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COIMBRA

Madania Gafur Amorim

Tears Biomarkers for Diagnosis Strategy of Diabetic Retinopathy Onset

Dissertation presented to University of Coimbra as a requirement for the degree of MSc in Biomedical Research and performed under supervision of Doctor Rosa Cristina Simões Fernandes and Professor Doctor Rufino Silva.

November 2020

Faculdade de Medicina
Universidade de Coimbra

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“Seek knowledge from the cradle to the grave”

- ***Muhammad (SAW)***

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CONTENTS

Agradecimentos v

Publications xi

Abbreviations xv

List of tables xix

List of figures xxi

Resumo xxiii

Abstract xxv

1. Introduction 3

1.1 The Human eye In Health 3

1.1.1 Anterior segment 3

1.1.2 Posterior segment 13

1.2 The human eye in disease 16

1.2.1 Diabetic retinopathy 16

1.2.2 The impact of Diabetes on ocular surface 23

1.2.3 The role of tear biomarkers on Diabetic Retinopathy 25

1.2.4 Extracellular vesicles 26

2. Aims 30

3. Materials and methods 28

3.1 Human subjects selection and recruitment 28

3.1.1 Criteria 29

3.2 Schirmer test 30

3.3 Tear breakup time test (TBUT) 31

3.4 Protein tear extraction from Schirmer strips 32

3.4.1 Determination of total protein concentration 32

3.4.2 Western Blotting 33

3.4.3 Liquid chromatography tandem mass spectrometry (LC-MS/MS) 34

3.4.4 Multiplex immunoassay 35
3.4.5 Gelatin zymography 36
3.5 Isolation of exosomes from tear fluid 37
3.5.1 Nanoparticle Tracking Analysis (NTA) 38
3.5.2 Transmission electron microscopy (TEM) 39
3.6 Statistical analysis 39
4. Results 41
4.1 Characterization of the study population 41
4.2 Tear proteomics 43
4.2.1 Analysis and correlation of differential expression with the stages of DR 48
4.3 Multiplex 73
4.4 Zymography 74
4.5 Extracellular vesicles 75
5. Discussion 80
6. Conclusions and future perspectives 86
7 References 90

ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
AGE	Advanced Glycation End product
AMP	Anti-Microbial Peptides
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BCA	Bicinchoninic Acid
BCVA	Best/Corrected Visual Acuity
BRB	Blood Retinal Barrier
BSA	Bovine Serum Albumin
CD	Difference Cluster
CNS	Central Nervous System
COX	Cyclooxygenase
DAMP	Damage-Associated Molecular Patterns
DDA	Data-Dependent Acquisition
DED	Dry Eye Disease
DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
DPP	Dipeptidyl Peptidase
DR	Diabetic Retinopathy
ECL	Enhanced Chemiluminescence Substrate
ELISA	Enzyme-linked Immunosorbent Assay
ESCRT	Endosomal Sorting Complexes Required for Transport
ETC	Electron Transport Chain
ETDRS	Early Treatment Diabetic Retinopathy Study
EV	Extracellular vesicle
FA	Fluorescein Angiography
FDA	Food and Drug Administration
FDR	False Discovering Rate
GABA	Gamma Amino Butyric Acid
GDP	Guanosine diphosphate
GO	Gene Ontology
GTP	Guanosine triphosphate

HDP	Host Defense Peptides
HIF-1	Hypoxia-Induced Factor-1
HNP	Human Neutrophil peptide
ICAM	Intercellular Adhesion Molecule
ID	Identifier
IDF	International Diabetes Federation
IL	Interleukin
LASEK	Laser Assisted Sub-Epithelial Keratectomy
LASIK	Laser-Assisted Stromal In-situ Keratomileusis
LC	Liquid Chromatography
LEAP	Liver-expressed Antimicrobial Peptides
LFQ	Label-free Quantification
LFU	Lacrimal Functional Unit
MCP	Monocytic Chemotactic Protein1
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
MMP	Metalloproteinase
MS	Mass Spectrometry
NAD	Nicotinamide Adenine Dinucleotide
NAN	Not A Number
NIH	National Institutes of Health
NO	Nitric Oxide
NOX	Nitrous Oxide
NPDR	Non-Proliferative Diabetic Retinopathy
NSAID	Non-Steroidal Anti-Inflammatory Drug
NTA	Nanoparticle Tracking Analysis
OCT	Optical Coherence Tomography
PAGE	Polyacrylamide Gel Electrophoresis
PANTHER	Protein Analysis Through Evolutionary Relationships
PBS	Phosphate-Buffered Saline
PC	Principal Component
PCA	Principal Component Analysis
PDR	Proliferative Diabetic Retinopathy
PE	Streptavidin-Phycoerythrin

PFA	Paraformaldehyde
PPPM	Predictive, Preventive and Personalized Medicine
PSM	Propensity Score Matching
PUFA	Polyunsaturated Fatty Acid
PVDF	Polyvinylidene Difluoride
RAGE	Receptors of Advanced Glycation End product
RAS	Renin–Angiotensin System
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPE	Retinal Pigment Epithelium
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
SPD	Surfactant Protein D
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline with Tween
TBUT	Tear Breakup Time
TEM	Transmission Electron Microscopy
TIMP	Tissue Inhibitor of Metalloproteinase
TLR	Toll Like Receptor
TNF	Tumor Necrosis Factor
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor

LIST OF TABLES

TABLE 1- PRIMARY ANTIBODIES USED IN WESTERN BLOTTING INCUBATION 34

TABLE 2- DEMOGRAPHIC DATA OF CONTROL AND T2D PATIENTS 41

TABLE 3- TEAR FUNCTION TESTS IN CONTROL AND T2D PATIENTS.. 42

TABLE 4- DEMOGRAPHIC DATA OF CONTROL AND T2D PATIENTS 43

TABLE 5- DESCRIPTION OF PROTEINS WITH INTERACTIONS BETWEEN EACH OTHER AMONG HEALTH SUBJECTS AND DIABETIC SUBJECTS WITHOUT AND WITH RETINOPATHY... 71

TABLE 6- CONCENTRATION, MEAN AND MODE OF EXOSOMES ISOLATED FROM TEARS FROM CONTROL, T2D, NPDR AND PDR GROUPS 75

LIST OF FIGURES

FIGURE 1.1 SCHEMATIC REPRESENTATION OF OCULAR SURFACE CONSTITUENTS. 5
FIGURE 1.2 SCHEMATIC REPRESENTATION OF TEAR FILM LAYERS. 8
FIGURE 1.3 CELLS AND LAYERS OF RETINA. 14
FIGURE 1.4 PATHOLOGICAL ALTERATIONS LEADING TO DIFFERENT STAGES OF DIABETIC RETINOPATHY. 20
FIGURE 4.1 PCA SCATTER PLOT.. 44
FIGURE 4.2 GO MOLECULAR FUNCTION COMPLETE.. 45
FIGURE 4.3 PANTHER GO-SLIM MOLECULAR FUNCTION. 46
FIGURE 4.4 GO BIOLOGICAL PROCESS COMPLETE. 46
FIGURE 4.5 PANTHER GO-SLIM BIOLOGICAL PROCESS. 47
FIGURE 4.6 GO CELLULAR COMPONENT COMPLETE. 47
FIGURE 4.7 PANTHER GO-SLIM CELLULAR COMPONENT. 48
FIGURE 4.8 VOLCANO PLOT OF DISTRIBUTION OF DIFFERENTIALLY EXPRESSED PROTEINS BETWEEN T2D AND CONTROL GROUPS.. 49
FIGURE 4.9 COMPARISON OF LFQ INTENSITIES OF THE HEMOGLOBIN SUBUNIT BETA.. 50
FIGURE 4.10 VOLCANO PLOT OF DISTRIBUTION OF DIFFERENTIALLY EXPRESSED PROTEINS BETWEEN NPDR AND CONTROL GROUPS. 51
FIGURE 4.11 COMPARISON OF LFQ INTENSITIES OF THE 42 PROTEINS.. 55
FIGURE 4.12 PANTHER GO-SLIM MOLECULAR FUNCTION. 56
FIGURE 4.13 PANTHER GO-SLIM BIOLOGICAL PROCESS. 56
FIGURE 4.14 PANTHER GO-SLIM CELLULAR COMPONENT. 57
FIGURE 4.15 PANTHER GO-SLIM PROTEIN CLASS.. 57
FIGURE 4.16 PANTHER PATHWAYS.. 58
FIGURE 4.17 VOLCANO PLOT OF DISTRIBUTION OF DIFFERENTIALLY EXPRESSED PROTEINS BETWEEN PDR AND CONTROL GROUPS. 58
FIGURE 4.18 COMPARISON OF LFQ INTENSITIES OF THE 26 PROTEINS.. 61
FIGURE 4.19 PANTHER GO-SLIM MOLECULAR FUNCTION. 62
FIGURE 4.20 PANTHER GO-SLIM BIOLOGICAL PROCESS. 62
FIGURE 4.21 PANTHER GO-SLIM CELLULAR COMPONENT. 63
FIGURE 4.22 PANTHER GO-SLIM PROTEIN CLASS.. 63
FIGURE 4.23 PANTHER PATHWAYS. 64
FIGURE 4.24 COMMON PROTEINS BETWEEN THE T2D, NPDR AND PDR GROUPS. 65
FIGURE 4.25 MULTIPLE COMPARISON OF LFQ INTENSITIES OF THE 32 PROTEINS. 68

FIGURE 4.26 COMMON STATISTICALLY SIGNIFICANT PROTEINS. 69
FIGURE 4.27 INTERACTOME OF COMMON PROTEINS BETWEEN NPDR AND PDR. 70
FIGURE 4.28 INTERACTOME OF COMMON PROTEINS BETWEEN NPDR AND PDR. 70
FIGURE 4.29 THE LEVELS OF MMP2, 3 AND 9 OF CONTROL, T2D WITHOUT AD WITH NPDR OR PDR. 74
FIGURE 4.30 MMP-2 AND -9 ACTIVITY IN TEAR SAMPLES FROM CONTROL, T2D WITHOUT AND WITH NPDR OR PDR. 75
FIGURE 4.31 NANOPARTICLE TRACKING ANALYSIS SHOWING THE CONCENTRATION AND SIZE OF EXOSOMES FROM HUMAN TEARS USING TOTAL EXOSOME KIT 76
FIGURE 4.32 WESTERN BLOT DETECTION OF CD63 (EXOSOME MARKER) IN EXOSOMES ISOLATED FROM HUMAN TEARS. 76
FIGURE 4.33 SUBMICROSCOPIC STRUCTURES IN A TEAR SAMPLES OF T2D PATIENT. 77
FIGURE 4.34 MORPHOLOGICAL STRUCTURES FOUND IN TEARS OR ISOLATED FROM TEARS USING THE TOTAL EXO KIT. 77
FIGURE 4.35 WESTERN BLOT DETECTION OF MMP-9. 78

RESUMO

A Diabetes Mellitus (DM) constitui atualmente um grave problema de saúde pública a nível mundial, com impacto negativo sobre as sociedades. Cerca de 463 milhões de adultos em idades economicamente ativas vivem com a doença e espera-se que este número duplique nos próximos anos. A retinopatia diabética (RD), a complicação microvascular mais comum da diabetes, é a principal causa de cegueira em indivíduos dos 20-79 anos em todo mundo, afetando cerca de 149 milhões de pessoas. Um em cada 3 doentes diabéticos tem RD e a incidência desta doença está intimamente associada com a duração da diabetes. Apesar de não ser mortal, a RD afeta significativamente a qualidade de vida, tendo um impacto socioeconómico negativo. Trata-se de uma condição patológica dinâmica e progressiva, a qual é, nas suas fases iniciais, assintomática e caracterizada por alterações bioquímicas e histológicas nos vasos sanguíneos, neurónios e células gliais da retina. Numa fase seguinte e sintomática, a doença carateriza-se por alterações morfológicas e estruturais que ditam o diagnóstico clínico da doença. Assim, a falta de biomarcadores úteis para estratificação de risco e diagnóstico dos estádios iniciais da doença em doentes assintomáticos, associada a modalidades invasivas, de elevado custo, morosas e com necessidade de pessoal qualificado, são fatores que dificultam um diagnóstico mais atempado da RD. Desta forma, num diagnóstico e tratamento tardio da RD, as hipóteses de sucesso para reduzir a progressão da doença para os estádios mais avançados, incluindo a prevenção da perda de visão, ficam limitadas.

Embora a RD seja a complicação ocular mais conhecida da DM, as complicações do segmento anterior do olho muitas vezes são ignoradas, embora uma elevada proporção de doentes diabéticos desenvolva complicações a esse nível. A hiperglicemia pode conduzir a alterações na osmolaridade e estabilidade do filme lacrimal e da superfície ocular de pacientes diabéticos. Essas alterações contribuem para a disrupção dos eventos bioquímicos locais envolvidos na manutenção da homeostase da superfície ocular, conduzindo à lesão tecidual.

O fluído lacrimal é um fluído relativamente menos complexo que outros fluídos do organismo como o soro e o plasma, cuja composição varia constantemente em função de diferentes estímulos, de modo a proteger o olho de forma eficiente e eficaz. Este fluído exerce importantes funções como lubrificar, manter a homeostasia da superfície ocular e

preservar a alta qualidade ótica da córnea. Para além destas, o fluído lacrimal possui componentes com qualidades imuno-moduladoras, constituindo a primeira linha de defesa contra agentes patogénicos. Assim, o acesso de forma não invasiva dos componentes presentes no delicado equilíbrio das lágrimas pode contribuir para um melhor diagnóstico e estudo da fisiopatologia da RD. A hipótese é que as lágrimas podem constituir uma fonte de biomarcadores com papel no diagnóstico precoce, estadiamento e monitorização da progressão da RD, e para tal pretendeu-se neste estudo avaliar de que forma a composição lacrimal se relaciona com a evolução natural da RD. Numa amostragem relativamente pequena, composta por indivíduos saudáveis, diabéticos sem retinopatia e diabéticos com retinopatia diabética não proliferativa e retinopatia diabética proliferativa foi utilizado o teste de Schirmer para avaliação da função lacrimal e recolha de lágrimas, com subsequente estudo da respetiva proteómica e pesquisa por imuno-ensaio das MMPs. Por outro lado, foi efetuado um ensaio de zimografia para avaliar a actividade das MMPs e um estudo piloto das vesículas extracelulares presentes nas lágrimas. Os resultados sugerem suscetibilidade para o olho seco em indivíduos diabéticos com retinopatia, diretamente associada a progressão da doença. Além disso, foram identificadas várias proteínas, AMPs e mediadores inflamatórios diferentemente expressas nos indivíduos diabéticos com retinopatia, que parecem estar associadas as alterações fisiopatológicas no contexto da DR. Embora os resultados sejam interessantes e estatisticamente significativos, mais estudos precisam ser feitos com um maior número de amostras, de modo a validar os resultados obtidos. A identificação de um conjunto de biomarcadores pode melhorar o diagnóstico precoce da RD e garantir o tratamento imediato para essa doença que ameaça a visão.

Palavras-chave: Lágrimas; Biomarcadores; Retinopatia Diabética (DR); Vesículas extracelulares (EVs); Mediadores inflamatórios; Peptídeos Antimicrobianos (AMPs).

ABSTRACT

Diabetes Mellitus (DM) is currently a worldwide serious public health problem, with a high negative impact on societies. About 463 million adults of economically active age are living with the disease and this number is expected to double in the coming years. Diabetic retinopathy (DR), the most common microvascular complication of diabetes, is the main cause of blindness in individuals aged 20-79 years worldwide, affecting about 149 million people One in 3 diabetic patients has DR and the incidence of this disease is closely associated with the duration of diabetes. DR is a dynamic and progressive pathological condition, with an initial (and asymptomatic) stage characterized by biochemical and histological changes in blood vessels, neurons and glial cells of the retina, followed by a symptomatic stage with morphological and structural changes leading to the clinical diagnosis of the disease. Thus, the lack of useful biomarkers for risk stratification for time to developing DR and, diagnosis in early stage of the disease in asymptomatic patients, associated with the invasive retina monitoring modalities, which are expensive, time consuming and need for qualified personnel, are factors that make the early diagnosis of DR more difficult. Consequently, in a delayed diagnosis and treatment of DR, the chances of success of reducing the disease progression to advanced stages, including prevention of vision loss, are limited. Although DR is the most well-known ocular complication of DM, complications of the anterior segment of the eye are often overlooked. In fact, ocular surface complications due to diabetes are common among diabetic patients. Hyperglycemia can lead to changes in osmolarity and stability of the tear film and ocular surface in diabetic patients. These changes can contribute to inflammation, which impairs local biochemical events involved in ocular surface homeostasis, leading to tissue damage. Ocular surface microenvironment changes due to diabetes, namely on tear stability, volume and composition, can reflect changes in diabetic retina. Tear fluid is a relatively less complex body fluid (than plasma or serum) when compared to others of the human body, whose composition constantly varies depending on different stimuli, in order to protect the eye efficiently and effectively. This fluid has important functions such as lubricating, maintaining the homeostasis of the ocular surface and preserving the cornea high optical quality. In addition to these, tear fluid has components with immunomodulating qualities, constituting the first line of defense against pathogens.

Thus, non-invasive access to the components present in the delicate balance of tears can contribute to a better diagnosis and study of the pathophysiology of DR. The hypothesis of this study was to investigate whether tears can be a source of biomarkers for early diagnosis, staging and monitoring of DR progression, and for this purpose, this study aimed to assess how the tear composition is related to the natural history of DR. This work was carried out on healthy individuals, T2D patients without retinopathy and T2D patients with different stages of retinopathy. Schirmer test was used to assess tear function and tear collection, with subsequent mass-spectrometry-based proteomic study, as well as western blotting and bead based multiplex assay to detect multiple analytes in tear film. On other hand, a gelatin zymography to access the metalloproteinases activity and a pilot study of extracellular vesicles from tears were performed. The results suggest susceptibility to dry eye in diabetic individuals with retinopathy, directly associated with disease progression. In addition, several proteins, AMPs and inflammatory mediators differently expressed in diabetic individuals with retinopathy have been identified, which appear to be associated with pathophysiological changes in the context of DR. Although the results are very interesting and statistically significant, more studies need to be done with a larger number of samples, in order to validate the results obtained. The identification of a set of biomarkers can improve early diagnosis of DR and ensure prompt treatment for this vision-threatening disease.

Keywords: Tears; Biomarkers; Diabetic Retinopathy (DR); Extracellular vesicles (EVs); Inflammatory mediators; Antimicrobial Peptides (AMPs).

INTRODUCTION

1. INTRODUCTION

1.1 THE HUMAN EYE IN HEALTH

1.1.1 Anterior segment

1.1.1.1 Ocular Surface System

The human eye is a highly complex structure, with various components that work in well-orchestrated mechanisms making it an immune-privileged organ. Both the protective mucosal environment of the ocular surface and internal compartments (anterior and posterior poles of the eye) contain many soluble immunoregulatory and immunosuppressive molecules that regulate apoptosis, induce the production of anti-inflammatory cytokines and influence the activity of immune cells (AW. 2009). The external surface of the eye composed by a mucosal tissue (conjunctiva and cornea) is constantly exposed to external threats. The cornea is an optically transparent tissue of the anterior part of the eye that functions as a chemical and mechanical barrier, preventing the penetration of foreign bodies (AW. 2009). The external surface of the eye composed by a mucosal tissue (conjunctiva and cornea) is constantly exposed to external threats. The cornea is an optically transparent tissue of the anterior part of the eye that functions as a chemical and mechanical barrier, preventing the penetration of foreign bodies. Protection of the ocular surface and integrity of the barrier is due mainly to the presence of substances with antibacterial function that are present in the tear film and are produced by immune cells and cells at ocular surface (Caspi 2013). Any perturbations by environment factors and/or pathogens can cause inflammation or ocular infection, leading to disruption of immune system homeostasis at ocular surface, thus threatening vision.

The ocular surface comprises the surface and epithelia of the cornea, conjunctiva, accessory lacrimal gland, meibomian gland and the eyelid structures (Fig.1.1). In combination with the lacrimal apparatus and an interconnecting innervation (sensory and motor nerves) constitute the lacrimal functional unit (LFU). LFU is formed by distinct

components that work in an integrated manner to regulate the volume and composition of the tear film through activation of corneal sensory inputs, which is processed by the secretory apparatus, thus contributing to maintain the ocular surface health (Gipson 2007, Rodrigo Bolaños-Jiménez 2015). All constituents of the ocular surface system are linked functionally by continuity of the epithelia, innervation, and by the endocrine, circulatory and immune systems, and are supported by the connective tissue with its resident cells and blood vessels. (Gipson 2007) (Fig.1.2). Signals from one region influences the blink, goblet cell secretion, lacrimation, and/or lacrimal gland gene expression, making the ocular surface a closed integrated system (Gipson 2007).

The term ocular surface is related to its functions. Firstly, the main function is to protect, nourish and assure a smooth refractive surface on the cornea. Secondly, another function is related to the maintenance of a continuous corneal and conjunctival epithelia with no breaks, as well as the communication along these epithelia through gap junctions and cytokines (S Kinoshita 2001, Benjamin Walcott 2002, Keven Williams 2002). Thirdly, all cells present at ocular surface are responsible for production of the components of the tear film (Gipson 2007).

The eye is the window that allow the body to contact with the world around it, having a distinct role in the acquisition of this external information. Therefore, the ocular surface, the most external component of the eye, is directly exposed to various agents (Rodrigo Bolaños-Jiménez 2015, Anna M. Ambroziak 2016). Thus, vision is well preserved, in order to be shielded from injury (Carlos Belmonte 2004). The main function of the bony orbit and eyelids is to provide protection for the external surface of the eye, especially against traumatic events and airborne particles (Rodrigo Bolaños-Jiménez 2015). In order to avoid exaggerated, chronic and potentially harmful reactions, the tear film has immunomodulating properties so that can be able to respond to stimuli and modulate the responses. On other hand, the corneal and conjunctiva epithelium constitute an anatomical barrier to the eye, which is considered the first barrier line (Rodrigo Bolaños-Jiménez 2015) and include tight junctions, desmosomes, adherents junctions and gap junctions (Fig.1.1) (Flavio Mantelli 2013). Beyond that, an additional layer of protection is given by the apical cell membranes on superficial cells, (Flavio Mantelli 2013) composed by a hydrophilic, heavily glycosylated glycocalyx (Gipson 2007) that consists of transmembrane mucins, providing surface protection through association with

carbohydrate-binding proteins. For example, interaction of carbohydrate-binding protein galectin-3 with carbohydrate residues on MUC1 and MUC16, contributes to the integrity of the epithelial barrier (Flavio Mantelli 2013). Mucins can also play a role in epithelial surface hydration and lubrication. Besides epithelial cells, fibroblasts and Langerhan's cells are the main components of the corneal innate immune system (Rodrigo Bolaños-Jiménez 2015). The cornea also possesses the richest sensory innervation of the body, with a nerve density of 300 to 600 times greater than the skin (Takefumi Yamaguchi 2016). In fact, it is particularly sensitive, capable to provide a potent response to harmful physical and chemical stimuli, while the number of sensory fibers responsible for acquiring information about innocuous environmental changes is comparatively small (Carlos Belmonte 2004).

