinazo-Durán, M. D. (2023). Oxidative Stress, Inflammatory, Angiogenic, and Apoptotic molecules in Proliferative Diabetic Retinopathy and Diabetic Macular Edema Patients. *International Journal of Molecular Sciences*, *24*(9), 8227. https://doi.org/10.3390/ijms24098227

Augustine, J., Troendle, E. P., Barabas, P., McAleese, C. A., Friedel, T., Stitt, A. W., and Curtis, T. M. (2021). The Role of Lipoxidation in the Pathogenesis of Diabetic Retinopathy. *Frontiers in Endocrinology*, *11*. https://doi.org/10.3389/fendo.2020.621938

Auld, D. S. (1999). Fluorometric Assays. In *Proteolytic Enzymes* (pp. 30–48). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-59816-6_3

Aydin, S. (2015). A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. *Peptides*, *72*, 4–15. https://doi.org/10.1016/j.peptides.2015.04.012

Bading, H. (2017). Therapeutic targeting of the pathological triad of extrasynaptic NMDA receptor signaling in neurodegenerations. *Journal of Experimental Medicine*, *214*(3), 569–578. https://doi.org/10.1084/jem.20161673

Bai, N., Aida, T., Yanagisawa, M., Katou, S., Sakimura, K., Mishina, M., and Tanaka, K. (2013). NMDA receptor subunits have different roles in NMDA-induced neurotoxicity in the retina. *Molecular Brain*, *6*(1). https://doi.org/10.1186/1756-6606-6-34

Bandello, F., Battaglia Parodi, M., Lanzetta, P., Loewenstein, A., Massin, P., Menchini, F., and Veritti, D. (2017). Diabetic macular edema. *Developments in Ophthalmology*, *58*, 102–138. https://doi.org/10.1159/000455277

Barber, A. J., and Baccouche, B. (2017). Neurodegeneration in diabetic retinopathy: Potential for novel therapies. *Vision Research*, *139*, 82–92. https://doi.org/10.1016/j.visres.2017.06.014

Barber, A. J., Gardner, T. W., and Abcouwer, S. F. (2011). The Significance of Vascular and Neural Apoptosis to the Pathology of Diabetic Retinopathy. *Investigative Opthalmology and Visual Science*, *52*(2), 1156. https://doi.org/10.1167/iovs.10-6293

Barcelona, P. F., Sitaras, N., Galan, A., Esquiva, G., Jmaeff, S., Jian, Y., Sarunic, M. V., Cuenca, N., Sapieha, P., and Saragovi, H. U. (2016). p75 ^{NTR} and Its Ligand ProNGF Activate Paracrine Mechanisms Etiological to the Vascular, Inflammatory, and Neurodegenerative Pathologies of Diabetic Retinopathy. *The Journal of Neuroscience*, *36*(34), 8826–8841. https://doi.org/10.1523/JNEUROSCI.4278-15.2016

Beebe, D. C., Latker, C. H., Jebens, H. A. H., Johnson, M. C., Feagans, D. E., and Feinberg, R. N. (1986). Transport and steady-state concentration of plasma proteins in the vitreous humor of the chicken embryo: Implications for the mechanism of eye growth during early development. *Developmental Biology*, *114*(2), 361–368. https://doi.org/10.1016/0012-1606(86)90200-9

Behl, T., and Kotwani, A. (2015). Exploring the various aspects of the pathological role of vascular endothelial growth factor (VEGF) in diabetic retinopathy. *Pharmacological Research*, *99*, 137–148. https://doi.org/10.1016/j.phrs.2015.05.013

Behl, T., and Kotwani, A. (2017). Downregulated Brain-Derived Neurotrophic Factor-Induced Oxidative Stress in the

Pathophysiology of Diabetic Retinopathy. *Canadian Journal of Diabetes*, *41*(2), 241–246. https://doi.org/10.1016/j.jcjd.2016.08.228

Bhaskaran, A., Babu, M., Sudhakar, N. A., Kudlu, K. P., and Shashidhara, B. C. (2023). Study of retinal nerve fiber layer thickness in diabetic patients using optical coherence tomography. *Indian Journal of Ophthalmology*, *71*(3), 920–926. https://doi.org/10.4103/ijo.IJO_1918_22

Bianco, L., Arrigo, A., Aragona, E., Antropoli, A., Berni, A., Saladino, A., Battaglia Parodi, M., and Bandello, F. (2022). Neuroinflammation and neurodegeneration in diabetic retinopathy. In *Frontiers in Aging Neuroscience* (Vol. 14). Frontiers Media S.A. https://doi.org/10.3389/fnagi.2022.937999

Bikbova, G., Oshitari, T., Baba, T., and Yamamoto, S. (2014). Neurotrophic Factors for Retinal Ganglion Cell Neuropathy - With a Special Reference to Diabetic Neuropathy in the Retina. *Current Diabetes Reviews*, *10*(3), 166–176. https://doi.org/10.2174/1573399810666140508121927

Boccuni, I., and Fairless, R. (2022). Retinal Glutamate Neurotransmission: From Physiology to Pathophysiological Mechanisms of Retinal Ganglion Cell Degeneration. In *Life* (Vol. 12, Issue 5). MDPI. https://doi.org/10.3390/life12050638

Bonfiglio, V., Platania, C. B. M., Lazzara, F., Conti, F., Pizzo, C., Reibaldi, M., Russo, A., Fallico, M., Ortisi, E., Pignatelli, F., Longo, A., Avitabile, T., Drago, F., and Bucolo, C. (2020). TGF-β Serum Levels in Diabetic Retinopathy Patients and the Role of Anti-VEGF Therapy. *International Journal of Molecular Sciences*, *21*(24), 9558. https://doi.org/10.3390/ijms21249558

Boutahar, N., Reynaud, E., Lassabliere, F., and Borg, J. (2010). Brain-derived neurotrophic factor inhibits cell cycle reentry but not endoplasmic reticulum stress in cultured neurons following

oxidative or excitotoxic stress. *Journal of Neuroscience Research*, *88*(10), 2263–2271. https://doi.org/10.1002/jnr.22384

Bryl, A., Mrugacz, M., Falkowski, M., and Zorena, K. (2022). The Effect of Hyperlipidemia on the Course of Diabetic Retinopathy—Literature Review. *Journal of Clinical Medicine*, *11*(10), 2761. https://doi.org/10.3390/jcm11102761

Busik, J. V. (2021). Lipid metabolism dysregulation in diabetic retinopathy. *Journal of Lipid Research*, *62*, 100017. https://doi.org/10.1194/jlr.TR120000981

Castillo Benítez, V. E., Castro Matto, I., Mello Román, J. C., Vázquez Noguera, J. L., García-Torres, M., Ayala, J., Pinto-Roa, D. P., Gardel-Sotomayor, P. E., Facon, J., and Grillo, S. A. (2021). Dataset from fundus images for the study of diabetic retinopathy. *Data in Brief*, *36*. https://doi.org/10.1016/j.dib.2021.107068

Cervantes-Villagrana, A. R., Garcia-Román, J., González-Espinosa, C., and Lamas, M. (2010). Pharmacological Inhibition of N-Methyl D-Aspartate Receptor Promotes Secretion of Vascular Endothelial Growth Factor in Müller Cells: Effects of Hyperglycemia and Hypoxia. *Current Eye Research*, *35*(8), 733–741. https://doi.org/10.3109/02713683.2010.483312

Chaudhary, S., Zaveri, J., and Becker, N. (2021). Proliferative diabetic retinopathy (PDR). *Disease-a-Month*, *67*(5). https://doi.org/10.1016/j.disamonth.2021.101140

Chen, C., Chen, H., Xu, C., Zhong, Y., and Shen, X. (2014). Role of interleukin-1β in hypoxia-induced depression of glutamate uptake in retinal Müller cells. *Graefe's Archive for Clinical and Experimental Ophthalmology*, *252*(1), 51–58. https://doi.org/10.1007/s00417-013-2516-z