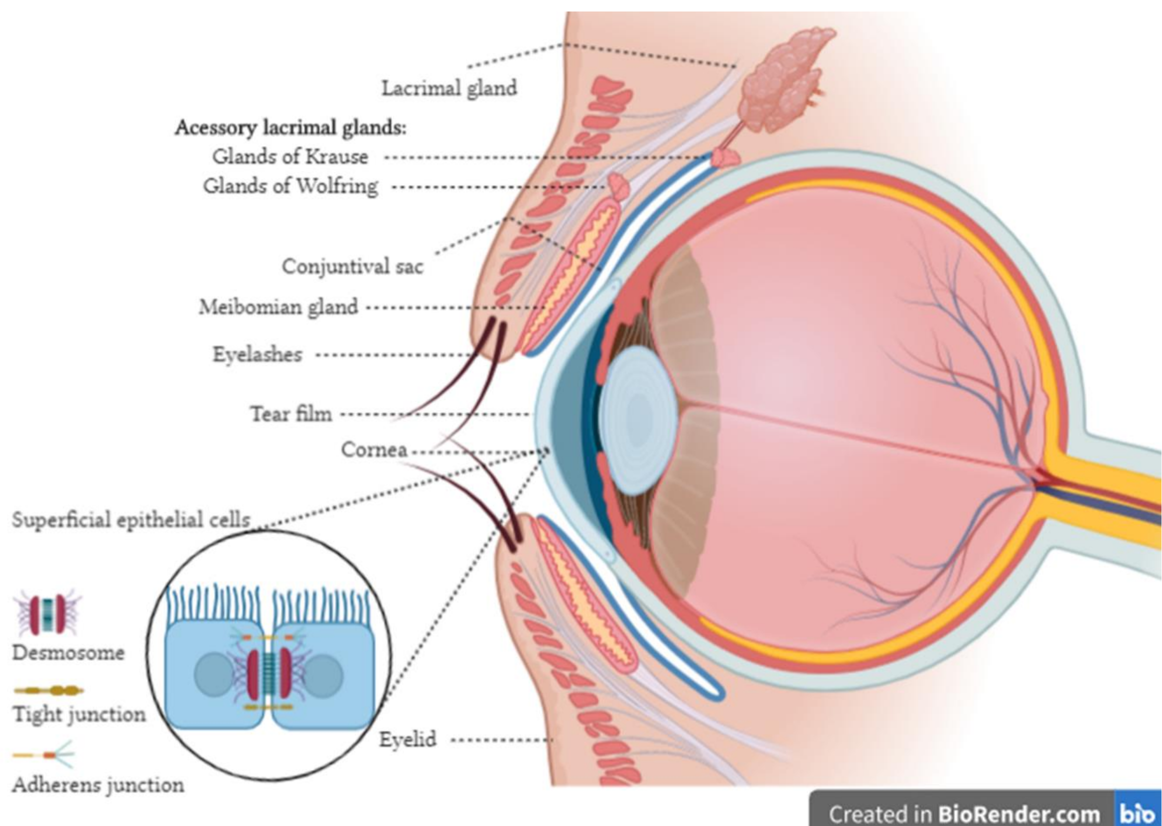


Figure 1.1 Schematic representation of ocular surface constituents. *Created in BioRender.com-*

1.1.1.1.1 Tear film

The LFU controls the volume and composition of the tears (C. Albarrdn 1997, Lei Zhou 2012) and has the function of work harmoniously to supply, regulate and control the tear film. (Aisling Mann 2019, Mitalee Tamhane 2019) The tear fluid present as the precorneal film and in the conjunctival sac is different from the one that is in the surface of the eye most directly in contact with the environment. (Haeringen 1981) Most of tears (95%) is produced by the lacrimal gland and the remainder 5% by goblet cells and accessory lacrimal glands of conjunctiva (Haeringen 1981). Stimuli of the ocular surface trigger responses by the LFU through the neural pathway, in which the nerve endings of the cornea emit afferent impulses to the central nervous system through the ophthalmic branching of the trigeminal nerve, triggering an efferent response culminating in stimulation of the lacrimal secretion (Fig.1.2) (Anna M. Ambroziak 2016). Except for those that evaporate, tears exit through the lacrimal puncta and canaliculi to the lacrimal sac which communicates with the nasal cavity (Haeringen 1981). The tear film has a thickness ranging from 6 to 20 µm while the eye is open, and some authors claimed that the thickness is around the range 5 to 10 µm (C. Albarrdn 1997, Mitalee Tamhane 2019). Under normal conditions, the volume of tear fluid is around 5-10 µl (C. Albarrdn 1997, Mitalee Tamhane 2019) in which 4.5 µL is in the conjunctival sac and approximately 2.9 µL and 1.1 µL are in the tear menisci and preocular tear film, respectively (Haeringen 1981). The secretion rate is about 1.2 µl/min, with a turnover rate of approximately 16% per minute (Lei Zhou 2012). The tear's pH is 7.45, with an evaporation rate of 9,5x 10^{-7} g/sec and 4-8 mPa/sec of viscosity (C. Albarrdn 1997).

1.1.1.1.2 Composition

The well-known classic three-layer (or components) model of the tear film consists of an inner mucin layer, a middle aqueous layer and an outer lipid layer (Fig.1.2) (Lei Zhou 2012).

Mucous layer is mainly secreted by the goblet cells and the crypts of Henle, and also by epithelial cells of the cornea and conjunctiva and lacrimal glands (Lei Zhou 2012). It is composed of mucins, which are carbohydrate-protein complexes of high molecular weight glycoproteins which contain O-linked carbohydrates and a protein core, (Lei Zhou 2012) characterized by the presence of hexosamines, hexoses and sialic acid (Haeringen 1981). These mucins form glycocalyx, which ensures cell adhesion (Anna M. Ambroziak 2016), cover the ocular surface and lower the hydrophobicity of the epithelial cells (Mitalee Tamhane 2019). Wherefore, enable the tear film to attach to the cornea (C. Albarrdn 1997), making this layer the interface between the ocular surface epithelium and aqueous layer (Lei Zhou 2012). This contributes to the stability of the tear film and that the aqueous layer is constantly distributed over the ocular surface. Thus, the mucins present in the tear film maintain ocular surface hydrated, provide lubrication and prevent friction of the ocular surface against the conjunctiva during a blink of the eye. Additionally, they form a protective physical barrier preventing microbes adhesion and invasion (Lei Zhou 2012, Anna M. Ambroziak 2016). Both corneal epithelium and conjunctival non-goblet cells express membrane-bound mucins, such as MUC1, MUC2 and MUC4, while the conjunctival goblet cells produce secreted mucins such as MUC5AC (Anna M. Ambroziak 2016). Mucins production can also be induced by the inflammatory cytokines, as well as, the stimulation of Toll-like receptors (TLR) in the corneal epithelium (Anna M. Ambroziak 2016).

Aqueous layer is the thickest layer contributing to around 90% of the total tear film thickness and is secreted by the principal and accessory lacrimal glands (C. Albarrdn 1997, Lei Zhou 2012); this layer is composed basically of 98% of water, organic components such proteins/peptides (enzymes, growth factors, cytokines, antimicrobial peptides (AMPs)), small molecule metabolites, and inorganic electrolytes (C. Albarrdn 1997, Lei Zhou 2012, Aniko Rentka 2017, Mitalee Tamhane 2019) Corneal and conjunctival cells also secrete proteins/peptides and small molecule metabolites into the tear film (Lei Zhou 2012). The main function of this layer is to form a smooth refractive surface together with the corneal epithelium (Mitalee Tamhane 2019). It is now believed that the mucin and the aqueous layers are a single layer that forms the muco-aqueous layer (Mitalee Tamhane 2019).

The presence of electrolytes, mainly sodium, potassium, chloride, bicarbonate, and also magnesium and calcium in lower levels in the aqueous layer contribute to the maintenance of the osmolality of the tear film (300-305 mOsm/kg in healthy eyes), which represents a measurement for the balance between tear production, evaporation, drainage and absorption (Ulrike Stahl 2012). Besides that, electrolytes within the aqueous layer play a critical role in maintenance of epithelial integrity and contribute to regulate the pH of the tear fluid (L G Carney 1976, W G Bachman 1985, Ulrike Stahl 2012).

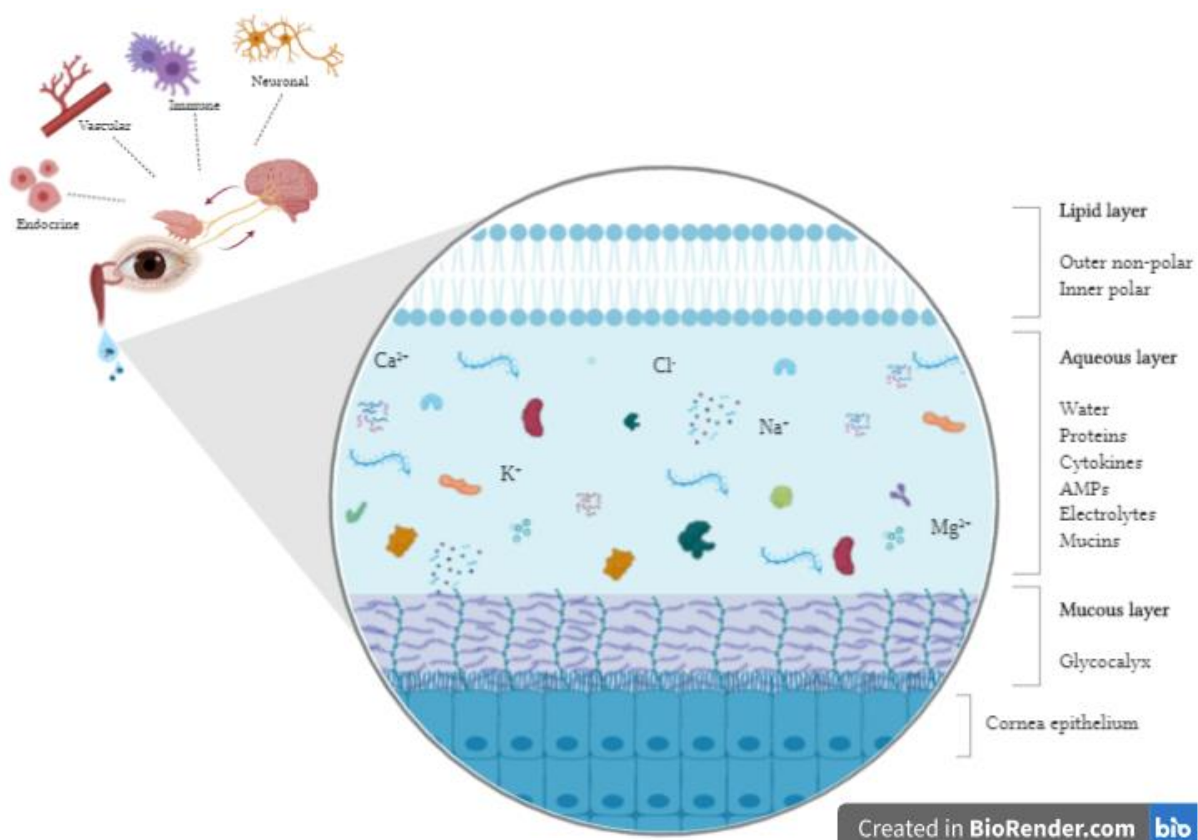


Figure 1.2 Schematic representation of tear film layers. *Created in BioRender.com*

Lipid layer forms the outermost layer of tear fluid and is approximately 42 nm thick (P Ewen King-Smith 2010). Lipids are secreted by the Meibomian, Moll, and Zeis Glands (C. Albarrdn 1997, Lei Zhou 2012). Lipid layer is mainly composed of cholesterol, ester and ether lipids, whose main function is to prevent rapid evaporation of the aqueous layer of the tear film (C. Albarrdn 1997, Lei Zhou 2012, Aniko Rentka 2017, Mitalee Tamhane 2019). So, lipid layer is crucial for preserving stability and integrity of the tear film. It may also provide a barrier against pathogens (Lei Zhou 2012). A study by Green-Church et al

(2011) has been proposed a model of the precorneal tear film, in which lipid layer is further divided into two sublayers-the outer non-polar lipid layer and inner polar lipid layer with intercalated proteins (Lei Zhou 2012).

The tear film is a complex biological fluid. In fact, analysis of the human tear proteome has revealed that it contains approximately 1500 proteins(Lei Zhou 2012). The proteins are secreted from lacrimal gland, its ductal epithelium and associated plasma cells (Aniko Rentka 2017). Proteins such lactoferrin, lysozyme, secretory immunoglobulin A (sIgA), lipocalin, superoxide dismutase, cystatins, alpha-1 protease inhibitor are highly present, comprise more than 90% of the total amount of tear proteins. The remainder protein components include growth factors, neurotrophic factors, cytokines, cell adhesion, molecules, matrix metalloproteinases, immunoglobulins and insulin (Aniko Rentka 2017). Several lysosomal enzymes (acid hydrolases) are present in tears in concentrations of 2-10 times higher than those of the serum. The lacrimal gland is the main source of the lysosomal enzyme. Two of the more abundant proteins of the tear film are lysozyme, an antibacterial enzyme, and lactoferrin, an iron-binding protein which function is to inhibit bacterial growth. Lipocalin binds and transports small hydrophobic molecules and acts as a lipid scavenger from the corneal surface. The sIgA is the primary antibody in tears and appears to play a key role in host ocular immune response (Aniko Rentka 2017).
Lipids are secreted by Meibomian glands (also known as palpebral glands, tarsal or tarso-conjunctival glands) (Butovich 2011). Lipidomic analysis of secretions from Meibomian glands revealed that almost all lipid classes are represented, mainly hydrocarbons, wax esters, cholesterol esters, triglycerides, and in lesser amount diglycerides, monoglycerides, free fatty acids, free cholesterol and phospholipid (Haeringen 1981, Butovich 2011). Moreover, it has been shown that the lipid profiles in tears and Meibomian glands are nearly identical (Simon H J Brown 2013).
There is growing evidence that the normal tear film contains several pro- and anti-inflammatory cytokines. These molecules are secreted not only by cells of the immune system but also by epithelial cells of ocular surface. Although secretion of cytokines and chemokines by epithelial cells is normally increased upon cell stimulation, in basal (unstimulated) tears, secretion of those mediators also occurs (Ester Carreño 2010).

1.1.1.1.3 Functions

The ocular surface is in constant balance in which the production, evaporation, drainage and absorption of tears occurs dynamically, with a normal tear osmolarity, indicating adequate dynamic (Mitalee Tamhane 2019). Tears must be viscous enough to protect and lubricate the ocular surface, but not too viscous, to avoid ocular surface damages during blinking (Lei Zhou 2012). Three fundamental functions can be attributed to the tear film: optical, lubricant, and protective (Mitalee Tamhane 2019).

As an optical refractive medium: tear film neutralizes the small irregularities present at the ocular surface, providing the optically smooth surface necessary for refraction of light. The tear film also has refractive power, which is 48.35 D alone and 42.366 D when coupled to the cornea. Thus, changes in the thickness of the tear film will have a significant impact on the refractive power (C. Albarrdn 1997, Lei Zhou 2012).

As a lubricant: tear film prevents the dehydration of the mucous membrane of the ocular surface (Aniko Rentka 2017, Mitalee Tamhane 2019). It lubricates the eye surface and nourishes also the avascular tissues of the cornea (Lei Zhou 2012).

Protective: tear film supports the innate and acquired immune ocular defense, playing a range of important roles in ocular immunity and homeostasis (Ester Carreño 2010). It acts as mechanical and antimicrobial barrier (Lei Zhou 2012), protecting the eye from external influences and maintaining the health of cornea and conjunctiva (Aisling Mann 2019).

Nutritive: Although the nutritional function of tears is debatable, they are responsible for providing nutrients to the cells of the ocular surface, as well as, drainage of the products resulting from the metabolism (Aniko Rentka 2017). They carry secreted molecules from corneal epithelial cells and tissues producing tear components and nourish the underlying cells (Aniko Rentka 2017).

1.1.1.2 Innate immune system of the anterior segment

Innate immune system is comprised of host components and resident microbiome (Nesrin Büyüktortop Gökçınar 2018). The host components are physical barriers such as tight

junctions in the epithelial and mucous membrane surfaces; pattern recognition receptors (PRRs) such as toll-like receptors (TLR); phagocytes including neutrophils, monocytes, and macrophages; PRR expressing cells such as epithelial cells, mast cells, platelets, and dendritic cells; special lymphoid cells including natural killer, and innate lymphoid cells; inflammation-related serum proteins such as complement system, cytokines, and C-reactive protein (Nesrin Büyüktortop Gökçınar 2018). The innate immune system identifies pathogen-associated molecular patterns through PRRs, and eliminates them with inflammation, phagocytosis, cytokine release, destruction by natural killer cells, and/or a complement system (Alison M. McDermott 2003).

1.1.1.2.1 Ocular microbiota

The microbiota of the ocular surface is composed by microorganisms that colonize commensally the cornea and conjunctiva, excluding the ones that colonize the eyelid, which are considered as components of skin microbiota (Louise J. Lu 2016). These microorganisms are not the target of ocular surface epithelial cells, which recognize and act selectively against ocular pathogenic microorganisms, producing pro-inflammatory cytokines (Louise J. Lu 2016) therefore, the ocular surface plays a relevant protective role in the suppression of pathogens (Qunfeng Dong 2011). These could involve a unique innate immune response of the ocular surface epithelium that supports the colonization of a resident microbiota, probably an interaction between immune system and microbiota (Qunfeng Dong 2011, Louise J. Lu 2016). Several factors can alter the ocular surfaces microbiota, such as dry eye syndrome, contact lens wear, antibiotics, and infection (Louise J. Lu 2016). This microbiota was proposed to be dominated by Gram-positive species (Qunfeng Dong 2011, Louise J. Lu 2016), some Gram-negative species, as well as fungi (Louise J. Lu 2016).

1.1.1.2.2 Antimicrobial peptides

Antimicrobial peptides (AMPs) are also known as host defense peptides (HDP) (Fern Findlay 2017, Imran Mohammed 2017, Nesrin Büyüktortop Gökçınar 2018) and, they are a group of secreted PRRs, which are secreted in the epithelial cells in their respective injured points, so that they form a protective layer which damages microorganisms before fixation and invasion (Fabian Garreis 2011). AMPs are the major component of the innate immune system (Fern Findlay 2017), being considered as an ancient or one of the primary defense form of the innate immune system (Fabian Garreis 2010, Prerana Sharma 2018). They are less than 100 amino acids in length coded by individual genes, (Fabian Garreis 2010) and have potent immunomodulatory and antimicrobial activities (Fabian Garreis 2010, Fern Findlay 2017). According to their secondary structure, AMPs are classified into 4 groups, and is important to say that defensins, cathelicidin and S100A proteins are the major classes of AMPs present in humans (Prerana Sharma 2018).
AMPs have a positive net charge due to the high amount of cationic amino acids and, their structure is characterized by an amphipathic character, in which the active principle is based on the charge-dependent interaction of the positively charged AMPs with the negatively charged phospholipids in the membrane of microorganisms (Fabian Garreis 2010, Fabian Garreis 2011) (Prerana Sharma 2018). This electrostatic interaction seems to cause permeabilization of the microbial cell membrane, which leads to loss of essential intracellular components and cell death (Fabian Garreis 2010, Fabian Garreis 2011, Prerana Sharma 2018). Authors suggests that AMPs also have functions regarding proliferation, migration, chemotaxis and cytokine production, therefore linking AMPs with the adaptive immune system (Fabian Garreis 2010, Prerana Sharma 2018). Several studies have showed that ocular surface cells express several AMPs, which some of them are constitutive, and others are induced by infection, inflammation and in response to microorganisms (Prerana Sharma 2018). α-defensins (defensins 1, 2, and 3 that are produced by resident or non-resident neutrophils and possibly by secretion from lacrimal ductular epithelia), β-defensins (defensin 1 and 2 produced by the ocular surface) and human cathelicidin LL-37 are the major AMPs present at the ocular surface (Fabian Garreis 2010, Fern Findlay 2017). HNPs 1–3, MUC7, histatin, surfactant protein D (SPD), liver-

expressed antimicrobial peptides (LEAP)-1 and -2, macrophage inflammatory protein (MIP)-3, DEFB109, and RNase-7 are other AMPs less present (Marcia M. Jumblatt 2006, Fabian Garreis 2011, Imran Mohammed 2017).These AMPs have been detected in cells from the lacrimal gland, glands of Krause and Moll, and also from cornea and conjunctiva epithelia (Fabian Garreis 2010).

Defensins have a wide range of antimicrobial activity against Gram positive and Gram-negative bacteria, fungi, and viruses creating voltage sensitive channels in the plasma membrane of the microorganism. They also promote a quickly cellular immune response to infection via a chemotactic effect and, accelerate wound healing by mitogenic effect on epithelial cells and fibroblast (Richard John Haynes 1999). Human beta-defensin 1 (hBD1) is constitutively expressed, whereas human beta-defensin 2 (hBD2) is inducible by exposure to lipopolysaccharide and peptidoglycan from bacteria, proinflammatory cytokines and in response to injury (Fabian Garreis 2011).
The cathelicidin, LL-37, is naturally present in conjunctiva and cornea and its expression increases in response to corneal injury, infections and other inflammatory processes (Huang LC 2007). Recruitment of inflammatory cells, wound healing, promotion of angiogenesis, proprieties as chemotactic agent for neutrophils, monocytes, and T cells, as well as regulation of cell death pathways, and antimicrobial activity are some of the functions (Sorensen 2011, Fern Findlay 2017).

1.1.2 Posterior segment

1.1.2.1 Retina

Retina is a thin, transparent tissue derived from the neuroectoderm, comprised of retinal pigment epithelium (RPE) and neurosensory retina (Mrinali Patel Gupta 2016). This tissue is important to normal vision and among several functions, it is responsible for convert photochemical energy from photons of light into electrical energy that generates action potential, which furthermore are interpreted in the occipital lobe of the brain into images

(David A. Antonetti 2006). This process depends on intact cell-cell communication among retinal cells (David A. Antonetti 2006).

The first layer of the retina is the retinal pigment epithelium (RPE), a monolayer of cuboidal cells (Fig.1.3) containing melanosomes. The basal membrane of these cells comprises the inner layer of Bruch´s membrane, which separates the retina from the choroid, located underneath (Mrinali Patel Gupta 2016). The RPE facilitates the diffusion of oxygen and nutrients from the choroid to the outer retina, (David A. Antonetti 2006, Mrinali Patel Gupta 2016). It is also responsible for the constant removal of retinal waste or stacks of membrane disks of photoreceptors outer segments (Mrinali Patel Gupta 2016). RPE is also important to absorb light, secret trophic factors and, together with the photoreceptors, participates in the vitamin A metabolism (Strauss 2005).