Chen, T. A., Yang, F., Cole, G. M., and Chan, S. O. (2001). Inhibition of caspase-3-like activity reduces glutamate induced cell death in

adult rat retina11Published on the World Wide Web on 9 May 2001. *Brain Research*, *904*(1), 177–188. https://doi.org/10.1016/S0006-8993(01)02485-4

Cheung, N., Mitchell, P., and Wong, T. Y. (2010). Seminar Diabetic retinopathy. *The Lancet*, *376*, 124–136. https://doi.org/10.1016/S0140

Chiarelli, F., Santilli, F., and Mohn, A. (2000). Role of Growth Factors in the Development of Diabetic Complications. *Hormone Research in Paediatrics*, *53*(2), 53–67. https://doi.org/10.1159/000023515

Choi, D. W. (2020). Excitotoxicity: Still Hammering the Ischemic Brain in 2020. In *Frontiers in Neuroscience* (Vol. 14). Frontiers Media S.A. https://doi.org/10.3389/fnins.2020.579953

Chong, D. D., Das, N., and Singh, R. P. (2024). Diabetic retinopathy: Screening, prevention, and treatment. *Cleveland Clinic Journal of Medicine*, *91*(8), 503–510. https://doi.org/10.3949/ccjm.91a.24028

Ciprés, M., Satue, M., Melchor, I., Gil-Arribas, L., Vilades, E., and Garcia-Martin, E. (2022). Neurodegeneración retiniana en pacientes diabéticos tipo 2 sin retinopatía diabética. *Archivos de La Sociedad Española de Oftalmología*, *97*(4), 205–218. https://doi.org/10.1016/j.oftal.2021.04.004

Cusato, K., Bosco, A., Linden, R., and Reese, B. E. (2002). Cell death in the inner nuclear layer of the retina is modulated by BDNF. *Developmental Brain Research*, *139*(2), 325–330. https://doi.org/10.1016/S0165-3806(02)00570-9

Dai, M., Xia, X., and Xiong, S.-Q. (2012). BDNF regulates GLAST and glutamine synthetase in mouse retinal Müller cells. *Journal of Cellular Physiology*, *227*(2), 596–603. https://doi.org/10.1002/jcp.22762

D'Amelio, M., Cavallucci, V., and Cecconi, F. (2010). Neuronal caspase-3 signaling: not only cell death. *Cell Death and Differentiation*, *17*(7), 1104–1114. https://doi.org/10.1038/cdd.2009.180

Danek, D., Larsen, B., and Anderson-Nelson, S. (2021). Non-proliferative diabetic retinopathy. *Disease-a-Month*, *67*(5). https://doi.org/10.1016/j.disamonth.2021.101139

Das, T., Takkar, B., Sivaprasad, S., Thanksphon, T., Taylor, H., Wiedemann, P., Nemeth, J., Nayar, P. D., Rani, P. K., and Khandekar, R. (2021). Recently updated global diabetic retinopathy screening guidelines: commonalities, differences, and future possibilities. *Eye*, *35*(10), 2685–2698. https://doi.org/10.1038/s41433-021-01572-4

Deng, J., Wu, D. Z., and Gao, R. (2000). Detection of glutamate and gamma-aminobutyric acid in vitreous of patients with proliferative diabetic retinopathy. *Yan Ke Xue Bao = Eye Science*, *16*(3), 199–202.

Diaz, J. D., and Arroyo, J. G. (2020). Modern Clinical Applications of Endoscopic Pars Plana Vitrectomy in Vitreoretinal Surgery. *International Ophthalmology Clinics*, *60*(1), 25–33. https://doi.org/10.1097/IIO.0000000000000295

Diederen, R. M. H., La Heij, E. C., Deutz, N. E. P., Kijlstra, A., Kessels, A. G. H., van Eijk, H. M. H., Liem, A. T. A., Dieudonné, S., and Hendrikse, F. (2006). Increased glutamate levels in the vitreous of patients with retinal detachment. *Experimental Eye Research*, *83*(1), 45–50. https://doi.org/10.1016/j.exer.2005.10.031

Ding, X., Sun, Z., Guo, Y., Tang, W., Shu, Q., and Xu, G. (2023). Inhibition of NF-κB ameliorates aberrant retinal glia activation and inflammatory responses in streptozotocin-induced diabetic

rats. *Annals of Translational Medicine*, *11*(5), 197–197. https://doi.org/10.21037/atm-22-2204

Dirani, M., Xie, J., Fenwick, E., Benarous, R., Rees, G., Wong, T. Y., and Lamoureux, E. L. (2011). Are Obesity and Anthropometry Risk Factors for Diabetic Retinopathy?: The Diabetes Management Project. *Investigative Opthalmology and Visual Science*, *52*(7), 4416. https://doi.org/10.1167/iovs.11-7208

dos Santos, F. M., Ciordia, S., Mesquita, J., de Sousa, J. P. C., Paradela, A., Tomaz, C. T., and Passarinha, L. A. P. (2023). Vitreous humor proteome: unraveling the molecular mechanisms underlying proliferative and neovascular vitreoretinal diseases. In *Cellular and Molecular Life Sciences* (Vol. 80, Issue 1). Springer Science and Business Media Deutschland GmbH. https://doi.org/10.1007/s00018-022-04670-y

El-Asrar, A. M. A., Dralands, L., Missotten, L., Al-Jadaan, I. A., and Geboes, K. (2004). Expression of Apoptosis Markers in the Retinas of Human Subjects with Diabetes. *Investigative Opthalmology and Visual Science*, *45*(8), 2760. https://doi.org/10.1167/iovs.03-1392

Elmore, S. (2007). Apoptosis: A Review of Programmed Cell Death. *Toxicologic Pathology*, *35*(4), 495–516. https://doi.org/10.1080/01926230701320337

Elshaer, S. L., Abdelsaid, M. A., Al-Azayzih, A., Kumar, P., Matragoon, S., Nussbaum, J. J., and El-Remessy, A. B. (2013). Pronerve Growth Factor Induces Angiogenesis via Activation of TrkA: Possible Role in Proliferative Diabetic Retinopathy. *Journal of Diabetes Research*, *2013*, 1–10. https://doi.org/10.1155/2013/432659

ETDRS. (1991). Fundus photographic risk factors for progression of diabetic retinopathy. ETDRS report number 12. Early Treatment

Diabetic Retinopathy Study Research Group. *Ophthalmology*, *98*(5 Suppl), 823–833.

Eyileten, C., Kaplon-Cieslicka, A., Mirowska-Guzel, D., Malek, L., and Postula, M. (2017). Antidiabetic Effect of Brain-Derived Neurotrophic Factor and Its Association with Inflammation in Type 2 Diabetes Mellitus. *Journal of Diabetes Research*, *2017*, 1–14. https://doi.org/10.1155/2017/2823671

Fang, J., Wang, H., Niu, T., Shi, X., Xing, X., Qu, Y., Liu, Y., Liu, X., Xiao, Y., Dou, T., Shen, Y., and Liu, K. (2023). Integration of Vitreous Lipidomics and Metabolomics for Comprehensive Understanding of the Pathogenesis of Proliferative Diabetic Retinopathy. *Journal of Proteome Research*, *22*(7), 2293–2306. https://doi.org/10.1021/acs.jproteome.3c00007

Faul, F., Erdfelder, E., Buchner, A., and Lang, A.-G. (2009). Statistical power analyses using G*Power 3.1: Tests for correlation and regression analyses. *Behavior Research Methods*, *41*(4), 1149–1160. https://doi.org/10.3758/BRM.41.4.1149

Feng, S., Yu, H., Yu, Y., Geng, Y., Li, D., Yang, C., Lv, Q., Lu, L., Liu, T., Li, G., and Yuan, L. (2018). Levels of Inflammatory Cytokines IL-1 β , IL-6, IL-8, IL-17A, and TNF- α in Aqueous Humour of Patients with Diabetic Retinopathy. *Journal of Diabetes Research*, *2018*, 1–6. https://doi.org/10.1155/2018/8546423

Fischer, F. (1989). The First Case of Diabetic Retinopathy. In *Diabetes Its Medical and Cultural History* (pp. 363–369). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-48364-6_26