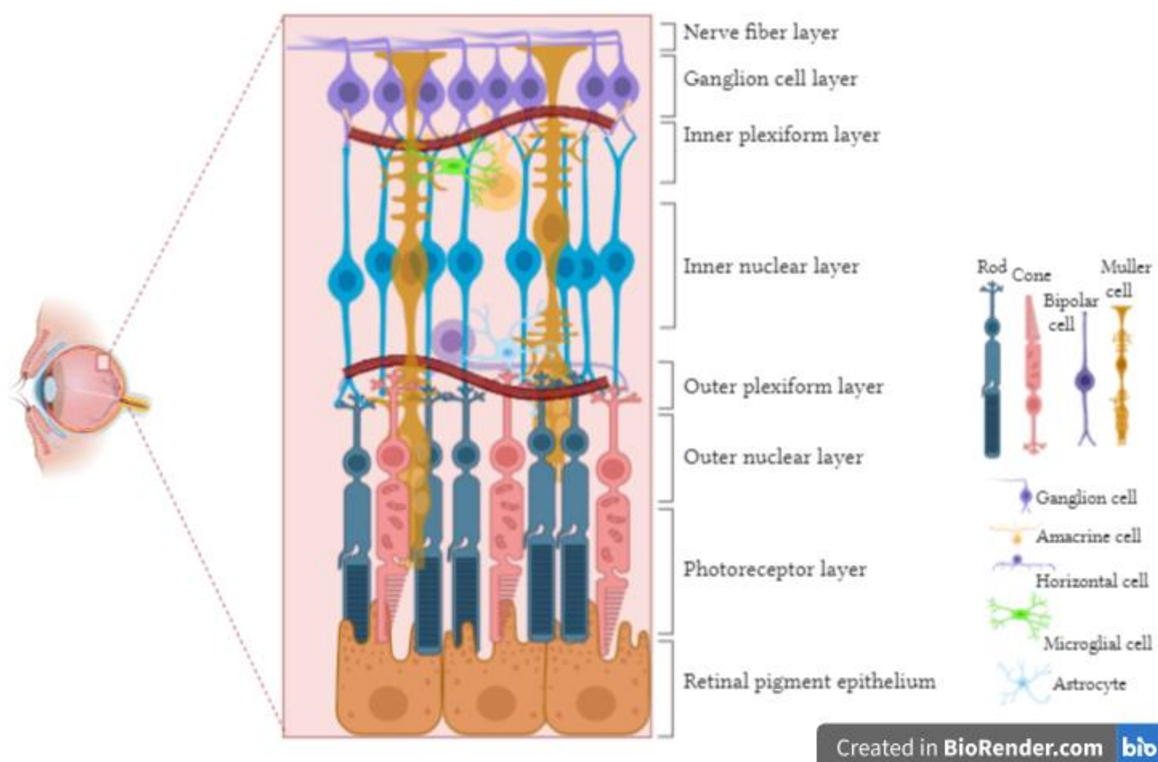


Figure 1.3 Cells and layers of retina. ***Created in BioRender.com***

The neurosensory area of the retina is rich in different types of cells. In total, there are five major cell types with different functions (Fig.1.3) (David A. Antonetti 2006). The neurons are comprised of photoreceptor, bipolar, horizontal, amacrine, and ganglion cells

which are responsible for sensory functions and define color perception, spatial resolution, and contrast discrimination (Masland 2001). Rods and cones are the photoreceptors; bipolar cells are glutamatergic or glycinergic neurons (Mrinali Patel Gupta 2016); horizontal cells are mainly GABAergic neurons that connect to either rods or cones cells and synapse with bipolar cells to regulate signal transduction (David A. Antonetti 2006); amacrine cells receive excitatory glutamatergic input from bipolar cells and primarily inhibitory input from other amacrine cells mediated by GABA receptors (David A. Antonetti 2006).

Beyond neurons, there are glial cells which have many functions, support the segments of the photoreceptors (David A. Antonetti 2006), protect them, provide nutritional support, lead to inter-cellular contact, regulate blood-retinal barrier properties (Gardner TW 1997), and have synaptic function (Newman 2003). Those are Muller cells and astrocytes. Microglial cells are another type of glial cells which are responsible for immunomodulatory functions. Those cells are resident macrophages that react to stress conditions by releasing proinflammatory cytokines or phagocytosis (Elward K 2003, Krady JK 2005). Constituting less than 5 % of the retinal mass, there are also vascular endothelial cells and pericytes, which are responsible for nutritional support and waste product removal from the inner retina (David A. Antonetti 2006). All of these cells interact with each other in a complex multi-layer scheme (Fig.1.3), in which neurons alternates with adjacent layers where they communicate with other cells between dendrites and axon-dendrites synapses (David A. Antonetti 2006). Those multilayers are comprised of the following nine layers: outer and inner segments of photoreceptors, external limiting membrane, outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiform layer, ganglion cell layer, nerve fiber layer and internal limiting membrane (Mrinali Patel Gupta 2016). The outer and inner segments of photoreceptors are formed by the rods and cones cells, which nuclei are localized in the outer nuclear layer. The following layer, the outer plexiform layer, is formed by the axons of rods and cones, as well as their synapses with bipolar cells and the dendrites of the ganglion cells. The inner nuclear layer has the nuclear bodies of bipolar cells, ganglion cells, amacrine cells and muller cells. The inner plexiform layer is the layer in which inner side has the terminations of “on” bipolar cells and the outer side has the terminations of “off” bipolar cells. This layer is where the

axons of the bipolar and amacrine cells connect to the dendrites of the ganglion cells. The ganglion cell layer contains the nuclei of the ganglion cells. The axons of those cells together with the optic nerve form the nerve fiber layer. Moreover, the last layer, the internal limiting membrane, is formed by the end-feet of the Muller cells, which extend since the RPE (Mrinali Patel Gupta 2016). Those Muller cells contact neurons and blood vessels in the plexiform and nerve fiber layers (David A. Antonetti 2006).

1.2 THE HUMAN EYE IN DISEASE

1.2.1 Diabetic retinopathy

1.2.1.1 Epidemiology

Diabetes Mellitus (DM) has become the epidemic of this century and currently, is one of the most common metabolic disorders being a major public health threat with high negative impact for societies (Federation 2019). In fact, according to estimates from the International Diabetes Federation (IDF) in 2019, approximately 463 million adults, between 20-79 years old, were living with diabetes and by 2045 this number is expected to reach to around 700 million (Federation 2019). The disease caused at least 10% of total spending amount of money in health expenditure on adults. An important vascular complication, which is intimately associated to the rise in the prevalence of diabetes, is Diabetic Retinopathy (DR), also becoming epidemic (Biswas, Sarabusky et al. 2019). It is a leading cause of blindness in the economically active population in developed countries (Wang, Chen et al. 2019). Worldwide, it affects more than 149 million individuals (Youngblood, Robinson et al. 2019) and contributes to the majority of blindness in working-age adults, therefore, economically active people, affecting more than four million individuals (Youngblood, Robinson et al. 2019). The global prevalence of DR has increased to epidemic proportions and is responsible for 50 000 new cases of retinal neovascularization and diabetic macular edema, as well as 10 000 cases of blindness every year, worldwide (Petrie, Guzik et al. 2018). So, around one-third of the diabetic patients is affected with DR, and one in ten will develop a vision threatening form of the disease

(Simo, Stitt et al. 2018, Rodriguez, Perez et al. 2019). Although DR is not a mortal illness, it impacts significantly in life quality, leading to emotional distress and, personal and socioeconomic consequences (Rodriguez, Perez et al. 2019). Furthermore, DR is associated with significant economic consequences for health public systems (Rodriguez, Perez et al. 2019).

1.2.1.2 Pathophysiology

DR is a progressive disease and develops in stages of growing severity (Li, Yu et al. 2020). The major risk factor is the diabetes duration and is well known that the incidence of DR increases with the duration of type 2 diabetes (T2D), which is the common form of diabetes. Within 20 years of T2D diagnosis, almost two-thirds of the patients will have some degree of retinopathy (Federation 2019). The pathophysiology of DR is not well-known. In fact, the whole scenario of changes is dynamic at different stages of the natural history of the disease and varies from individual to individual (Barrett, Liu et al. 2017). Although not enough, sustained hyperglycemia-damage induces the changes involved in the pathogenesis of DR, leading to the development of several adaptive abnormalities in the diabetic retina (Aboualizadeh 2017). Those changes include biochemical, physiological, rheological, hormonal and others, which leads to complex alterations on pathways and factors related and/or important for the interaction, communication and regulation of all retinal cells. Although endothelial cells are particularly sensitive (Liao, Lin et al. 2017, Wu, Yiang et al. 2018), functional and/or morphological changes are found in various retinal cell types in the diabetic retina, before clinical symptoms and diagnosis are attained (Al-Shabrawey M 2015). The pathogenesis of DR is very complex, and several biochemical pathways have been implicated (Santiago, Boia et al. 2018). Despite that, the dysregulation of the neuroglial vascular unit is considered to be the cause of the development of the disease (Fig1.4), although the precise mechanism remains unclear. It has been pointed as mechanisms involved in the disease development and progression, the hypoxia, oxidative stress and inflammation (Al-Kharashi 2018).

The retina is extremely sensitive to changes in oxygen levels (Rodriguez, Perez et al. 2019). In addition to having a high content of polyunsaturated fatty acids (PUFAs) (Aboualizadeh 2017, Liao, Lin et al. 2017, Al-Kharashi 2018), it needs to produce ATP by consuming large amounts of glucose and oxygen, through the mitochondrial electron transport chain (ETC) in the inner membranes. During this process, electrons leaving the ETC are captured by molecular oxygen, generating reactive oxygen species (ROS) (Liao, Lin et al. 2017, Al-Kharashi 2018, Santiago, Boia et al. 2018, Wu, Yiang et al. 2018). The photoreceptors contribute in a large scale, being the main source of superoxide and reactive oxygen in the retina, and thus, they contribute to the oxidative stress also because their limited capacity of mitochondrial reserve, being particularly vulnerable to changes in homeostasis. So, retina tries to compensate the overproduction of ROS by decreasing its metabolic activity; however, in the case of DM, this mechanism ends up failing. There are damage of mitochondrial membrane lipids / proteins and mitochondrial DNA (mtDNA), consequently resulting in mitochondria inefficient in producing ATP, but producing high amounts of ROS, which cause oxidative stress initiating the pathogenesis of DR (Wang and Lo 2018, Wu, Yiang et al. 2018, Yumnamcha, Devi et al. 2019).

When intracellular antioxidant enzymes fail to remove ROS and other free radicals, excessive ROS enters the cell nucleus and is listed as causing DNA strand (Al-Kharashi 2018, Wang and Lo 2018, Adhya and Sharma 2019, Chung, Kim et al. 2019, Rossino and Casini 2019, Yumnamcha, Devi et al. 2019), which cause reactive gliosis in Müller cells and astrocytes (Fig.1.4), as well as transcription of NF – κB. Once activated, this transcription factor is translated into the nucleus binds to nuclear DNA and promotes the expression of pro-inflammatory cytokines such as IL-1β, interleukin 6, interleukin 8 (IL-8), interferon and TNFα. On the other hand, PI3K / Akt / mTOR mediate the secretion of inflammatory cytokines by ROS induced by hyperglycemia itself. Moreover, these pro-inflammatory molecules contribute to Müller cells to induce inflammation by stimulating the difference cluster (CD) 40 and indirectly promoting inflammation of the microglia, releasing adenosine triphosphate. In this inflammation status, starts to occur up-regulation of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), monocytic chemotactic protein1 (MCP-1) and cyclooxygenase -2 (COX-2) (Chung, Kim et al. 2019, Rossino and Casini 2019). This last one, increases the synthesis of prostaglandins,

which stabilizes hypoxia-induced factor-1 (HIF-1), leading to vascular endothelial growth factor (VEGF) expression and NF-kB activation for COX-2 expression.

Mitochondrial ROS and oxidized mtDNA, when released into the cytosol, are recognized as damaged associated molecular patterns (DAMPs) by cytosolic pattern recognition receptors (PRRs), including TLR4, toll-like TLR9 receptors. Together with the pro-inflammation, this recognition trigger to cell death processes by different pathways: pyroptosis, apoptosis and autophagy. The contribution of mitochondria to diabetes-induced oxidative stress is well-known (Aboualizadeh 2017) and, according to unified theory of hyperglycemia-induced endothelial cell damage, from Brownlee, ROS overproduction is the common upstream event which can stimulate the biochemical pathways that have a pathogenic role in diabetic retinopathy. These pathways are: the polyol (sorbitol) pathway (Barrett, Liu et al. 2017), formation of advanced glycation end products (AGEs) intracellularly (Chung, Kim et al. 2019, Rossino and Casini 2019) (Aouiss, Anka Idrissi et al. 2019, Othman, Vaucher et al. 2019), expression of receptors (RAGE) and ligands (Aouiss, Anka Idrissi et al. 2019, Othman, Vaucher et al. 2019), activation of the kinase C (PKC) pathway (Berezin 2019, Cecilia, Jose Alberto et al. 2019), and increasing the activity of the hexosamine pathway.

Oxidative stress associated with chronic hyperglycemia and low-grade inflammation is considered to play critical roles in the onset and progression of diabetic retinopathy (Santiago, Boia et al. 2018), so that they participate together as a single mechanism, inducing BRB breakdown and neovascularization (Santiago, Boia et al. 2018). Cellular damage to the retina, the neovascularization process and the entire progressive pathogenesis of RD occur as a result of oxidative stress that directly or indirectly stimulates the release of pro-inflammatory cytokines, VEGF-α and nitric oxide (NO) (He, Long et al. 2019, Liu, Jiang et al. 2019). In summary, hyperglycemia leads to a series of successively triggered events that culminate in the development of diabetic retinopathy (Picconi, Parravano et al. 2017), where microvascular dysfunction involves an increase in impaired pro-inflammatory and pro-oxidant mediators or endogenous signaling pathways (Barrett, Liu et al. 2017, Sorrentino, Matteini et al. 2018, Fresta, Fidilio et al. 2020) (Kitamura 2019).

1.2.1.3 Classification and diagnosis

The natural history of DR is essential to understand its classification. As it is a dynamic and progressive condition (Barrett, Liu et al. 2017), there is a first stage (preclinical retinopathy) with significant biochemical and histological changes in retinal vessel (Othman, Vaucher et al. 2019) which include increased permeability of the blood retinal barrier (BRB), loss of vascular endothelial cell and pericyte, thickening of the vascular basement membrane with subsequent occlusion of capillaries, and abnormalities in retinal neurons and glial cells (Fig.1.4). There is a further stage (clinical stage) of morpho-structural and pathophysiological changes, intimately related to the progressive dysfunction of endothelial cells, which play a crucial role in this progression. This dysfunction lead to worsening of previous changes, culminating in neovascularization (Fig.1.4) (Sorrentino, Matteini et al. 2018).

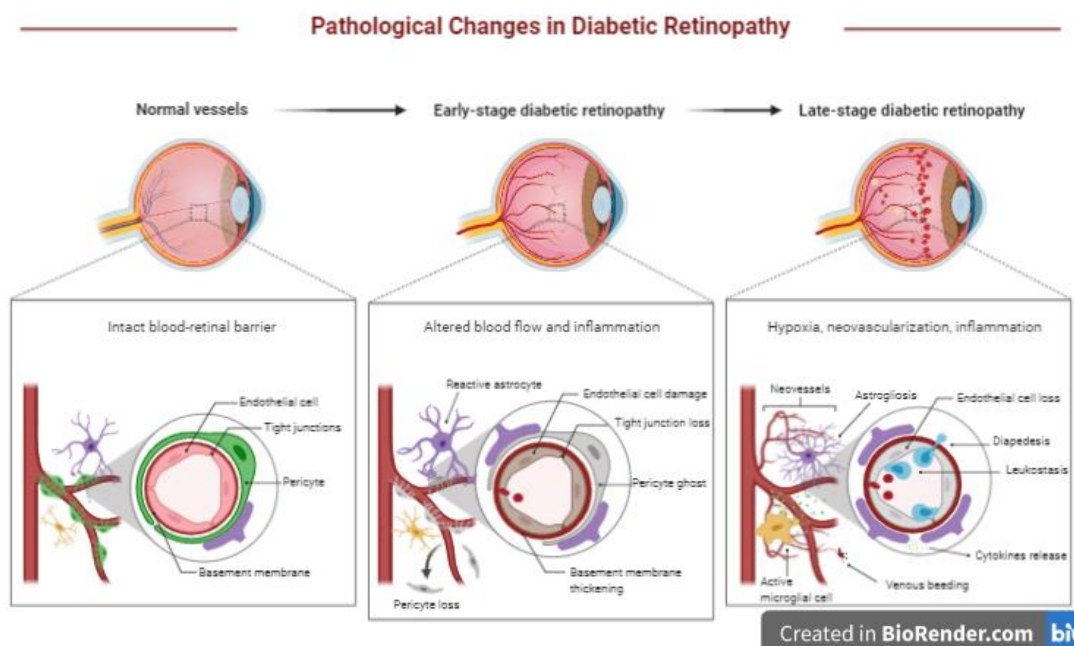


Figure 1.4 Pathological alterations leading to different stages of Diabetic Retinopathy. *Created in BioRender.com*

The classification and diagnosis of DR is based on the typical retinal microvascular lesions of the clinical stage, which become fundoscopically visible. Based on them, the disease is classified in stages as non-proliferative diabetic retinopathy (NPDR) and proliferative

diabetic retinopathy (PDR), according to the Multicenter Early Treatment Diabetic Retinopathy Study (ETDRS) (Rodriguez, Perez et al. 2019).

Mild non-proliferative retinopathy is characterized by the presence of a few microaneurysms and moderate non-proliferative retinopathy is characterized by the presence of microaneurysms, intraretinal hemorrhages, or venous beading (Santiago, Boia et al. 2018). Then, retinopathy may progress with the appearance of retinal hard exudates (lipid deposits in the retina resulting from lipoprotein leakage from the retinal microvasculature), cotton wool spots (small localized infarctions of the nerve fiber layer of the retina), intraretinal microvascular abnormalities (collateral dilated capillary channels in areas of retinal ischemia), and venous beading (irregular dilation of retinal veins associated with significant retinal ischemia). The disease may further progress to the proliferative stage, characterized by the development of new retinal blood vessels through upregulation of angiogenic factors in response to oxygen deprivation (Abu El-Asrar, Ahmad et al. 2019). A fibrous tissue is also developed at the optic disc or near venules elsewhere in the retina. These new retinal blood vessels may bleed, resulting in preretinal and vitreous hemorrhage, and the fibrovascular tissue can cause traction on the macula, resulting in loss of vision (Barrett, Liu et al. 2017, Aouiss, Anka Idrissi et al. 2019). An important additional category in diabetic retinopathy is diabetic macular edema, characterized by retinal thickening resulting from leaky blood vessels, which represents the most common cause of vision loss in patients with diabetes. (Barrett, Liu et al. 2017, Wang and Lo 2018) Diabetic macular edema, can develop at all stages of retinopathy, although it is more prevalent during the later phases (Santiago, Boia et al. 2018).

Due to a lack of suited biomarkers, the diagnosis of asymptomatic patients is insufficient, as well as the risk stratification to predict DR. Many patients without the traditional risk factors (long duration of DM, poor blood glucose and blood pressure control and dyslipidemia) develop DR. Furthermore, there are persons with long diabetes duration with poor glycemic control who do not develop DR. On other hand, clinical diagnostic tools for the disease screening can be invasive or mainly limited to a qualitative assessment, expensive, time-consuming and dependent on the personal experience of ophthalmologists. Regular follow-up of diabetic patients could result in early detection and treatment of vision-threating DR. Therefore, early diagnosis is critical.

1.2.1.4 Treatment

Although over the past several decades significant advances have been made and a variety of treatments for DR are currently available, none of them are yet curative and the existing damage related to the late stage of the disease are typically irreversible (Mansour, Browning et al. 2020). In the early stages, a tight blood glucose control and regular monitoring can help prevent its progression to more advanced stages. In advanced stages, the main treatment of DR includes immunotherapy with intravitreal injections of anti-VEGF antibodies, photocoagulation with argon laser, and vitrectomy (Mansour, Browning et al. 2020).

There are also new possible approaches, which have been under investigation, such as the use of corticosteroids (Semeraro, Morescalchi et al. 2019), currently used as a second-line therapy for DR patients poor responsive to anti-VEGF therapy (Lattanzio, Cicinelli et al. 2017). They lead the production of several proteins involved in inflammation and cell metabolism, which can induce anti-inflammatory properties, preservation of BRB, induction of vasoconstriction (Stewart 2012) (Semeraro, Morescalchi et al. 2019), and protection of tight junction integrity. Another approach, is the use of non-steroidal anti-inflammatory drugs (NSAIDs) (Ayalasomayajula, Amrite et al. 2004, Zhang, Liu et al. 2011), which prevent capillary cell apoptosis and vessel degeneration (Kern and Engerman 2001, Sun, Gerhardinger et al. 2005).

There are other therapeutic strategies under evaluation including TNF-α and IL-1β, such infliximab or canakinumab, mainly evaluated in preclinical studies (Tsilimbaris, Panagiotoglou et al. 2007, Stahel, Becker et al. 2016). The renin–angiotensin system (RAS) blockers such losartan and candesartan, which are angiotensin II type 1 receptor (AT1R) blockers, and enalapril, an angiotensin-converting enzyme (ACE) inhibitor are also under evaluation, as well as dipeptidyl peptidase 4 (DPP-4) inhibitors (gliptins) and glucagon-like peptide 1 receptor agonists (GLP1RAs) (Martins, Amorim et al. 2020). On other hand, vitamins, NAD(P)H-oxidase (NOX) inhibitors, nutraceutical agents among polyphenols, anthocyanins, sesamin, bromelain, alpha-lipoic acid and lutein are also being considered promises for DR management (Martins, Amorim et al. 2020).

1.2.2 The impact of Diabetes on ocular surface

The first layer of the eye needs to be constantly regenerated. So, any process that affects wound healing, or the speed of epithelial regeneration will have physiological impact and increased morbidity (K Co Shih 2017). It is well known that DM is a risk factor and/or a common cause for ocular disease (David L. DeMill 2016). Studies reported that DM is associated with decreased corneal oxygen consumption, abnormal collagen formation, altered glycosaminoglycans metabolism and thickening of the corneal basement membrane (Ismail 2014). Both types of DM have been associated with reduction of corneal nerve density which is a characteristic manifestation of diabetic corneal neuropathy, responsible for decreased corneal sensitivity (K Co Shih 2017). Regarding this, it has been proposed that demyelination of the nerve secondary to abnormal lipid metabolism and sorbitol accumulation within the Schwann cell, leads to demyelination of the nerve, and therefore, decrease of corneal sensitivity (Ismail 2014). Several studies reported that type 1 and type 2 DM patients have higher hysteresis, which suggests a more rigid and less deformable cornea. Another studies, found DM to be associated with a greater corneal thickness (K Co Shih 2017), and others found that structural and metabolic corneal abnormalities contribute to ocular surface disease in patients with DM (David L. DeMill 2016). Although the reason why DM is associated with greater corneal hysteresis or thickness is not completely known. Nevertheless, it has been speculated an accumulation of AGE in the cornea stroma of diabetics, together with non-enzymatic crosslinking between collagen molecules and proteoglycans, can contribute to those corneal changes (K Co Shih 2017). Another problem that is associated with Diabetes, is epithelial keratopathy which is characterized by an impairment in epithelial healing, increased endothelial permeability and fragility, keratitis, glycogen and glucose accumulation, edema and bleb formation. There are also subconjunctival hemorrhages and/or microaneurysms in diabetics patients (Ismail 2014).