Flaxman, S. R., Bourne, R. R. A., Resnikoff, S., Ackland, P., Braithwaite, T., Cicinelli, M. V, Das, A., Jonas, J. B., Keeffe, J., Kempen, J. H., Leasher, J., Limburg, H., Naidoo, K., Pesudovs, K., Silvester, A., Stevens, G. A., Tahhan, N., Wong, T. Y., Taylor, H. R., … Zheng, Y. (2017). Global causes of blindness and distance vision

impairment 1990–2020: a systematic review and meta-analysis. *The Lancet Global Health*, *5*(12), e1221–e1234. https://doi.org/10.1016/S2214-109X(17)30393-5

Forrester, J. V., Kuffova, L., and Delibegovic, M. (2020). The Role of Inflammation in Diabetic Retinopathy. *Frontiers in Immunology*, *11*. https://doi.org/10.3389/fimmu.2020.583687

Friedrich, J., Steel, D. H. W., Schlingemann, R. O., Koss, M. J., Hammes, H.-P., Krenning, G., and Klaassen, I. (2020). microRNA Expression Profile in the Vitreous of Proliferative Diabetic Retinopathy Patients and Differences from Patients Treated with Anti-VEGF Therapy. *Translational Vision Science and Technology*, *9*(6), 16. https://doi.org/10.1167/tvst.9.6.16

Frith, P., and Mehta, A. R. (2021). The retina as a window into the brain. *The Lancet Neurology*, *20*(11), 892. https://doi.org/10.1016/S1474-4422(21)00332-X

Garcia-Medina, J. J., Rubio-Velazquez, E., Foulquie-Moreno, E., Casaroli-Marano, R. P., Pinazo-Duran, M. D., Zanon-Moreno, V., and del-Rio-Vellosillo, M. (2020). Update on the Effects of Antioxidants on Diabetic Retinopathy: In Vitro Experiments, Animal Studies and Clinical Trials. *Antioxidants*, *9*(6), 561. https://doi.org/10.3390/antiox9060561

Gasecka, A., Siwik, D., Gajewska, M., Jaguszewski, M. J., Mazurek, T., Filipiak, K. J., Postuła, M., and Eyileten, C. (2020). Early biomarkers of neurodegenerative and neurovascular disorders in diabetes. In *Journal of Clinical Medicine* (Vol. 9, Issue 9, pp. 1–24). MDPI. https://doi.org/10.3390/jcm9092807

Ge, K., Wang, Y., Li, P., Li, M., Zhang, W., Dan, H., Hu, X., Zhou, J., Yang, Q., Wang, J., and Song, Z. (2022). Down-expression of the NLRP3 inflammasome delays the progression of diabetic

retinopathy. *Microvascular Research*, *139*, 104265. https://doi.org/10.1016/j.mvr.2021.104265

Ghaseminejad, F., Kaplan, L., Pfaller, A. M., Hauck, S. M., and Grosche, A. (2020). The role of Müller cell glucocorticoid signaling in diabetic retinopathy. In *Graefe's Archive for Clinical and Experimental Ophthalmology* (Vol. 258, Issue 2, pp. 221–230). Springer. https://doi.org/10.1007/s00417-019-04521-w

Glassman, A. R., Elmasry, M. A., Baskin, D. E., Brigell, M., Chong, V., Davis, Q., Lesmes, L., Levin, L. A., Maddess, T., Taylor, L. J., and Wenzel, A. (2024). Visual Function Measurements in Eyes With Diabetic Retinopathy: An Expert Opinion on Available Measures. *Ophthalmology Science*, *4*(5), 100519. https://doi.org/10.1016/j.xops.2024.100519

Graeber, M. B., Li, W., and Rodriguez, M. L. (2011). Role of microglia in CNS inflammation. *FEBS Letters*, *585*(23), 3798–3805. https://doi.org/10.1016/j.febslet.2011.08.033

Grimes, W. N., Berson, D. M., Sabnis, A., Hoon, M., Sinha, R., Tian, H., and Diamond, J. S. (2024). *The retina's neurovascular unit: Müller glial sheaths and neuronal contacts*. https://doi.org/10.1101/2024.04.30.591885

Grossniklaus, H. E., Geisert, E. E., and Nickerson, J. M. (2015). Introduction to the Retina. *Progress in Molecular Biology and Translational Science*, *134*, 383–396. https://doi.org/10.1016/bs.pmbts.2015.06.001

Guerra-Farfan, E., Garcia-Sanchez, Y., Jornet-Gibert, M., Nuñez, J. H., Balaguer-Castro, M., and Madden, K. (2023). Clinical practice guidelines: The good, the bad, and the ugly. *Injury*, *54*, S26–S29. https://doi.org/10.1016/j.injury.2022.01.047

Häggström, M. (2014). Medical gallery of Mikael Häggström 2014. *Wikiversity Journal of Medicine*, *1*(2).

He, S., Sun, L., Chen, J., and Ouyang, Y. (2023). Recent Advances and Perspectives in Relation to the Metabolomics-Based Study of Diabetic Retinopathy. *Metabolites*, *13*(9), 1007. https://doi.org/10.3390/metabo13091007

Hernández, C., and Simó, R. (2012). Neuroprotection in Diabetic Retinopathy. *Current Diabetes Reports*, *12*(4), 329–337. https://doi.org/10.1007/s11892-012-0284-5

Hombrebueno, J. R., Ali, I. H., Xu, H., and Chen, M. (2015). Sustained intraocular VEGF neutralization results in retinal neurodegeneration in the Ins2 Akita diabetic mouse. *Scientific Reports*, *5*. https://doi.org/10.1038/srep18316

Hoon, M., Okawa, H., Della Santina, L., and Wong, R. O. L. (2014). Functional architecture of the retina: Development and disease. In *Progress in Retinal and Eye Research* (Vol. 42, pp. 44–84). Elsevier Ltd. https://doi.org/10.1016/j.preteyeres.2014.06.003

Ibrahim, A. S., El-Remessy, A. B., Matragoon, S., Zhang, W., Patel, Y., Khan, S., Al-Gayyar, M. M., El-Shishtawy, M. M., and Liou, G. I. (2011). Retinal Microglial Activation and Inflammation Induced by Amadori-Glycated Albumin in a Rat Model of Diabetes. *Diabetes*, *60*(4), 1122–1133. https://doi.org/10.2337/db10-1160

Ishikawa, M. (2013). Abnormalities in Glutamate Metabolism and Excitotoxicity in the Retinal Diseases. *Scientifica*, *2013*, 1–13. https://doi.org/10.1155/2013/528940

Jampol, L. M., Glassman, A. R., and Sun, J. (2020). Evaluation and Care of Patients with Diabetic Retinopathy. *New England Journal of Medicine*, *382*(17), 1629–1637. https://doi.org/10.1056/nejmra1909637

Jha, M. K., Seo, M., Kim, J.-H., Kim, B.-G., Cho, J.-Y., and Suk, K. (2013). The secretome signature of reactive glial cells and its pathological implications. *Biochimica et Biophysica Acta (BBA) - Proteins and*

Proteomics, *1834*(11), 2418–2428. https://doi.org/10.1016/j.bbapap.2012.12.006

Jiang, Y., Zhou, L., and Yao, L. (2023). Serum levels of interleukin-18 in diabetic retinopathy patients: A meta-analysis. *European Journal of Ophthalmology*, *33*(6), 2259–2266. https://doi.org/10.1177/11206721231163900

Jindal, V. (2015). Neurodegeneration as a Primary Change and Role of Neuroprotection in Diabetic Retinopathy. *Molecular Neurobiology*, *51*(3), 878–884. https://doi.org/10.1007/s12035-014-8732-7

Joussen, A. M., Doehmen, S., Le, M. L., Koizumi, K., Radetzky, S., Krohne, T. U., Poulaki, V., Semkova, I., and Kociok, N. (2009). TNF-alpha mediated apoptosis plays an important role in the development of early diabetic retinopathy and long-term histopathological alterations. *Molecular Vision*, *15*, 1418–1428.

Jun, Y. H., and Kim, S. T. (2021). Brain-derived neurotrophic factor in non-proliferative diabetic retinopathy with diabetic macular edema. *European Journal of Ophthalmology*, *31*(4), 1915–1919. https://doi.org/10.1177/1120672120944801

Jung, K. I., Woo, J. E., and Park, C. K. (2020). Intraocular pressure fluctuation and neurodegeneration in the diabetic rat retina. *British Journal of Pharmacology*, *177*(13), 3046–3059. https://doi.org/10.1111/bph.15033

Karlstetter, M., Scholz, R., Rutar, M., Wong, W. T., Provis, J. M., and Langmann, T. (2015). Retinal microglia: Just bystander or target for therapy? *Progress in Retinal and Eye Research*, *45*, 30–57. https://doi.org/10.1016/j.preteyeres.2014.11.004

Kashyap, D., Garg, V. K., and Goel, N. (2021). *Intrinsic and extrinsic pathways of apoptosis: Role in cancer development and prognosis* (pp. 73–120). https://doi.org/10.1016/bs.apcsb.2021.01.003

Kaviarasan, K., Jithu, M., Arif Mulla, M., Sharma, T., Sivasankar, S., Das, U. N., and Angayarkanni, N. (2015). Low blood and vitreal BDNF, LXA4 and altered Th1/Th2 cytokine balance are potential risk factors for diabetic retinopathy. *Metabolism*, *64*(9), 958–966. https://doi.org/10.1016/j.metabol.2015.04.005

Kels, B. D., Grzybowski, A., and Grant-Kels, J. M. (2015). Human ocular anatomy. *Clinics in Dermatology*, *33*(2), 140–146. https://doi.org/10.1016/j.clindermatol.2014.10.006

Kim, S. T., Chung, Y. Y., Hwang, H.-I., Shin, H.-K., Choi, R., and Jun, Y. H. (2021). Differential Expression of BDNF and BIM in Streptozotocin-induced Diabetic Rat Retina After Fluoxetine Injection. *In Vivo*, *35*(3), 1461–1466. https://doi.org/10.21873/invivo.12398

Kovács-Valasek, A., Rák, T., Pöstyéni, E., Csutak, A., and Gábriel, R. (2023). Three Major Causes of Metabolic Retinal Degenerations and Three Ways to Avoid Them. In *International Journal of Molecular Sciences* (Vol. 24, Issue 10). Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/ijms24108728

Krijnen, P. A. J., Simsek, S., and Niessen, H. W. M. (2009). Apoptosis in diabetes. *Apoptosis*, *14*(12), 1387–1388. https://doi.org/10.1007/s10495-009-0419-6

Kropp, M., Golubnitschaja, O., Mazurakova, A., Koklesova, L., Sargheini, N., Vo, T.-T. K. S., de Clerck, E., Polivka, J., Potuznik, P., Polivka, J., Stetkarova, I., Kubatka, P., and Thumann, G. (2023). Diabetic retinopathy as the leading cause of blindness and early predictor of cascading complications—risks and mitigation. *EPMA Journal*, *14*(1), 21–42. https://doi.org/10.1007/s13167-023-00314-8

Kueck, P., Morris, J., and Stanford, J. (2024). Current Perspectives: Obesity and Neurodegeneration - Links and Risks. *Degenerative Neurological and Neuromuscular Disease*, *Volume 13*, 111–129. https://doi.org/10.2147/DNND.S388579

Kuranaga, E. (2012). Beyond apoptosis: caspase regulatory mechanisms and functions *in vivo*. *Genes to Cells*, *17*(2), 83–97. https://doi.org/10.1111/j.1365-2443.2011.01579.x

Lau, A., and Tymianski, M. (2010). Glutamate receptors, neurotoxicity and neurodegeneration. In *Pflugers Archiv European Journal of Physiology* (Vol. 460, Issue 2, pp. 525–542). https://doi.org/10.1007/s00424-010-0809-1

Leist, M., and Nicotera, P. (1998). Apoptosis, Excitotoxicity, and Neuropathology. *Experimental Cell Research*, *239*(2), 183–201. https://doi.org/10.1006/excr.1997.4026

Li, G., Wang, L., and Feng, F. (2025). A systematic meta-analysis of the prevalence of diabetic retinopathy. *Technology and Health Care*. https://doi.org/10.1177/09287329241295877

Li, X., Zhang, L., Tang, F., and Wei, X. (2021). Retinal Organoids: Cultivation, Differentiation, and Transplantation. *Frontiers in Cellular Neuroscience*, *15*. https://doi.org/10.3389/fncel.2021.638439

Lim, R. R., Hainsworth, D. P., Mohan, R. R., and Chaurasia, S. S. (2019). Characterization of a functionally active primary microglial cell culture from the pig retina. *Experimental Eye Research*, *185*, 107670. https://doi.org/10.1016/j.exer.2019.05.010

Liu, C., Dong, W., Lv, Z., Kong, L., and Ren, X. (2022). Thioredoxin-interacting protein in diabetic retinal neurodegeneration: A novel potential therapeutic target for diabetic retinopathy. In *Frontiers in Neuroscience* (Vol. 16). Frontiers Media S.A. https://doi.org/10.3389/fnins.2022.957667

Liu, Y., Li, L., Pan, N., Gu, J., Qiu, Z., Cao, G., Dou, Y., Dong, L., Shuai, J., and Sang, A. (2021). TNF-α released from retinal Müller cells aggravates retinal pigment epithelium cell apoptosis by upregulating mitophagy during diabetic retinopathy. *Biochemical and Biophysical Research Communications*, *561*, 143–150. https://doi.org/10.1016/j.bbrc.2021.05.027

Liu, Y., Tao, L., Fu, X., Zhao, Y., and Xu, X. (2013). BDNF protects retinal neurons from hyperglycemia through the TrkB/ERK/MAPK pathway. *Molecular Medicine Reports*, *7*(6), 1773–1778. https://doi.org/10.3892/mmr.2013.1433

Lorenzi, M. (2007). The polyol pathway as a mechanism for diabetic retinopathy: Attractive, elusive, and resilient. In *Experimental Diabesity Research* (Vol. 2007). https://doi.org/10.1155/2007/61038

Lovic, D., Piperidou, A., Zografou, I., Grassos, H., Pittaras, A., and Manolis, A. (2020). The Growing Epidemic of Diabetes Mellitus. *Current Vascular Pharmacology*, *18*(2), 104–109. https://doi.org/10.2174/1570161117666190405165911

Mason, R. H., Minaker, S. A., Lahaie Luna, G., Bapat, P., Farahvash, A., Garg, A., Bhambra, N., and Muni, R. H. (2022). Changes in aqueous and vitreous inflammatory cytokine levels in proliferative diabetic retinopathy: a systematic review and meta-analysis. *Eye*. https://doi.org/10.1038/s41433-022-02127-x

Mason, R. H., Minaker, S. A., Lahaie Luna, G., Bapat, P., Farahvash, A., Garg, A., Bhambra, N., and Muni, R. H. (2025). Changes in aqueous and vitreous inflammatory cytokine levels in nonproliferative diabetic retinopathy: systematic review and meta-analysis. *Canadian Journal of Ophthalmology*, *60*(1), e100–e116. https://doi.org/10.1016/j.jcjo.2024.05.031

Massagué, J., and Sheppard, D. (2023). TGF-β signaling in health and disease. *Cell*, *186*(19), 4007–4037. https://doi.org/10.1016/j.cell.2023.07.036

Matsuzaki, H., Tamatani, M., Yamaguchi, A., Namikawa, K., Kiyama, H., Vitek, M. P., Mitsuda, N., and Tohyama, M. (2001). Vascular endothelial growth factor rescues hippocampal neurons from glutamate-induced toxicity: signal transduction cascades. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *15*(7), 1218–1220.