Besides ocular surface, DM also has an impact on tear film homeostasis (Fanglin He 2018), and studies reported that diabetic patients have decreased tear secretion and impaired tear film function, which is aggravated with progression of DR (Ling Yu 2008). So, altered tear film quality and quantity is observed in diabetic patients (Zhenjun Zhao 2010). And

those alterations lead to dry eye disease, a condition that is very common among diabetic patients (Fanglin He 2018). Alteration on tear film homeostasis seem to be negatively correlated with diabetes duration (Fanglin He 2018), and it has been also shown to be correlated with severity of DR (Srutarshi Ghosh 2014, Fanglin He 2018). Regarding the DM factors that could contribute to the impact on tear film dynamics, it was reported that DM contribute to impaired tear film production and function, due to the damage to the microvasculature that supply the lacrimal gland together with autonomic neuropathy abnormalities. (Fanglin He 2018)Abnormalities in innervation leads to alterations in tear production which can impair motor and vegetative stimulus, suggesting that neurotrophic lesions are correlated with disruption of tear dynamics and ocular surface homeostasis (Garzón P. Sandra Johanna 2019). On other hand, lesions in corneal innervation interrupt the anti-inflammatory neural feedback (Kern 2007), and leads to a reduction in globet cells and mucin proteins (E. M. Messmer 2010, Beckman 2014). This can cause alterations in tear film composition and therefore, its stability and functions. Studies have been reported that long-term hyperglycemia and poorly controlled diabetes lead to impaired meibomian gland function. Seems that insulin resistance/deficiency and hyperglycemia impacting the steroids and lipid receptors in the glands (Garzón P. Sandra Johanna 2019). Insulin supports the metabolism and growth of lacrimal gland, being important to metabolic and mitogenic effects through the mediation of nutrient influx, energy storage, gene expression and protein synthesis (Mônica de Cássia Alves 2008). So, isolated or altogether, peripheral nervous lesions, glucometabolic disorder with chronic hyperglycemia and impairment on insulin action may create an environment fostering inflammation (Mônica de Cássia Alves 2008), impairing tear film homeostasis in diabetic patients and can lead to alteration in tear film secretion and function and, changes in their proteins profile.

1.2.3 The role of tear biomarkers on Diabetic Retinopathy

Nowadays, with the concept of predictive, preventive and personalized medicine (PPPM), biomarkers have gained more and more space in science, as they are playing a raised relevance in the discovery and development of new drugs, new diagnostic tools or important role in clinical trials (Suzanne Hagan 2016). A biomarker was initially defined by the National Institutes of Health (NIH) as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Group 2001) afterwards as “ a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to a exposure or intervention, including therapeutic interventions” (BEST (Biomarkers 2018). According to the different functions that they can perform, biomarkers are distinguished in several types, among them molecular, histologic, radiographic and physiologic. These, therefore, fall into the following categories: diagnostic, prognostic, monitoring, predictive, response, safety and susceptibility or risk (BEST (Biomarkers 2018). A useful biomarker has to be correlated with specific symptoms or clinical signs, validated diagnostic test and be non-invasive as possible (Suzanne Hagan 2016).

In pathologies of the ocular forum, it is considered an ideal biomarker, that one which is easy to acquire and quantify, obtained from a biological tissue or source of interest, other than blood or urine (Mitalee Tamhane 2019). So, in the eye, there are the tears, ocular surface tissues such as cornea and conjunctiva, aqueous humor and vitreous humor (Mitalee Tamhane 2019). In the last two, the access is invasive. In the case of tears, they have the advantage of being easily accessible, can be obtained more frequently, in a mini-invasive way, which composition or dynamic may reflect local changes in eye diseases (Mitalee Tamhane 2019). Despite that, there are many challenges regarding the validation of the emerged promising biomarkers candidates (Daniel Shu Wei Ting 2016). Few biomarkers in tears, were approved by the US Food and Drug Administration (FDA). Among those, there are point of care such as the Advanced Tear Diagnostics ocular lactoferrin tear test, Tear lab osmolarity test and InflammaDry (measure levels of MMP-

9) for DED, Total Immunoglobulin E diagnostic kit for allergic conjunctivitis (Mitalee Tamhane 2019).

In the context of DR, it is known that several studies reported changes in tears proteins of diabetic patients, for example, a significant increase in tear film proteomic was demonstrated by Herber et al, while Yu et al shown increased levels of secretory immunoglobulin A (sIgA) in diabetic patients and increased levels of lysozyme and decreased levels of lipocalin in DR patients when compared with healthy controls (Terry Nguyen-Khuong 2015). They also reported decreased levels of lactoferrin and lipocalin on onset diabetic proliferative retinopathy, with a decreased tear film function (Terry Nguyen-Khuong 2015). Kawai et al reported that diabetic and diabetic retinopathy patients have increased levels of apolipoprotein A-1(Terry Nguyen-Khuong 2015). It is known that in DM and its complications, including diabetic retinopathy, tear proteins could undergo glycation (non-enzymatic glycosylation) (Negre-Salvayre A 2009), which results in the production of advanced glycation end product (AGE) modified proteins which might impair protein function (Zhenjun Zhao 2010).

As tears are a complex fluid, although less complex than other biological fluids it was also reported that cytokines of tear film are also changed due to diabetes, and diabetic retinopathy. (Srutarshi Ghosh 2014)

As DR is a progressive disease with a long pre-clinical stage, ideal biomarkers for the disease could be: those that can help in risk stratification in order to guarantee better screenings; those that can predict the progression of the disease so, preventive interventions can be taken; those that provides prognostic, predicts response to treatment and quantifies treatment response accurately (Daniel Shu Wei Ting 2016). All of these characteristics could favor or enable an adequate, better and cost-effective therapeutic option for each patient.

1.2.4 Extracellular vesicles

Extracellular vesicles (EVs) consist of vesicles bound to lipids that are secreted by cells in the extracellular space, which according to biogenesis, secretion via, size, content and function are distinguished in microvesicles, exosomes and apoptotic bodies. Exosomes,

also known as intraluminal vesicles, are vesicles of endosomal origin that are secreted in the extracellular space after the fusion of early multivesicular endosomes with the plasma membrane. Which, in turn, originates from the inward budding of cell´s plasma membrane (María Yáñez-Mó 2015). The subsequent conversion of endosomes into exosomes or multivesicular bodies is regulated through the endosomal sorting complexes required for transport (ESCRT) pathway (Thomas Wollert 2010), or by an alternative route dependent on the sphingomyelinase enzyme. Exosomes are secreted by all types of cells and have also been described as present in 14 body fluids, including tear fluid, and are involved in sorting, recycling, storage, transport and release of proteins (F.T. Borges 2013). It is known that the contents of the vesicles range from lipids, nucleic acids and proteins that are directly related to the plasma membrane, cytosol, lipid metabolism as well as messengers of cell activation and apoptosis.

The proteins of the ESCRT pathway and their accessors, among Alix, TSG101, HSC70 and HSP90B, as they are found in vesicles provide information about the origin cell, allowing a better understanding of their content. The proteins responsible for the formation and release of exosomes (RAB27A, RAB11B, ARF6), transmembrane proteins of the tetraspanin family (CD63, CD9, CD81) and other proteins associated with the plasma membrane (LAMP1, TfR), as well as proteins involved in the transduction of signal (EGFR) and antigenic presentation (MHC 1 and 2) are commonly found in exosomes (Erik R Abels 2016). Regarding lipid content, contain sphingomyelin, cholesterol, GM3 ganglioside, desaturated lipids, phosphatidylserine and ceramide. EVs 3 In addition to proteins and lipids, exosomes also have genetic material as content, from genomic and mitochondrial DNA, to mRNA and miRNA (Erik R Abels 2016).

Exosomes play an active role in various physiological and pathological biological processes, including intercellular communication, cell maintenance, immunomodulatory response, as well as disease onset and progression. Thus, the study and a better understanding of the exosomes with regard to the identification of the cells of origin and the subpopulations, as well as the molecular content they have, can be very useful for a better understanding of intercellular communication in pathological conditions, and allow the identification of diagnosis biomarkers for various diseases, including diabetes and diabetic retinopathy (Laura M Doyle 2019).

It is known that distinct molecular profiles of EVs with procoagulant, proinflammatory and proangiogenic properties, are associated with diabetes and its complications (M Garcia-Contreras 2017, Fan Zhou 1 2019). In DR, early diabetes-related endothelial dysfunction can lead to impaired intercellular communication between retinal endothelial cells, leukocytes and neuroglial cells, leading in increased BRB permeability, which is closely related to the release of EVs with different profiles. Regarding DR progression, it has been reported that retinal vascular dysfunction may be mediated by exosomes that carry cPWW2P2A circRNA from pericytes to endothelial cells (Chang Liu 2019) Other studies have shown that in conditions similar to mesenchymal diabetes stem cells-derived EVs were able to cause pericyte detachment and endothelial cell proliferation with consequent disruption of BRB, mediated by MMP-2 (Elena Beltramo 2014). In this study, the authors have also addressed the involvement of EVs-derived MMPs in the progression of DR. In fact, several MMPs, such as MMP-2, MMP-9 and MMP-14 have been described as being increased in the vitreous fluid and in the retina of both patients with DR and animal models of DR, contributing to vessel destabilization and consequent BRB breakdown (Wei Zhang 2018).

Other important mediators involved in DR onset and progression appear to be closely related with EVs-mediated communication. In fact, TNF-α, C-reactive protein and thrombin, commonly increased in the eye of diabetic patients, can stimulate the formation of endothelial microvesicles in vitro. As consequence, the increased release of endothelial EVs can stimulate the production of ROS in the target cells, which may contribute to retinal vascular damage in the context of DR progression (Dylan Burger 2011). Nonetheless, in the early stages of the disease, EVs can also exhibit protective effects, preventing the rapid progression of the retinal damage. For example, in vitro and in vivo studies have demonstrated that EVs derived from microglial cells were able to inhibit hypoxia-induced photoreceptor apoptosis, thus preventing neovascularization and alleviating visual injury (Wenqin Xu 2019).

All these studies highlighted the importance of a tight regulated intercellular communication between retinal cells and the role of retinal-derived EVs in the progression of retinal disorders, namely DR.

AIMS

2. AIMS

Although tear composition in DR has been investigated, most of the studies did not assess the progression of the disease, or had a small sample size, or the assessment was made in pool of tear samples prepared with different amounts of each sample. A detailed examination of proteins, AMP´s and inflammatory mediators from tears of patients with DM and their relationship to DR stages has not yet been performed. Changes in those molecules that may able to discriminate between subjects without retinopathy, with non-proliferative diabetic retinopathy (NPDR) and with proliferative diabetic retinopathy (PDR), the various stages of DR, will have the potential not only to identify novel biomarkers in the tear film for the different stages of DR but also provide a better understanding of disease progression. In the past few years, there has been an increasing research interest in extracellular vesicles due to their role in intercellular communication and as a potential source of biomarkers for diagnosis and monitoring of human diseases. Moreover, the presence in peripheral body fluids, including the tears, of extracellular vesicles (EVs) shed from neurons and microglia of the CNS (Damiana Pieragostino 2019), suggests that these vesicles can well mirror the changes occurring in the retina. In fact, EVs carry a specific cargo, various RNA, proteins and lipids, which is related to the parental cell. Therefore, EVs can be also used as a potential source of biomarkers for diagnosis and staging of DR.

The main goal of this work was to investigate whether the composition of the tear fluid is changed in DR. In order to accomplish this, we proposed to:

1. Recruit individuals and collect data and tear fluid from healthy controls and patients with T2D without retinopathy, with NPDR and with PDR;
2. Evaluate changes in tear protein profiles, with respect to proteins, AMP´s and inflammatory mediators, that can act as early biomarkers for diagnosis of DR onset and be useful do predict or monitor its progression;
3. To characterize exosomes, a type of EVs, obtained from tears of healthy controls and T2D patients without DR and with NPDR and PDR.
4. Establish associations between changes in the composition and stability of the tear fluid, as well as, with the two types of DR.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 HUMAN SUBJECTS SELECTION AND RECRUITMENT

This study took place at the Institute of Pharmacology and Experimental Therapeutics of the Faculty of Medicine of the University of Coimbra (FMUC)/ Institute of Clinical and Biomedical Research of Coimbra (iCBR, Coimbra, Portugal) in collaboration with the Centro Hospitalar e Universitário de Coimbra (CHUC, Coimbra, Portugal) and Association for Innovation and Biomedical Research on Light and Image (AIBILI, Coimbra, Portugal). There was a previous approval of the Ethics Committee of CHUC (CHUC-059-18), in order to guarantee the well-being, safety and protection of the rights of all subjects, and to guarantee public proof of this protection. All participants gave their informed consent after a detailed description of the aim of the study and procedures that were going to be used, as well as the possible consequences of the study. Ethical principles and all applicable international, European and national law were respected. The clinical study was conducted in accordance with the Declaration of Helsinki, the following the Convention for the Protection of Human Rights and Dignity of the Human Being with regard to the Application of Biology and Medicine: Convention on Human Rights and Biomedicine (Oviedo, 4 April 1997) (Oviedo Bioethics Convention) and according to the International Conference on Harmonization: E6 (R2): Guideline for Good Clinical Practice (IHC-GCP).

A cross-sectional, non-interventional study was performed. It was designed in order to describe and analyze the subject's characteristics and to correlate them with the natural history of DR. Healthy control participants were recruited from medical check-up at the CHUC and some were volunteers at the iCBR-FMUC and Association for Innovation and Biomedical Research on Light and Image (AIBILI). T2D patients attending regular screening appointments at the Department of Ophthalmology at the CHUC were also included. In a systematic and standardized way, data and tears samples of (a) healthy control subjects and patients with T2D (b) without retinopathy, (c) with NPDR and (d) with PDR, were collected between May 2019 and February 2020. Background information and data concerning duration of diabetes, type of diabetes, treatments, retinopathy and other

diabetes complications as well as other co-morbidities were collected from patient records and, complemented with self-reported information by the patients during interviews. All subjects signed and dated the informed consent after receiving information about the purpose of the study, the procedures that would be used as well as the possible benefits and risks. They were anonymized with a code attributed once to each one and not reused, and the procedures were always used in the same order.

3.1.1 Criteria

3.1.1.1 Inclusion criteria

Participants with T2D, between 40-75 years old, diagnosed with T2D for more than five years, without or with DR (NPDR and PDR) were enrolled in this study. Patients diagnosed with diabetes under insulin therapy and/or other oral antidiabetic agents were included.

3.1.1.2 Exclusion criteria

After patient records were examined, patients who had cataract, glaucoma, or other eye diseases compromising visual acuity were excluded from the study. The following patients were also excluded: patients with other systemic diseases potentially associated with tears abnormalities including Rheumatoid arthritis, Lupus, Sjögren's syndrome, thyroid related disorders such as Grave's disease, Hashimoto's and thyroiditis, asthma, allergies; patients who reported administration of anti-inflammatory, anti-bacterial or immunomodulatory drugs in the last 3 months, patients with previous punctual plug, ocular surgery or trauma, active ocular infections or inflammations, use of contact lens wear within the previous 3 months; individuals with eyelid problems (entropion, ectropion, Meibomian Gland Dysfunction and other anterior segment disorders); patients with glaucoma; patients that were subjected to kerato-refractive procedures (LASIK, LASEK, PRK) in the last year.

Non-diabetics and diabetics subjects were submitted to an ophthalmology screening appointment made by an ophthalmologist, to assure that inclusion and exclusion criteria were met. During the ocular examinations, distance best/corrected visual acuity (BCVA) was checked and both eyes were examined using a Slit-Lamp Biomicroscope (Takagi Nagano Ken, model SM 10N), paying particular attention to lid margins, tarsal and bulbar conjunctiva and cornea.

The diabetic patients were classified according to the presence and extent of retinal lesions, based on views assessed by dilated slit-lamp anterior segment and fundus biomicroscopy using tropicamide drops (5%) for evaluation of the papilla, macula and retinal vessels. In some cases, fluorescein angiography (FA) and/or optical coherence tomography (OCT) were performed.

3.2 SCHIRMER TEST

Principle

Schirmer´s test is an auxiliary diagnostic tool used in clinical practice by ophthalmologists, in order to assess the tears production and secretion. This evaluation can be done in two different ways: Schirmer test type 1 and Schirmer test type 2, which differ each other in the use of anesthetic and reflex stimulation (Oftalmologia 2012). Schirmer I measure (basal and reflex) total tear secretion and is performed without anesthesia (Mitalee Tamhane 2019). However, if a drop of topical anesthetic is applied prior to the test, it serves to measure only the basal tears (Oftalmologia 2012). Although there is great intra and inter individual variability, values less than 5 mm in five minutes are considered pathological (Schein OD 1997). Schirmer II evaluates the reflex secretion, in which there is a nasal mucosa stimulation with the insertion of the strip. In this test, values less than 15 mm in five minutes are considered abnormal and suggest changes in the reflex tear (Oftalmologia 2012). According to the production mode, 4 types of tears can be considered: basal tears, reflex tears, closed eyes tears and emotional tears (Mitalee Tamhane 2019). In addition to a diagnostic tool, the Schirmer test is useful to collect tear fluid.

Procedure

In order to measure total tear secretion and to collect tear samples, we performed a Schirmer test type 1 using Schirmer filter paper strips (Dina strip Schirmer-Plus, Dina-Hitex, Bucovice, Czech Republic). In this test, the samples were collected from each eye by placing the Schirmer filter paper strips without anesthetic, at the junction of the lateral and middle thirds of the lower eyelid and kept in place for 5 min while subjects closed their eyes. Then, Schirmer strips were removed with gloves, and the wet length of Schirmer strips was registered, which indicates the tear volume in mm. Immediately after sampling, proteins were extracted from Schirmer strips.

3.3 TEAR BREAKUP TIME TEST (TBUT)

Principle

The TBUT test is a method used to investigate tear fluid stability and it is usually used for dry eye diagnosis. It gives an indication of the quality of the tear fluid and its vulnerability to evaporate. This test consists on the instillation of fluorescein onto the ocular surface to the bottom of the lower conjunctival sac, followed by several quick blinks so that it can be dispersed throughout the ocular surface (Oftalmologia 2012). Then, a cobalt blue light is used to observe the tear film rupture or breakup, which will be visible with the appearance of dry spots on the cornea. Values below ten seconds are considered pathological (Abelson M 2002).

Procedure

TBUT was assessed by an ophthalmologist. Fluorescein (0.5%) was instilled into patient´s tear film and the interval between instillation and appearance of the first dry spots on the cornea was measured using a broad beam of slit lamp with a blue filter, as described. The time was then registered.

3.4 PROTEIN TEAR EXTRACTION FROM SCHIRMER STRIPS

It is known that strip retention of proteins is related to their molecular weight and hydrophobic surface area, thus meaning that extraction efficiency varies according to a range of factors, including the solution that is used in that procedure. Despite that, the extraction solution elected and used throughout the study was 0.9% NaCl saline solution. Each Schirmer strip was immersed in0.9% NaCl saline solution as the extraction buffer. After 1h of incubation, the samples were centrifuged at 10,000 g x 5 minutes. Then, the extracts were divided into several aliquots for further experiments and stored at -80ºC.

3.4.1 Determination of total protein concentration

Principle

The tear protein concentration of all samples was measured with the bicinchoninic acid (BCA) total protein assay. The principle of this BCA assay is based on the formation of a complex comprised of protein and Cu^{2+} under alkaline conditions, a reaction known as biuret reaction, which is followed by reduction of the Cu^{2+} to Cu^{+} (Smith 1985). In those conditions, 2 molecules of BCA form a complex with Cu^{+} which have a strong purple-blue coloration, so it is possible to observe the reduction of alkaline Cu^{2+} by proteins (Smith 1985). This complex is water-soluble and at 562 nm shows strong absorbance, therefore the absorbance can be measured between 540 nm and 590 nm. As the amount of reduction is proportional to the amount of protein present, BCA relies on colorimetric detection of protein-induced Cu reduction and thus measures total protein in a sample.

Procedure

The protein concentration in tear fluid was measured spectrophotometrically using the BCA assay kit (Thermo Fisher Scientific). A standard curve was performed for the BCA protein assay using bovine serum albumin as a standard (prepared at concentrations ranging from 12.5 - 800 µg/mL using the BSA standard at 2 mg/mL). Samples were diluted in 1:9 proportion. After standards and samples dilutions, 25 µL of each were transferred into respective wells of 96-well plate. Then, 25 µL of tear extraction buffer (0.9% NaCl

saline solution) were added to the standard wells, 25 µL of deionized water to the sample wells, and 200 µL of BCA Reagent mixture with ratio of 50A:1B to all wells of the plate. After that, the plate was covered and incubated at 37º C for 30 min. Absorbance measurements were obtained at 570 nm using Gen5 Data Analysis Software. Standard curves were generated and were used to calculate the protein concentration in samples.

3.4.2 Western Blotting

Principle

Western Blotting consists in the detection of specific proteins in each sample. First, proteins are separated on a SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to their molecular weight and then are transferred onto a solid matrix support, where they are stained with specific antibodies. This methodology enables the identification of a target protein in a complex mixture of proteins as well as quantification of its levels.

Procedure

Samples were denatured with Laemmli sample buffer 4x [0.5 M Tris-HCl (pH: 6.8), 10% SDS (w/v), 30% glycerol (w/v), 0.6 M 2-mercaptoethano, 0.012% (w/v) bromophenol blue) and 5x [125 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (w/v) glycerol, 0.01% (w/v) bromophenol blue] followed by heating for 5 minutes at 95º. Thirty µg of protein from the samples were loaded on polyacrylamide gels and proteins were separated by SDS-PAGE. The electrophoresis was stopped when the front of the dye reached the bottom of the gel. The separated proteins were transferred to a solid support, 0.45 µm hydrophobic polyvinylidene difluoride Amersham™ Hybond™ PVDF membranes (GE Healthcare, USA), using ice cold transfer buffer [25 mM Tris,192 mM glycine, 0.005% (w/v) SDS, 20% (v/v) methanol, deionized water] at 320 mAmp, 4ºC for 90 minutes. Then, membranes were blocked with blocking solution (5% non-fat milk prepared in TBS-T [20 mM Tris base, 150 mM NaCl, 0.1% (v/v) Tween 20, pH 7.60] for 1 hour with agitation, at room temperature, in order to avoid unspecific binding of antibodies. After that, an incubation of the membranes was performed using primary antibodies diluted in the blocking solution, at 4ºC overnight. Then, the membranes were washed in TBS-T (3 x10 min) and then

incubated with the respective secondary antibody, with agitation for 1 hour at room temperature. After incubation with the secondary antibodies, the membranes were washed in TBST (3 x10 min), with agitation. Detection of bands intensity was performed using enhanced chemiluminescence substrate (ECL) by ImageQuant^{TM}LAS500. Then, the chemiluminescent detection of the membranes reflected in optical density of the bands, was measured by densitometry, using the Image J software. To demonstrate equivalent protein loading, all the membranes were re-probed for the protein β-actin.

Table 1- Primary antibodies used in western blotting incubation

Antibody	Molecular weight	Dilution	Company
Goat anti-CD63 Polyclonal antibody	40-60 kDa	1:500	SICGEN Antibodies (AB0047-200)
Rabbit anti-MMP9 Polyclonal antibody	92-95 kDa (pro) 82 kDa (active)	1:500	Abcam (ref: ab38898)

3.4.3 Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Principle

In LC-MS/MS technique, peptides from digested proteins extracted from the samples are separated by liquid chromatography (LC) in order to reduce complexity. In shotgun MS/MS approach, data-dependent acquisition (DDA) is used to convert the peptides to charged state, thus becoming ionized. Subsequently, these ions are analyzed in a mass analyzer, which sorts them based on their mass-to-charge ratio (m/z), a process known as ionization. By collision-induced dissociation, the ions with specific m/z are isolated and

fragmented. Those fragments follow another separation process based on m/z. Later on, a detector measures the number of ions at each m/z. Based on that, a spectra of m/z is generated and after that is used in association with intensity and retention time, in order to identify the precursor fragmented peptide (original). This identification could be made by database search, and the identified peptides and proteins are then quantified, for example using spectral counting (Pitt 2009).

Procedure

Here, in this study, LC-MS/MS was performed under service contract at VIB proteomics Core facility. Briefly, tear proteins were digested with trypsin and the resulting peptide were analyzed by LC-MS/MS using state-of-the -art orbitrap MS instruments. The LC-MS/MS runs were searched together using the MaxQuant algorithm (version 1.6.11.0) with mainly default search settings, including a false discovery rate set at 1% on PSM, peptide and protein level. Spectra were then searched against the human protein sequences in the Swiss-Prot database (database release version of 2020_01), containing 20,365 sequences www.uniprot.org.