Mendonca, H., Carpi-Santos, R., Da Costa Calaza, K., and Blanco Martinez, A. (2020). Neuroinflammation and oxidative stress act in concert to promote neurodegeneration in the diabetic retina and optic nerve: Galectin-3 participation. In *Neural Regeneration Research* (Vol. 15, Issue 4, pp. 625–635). Wolters Kluwer Medknow Publications. https://doi.org/10.4103/1673-5374.266910

Meyers, E. A., and Kessler, J. A. (2017). TGF-β Family Signaling in Neural and Neuronal Differentiation, Development, and Function. *Cold Spring Harbor Perspectives in Biology*, *9*(8), a022244. https://doi.org/10.1101/cshperspect.a022244

Mrugacz, M., Bryl, A., and Zorena, K. (2021). Retinal vascular endothelial cell dysfunction and neuroretinal degeneration in diabetic patients. *Journal of Clinical Medicine*, *10*(3), 1–18. https://doi.org/10.3390/jcm10030458

Nagineni, C. N., Samuel, W., Nagineni, S., Pardhasaradhi, K., Wiggert, B., Detrick, B., and Hooks, J. J. (2003). Transforming growth factor-β induces expression of vascular endothelial growth factor in human retinal pigment epithelial cells: Involvement of mitogen-activated protein kinases. *Journal of Cellular Physiology*, *197*(3), 453–462. https://doi.org/10.1002/jcp.10378

Narayanan, S. P., Shosha, E., and D Palani, C. (2019). Spermine oxidase: A promising therapeutic target for neurodegeneration in diabetic retinopathy. *Pharmacological Research*, *147*, 104299. https://doi.org/10.1016/j.phrs.2019.104299

Nebbioso, M., Lambiase, A., Armentano, M., Tucciarone, G., Sacchetti, M., Greco, A., and Alisi, L. (2022). Diabetic retinopathy, oxidative stress, and sirtuins: an in depth look in enzymatic patterns and new therapeutic horizons. In *Survey of Ophthalmology* (Vol. 67, Issue 1, pp. 168–183). Elsevier Inc. https://doi.org/10.1016/j.survophthal.2021.04.003

Nian, S., Lo, A. C. Y., Mi, Y., Ren, K., and Yang, D. (2021). Neurovascular unit in diabetic retinopathy: pathophysiological roles and potential therapeutical targets. *Eye and Vision*, *8*(1), 15. https://doi.org/10.1186/s40662-021-00239-1

Nishijima, K., Ng, Y. S., Zhong, L., Bradley, J., Schubert, W., Jo, N., Akita, J., Samuelsson, S. J., Robinson, G. S., Adamis, A. P., and Shima, D. T. (2007). Vascular endothelial growth factor-A is a survival factor for retinal neurons and a critical neuroprotectant during the adaptive response to ischemic injury. *American Journal of Pathology*, *171*(1), 53–67. https://doi.org/10.2353/ajpath.2007.061237

Ola, M. S., Nawaz, M. I., El-Asrar, A. A., Abouammoh, M., and Alhomida, A. S. (2013). Reduced Levels of Brain Derived Neurotrophic Factor (BDNF) in the Serum of Diabetic Retinopathy Patients and in the Retina of Diabetic Rats. *Cellular and Molecular Neurobiology*, *33*(3), 359–367. https://doi.org/10.1007/s10571-012-9901-8

O'Leary, F., and Campbell, M. (2023). The blood–retina barrier in health and disease. *The FEBS Journal*, *290*(4), 878–891. https://doi.org/10.1111/febs.16330

Ong, C. H., Tham, C. L., Harith, H. H., Firdaus, N., and Israf, D. A. (2021). TGF-β-induced fibrosis: A review on the underlying mechanism and potential therapeutic strategies. *European Journal of Pharmacology*, *911*, 174510. https://doi.org/10.1016/j.ejphar.2021.174510

Oshitari, T., Yamamoto, S., Hata, N., and Roy, S. (2008). Mitochondria- and caspase-dependent cell death pathway involved in neuronal degeneration in diabetic retinopathy. *British Journal of Ophthalmology*, *92*(4), 552–556. https://doi.org/10.1136/bjo.2007.132308

Owsley, C., McGwin, G., Lee, D. J., Lam, B. L., Friedman, D. S., Gower, E. W., Haller, J. A., Hark, L. A., and Saaddine, J. (2015). Diabetes Eye Screening in Urban Settings Serving Minority Populations. *JAMA Ophthalmology*, *133*(2), 174. https://doi.org/10.1001/jamaophthalmol.2014.4652

Pan, W. W., Lin, F., and Fort, P. E. (2021). The innate immune system in diabetic retinopathy. In *Progress in Retinal and Eye Research* (Vol. 84). Elsevier Ltd. https://doi.org/10.1016/j.preteyeres.2021.100940

Panda, S. P., Reddy, P. H., Gorla, U. S., and Prasanth, D. (2023). Neuroinflammation and neovascularization in diabetic eye diseases (DEDs): identification of potential pharmacotherapeutic targets. In *Molecular Biology Reports* (Vol. 50, Issue 2, pp. 1857–1869). Springer Science and Business Media B.V. https://doi.org/10.1007/s11033-022-08113-6

Paneque, A., Fortus, H., Zheng, J., Werlen, G., and Jacinto, E. (2023). The Hexosamine Biosynthesis Pathway: Regulation and Function. In *Genes* (Vol. 14, Issue 4). Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/genes14040933

Park, S. S. (2023). Retinal glia and NF-κB in diabetic retinopathy pathogenesis. *Annals of Translational Medicine*, *11*(9), 307–307. https://doi.org/10.21037/atm-23-1166

Patel, C., Pande, S., Sagathia, V., Ranch, K., Beladiya, J., Boddu, S. H. S., Jacob, S., Al-Tabakha, M. M., Hassan, N., and Shahwan, M. (2023). Nanocarriers for the Delivery of Neuroprotective Agents in the Treatment of Ocular Neurodegenerative Diseases. In *Pharmaceutics* (Vol. 15, Issue 3). Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/pharmaceutics15030837

Pekny, M., and Pekna, M. (2016). Reactive gliosis in the pathogenesis of CNS diseases. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1862*(3), 483–491. https://doi.org/10.1016/j.bbadis.2015.11.014

Peña, J. S., and Vazquez, M. (2022). Harnessing the Neuroprotective Behaviors of Müller Glia for Retinal Repair. In *Frontiers in Bioscience - Landmark* (Vol. 27, Issue 6). Bioscience Research Institute. https://doi.org/10.31083/j.fbl2706169

Peng, L., Parpura, V., and Verkhratsky, A. (2014). Editorial (Thematic Issue: Neuroglia as a Central Element of Neurological Diseases: An Underappreciated Target for Therapeutic Intervention). *Current Neuropharmacology*, *12*(4), 303–307. https://doi.org/10.2174/1570159X12999140829152550

Pichavaram, P., Palani, C. D., Patel, C., Xu, Z., Shosha, E., Fouda, A. Y., Caldwell, R. B., and Narayanan, S. P. (2019). Targeting polyamine oxidase to prevent excitotoxicity-induced retinal neurodegeneration. *Frontiers in Neuroscience*, *13*(JAN). https://doi.org/10.3389/fnins.2018.00956

Pillar, S., Moisseiev, E., Sokolovska, J., and Grzybowski, A. (2020). Recent Developments in Diabetic Retinal Neurodegeneration: A

Literature Review. In *Journal of Diabetes Research* (Vol. 2020). Hindawi Limited. https://doi.org/10.1155/2020/5728674

Podestà, F., Romeo, G., Liu, W.-H., Krajewski, S., Reed, J. C., Gerhardinger, C., and Lorenzi, M. (2000). Bax Is Increased in the Retina of Diabetic Subjects and Is Associated with Pericyte Apoptosis in Vivo and in Vitro. *The American Journal of Pathology*, *156*(3), 1025–1032. https://doi.org/10.1016/S0002-9440(10)64970-X

Podolenau, A. Gh. (2012). Optical coherence tomography. *Journal of Microscopy*, *247*(3), 209–219. https://doi.org/10.1111/j.1365-2818.2012.03619.x

Poshtchaman, F., Dehnabi, A., Poshtchaman, Z., and Birjandi, B. (2023). HbA1C, proliferative and non-proliferative retinopathy in diabetic patients. *Medicina Clínica Práctica*, *6*(3), 100371. https://doi.org/10.1016/j.mcpsp.2023.100371

Qian, J., and Huang, Y. (2022). Expression of TNF-α and IL-1β in Peripheral Blood of Patients with T2DM Retinopathy. *Computational and Mathematical Methods in Medicine*, *2022*, 1–7. https://doi.org/10.1155/2022/9073372

Qingzheng, K., Haiyu, D., Suwei, J., and Li, Y. (2022). Advanced glycation end products in diabetic retinopathy and phytochemical therapy. *Frontiers in Nutrition*, *9*(18).

Quiroz, J., and Yazdanyar, A. (2021). Animal models of diabetic retinopathy. *Annals of Translational Medicine*, *9*(15), 1272–1272. https://doi.org/10.21037/atm-20-6737

Rai, N. K., Tripathi, K., Sharma, D., and Shukla, V. K. (2005). Apoptosis: A Basic Physiologic Process in Wound Healing. *The International Journal of Lower Extremity Wounds*, *4*(3), 138–144. https://doi.org/10.1177/1534734605280018

Ram, V., Siddiqi, S. S., Debbarman, T., Mukherjee, A., Akhtar, N., Waris, A., Thacker, D., and . S. (2024). Serum Brain-Derived Neurotrophic Factor: An Emerging Marker for Diabetic Retinopathy. *Cureus*. https://doi.org/10.7759/cureus.74153

Ramirez, A., Wolfsgruber, S., Lange, C., Kaduszkiewicz, H., Weyerer, S., Werle, J., Pentzek, M., Fuchs, A., Riedel-Heller, S. G., Luck, T., Mösch, E., Bickel, H., Wiese, B., Prokein, J., König, H.-H., Brettschneider, C., Breteler, M. M., Maier, W., Jessen, F., and Scherer, M. (2015). Elevated HbA1c is Associated with Increased Risk of Incident Dementia in Primary Care Patients. *Journal of Alzheimer's Disease*, *44*(4), 1203–1212. https://doi.org/10.3233/JAD-141521

Ramirez, M. L. G., and Salvesen, G. S. (2018). A primer on caspase mechanisms. *Seminars in Cell and Developmental Biology*, *82*, 79–85. https://doi.org/10.1016/j.semcdb.2018.01.002

Ramon y Cajal, S. (1893). La rétine des Vertébrés. *La Cellule*, *IX*, 121–258.

Ramon y Cajal, S. (1896). Sobre la relación de las células nerviosas con las neuróglicas. *Revista Trimestral Micrográfica*, *1*, 123–126.

Rashid, R. R., Fazal, A., Khan, Q. U., Misbah-ul-Qamar, Dr., Ahmed, F., and Laique, T. (2020). Comparison of Serum Brain-Derived Neurotrophic Factor Levels in Diabetic Patients With and Without Retinopathy. *Cureus*. https://doi.org/10.7759/cureus.12028

Rasmussen, R., and Samson, A. J. (2017). Astrocytes: Tailored to Support the Demand of Neural Circuits? *Cell Systems*, *5*(3), 165–167. https://doi.org/10.1016/j.cels.2017.09.008

Roig-Revert, M. J., Lleó-Pérez, A., Zanón-Moreno, V., Vivar-Llopis, B., Marín-Montiel, J., Dolz-Marco, R., Alonso-Muñoz, L., Albert-Fort, M., López-Gálvez, M. I., Galarreta-Mira, D., García-Esparza,

M. F., Galbis-Estrada, C., Marco-Ramirez, C., Shoaie-Nia, K., Sanz-González, S. M., Vila-Bou, V., Bendala-Tufanisco, E., García-Medina, J. J., Nucci, C., … Valencia Study on Diabetic Retinopathy (VSDR). (2015). Enhanced Oxidative Stress and Other Potential Biomarkers for Retinopathy in Type 2 Diabetics: Beneficial Effects of the Nutraceutic Supplements. *BioMed Research International*, *2015*, 1–12. https://doi.org/10.1155/2015/408180

Rossino, M. G., Dal Monte, M., and Casini, G. (2019). Relationships Between Neurodegeneration and Vascular Damage in Diabetic Retinopathy. In *Frontiers in Neuroscience* (Vol. 13). Frontiers Media S.A. https://doi.org/10.3389/fnins.2019.01172

Rudraraju, M., Narayanan, S. P., and Somanath, P. R. (2020). Regulation of blood-retinal barrier cell-junctions in diabetic retinopathy. In *Pharmacological Research* (Vol. 161). Academic Press. https://doi.org/10.1016/j.phrs.2020.105115

Ruiz, A., Matute, C., and Alberdi, E. (2009). Endoplasmic reticulum Ca2+ release through ryanodine and IP3 receptors contributes to neuronal excitotoxicity. *Cell Calcium*, *46*(4), 273–281. https://doi.org/10.1016/j.ceca.2009.08.005

Runkle, E. A., and Antonetti, D. A. (2011). The Blood-Retinal Barrier: Structure and Functional Significance. In *Methods in Molecular Biology* (Vol. 686, pp. 133–148). Humana Press Inc. https://doi.org/10.1007/978-1-60761-938-3_5

Sachdeva, M. M. (2021). Retinal Neurodegeneration in Diabetes: an Emerging Concept in Diabetic Retinopathy. In *Current Diabetes Reports* (Vol. 21, Issue 12). Springer. https://doi.org/10.1007/s11892-021-01428-x

Saint-Geniez, M., Maharaj, A. S. R., Walshe, T. E., Tucker, B. A., Sekiyama, E., Kurihara, T., Darland, D. C., Young, M. J., and

D'Amore, P. A. (2008). Endogenous VEGF is required for visual function: Evidence for a survival role on Müller cells and photoreceptors. *PLoS ONE*, *3*(11). https://doi.org/10.1371/journal.pone.0003554

Santovito, D., Toto, L., De Nardis, V., Marcantonio, P., D'Aloisio, R., Mastropasqua, A., De Cesare, D., Bucci, M., Paganelli, C., Natarelli, L., Weber, C., Consoli, A., Mastropasqua, R., and Cipollone, F. (2021). Plasma microRNA signature associated with retinopathy in patients with type 2 diabetes. *Scientific Reports*, *11*(1), 4136. https://doi.org/10.1038/s41598-021-83047-w

Sanz-González, S., García-Medina, J., Zanón-Moreno, V., López-Gálvez, M., Galarreta-Mira, D., Duarte, L., Valero-Velló, M., Ramírez, A., Arévalo, J., and Pinazo-Durán, M. (2020). Clinical and Molecular-Genetic Insights into the Role of Oxidative Stress in Diabetic Retinopathy: Antioxidant Strategies and Future Avenues. *Antioxidants*, *9*(11), 1101. https://doi.org/10.3390/antiox9111101

Savitskaya, M. A., and Onishchenko, G. E. (2015). Mechanisms of apoptosis. *Biochemistry (Moscow)*, *80*(11), 1393–1405. https://doi.org/10.1134/S0006297915110012

Semba, R. D., Huang, H., Lutty, G. A., Van Eyk, J. E., and Hart, G. W. (2014). The role of O-GlcNAc signaling in the pathogenesis of diabetic retinopathy. *Proteomics - Clinical Applications*, *8*(3–4), 218–231. https://doi.org/10.1002/prca.201300076

Shyam, M., Sidharth, S., Veronica, A., Jagannathan, L., Srirangan, P., Radhakrishnan, V., and Sabina, E. P. (2025). Diabetic retinopathy: a comprehensive review of pathophysiology and emerging treatments. *Molecular Biology Reports*, *52*(1), 380. https://doi.org/10.1007/s11033-025-10490-7

Sies, H. (2015). Oxidative stress: a concept in redox biology and medicine. *Redox Biology*, *4*, 180–183. https://doi.org/10.1016/j.redox.2015.01.002

Simó, R., Simó-Servat, O., Bogdanov, P., and Hernández, C. (2021). Neurovascular unit: A new target for treating early stages of diabetic retinopathy. In *Pharmaceutics* (Vol. 13, Issue 8). MDPI. https://doi.org/10.3390/pharmaceutics13081320

Simó, R., Simó-Servat, O., Bogdanov, P., and Hernández, C. (2022). Diabetic Retinopathy: Role of Neurodegeneration and Therapeutic Perspectives. In *Asia-Pacific Journal of Ophthalmology* (Vol. 11, Issue 2, pp. 160–167). Lippincott Williams and Wilkins. https://doi.org/10.1097/APO.0000000000000510

Smith, S. B. (2002). Diabetic Retinopathy and the NMDA receptor. *Drugs News Perspect*, *15*(4), 226–232.