3.4.4 Multiplex immunoassay

Principle

Multiplex immunoassay is a sandwich type assay, using the principle of other two main techniques: flow cytometry and ELISA. The idea is that the analytes of certain samples are detected when they sandwich between color-coded magnetic beads, coated with specific antibody and biotinylated detection antibody. When that happens, a complex of analyte, magnetic beads with specific antibody and biotinylated detection antibody is detected by Streptavidin-Phycoerythrin (PE), which serves as a reporter molecule. In a double laser system, while a laser excites the dyes of the beads, which signal represents the specific antibody coating, the second laser excites the Streptavidin-PE conjugate of the reporting molecule linked to the detection antibody complex. The signal strength represents the analyte concentration (Khalifian S 2015).

Procedure

A set of three metalloproteinases (MMP-2, MMP-3 and MMP-9) were analyzed using a preconfigured panel (ProcartaPlex Human MMP Panel II 3plex, Thermo Fisher Scientific, Austria) using xMAP-based technology (Luminex) at the Immune Monitoring Core at FMUC. Samples were thawed on ice and mixed firstly by vortex and then centrifuged at 10 000 x g for 10 min, in order to remove eventual particulates. The supernatants were then transferred to new Eppendorf microcentrifuge tubes. Then, the reagents were prepared according to the manufacturer's instructions as well as the assay protocol. Standards were diluted in 4-fold serial dilution.

Bead mix was added to each well (50 µL/well) of the 96-wells plate supplied by the kit, and then the magnetic beads were washed. Subsequently, 25 µL of Universal Assay Buffer (supplied in the kit) plus 25 µL of standards or samples were added to the respective wells and then the plate was sealed, covered and incubated for 120 minutes at room temperature, with gentle agitation. After that, the plate was washed and incubated with detection antibody mixture 1X (25 µL/well), sealed, cover and incubated for 30 minutes at room temperature, with gentle agitation. Afterwards, the plate was washed and incubated with Streptavidin-PE (50 µL/well) with the same previous conditions. Later, the plate was washed again and 120 µL of reading buffer were added to each well, then the plate was sealed, covered and incubated for 5 minutes (with agitation) at room temperature, followed by run on Luminex instrument. Standard curves were generated by using the reference cytokine sample supplied in the kit and were used to calculate the cytokine concentrations in tear samples by ProcartaPlex Analyst 1.0 Software.

3.4.5 Gelatin zymography

Principle

Gelatin zymography is an electrophoretic technique used to assess gelatinolytic activity of MMPs. For that, gelatin is incorporated in the preparation of the running gel, as it functions as MMPs substrate. During gel electrophoresis, an inactivation of the MMPs occurs because during the denaturation SDS was used. However, washes in Triton X-100 allow a partial renaturing with restoration of the MMPs activity, which results in gelatin

degradation. This can be detected with the use of blue dye in the gel, in which the unstained clear regions indicate the activity of the MMPs (T.Jones 2014).

Procedure

Samples were thawed on ice and then denaturized with sample buffer 5X [4% (w/v) SDS, 20% (w/v) glycerol, 0.01% (w/v) bromophenol blue,125 mM Tris-HCl (pH 6.8)] without reducing agents. After preparation of the gel (10 %) containing 0.1% (1mg/mL) gelatin, 6 µg of proteins from the denatured samples were loaded into the wells. Protein separation was carried out using standard gel electrophoresis, at 90V for 15 minutes, followed by 140V until the molecular weight marker reached the bottom of the gel. After electrophoresis was completed, the gel was washed in the washing solution [Triton X-100 2.5% (w/v), 50mM Tris-HCl (pH7.5), 5mM $CaCl_2$, 1 μM $ZnCl_2$] for 30 minutes at room temperature with slow stirring, twice under the same conditions with a new solution. After that, the wash buffer was removed, and the gel was incubated with incubation buffer [Triton X-100 1% (w/v), 50mM Tris-HCl(pH7.5), 5mM $CaCl_2$, 1 μM $ZnCl_2$] for 30 minutes at room temperature, under gentle agitation. Then, an overnight incubation at 37ºC was performed with a new solution. A staining with 0.5% (w/v) Coomassie Blue R-250 was performed for 30 minutes followed by an incubation with destaining solution [Methanol: Acetic acid: Water (50: 10: 40)] until the bands started to become evident. After destaining, gels were immersed in distilled water and scanned. The gelatinolytic activity was assessed by densitometric analysis using Image J software.

3.5 ISOLATION OF EXOSOMES FROM TEAR FLUID

Procedure

The isolation of exosomes was performed using a Total Exosome Isolation kit from Invitrogen. Isolation reagent provided by the kit, was added to the samples, in a quantity corresponding to half of the volume of extracted tears. Then, the mixture was mixed well by vortexing until a homogenous solution was obtained. After incubation at 4ºC overnight, the samples were centrifuged at 10,000 x *g* at 4°C for 1 hour. Then, the pellets

containing the vesicles were resuspended in PBS or RIPA buffer for Nanoparticle Tracking Analysis (NTA) or Western Blotting, respectively. In addition, an aliquot of exosomes resuspended in PBS were fixed with 2% paraformaldehyde (PFA) for transmission electron microscopy.

3.5.1 Nanoparticle Tracking Analysis (NTA)

Principle

Nanoparticle tracking analysis (NTA) is a technique that uses the properties of light scattering microscopy at laser with a digital camera, allowing to view, measure and quantify nanoparticles with dimensions between 10 to 1000 nm, of samples in liquid suspension, based on Brownian motion. In this technique, the nanoparticles are loaded into a sample chamber, which is illuminated by a specially shaped laser beam. As they pass through the beam path, they disperse and spread the laser light, which is easily collected by the 20x microscope objective and viewed by the camera. The nanoparticles move randomly in all directions, and the camera captures a video of the movement, in real time, individually and simultaneously. This occurs under Brownian motion and, based on mathematical calculations (Stokes-Einstein equation), the software determines the hydrodynamic diameters, which will be used to determine the size of the individual particles. Also, by tracking the nanoparticles, the concentration can be determined.

Procedure

NTA was performed with exosomes isolated from tear fluid using NanoSight LM 10 Instrument (NanoSight Ltd). The settings were optimized and kept for the analysis of all samples. Each video was used to perform the analysis and estimate the mean size, modal and concentration of particles. The analysis was based on the NTA (version NTA 2.2) analytical software.

3.5.2 Transmission electron microscopy (TEM)

Principle

Transmission electron microscopy is a technique that allows nanoparticles to be visualized in high resolution images through electron beam. Therefore, the electrons that interact with the particles create shaded or dark areas on the fluorescent screen, thus producing the image.

Procedure

After deposition of paraformaldehyde-fixed exosomes on Formvar-carbon coated grids (TAAB Laboratories Equipment), grids were contrasted with uranyl acetate for 5 minutes. Observations were carried out under TECNAI G2 Spirit BioTWIN electron microscope (FEI) at 100 kV.

3.6 STATISTICAL ANALYSIS

The statistical analysis of each experiment will be described in the next chapter, in their respective results section.

RESULTS

4. RESULTS

4.1 CHARACTERIZATION OF THE STUDY POPULATION

In this study, a total of 66 participants [42 (64 %) men and 24 (36 %) women] were enrolled. Among them, 12 were healthy controls and 54 were patients with T2D. The latter group included 13 diabetic subjects without DR, 25 subjects with NPDR and16 subjects with PDR. Two of the subjects enrolled in this study had one eye with NPDR and another eye without retinopathy, and 1 another subject had one eye with NPDR and another eye with PDR. Whenever possible, we collected data and tear samples from both eyes of each subject. The data for the controls and diabetic patients are summarized in Table 2.

The subjects in the control group ranged in age from 42 to 75 years, while those in the T2D group were 40 to 75 years, with the average age of the all study population of 62 years. A statistically significant difference was observed between the 4 groups (p=0.0186). In fact, a significant difference was found between the control group and T2D with NPDR (p<0.05) in terms of age (Table 1). Also, there were differences in the gender distributions between the four groups (Table 2).

Table 2- Demographic data of control and T2D patients

Variables	Control (n= 12) Healthy subjects	Diabetic (n=54)		
		Without DR (n=13)	**NPDR** (n=25)	**PDR** (n=16)
Age (years)	54 ± 11	59 ± 11	65 ± 9*	66 ± 6
Gender (Male/Female)	3/9	7/6	18/7	13/3
DM duration (≤ 15 years/>15 years)	not applicable	9/4	10/15	3/13

Values are expressed as mean ± SD; *p<0.05 *versus* Control

In this study, the major proportion of T2D without DR had duration of diabetes ≤15 years while diabetics with retinopathy (non-proliferative and proliferative) had DM for more than 15 years. (Table 2). In fact, an average ± SD of 12 ± 8 years was found forT2D patients, 19 ± 9 years for NPDR subjects and 22 ± 9 for PDR subjects, with a statistically significant difference (p = 0.0103) by the Ordinary test one-way ANOVA. Most diabetic patients

without DR (69.2%) had a diabetes duration less than 15 years while T2D patients with the two stages of DR, NPDR and PDR, had a diabetes duration higher than 15 years, 60% and 81%, respectively (Table 2), which implies an association between the duration of DM and the stage of DR.

Schirmer I test and TBUT in the control, T2D, NPDR and PDR groups are presented in Table 2. The comparison between the 4 groups of the Schirmer I test generated a p= 0.0008, meaning that there are significant differences between groups regarding tear production/secretion. Schirmer I test value in T2D was decreased significantly ($p<0.05$), with 74% of the diabetic patients presenting values <10mm/ 5 min. Also, 68% of T2D with NPDR and 77% with PDR had a Schirmer test <10 mm/ 5 min, with Schirmer values also significantly reduced compared to the control group ($p<0.001$) (Table 3).

Compared with the control group, TBUT values in the NPDR and PDR were significantly decreased ($p<0.05$) (Table 3). The results of the TBUT test indicate that individuals with diabetic retinopathy (NPDR and PDR) have an average value below 10 seconds, reflecting changes in tear stability compared to controls.

Table 3- Tear function tests in control and T2D patients

	Control	Diabetic		
	Healthy subjects	Without DR	NPDR	PDR
Schirmer I test (mm)	15.38 ± 1.67	8.95 ± 1.77*	8.11 ± 0.96**	7.00 ± 0.79**
Tear film BUT (s)	13.16 ± 1.52	11.64 ± 0.97	9.18 ± 0.97*	9.62 ± 0.59*

Values are expressed as mean ± SEM; *p<0.05, **p<0.01 *versus* Control;

Regarding the total tear protein concentration, we found that in the control, T2D, NPDR and PDR was 0.79 ± 0.26, 0.55 ± 0.12, 0.44 ± 0.23 and 0.61 ± 0.19 µg/µL, respectively. **C**ontrol subjects showed a higher concentration of total protein, in comparison to T2D subjects ($p< 0.05$) and NPDR subjects ($p<0.0001$). On the other hand, individuals with PDR have a higher concentration of total protein compared to individuals with NPDR ($p< 0.05$).

4.2 TEAR PROTEOMICS

Our proteomics study included 8 control subjects and 24 T2D patients, and among those, 8 without DR, 8 with NPDR and 8 with PDR (Table 3). No age significant difference was observed between these 4 groups. However, regarding gender, 37% of healthy, 62.5% of T2D and NPDR and 75% PDR subjects were men (Table 4).

Table 4- Demographic data of control and T2D patients

Variables	Control (n= 8) Healthy subjects	Diabetic (n=24)		
		Without DR (n=8)	**NPDR** (n=8)	**PDR** (n=8)
Age average (years)	58 ± 12	64 ± 10	60 ± 9	67 ± 5
Gender (Male/Female)	3/5	5/3	5/3	6/2

Values are expressed as mean ± SD

After LC-MS/MS runs, spectra against human protein sequences were searched in the Swiss-Prot database. In all 32 samples, 312 428 peptide-to-spectrum matches (PSMs), 9707 peptides and 1407 protein groups were identified. Of the latter, 682 protein groups were reliably quantified, which are the protein groups with at least 3 LFQ intensity values valid in one of the experimental conditions. In order to explore the profile of a certain protein, as well as to identify patterns and clusters in the samples, a principal component analysis (PCA) was performed on the replicate samples using all quantified proteins as variables. The scatter plot below (Fig.4.1) shows sample mappings along the two principal components (PCs). Percentages of explained data variance for each PC are shown on the x and y axis.

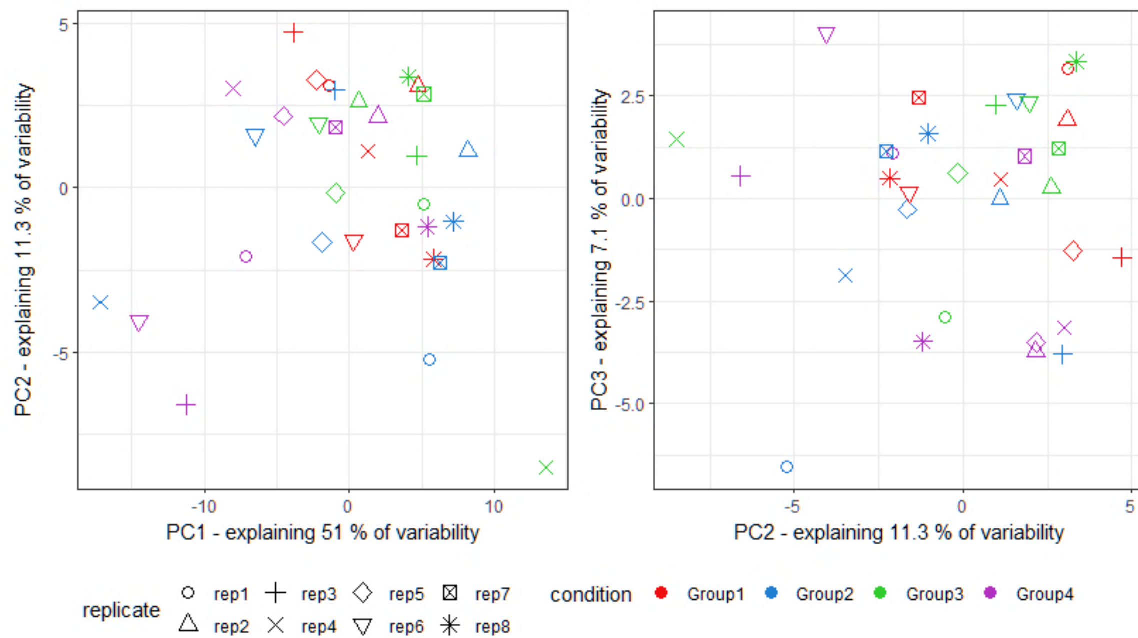


Figure 4.1 PCA scatter plot. The graph shown to the left represents the correlation between the original variables and the pairwise combination of PCs (PC1 on X axis and PC2 on Y axis). While the graph shown to the right represents the correlation between the variables and the pairwise combination of PCs (PC2 on X axis and PC3 on Y axis). A variation of 51%, 11.3% and 7.1% were captured from the data by PC1, PC2 and PC3, respectively. Each symbol represents the correlation of all quantified proteins with two PCs for each replicate of the 4 groups: healthy subjects, diabetic subjects without and with DR (NPDR and PDR).

Using the Gene Ontology (GO) knowledgebase, a database that until 10/September/2020 had 44272 GO terms and 1560690 gene products, a GO enrichment analysis with the 682 quantified proteins was carried out. Through the website, the input list of proteins was connected to an analyzing tool of the PANTHER Classification System. A PANTHER Overrepresentation Test (Releases 20200728) with Homo sapiens as reference list using the following Annotation Data Set: GO molecular function complete (Fig. 4.2) and PANTHER GO-Slim Molecular Function (Fig. 4.3), GO biological process complete (Fig. 4.4) and PANTHER GO-Slim Biological Process (Fig. 4.5), GO cellular component complete (Fig. 4.6) and PANTHER GO-Slim Cellular component (Fig. 4.7), PANTHER Protein class (Fig. 4.8), was performed. Fisher's Exact was the test type, with a correction using false discovering

rate (FDR). From a reference list containing 20851protein IDs, 511 proteins were identified from a list created with the 682 proteins, remaining 171 unidentified.

Molecular function

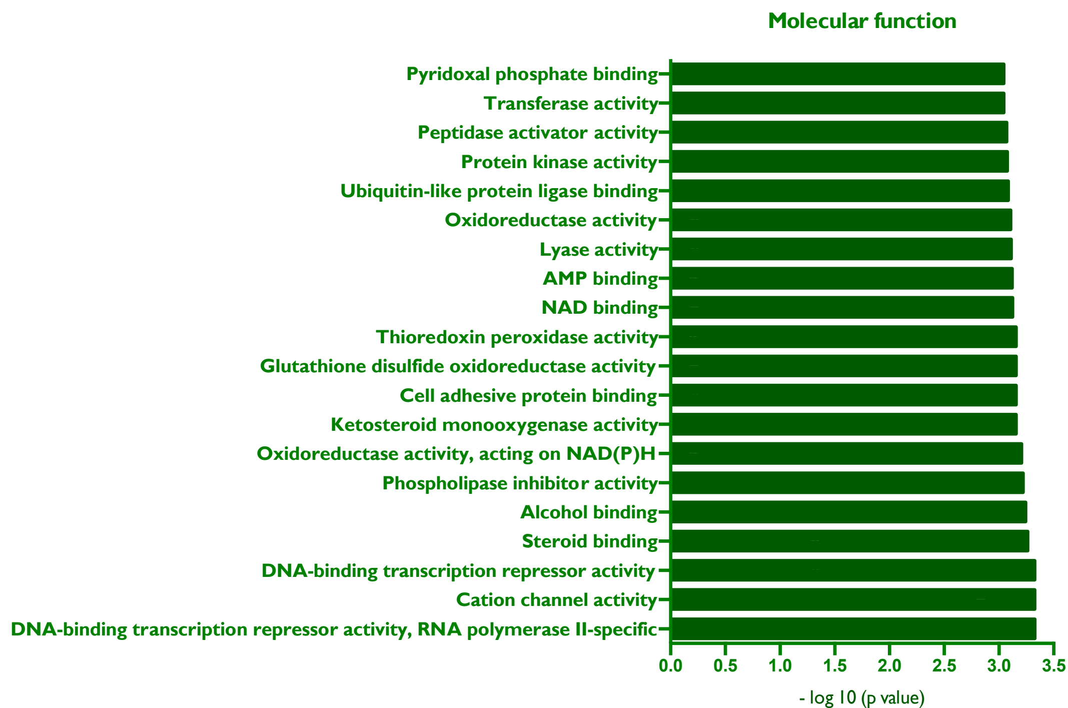


Figure 4.2 GO Molecular function complete. The graph presents the list of the 20 top-ranked molecular functions that were statistically significant, according to the GO Molecular function annotation data set. Based on the -log p, the base of the graph indicates the molecular function with the greatest statistical significance, which is the first function and the top indicates the molecular function with the least statistical significance. Therefore, DNA-binding transcription repressor activity and RNA polymerase II-specific is the top molecular function of the 511 identified proteins (GO enrichment) from 682 quantified by proteins (LC-MS/MS).

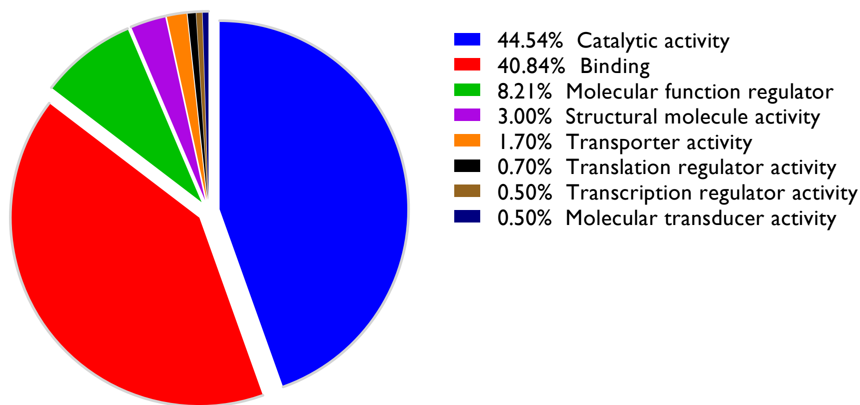


Figure 4.3 PANTHER GO-Slim Molecular Function. The graph represents the percentages of identified molecular functions based on the annotations data set in the PANTHER GO-Slim Molecular Function. For the 511 identified proteins, this analysis identified proteins, this analysis identified and grouped 402 functions hits. The function of catalytic activity, followed by binding, is performed by about 85% of the protein

Biological process

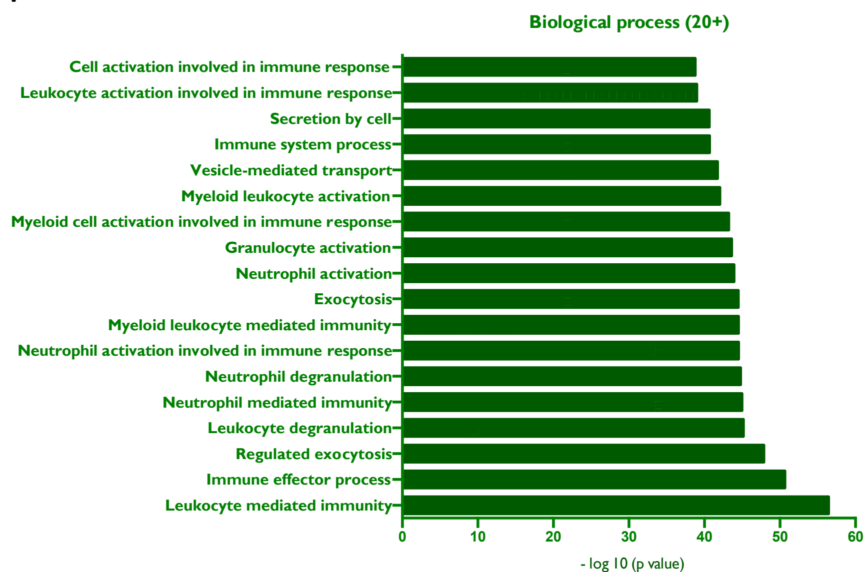


Figure 4.4 GO Biological Process complete. The graph presents the list of the 20 top-ranked biological processes that were statistically significant, according to the GO Biological Process annotation data set. Based on the -log p, the Leukocyte mediated immunity is the biological process with the greatest statistical significance, while cell activation involved in immune response is the biological process with the least statistical significance. Most of the biological processes to which the 511 identified proteins are connected, seem to be related to the immune system.

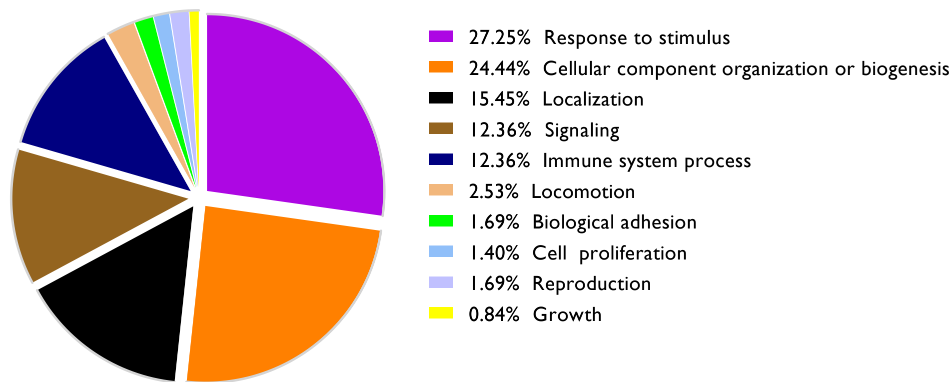


Figure 4.5 PANTHER GO-Slim Biological Process. The graph represents the percentages of identified biological process based on the annotations data set in the PANTHER GO-Slim Biological Process. 878 biological process hits were identified for the 511 proteins, with the highest percentage (27.25%) of proteins being related to the stimulus response process. It should be noted that in this analysis, immune system processes have 12.36% of proteins.

Cellular component

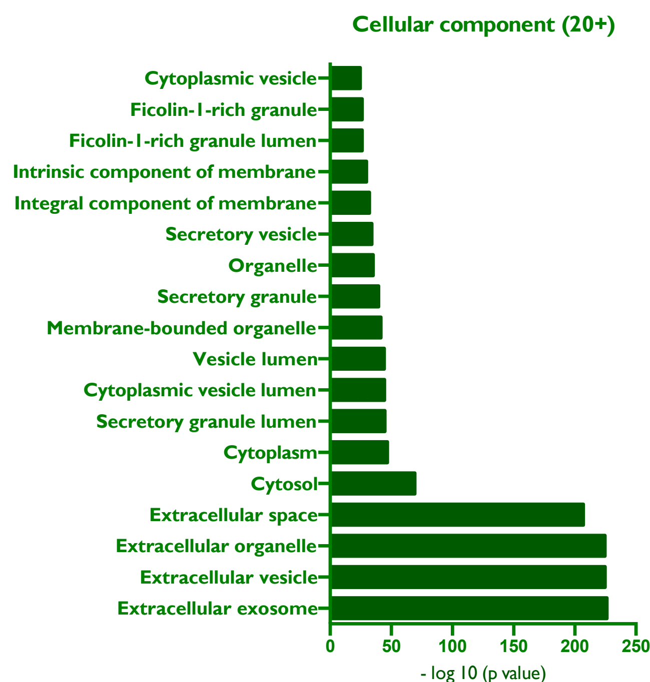


Figure 4.6 GO Cellular component complete. The graph presents the list of the 20 top-ranked cellular components that were statistically significant, according to the GO Cellular component annotation data set. The first cellular component listed according to the statistically significant -lop p, is extracellular exosome followed by extracellular vesicle. On other hand, the least statistically significant cellular component is cytoplasmic vesicle.

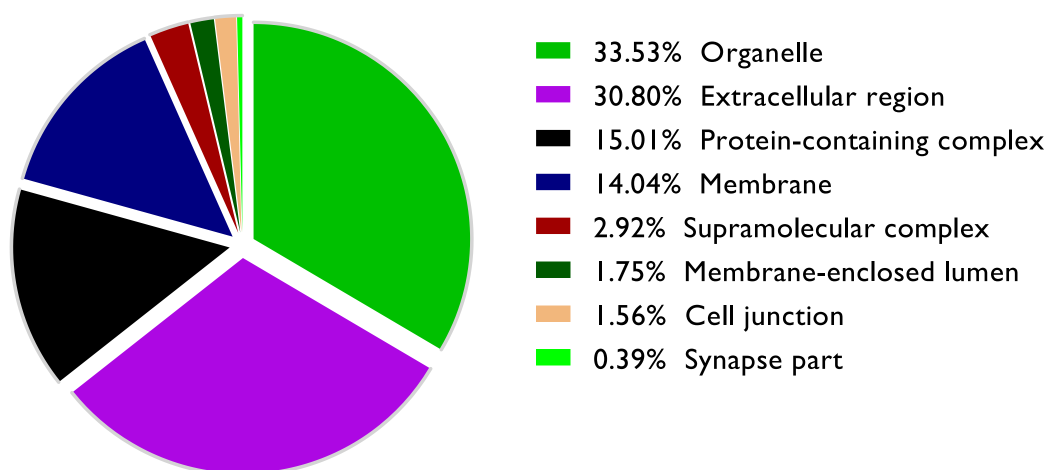


Figure 4.7 PANTHER GO-Slim Cellular Component. The graph represents the percentages of identified cellular components based on the annotations data set in the PANTHER GO-Slim Cellular Component. This analysis showed that 1051 cellular component are attributed to the 511 identified proteins. The highest percentage (33.53%) of proteins are from organelle, followed by extracellular region (30.80%).

4.2.1 Analysis and correlation of differential expression with the stages of DR

Since we had a small number of samples per group (n=8), as a first approach we assessed whether there were significant differences between the control group and the experimental groups. For this analysis, the intensities of the quantified proteins were considered (n = 682), for which a comparison was made between the samples of each group in relation to the control group using the Student t test with a correction by Benjamin and Hochberg, with the parameters false discovery rate (FDR) = 0.05 and S0 = 1 selected. In this context, the comparison for each protein between the two groups, was given as log 2fold-change. These values, as well as the statistical significance, given as a value -log p, for each protein, were plotted on a volcano graph, with fold change values shown on the X axis and -log p values on the Y axis. In certain samples, some proteins had low detection limits and therefore the LFQ values were listed as "NAN". In this context, to make it possible to perform the statistical test, these values were imputed, using a specific platform.

4.2.1.1 Control vs T2D

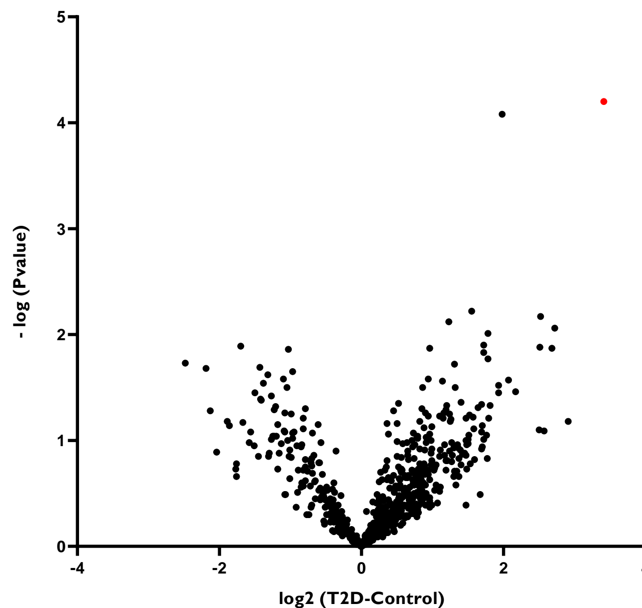


Figure 4.8 Volcano plot of distribution of differentially expressed proteins between T2D and control groups. The volcano plot shows that of 682 quantified proteins, only one had a statistically significant fold change. This protein is marked in red and within the values of the X axis, so it is upregulated.

According to this test, and the volcano plot, just one protein was significantly upregulated in T2D samples, hemoglobin subunit beta, also called LVV-hemorphin-7 or Spinorphin, whose gene is denominated HBB.

In another graphical representation comparing the LFQ intensities of the protein, the difference between the two groups is more evident and there is a relative consistency of values between the various samples of each group (Fig. 4.9).

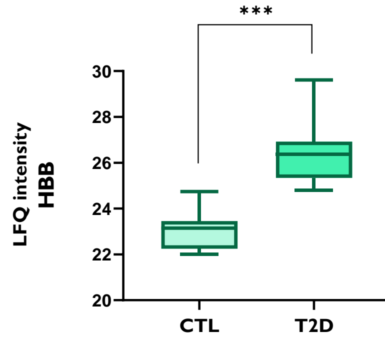


Figure 4.9 Comparison of LFQ intensities of the hemoglobin subunit beta. The graph above shows comparatively the LFQ intensities in the control subjects and in the T2D subjects. Since the data do not assume a normal distribution, according to the D'Agostino & Pearson test, an analysis by Mann-Whitney test, reveals a statistically significant increase in this protein in T2D samples. T2D subjects had a statistically significant increase ($p = 0.0002$) of LFQ intensities for hemoglobin subunit beta (HBB).

According to PANTHER GO-Slim Molecular Function, LVV-hemorphin-7 is a binding protein of organic acid, heme, protein and drugs, and has transporter activity and oxidoreductase activity as molecular functions. In addition, it acts as cofactor metabolic process and is involved in cellular catabolic process, reactive oxygen species metabolic process and drug metabolic process, according to PANTHER GO-Slim Biological Process.

4.2.1.2 Control vs NPDR

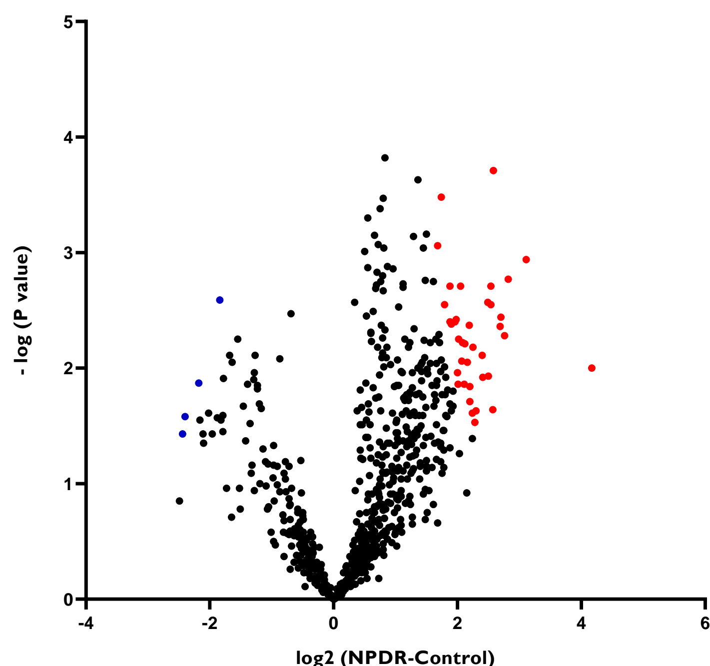
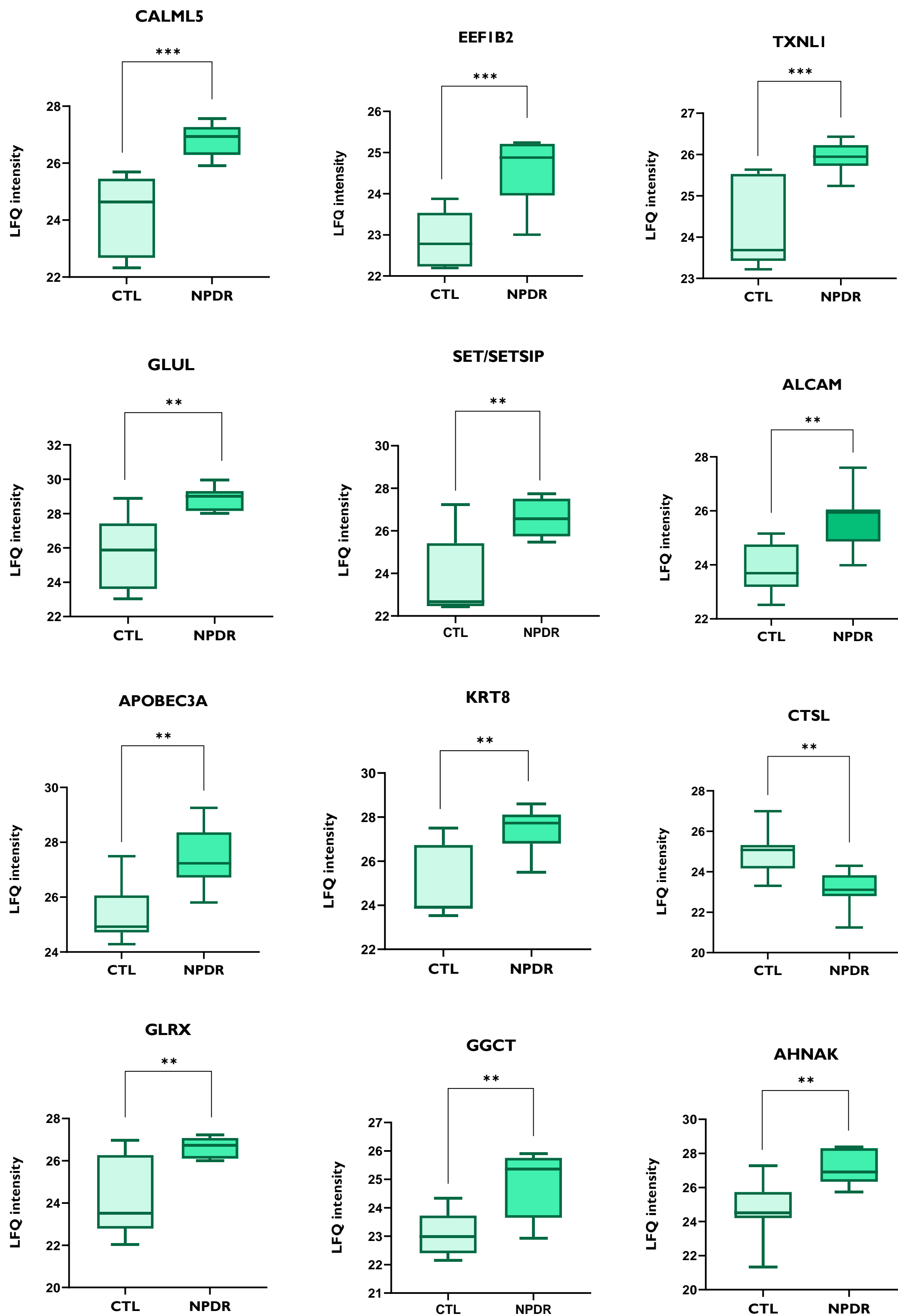


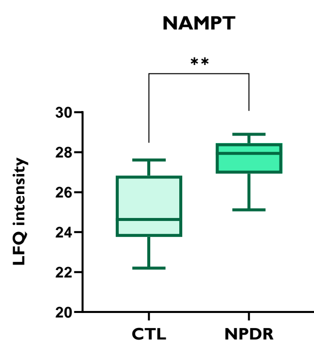
Figure 4.10 Volcano plot of distribution of differentially expressed proteins between NPDR and control groups. The volcano plot shows that of 682 quantified proteins, some proteins had a statistically significant fold change, in which the majority are marked in red and within the positive values of X axis, therefore upregulated and some, marked in blue and within the negative values of X axis, therefore downregulated.

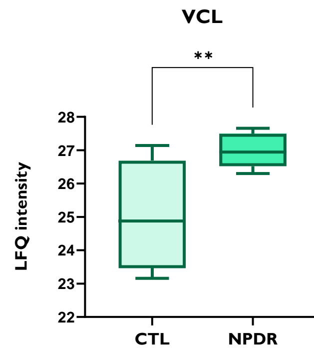
The volcano plot of comparison between NPDR samples and control samples, revealed 38 proteins (**CALML5, EEF1B2, TXNL1, GLUL, SET/SETSIP, ALCAM, APOBEC3A, KRT8, GLRX, GGCT, AHNAK, NAMPT, VCL, SH3BGRL, ABHD14B, GRHPR, TFF3, HNRNPA2B1, PDIA3, CRIP1, DDT/DDTL, NAPRT, AKR7A2, CAPS, GSTO1, TPT1, TMSB4X, GFPT1, CRYZ, PPP2R1A, TALDO1, GOT1, CAST, IQGAP1, RNPEP, CALML3, ADH1C, PPA1**) significantly upregulated and 4 proteins (**CTSL, CST2, PROL1, PRR27**) significantly downregulated.

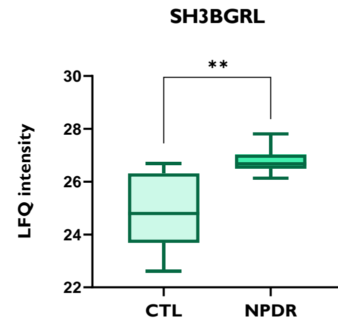
Below, the LFQ intensity comparisons of each of these proteins are represented also in box and whiskers graphs (Fig. 4.11). It is possible to observe clearly the differences and variability of the groups. It is interesting to note that, contrary to what was observed by

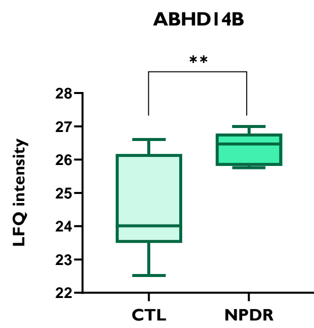
the volcano plot, for the proteins PPA1 and PRR27 there was no statistically significant difference.

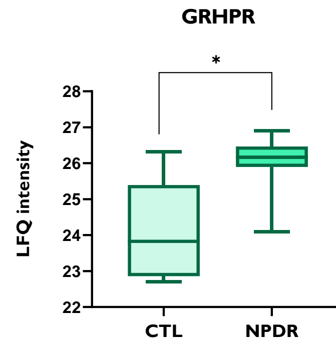


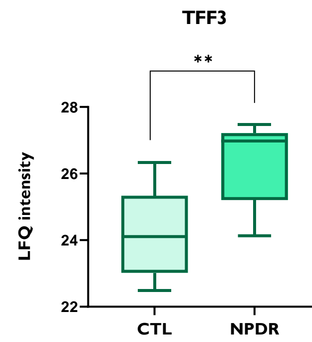

NAMPT
**
LFQ intensity
30
28
26
24
22
20
CTL
NPDR

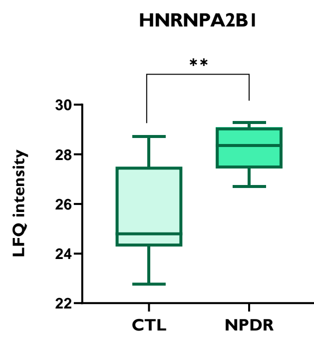

VCL
**
LFQ intensity
28
27
26
25
24
23
22
CTL
NPDR


SH3BGRL
**
LFQ intensity
30
28
26
24
22
CTL
NPDR


ABHD14B
**
LFQ intensity
28
27
26
25
24
23
22
CTL
NPDR


GRHPR
*
LFQ intensity
28
27
26
25
24
23
22
CTL
NPDR


TFF3
**
LFQ intensity
28
26
24
22
CTL
NPDR


HNRNPA2B1
**
LFQ intensity
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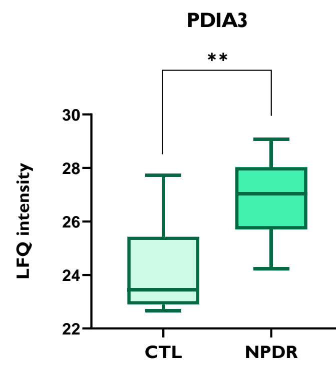
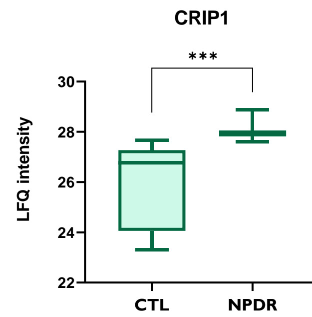
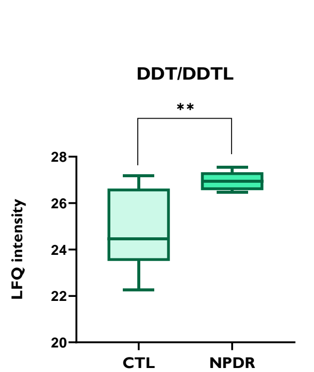
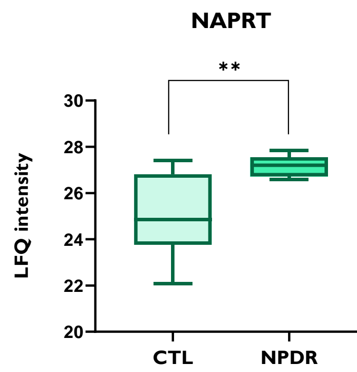
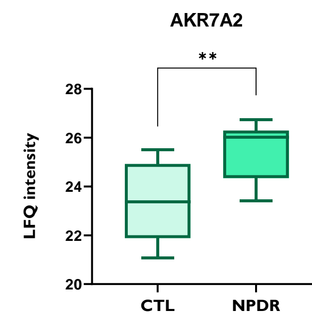
28
26
24
22
CTL
NPDR