Smit-McBride, Z., and Morse, L. S. (2021). MicroRNA and diabetic retinopathy—biomarkers and novel therapeutics. *Annals of Translational Medicine*, *9*(15), 1280–1280. https://doi.org/10.21037/atm-20-5189

Song, Z., Sun, M., Zhou, F., Huang, F., Qu, J., and Chen, D. (2014). Increased intravitreous interleukin-18 correlated to vascular endothelial growth factor in patients with active proliferative diabetic retinopathy. *Graefe's Archive for Clinical and Experimental Ophthalmology*, *252*(8), 1229–1234. https://doi.org/10.1007/s00417-014-2586-6

Soni, D., Sagar, P., and Takkar, B. (2021). Diabetic retinal neurodegeneration as a form of diabetic retinopathy. In *International Ophthalmology* (Vol. 41, Issue 9, pp. 3223–3248). Springer Science and Business Media B.V. https://doi.org/10.1007/s10792-021-01864-4

Srinivasan, V., Sandhya, N., Sampathkumar, R., Farooq, S., Mohan, V., and Balasubramanyam, M. (2007). Glutamine fructose-6-phosphate amidotransferase (GFAT) gene expression and activity in patients with type 2 diabetes: Inter-relationships with hyperglycaemia and oxidative stress. *Clinical Biochemistry*, *40*(13–14), 952–957. https://doi.org/10.1016/j.clinbiochem.2007.05.002

Sun, W., Su, M., Zhuang, L., Ding, Y., Zhang, Q., and Lyu, D. (2024). Clinical serum lipidomic profiling revealed potential lipid biomarkers for early diabetic retinopathy. *Scientific Reports*, *14*(1), 15148. https://doi.org/10.1038/s41598-024-66157-z

Sun, Y., Zou, H., Li, X., Xu, S., and Liu, C. (2021). Plasma Metabolomics Reveals Metabolic Profiling For Diabetic Retinopathy and Disease Progression. *Frontiers in Endocrinology*, *12*. https://doi.org/10.3389/fendo.2021.757088

Thoreson, W. (1999). Glutamate receptors and circuits in the vertebrate retina. *Progress in Retinal and Eye Research*, *18*(6), 765–810. https://doi.org/10.1016/S1350-9462(98)00031-7

Tian, M., Liu, S., Liu, L., Zhang, E.-K., Wang, H.-W., Deng, Y., and Yue, Y.-K. (2019). Correlations of the severity of diabetic retinopathy with EPO, Caspase-3 expression and oxidative stress. *European Review for Medical and Pharmacological Sciences*, *23*(22), 9707–9713. https://doi.org/10.26355/eurrev_201911_19532

Torjani, A., Mahmoudzadeh, R., Salabati, M., Cai, L., Hsu, J., Garg, S., Ho, A. C., Yonekawa, Y., Kuriyan, A. E., and Starr, M. R. (2023). Factors Associated with Fluctuations in Central Subfield Thickness in Patients with Diabetic Macular Edema Using Diabetic Retinopathy Clinical Research Protocols T and V. *Ophthalmology Science*, *3*(1), 100226. https://doi.org/10.1016/j.xops.2022.100226

Tsoka, P., Barbisan, P. R., Kataoka, K., Chen, X. N., Tian, B., Bouzika, P., Miller, J. W., Paschalis, E. I., and Vavvas, D. G. (2019). NLRP3 inflammasome in NMDA-induced retinal excitotoxicity. *Experimental Eye Research*, *181*, 136–144. https://doi.org/10.1016/j.exer.2019.01.018

Tsui, E., and Patel, P. (2020). Calculated Decisions: Visual acuity testing (Snellen chart). *Emergency Medicine Practice*, *22*(4), CD1–CD2.

Uyar, M., Lezius, S., Buhmann, C., Pötter-Nerger, M., Schulz, R., Meier, S., Gerloff, C., Kuhle, J., and Choe, C. (2022). Diabetes, Glycated Hemoglobin (<scp>HbA1c)</scp> , and Neuroaxonal Damage in Parkinson's Disease (<scp>MARK-PD Study</scp>). *Movement Disorders*, *37*(6), 1299–1304. https://doi.org/10.1002/mds.29009

Valdivia, A. O., He, Y., Ren, X., Wen, D., Dong, L., Nazari, H., and Li, X. (2023). Probable Treatment Targets for Diabetic Retinopathy Based on an Integrated Proteomic and Genomic Analysis. *Translational Vision Science and Technology*, *12*(2), 8. https://doi.org/10.1167/tvst.12.2.8

Vecino, E., Rodriguez, F. D., Ruzafa, N., Pereiro, X., and Sharma, S. C. (2016). Glia–neuron interactions in the mammalian retina. *Progress in Retinal and Eye Research*, *51*, 1–40. https://doi.org/10.1016/j.preteyeres.2015.06.003

Verma, M., Lizama, B. N., and Chu, C. T. (2022). Excitotoxicity, calcium and mitochondria: a triad in synaptic neurodegeneration. In *Translational Neurodegeneration* (Vol. 11, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s40035-021-00278-7

Waheed, N. K., Rosen, R. B., Jia, Y., Munk, M. R., Huang, D., Fawzi, A., Chong, V., Nguyen, Q. D., Sepah, Y., and Pearce, E. (2023).

Optical coherence tomography angiography in diabetic retinopathy. *Progress in Retinal and Eye Research*, *97*, 101206. https://doi.org/10.1016/j.preteyeres.2023.101206

Wajant, H., and Scheurich, P. (2011). TNFR1-induced activation of the classical NF-κB pathway. *The FEBS Journal*, *278*(6), 862–876. https://doi.org/10.1111/j.1742-4658.2011.08015.x

Wang, J., Xu, X., Elliott, M. H., Zhu, M., and Le, Y.-Z. (2010). Müller Cell-Derived VEGF Is Essential for Diabetes-Induced Retinal Inflammation and Vascular Leakage. *Diabetes*, *59*(9), 2297–2305. https://doi.org/10.2337/db09-1420

Wang, W., and Lo, A. C. Y. (2018). Diabetic retinopathy: Pathophysiology and treatments. In *International Journal of Molecular Sciences* (Vol. 19, Issue 6). MDPI AG. https://doi.org/10.3390/ijms19061816

Wei, L., Sun, X., Fan, C., Li, R., Zhou, S., and Yu, H. (2022). The pathophysiological mechanisms underlying diabetic retinopathy. In *Frontiers in Cell and Developmental Biology* (Vol. 10). Frontiers Media S.A. https://doi.org/10.3389/fcell.2022.963615

Wilkinson, C. P., Ferris, F. L., Klein, R. E., Lee, P. P., Agardh, C. D., Davis, M., Dills, D., Kampik, A., Pararajasegaram, R., and Verdaguer, J. T. (2003). Proposed international clinical diabetic retinopathy and diabetic macular edema disease severity scales. *Ophthalmology*, *110*(9), 1677–1682. https://doi.org/10.1016/S0161-6420(03)00475-5

Wong, T. Y., Cheung, C. M. G., Larsen, M., Sharma, S., and Simó, R. (2016). Diabetic retinopathy. *Nature Reviews Disease Primers*, *2*. https://doi.org/10.1038/nrdp.2016.12

World Medical Association. (2013). World Medical Association Declaration of Helsinki. *JAMA*, *310*(20), 2191. https://doi.org/10.1001/jama.2013.281053

Wu, H., Wang, M., Li, X., and Shao, Y. (2021). The Metaflammatory and Immunometabolic Role of Macrophages and Microglia in Diabetic Retinopathy. In *Human Cell* (Vol. 34, Issue 6, pp. 1617–1628). Springer Japan. https://doi.org/10.1007/s13577-021-00580-6