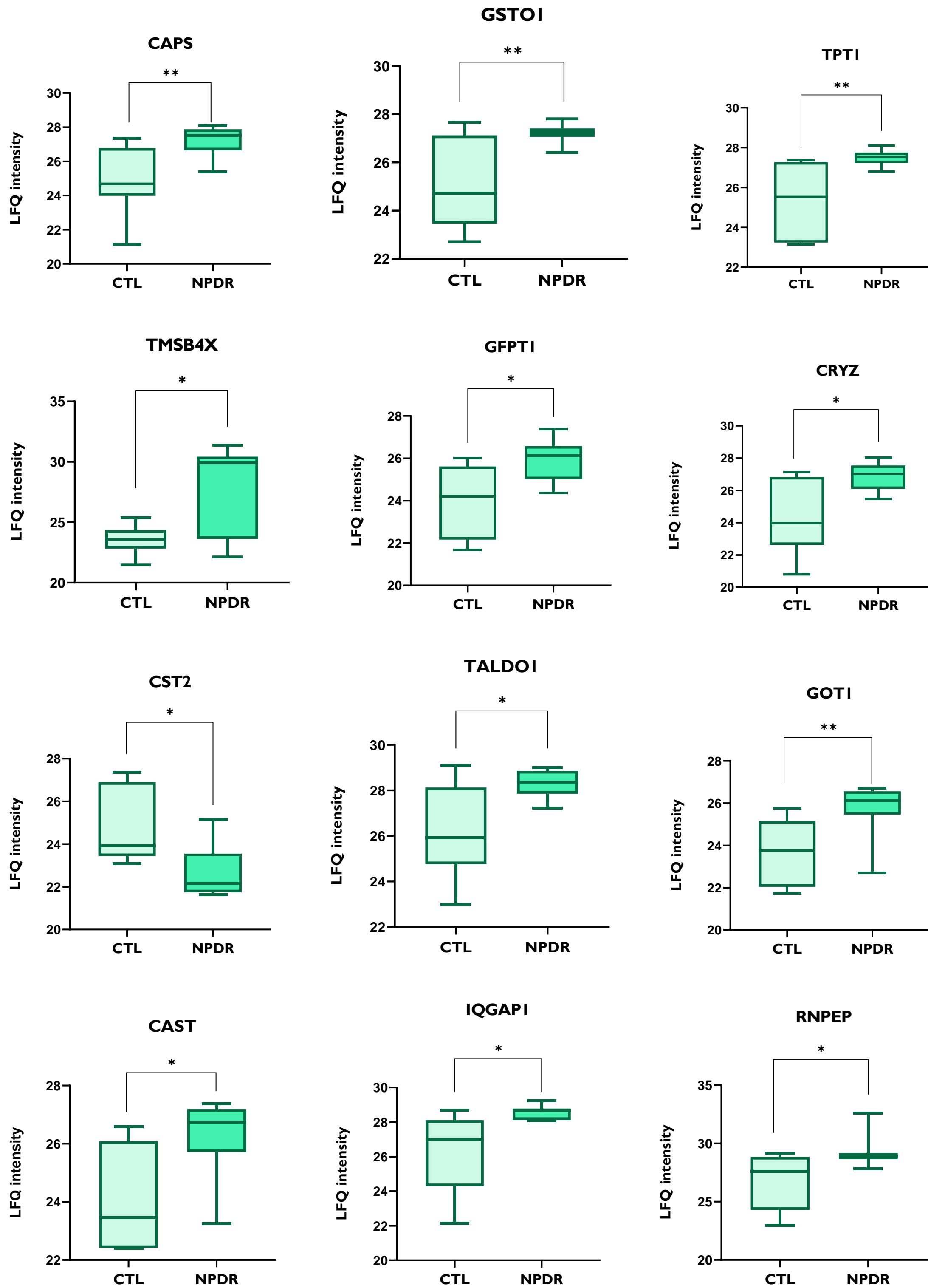

PDIA3
**
LFQ intensity
30
28
26
24
22
CTL
NPDR


CRIP1
***
LFQ intensity
30
28
26
24
22
CTL
NPDR


DDT/DDTL
**
LFQ intensity
28
26
24
22
20
CTL
NPDR


NAPRT
**
LFQ intensity
30
28
26
24
22
20
CTL
NPDR


AKR7A2
**
LFQ intensity
28
26
24
22
20
CTL
NPDR


CAPS
GSTO1
TPT1
TMSB4X
GFPT1
CRYZ
CST2
TALDO1
GOT1
CAST
IQGAP1
RNPEP
LFQ intensity
CTL
NPDR
**
*

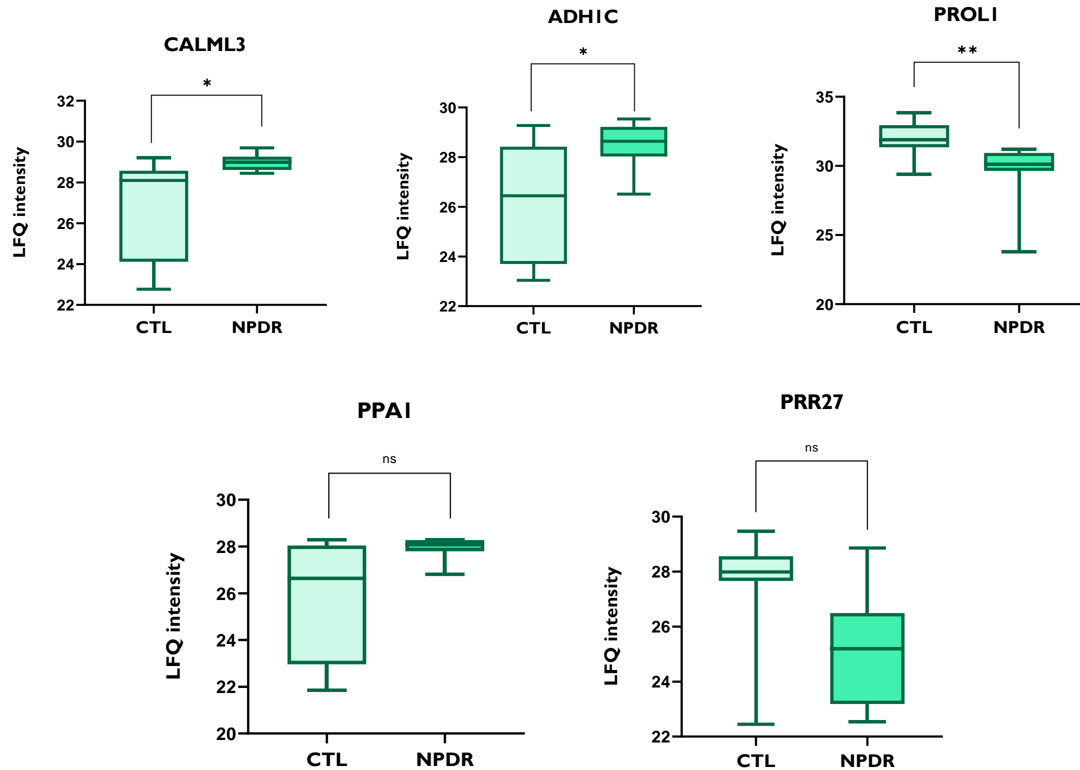


Figure 4.11 Comparison of LFQ intensities of the 42 proteins. The graph above shows comparatively the LFQ intensities in the control subjects and in the NPDR subjects for each one of the statistically significant altered proteins. In this analysis, among the 42 proteins, CALML5, EEF1B2, TXNL1 and CRIP1 stand out. An analysis of the data distribution was performed and for those who assumed a normal distribution, the statistical significance was assessed by unpaired t test, while those who did not assume a normal distribution, the statistical significance was assessed by the Mann-Whitney test. *P<0.05; **p<0.01; ***p<0.001 vs CTL.

A search was made for the proteins indicated as significantly altered by the volcano plot, by the enrichment analysis method for molecular functions, biological processes, cellular component, class of proteins and pathways (Fig. 4.12 a Fig. 4.16).

Molecular function

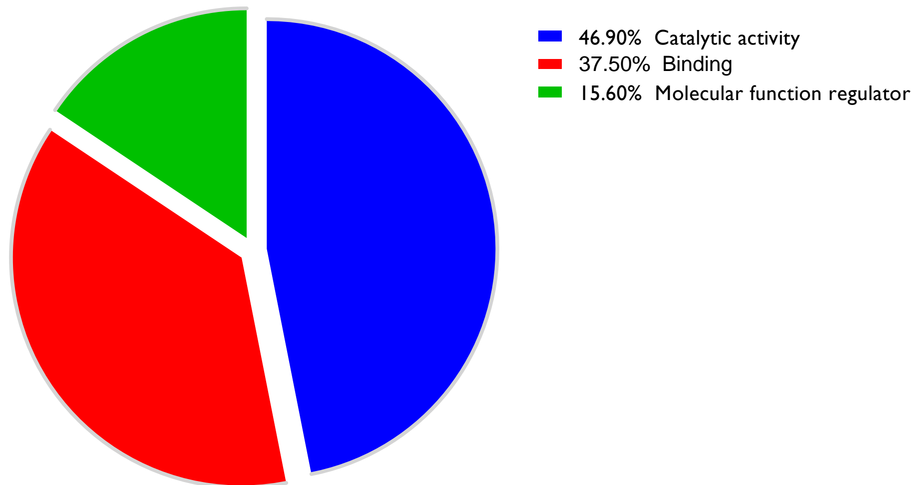


Figure 4.12 PANTHER GO-Slim Molecular Function. The graph represents the percentages of identified molecular functions based on the annotations data set in the PANTHER GO-Slim Molecular Function. This analysis showed that 32 molecular function hits are *attributed to the 42 proteins* statistically significant. 46.9% of these proteins have catalytic activity, followed by binding activity.

Biological process

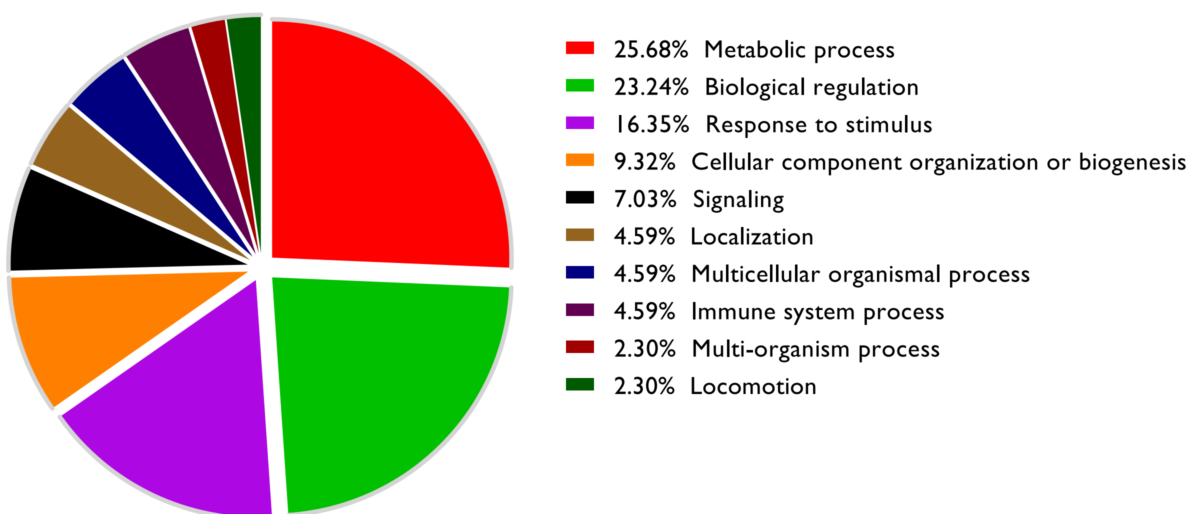


Figure 4.13 PANTHER GO-Slim Biological Process. The graph represents the percentages of identified biological processes based on the annotations data set in the PANTHER GO-Slim Biological Process. This analysis showed that 58 biological processes hits are related to the 42

proteins statistically significant. 48.92% of these proteins are involved in metabolic process and biological regulation, followed by response to stimulus (16.35%).

Cellular component

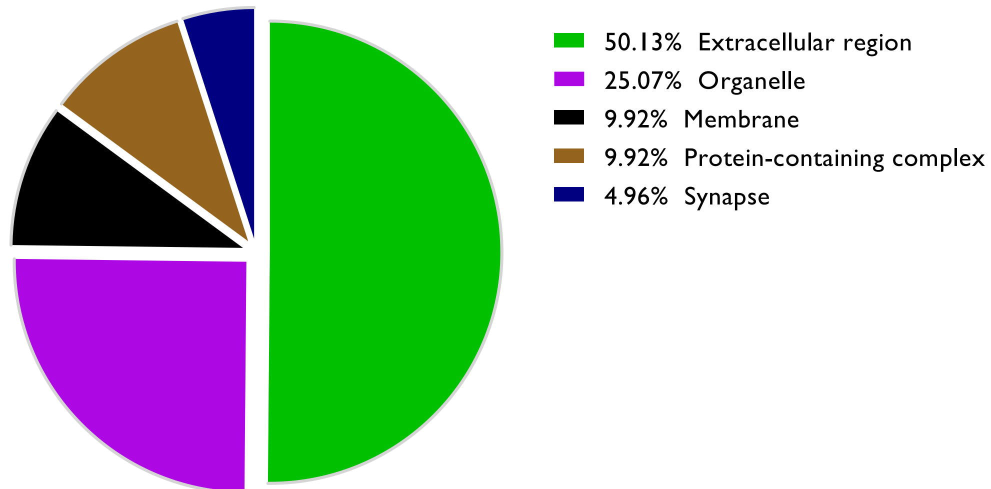


Figure 4.14 PANTHER GO-Slim Cellular Component. The graph represents the percentages of identified biological processes based on the annotations data set in the PANTHER GO-Slim Cellular Component. This analysis showed that 15 cellular component hits are related to the 42 proteins statistically significant. Half of these proteins are from extracellular region.

Protein class

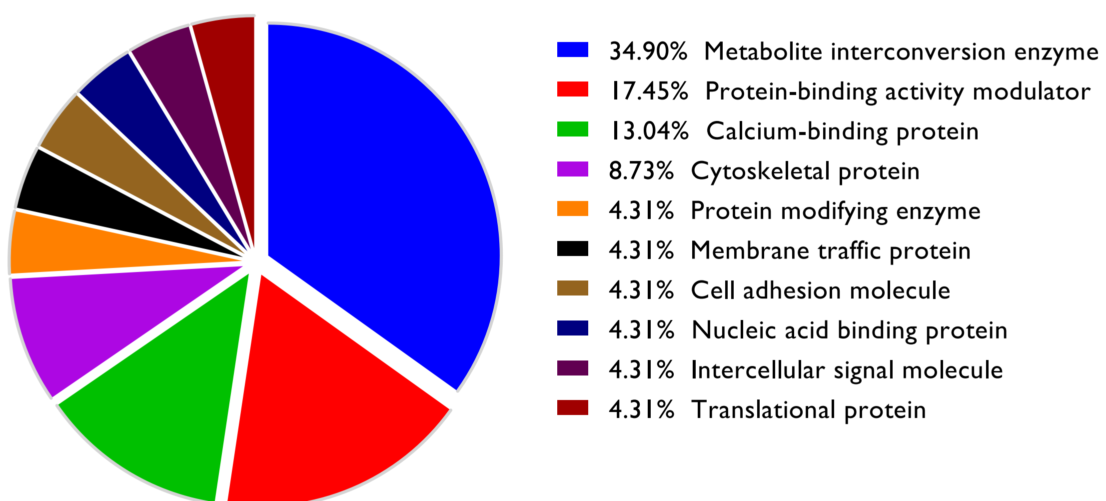


Figure 4.15 PANTHER GO-Slim Protein Class. The graph represents the percentages of identified protein classes based on the annotations data set in the PANTHER GO-Slim Protein Class. This analysis showed that 23 protein class hits are related to the 42 proteins statistically significant.

Metabolite interconversion enzyme, protein-binding activity modulator and calcium-binding protein are the most relevant.

Pathways

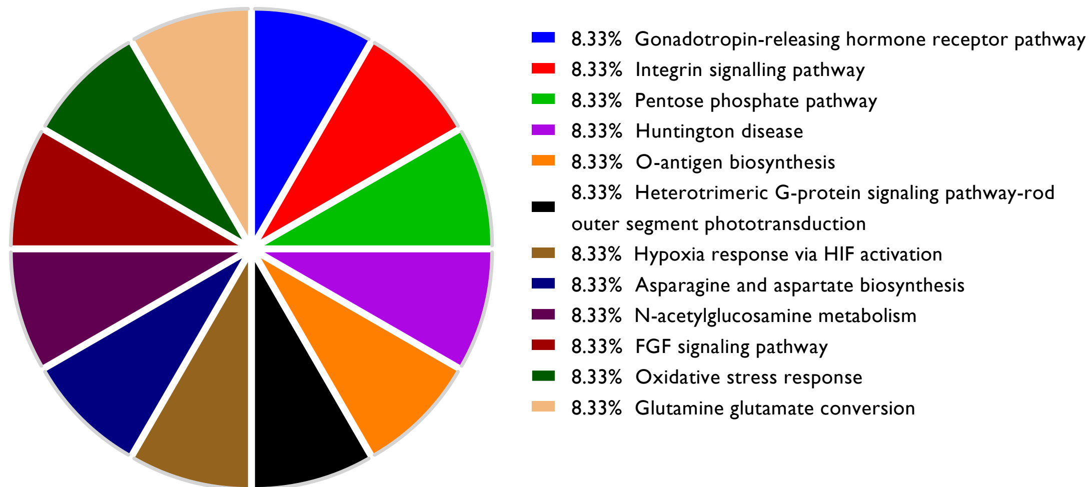


Figure 4.16 PANTHER Pathways. The graph represents the percentages of identified biological processes based on the annotations data set in the PANTHER Pathways. This analysis showed that 12 pathways hits are related to the 42 proteins statistically significant, which each one has the same percentage of relevance.

4.2.1.3 Controls vs PDR

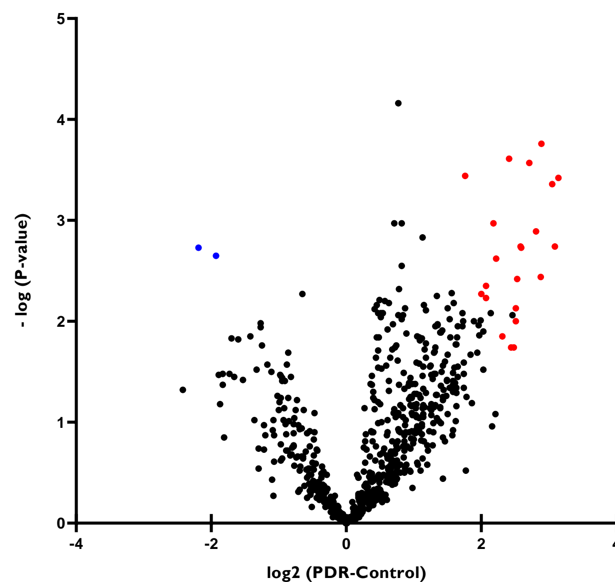
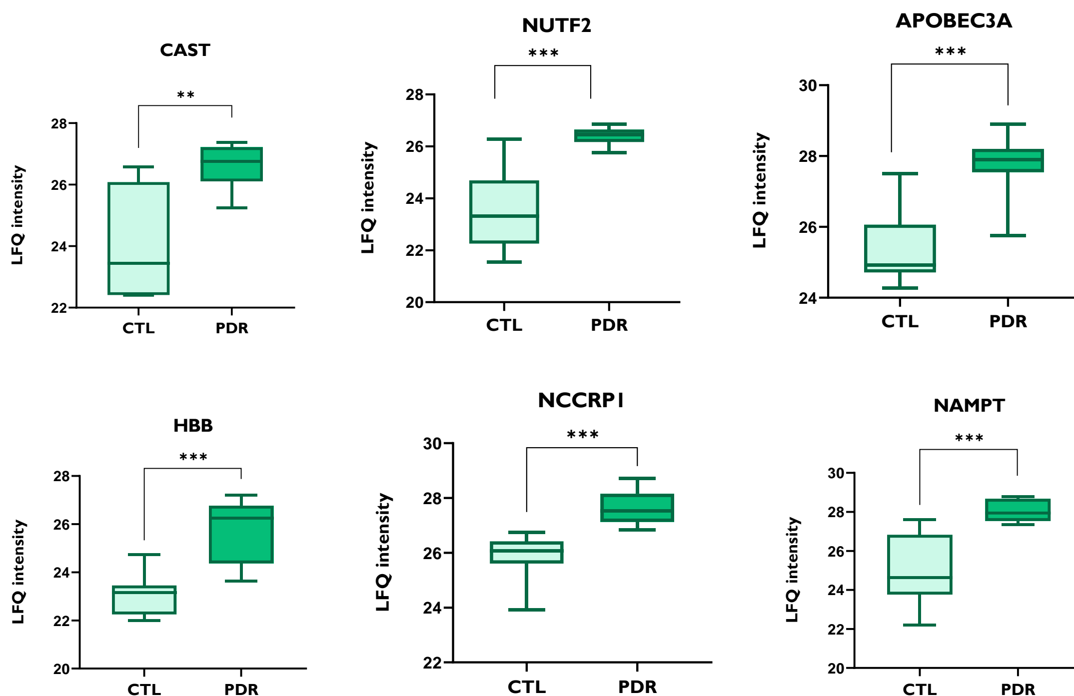


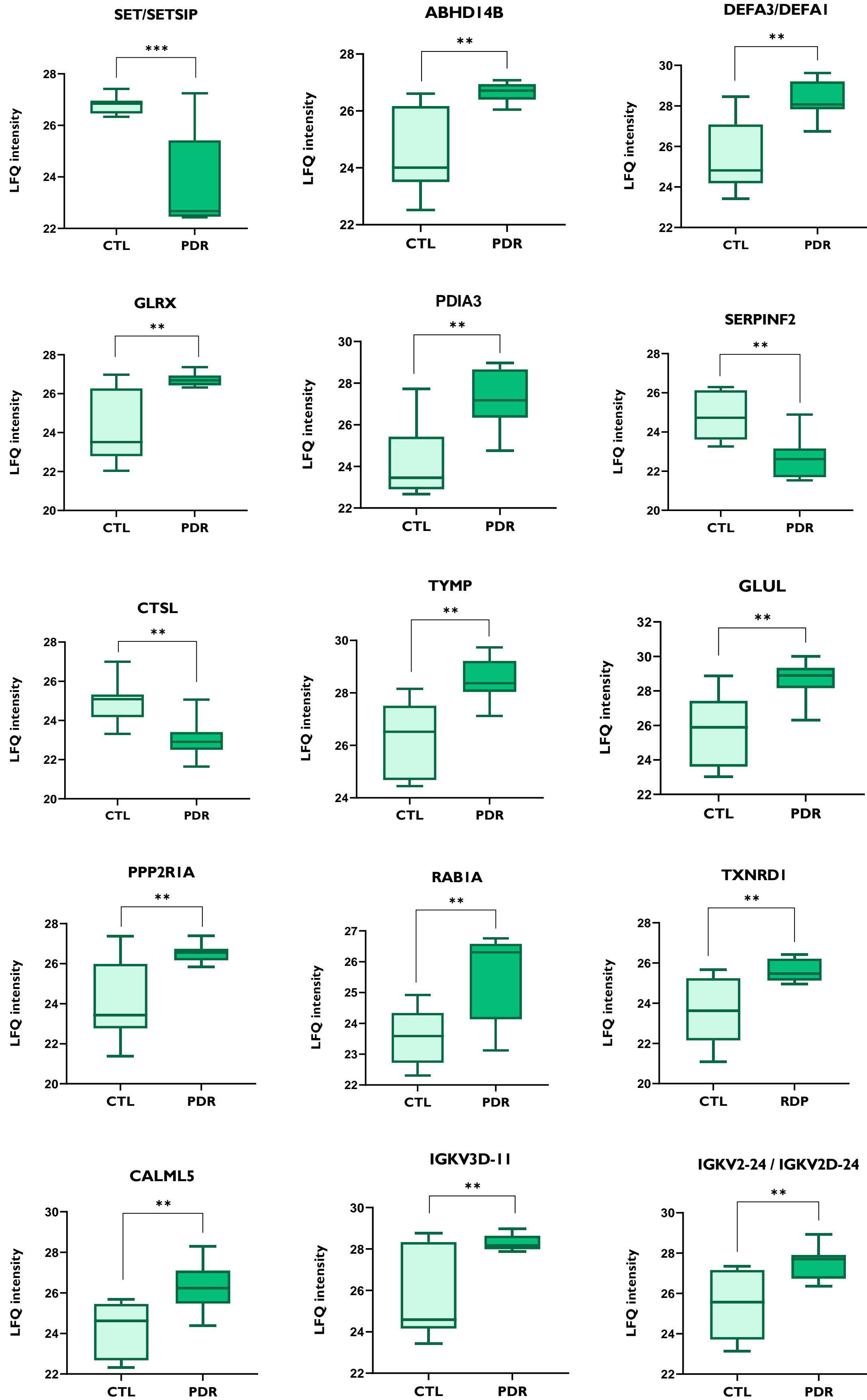
Figure 4.17 Volcano plot of distribution of differentially expressed proteins between PDR and control groups. The volcano plot shows that of 682 quantified proteins, some proteins had a

statistically significant fold change, in which the majority are marked in red and within the positive values of X axis, therefore upregulated and some, marked in blue and within the negative values of X axis, therefore downregulated.

In PDR samples, the volcano plot showed 24 proteins (**NUTF2, APOBEC3A, HBB, NCCRP1, NAMPT, SET/SETSIP, ABHD14B, DEFA3/DEFA1, GLRX, PDIA3, CAST, TYMP, GLUL, PPP2R1A, RAB1A, TXNRD1, CALML5, IGKV3D-11, IGKV2-24/IGKV2D-24, CALR, LAP3, WARS, CALML3, PPA1**) significantly upregulated and 2 proteins (**SERPINF2, CTSL**) significantly downregulated compared to those in control group.

Similar to the previous analyses, a comparison of the LFQ intensities of each of these proteins was established and represented by the graphs shown below (Fig. 4.18). The differences for each of the proteins are more clearly observed, as well as the inter/ intra variations and (or) consistency. There was a statistically significant difference for all, according to what was reported by the volcano plot.