Xiao, H., Xin, W., Sun, L. M., Li, S. S., Zhang, T., and Ding, X. Y. (2021). Comprehensive Proteomic Profiling of Aqueous Humor Proteins in Proliferative Diabetic Retinopathy. *Translational Vision Science and Technology*, *10*(6), 3. https://doi.org/10.1167/tvst.10.6.3

Xu, T., Wang, B., Liu, H., Wang, H., Yin, P., Dong, W., Li, J., Wang, Y. X., Yusufu, M., Briant, P., Reinig, N., Ashbaugh, C., Adelson, J., Vos, T., Bourne, R., Wang, N., and Zhou, M. (2020). Prevalence and causes of vision loss in China from 1990 to 2019: findings from the Global Burden of Disease Study 2019. *The Lancet Public Health*, *5*(12), e682–e691. https://doi.org/10.1016/S2468-2667(20)30254-1

Xu, Z., Bai, S., Wu, H., and Fang, M. (2023). Elevated retinal retinol-binding protein 4 levels in diabetic mice can induce retinal neurodegeneration through microglia. *Microscopy Research and Technique*, *86*(2), 223–231. https://doi.org/10.1002/jemt.24258

Yao, Y., Li, R., Du, J., Li, X., Zhao, L., Long, L., Li, D., and Lu, S. (2018). Tumor necrosis factor-α and diabetic retinopathy: Review and meta-analysis. *Clinica Chimica Acta*, *485*, 210–217. https://doi.org/10.1016/j.cca.2018.06.028

Yao, Y., Li, R., Du, J., Long, L., Li, X., and Luo, N. (2019). Interleukin-6 and Diabetic Retinopathy: A Systematic Review and Meta-Analysis. *Current Eye Research*, *44*(5), 564–574. https://doi.org/10.1080/02713683.2019.1570274

Ye, X., Fung, N. S. K., Lam, W. C., and Lo, A. C. Y. (2024). Nutraceuticals for Diabetic Retinopathy: Recent Advances and

Novel Delivery Systems. *Nutrients*, *16*(11), 1715. https://doi.org/10.3390/nu16111715

Yoshimura, A., Wakabayashi, Y., and Mori, T. (2010). Cellular and molecular basis for the regulation of inflammation by TGF- . *Journal of Biochemistry*, *147*(6), 781–792. https://doi.org/10.1093/jb/mvq043

Yun, J.-H. (2021). Interleukin-1β induces pericyte apoptosis via the NF-κB pathway in diabetic retinopathy. *Biochemical and Biophysical Research Communications*, *546*, 46–53. https://doi.org/10.1016/j.bbrc.2021.01.108

Zafar, S., Sachdeva, M., Frankfort, B. J., and Channa, R. (2019). Retinal Neurodegeneration as an Early Manifestation of Diabetic Eye Disease and Potential Neuroprotective Therapies. In *Current Diabetes Reports* (Vol. 19, Issue 4). Current Medicine Group LLC 1. https://doi.org/10.1007/s11892-019-1134-5

Zeng, Y., Mo, G., Wang, X., Yang, Y., Dong, Y., Zhong, R., and Tian, N. (2024). Investigating the relationship between blood metabolites and diabetic retinopathy using two-sample mendelian randomization and in vivo validation. *Scientific Reports*, *14*(1), 22947. https://doi.org/10.1038/s41598-024-73337-4

Zeppieri, M., and Gurnani, B. (2024). *Applanation Tonometry*.

Zhang, H., Liang, L., Huang, R., Wu, P., and He, L. (2020). Comparison of inflammatory cytokines levels in the aqueous humor with diabetic retinopathy. *International Ophthalmology*, *40*(10), 2763–2769. https://doi.org/10.1007/s10792-020-01463-9

Zhang, N., Cao, W., He, X., Xing, Y., and Yang, N. (2023). Long Non-Coding RNAs in Retinal Ganglion Cell Apoptosis. In *Cellular and Molecular Neurobiology* (Vol. 43, Issue 2, pp. 561–574). Springer. https://doi.org/10.1007/s10571-022-01210-x

Zheng, X., Wan, J., and Tan, G. (2023). The mechanisms of NLRP3 inflammasome/pyroptosis activation and their role in diabetic retinopathy. *Frontiers in Immunology*, *14*. https://doi.org/10.3389/fimmu.2023.1151185

Zhou, H., and Kuang, H. (2021). Circular RNAs: Novel target of diabetic retinopathy. *Reviews in Endocrine and Metabolic Disorders*, *22*(2), 205–216. https://doi.org/10.1007/s11154-021-09646-0

Zhou, J., and Chen, B. (2023). Retinal Cell Damage in Diabetic Retinopathy. In *Cells* (Vol. 12, Issue 9). MDPI. https://doi.org/10.3390/cells12091342

Zhou, J., Shen, X., Lu, Q., and Zhang, M. (2016). TXNIP Suppresses Expression of Glutamine Synthetase by Inducing Oxidative Stress in Retinal Muller Glia Under Diabetic Conditions. *Medical Science Monitor*, *22*, 1460–1466. https://doi.org/10.12659/MSM.895916

Zhou, Z., Ju, H., Sun, M., and Chen, H. (2019). Serum Vascular Endothelial Growth Factor Levels Correlate with Severity of Retinopathy in Diabetic Patients: A Systematic Review and Meta-Analysis. *Disease Markers*, *2019*, 1–15. https://doi.org/10.1155/2019/9401628

ANNEXE I

A/A.: Alex Gallego Martínez
Un. Inv. Oft. "Santiago Grisolía"

Título: - Neurodegeneration in diabetic retinopathy: a study of its signalling profile.
Tesis Doctoral _ Protocolo V2 de 01/09/2023
Código CEIm: 67.2

DICTAMEN DEL COMITÉ ETICO DE INVESTIGACIÓN CLÍNICA DEL HOSPITAL UNIVERSITARIO DOCTOR PESET DE VALENCIA

D. Jose Luis Rodrigo Pérez, Presidente del Comité Ético de Investigación con Medicamentos del Hospital Universitario Doctor Peset de Valencia

CERTIFICA:

Que este Comité, en la reunión ordinaria celebrada el día 28 de junio de 2023, ha evaluado la propuesta del proyecto de investigación del estudio arriba referenciado.

Y considera que:

Se cumplen los requisitos necesarios de idoneidad del protocolo en relación con los objetivos del estudio y están justificados los riesgos y molestias previsibles para el sujeto teniendo en cuenta los beneficios esperados.

La capacidad del investigador y los medios disponibles son apropiados para llevar a cabo el estudio.

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Que se han evaluado la compensaciones económicas previstas (cuando las haya) y su posible interferencia con el respeto a los postulados éticos y se consideran adecuadas.

Se cumplen los preceptos éticos formulados en la Declaración de Helsinki de la Asociación Médica Mundial sobre principios éticos para las investigaciones médicas en seres humanos y en sus posteriores revisiones, así como aquellos exigidos por la normativa aplicable en función de las características del estudio.

Que el proyecto se plantea siguiendo los requisitos del Real Decreto 957/2020, de 3 de noviembre, por el que se regulan los estudios observacionales con medicamentos de uso humano.

Son adecuados los procedimientos para obtener el Consentimiento Informado y el modo de reclutamiento previsto, así como la compensación prevista para los sujetos por daños que pudieran derivarse de su participación en el estudio

Que en la precitada reunión y tras el estudio del protocolo, este Ceim emite un Dictamen Favorable a la realización del estudio en el Centro y con **Alex Gallego Martínez** como **investigador de la Unidad de Inv. Oftalmológica Santiago Grisolia** del Hospital U. Dr. Peset, como investigador principal

Valencia 11 de septiembre de 2023.

Firmat per José Luis Rodrigo Pérez el
12/09/2023 13:13:24
Càrrec: Jefe de Servicio de Cirugía
Ortopédica y Traumatología

Fdo.: Dr. Jose Luis Rodrigo Pérez

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