SET/SETSIP
***
LFQ intensity
CTL
PDR
ABHD14B
**
LFQ intensity
CTL
PDR
DEFA3/DEFA1
**
LFQ intensity
CTL
PDR
GLRX
**
LFQ intensity
CTL
PDR
PDIA3
**
LFQ intensity
CTL
PDR
SERPINF2
**
LFQ intensity
CTL
PDR
CTSL
**
LFQ intensity
CTL
PDR
TYMP
**
LFQ intensity
CTL
PDR
GLUL
**
LFQ intensity
CTL
PDR
PPP2R1A
**
LFQ intensity
CTL
PDR
RAB1A
**
LFQ intensity
CTL
PDR
TXNRD1
**
LFQ intensity
CTL
RDP
CALML5
**
LFQ intensity
CTL
PDR
IGKV3D-11
**
LFQ intensity
CTL
PDR
IGKV2-24 / IGKV2D-24
**
LFQ intensity
CTL
PDR

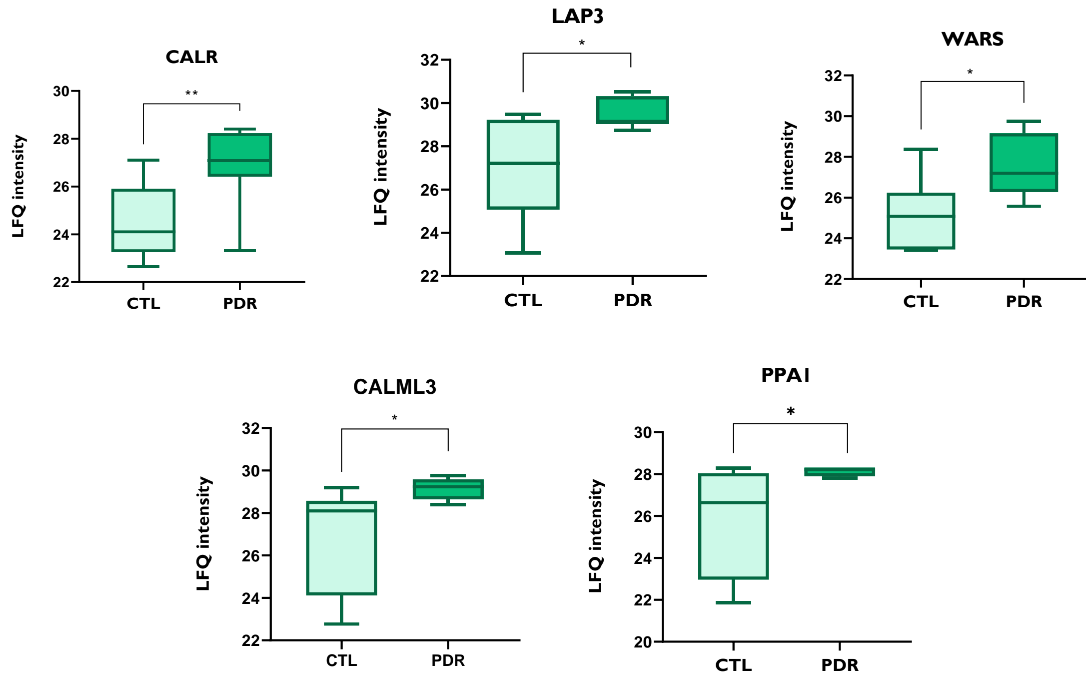


Figure 4.18 Comparison of LFQ intensities of the 26 proteins. The graph above shows comparatively the LFQ intensities in the control subjects and in the PDR subjects for each one of the statistically significant altered proteins. In this analysis, among the 42 proteins, NUTF2, APOBEC3A, HBB, NCCRP1, NAMPT and SET/SETSIP stand out. An unpaired t test was also performed for all data that assumed a normal distribution and a Mann-Whitney test for those that assumed the opposite. *P<0.05; **p<0.01; ***p<0.001 vs CTL.

A search was made for the proteins indicated as significantly altered by the volcano plot, by the enrichment analysis method for molecular functions, biological processes, cellular component, class of proteins and pathways (Fig. 4.19 to Fig. 4.23).

Molecular function

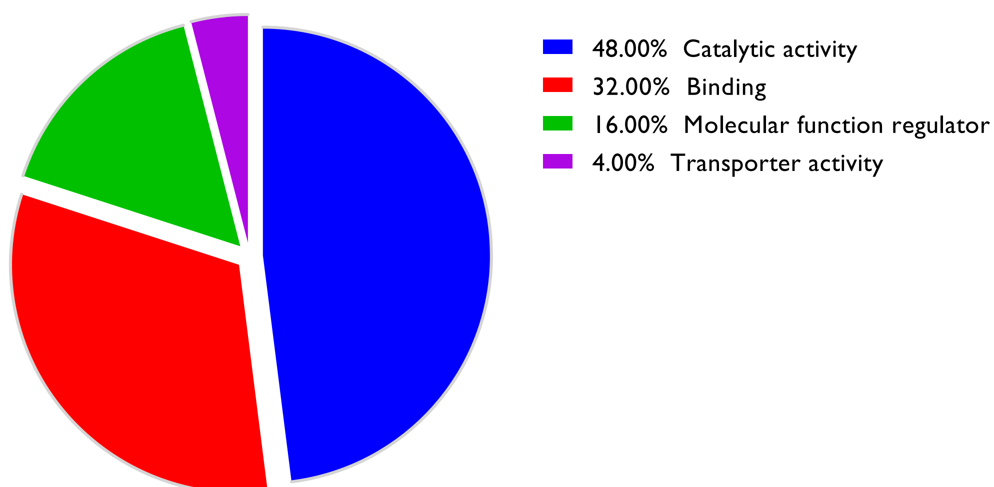


Figure 4.19 PANTHER GO-Slim Molecular Function. The graph represents the percentages of identified protein classes based on the annotations data set in the PANTHER GO-Slim Molecular Function. This analysis showed that from the 23 molecular function hits, catalytic and binding are the more relevant for the 26 proteins.

Biological process

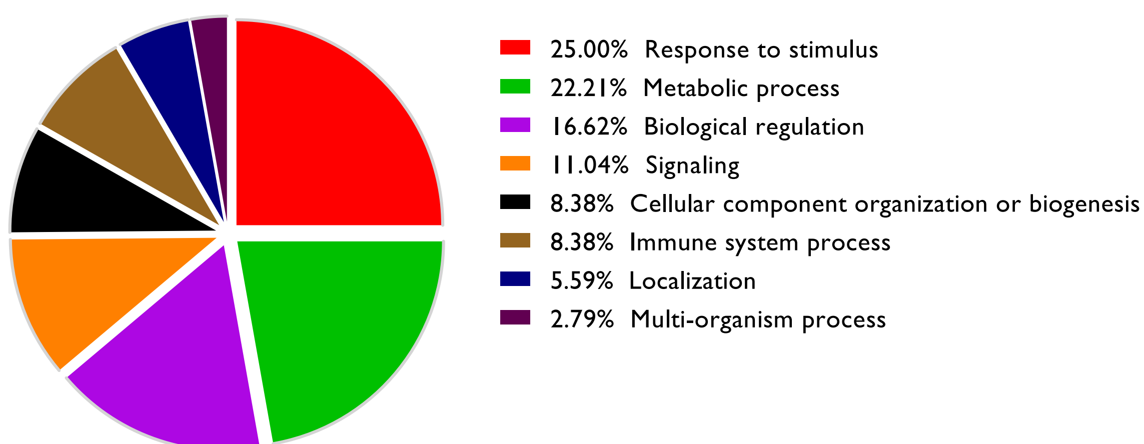


Figure 4.20 PANTHER GO-Slim Biological Process. The graph represents the percentages of identified protein classes based on the annotations data set in the PANTHER GO-Slim Biological Process. This analysis showed that 48 biological process hits are related to the 26 proteins. Response to stimulus and metabolic process are the first in the list, with 25 and 22.21%, respectively.

Cellular component

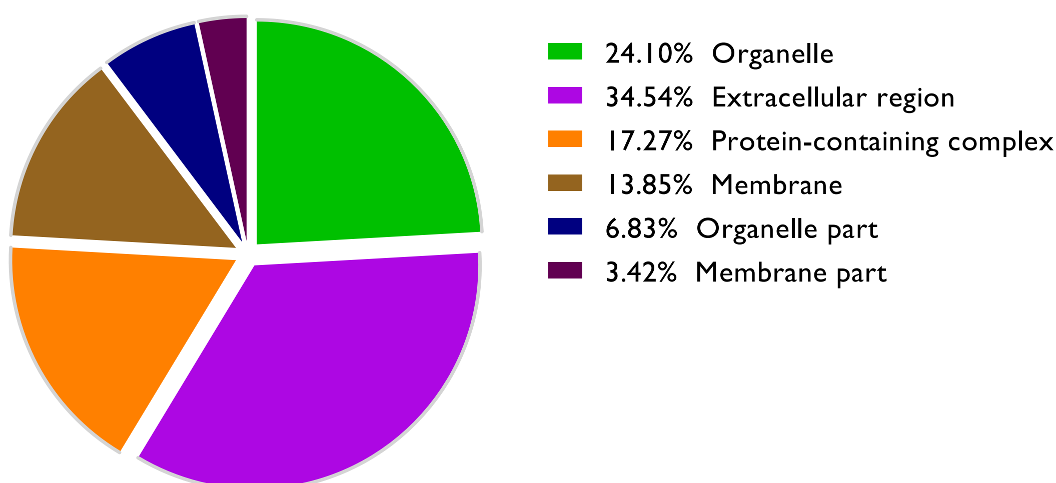


Figure 4.21 PANTHER GO-Slim Cellular Component. The graph represents the percentages of identified protein classes based on the annotations data set in the PANTHER GO-Slim Cellular Component. 55 cellular component hits were identified, which extracellular region and organelle are the most relevant for the 26 proteins.

Protein class

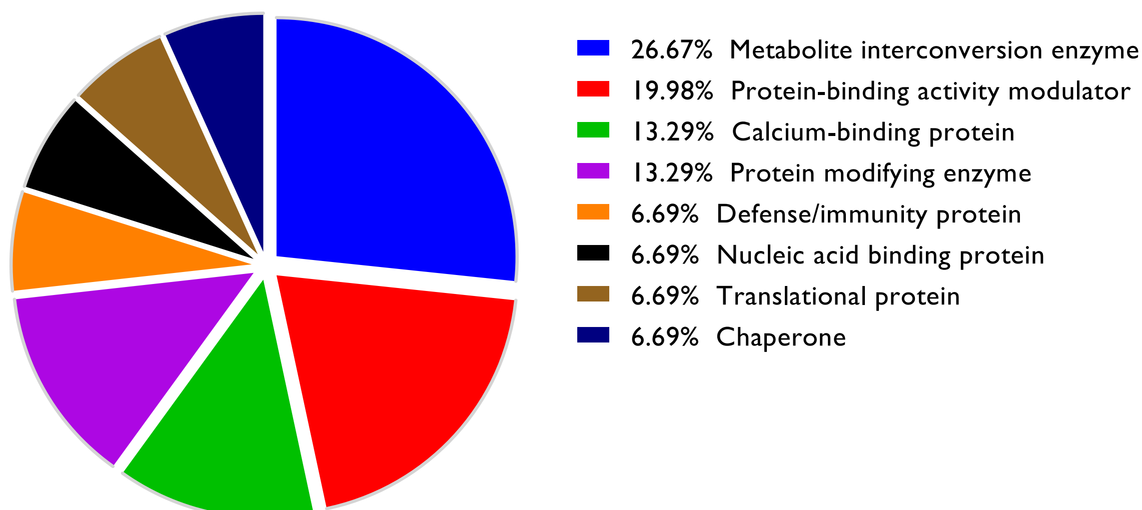


Figure 4.22 PANTHER GO-Slim Protein Class. The graph represents the percentages of identified protein classes based on the annotations data set in the PANTHER GO-Slim Protein Class. This analysis showed that 15 protein class hits are related to the 26 proteins statistically significant. Metabolite interconversion enzyme, protein-binding activity modulator and calcium-binding protein are the most relevant.

Pathways

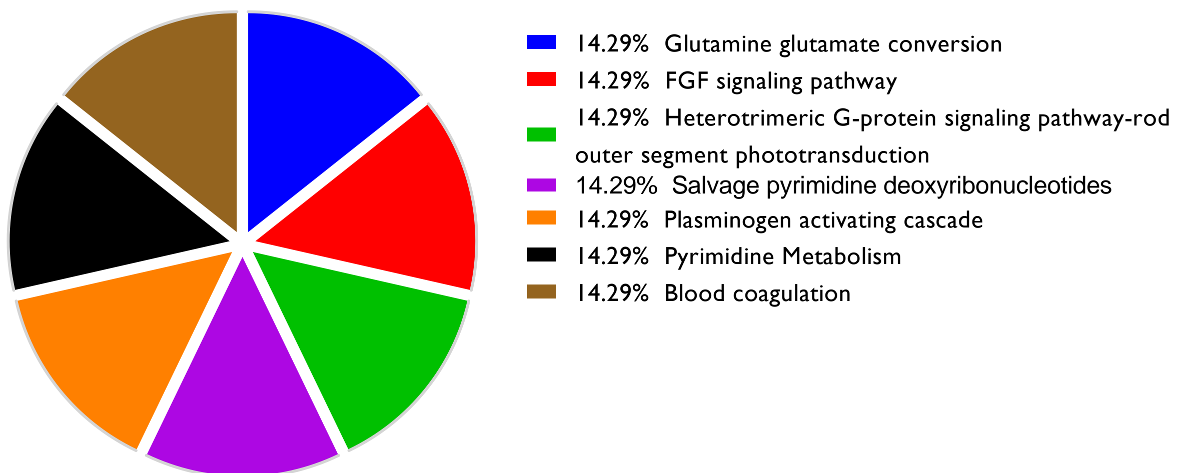
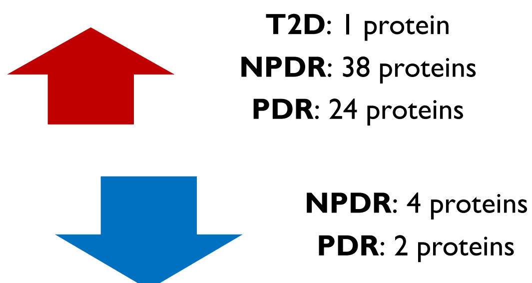


Figure 4.23 PANTHER Pathways. The graph represents the percentages of identified protein classes based on the annotations data set in the PANTHER Pathways. This analysis showed 7 pathways related to the 26 proteins, which each one has the same percentage (14.29%).



The upregulated and downregulated proteins for the three groups in relation to the control group are summarized above.

The T2D group had less changes, with only one upregulated protein, while the remaining two had a range of altered proteins, with a greater change observed in the NPDR group.

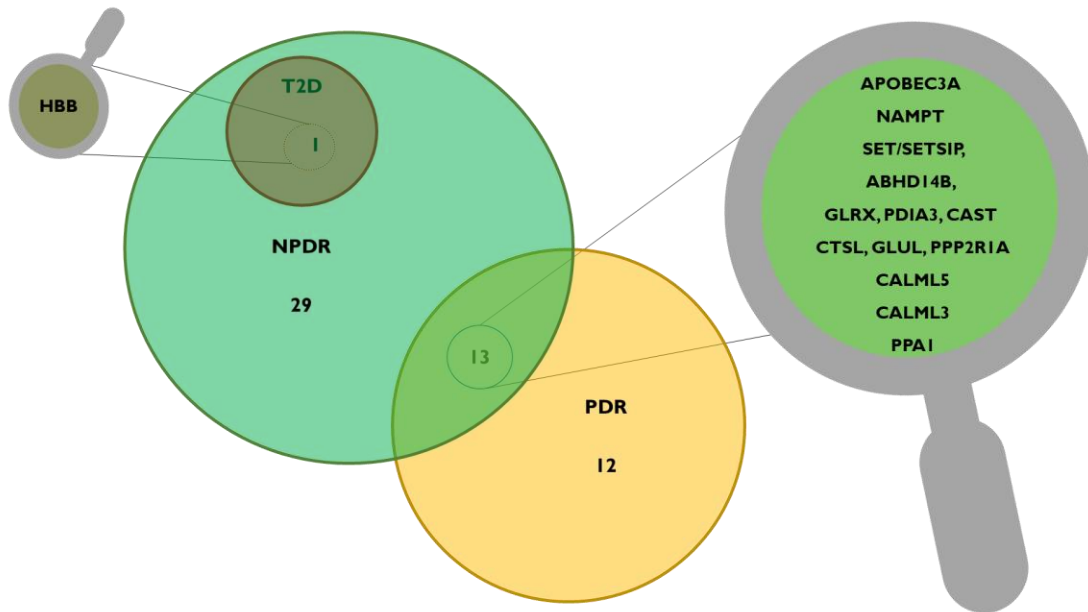
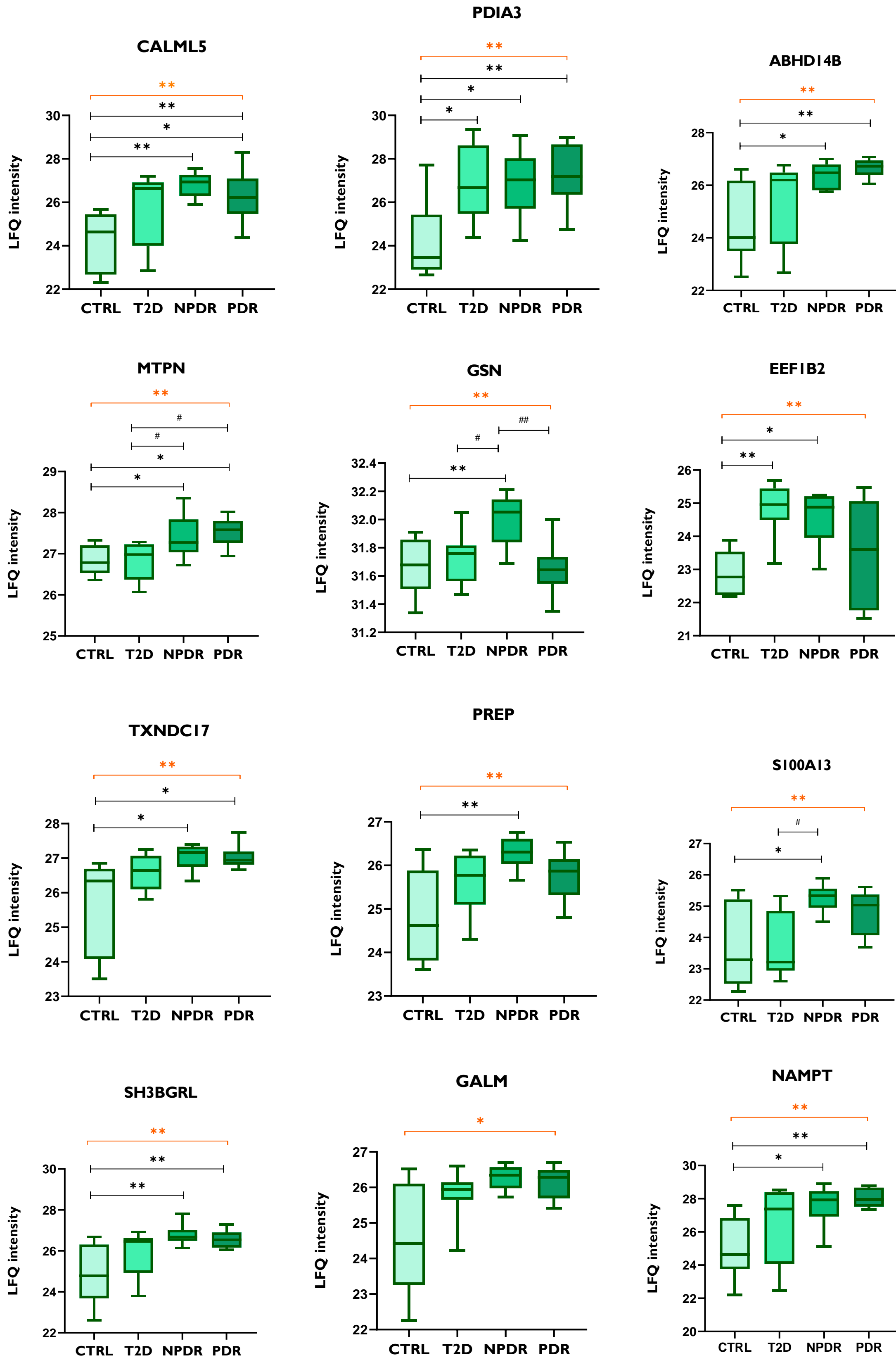
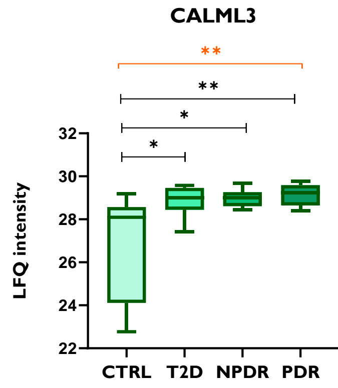


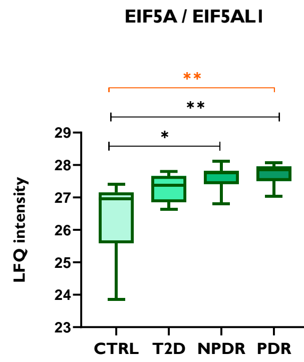
Figure 4.24 Common proteins between the T2D, NPDR and PDR groups. The figure indicates the number of statistically significant proteins for each of the 3 groups and mentions the respectively common ones. The NPDR and PDR group have 13 proteins in common.

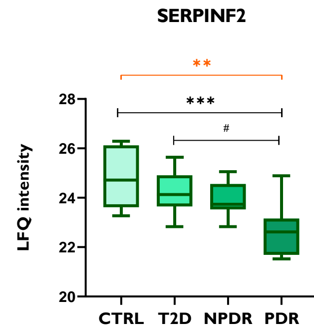
4.2.1.4 Multiple comparison

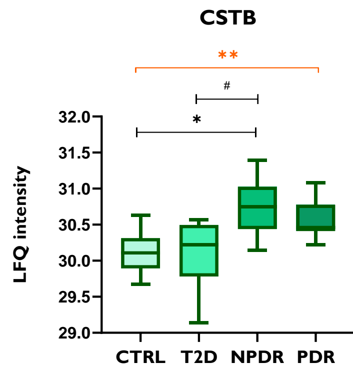
In order to compare all the groups with each other, an ANOVA test was performed, considering the LFQ intensities of the 682 quantified proteins, using S0=0 and FDR=0.05 as parameters. In this analysis, 32 proteins (**CALM5, PDIA3, ABHD14B, MTPN, GSN, EEF1B2, TXNDC17, PREP, S100A13, SH3BGRL, GALM, NAMPT, CALML3, EIF5A/ EIF5AL1, SERPINF2, CSTB, CTSL, PLIN3, S100P, TXNRD1, IMPA1, S100A11, PGD, HBB, GLUL, NQO2, APOBEC3A, CALM3, CFL1, SET\SETSIP, GLRX, NCCRP1**) were significantly changed (Fig. 4.25).

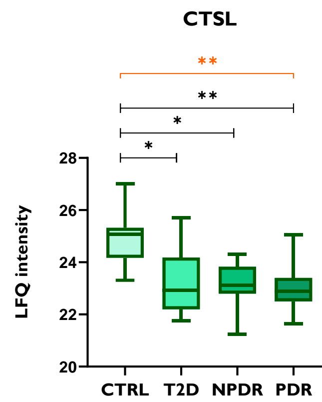

CALML5
PDIA3
ABHD14B
MTPN
GSN
EEF1B2
TXNDC17
PREP
S100A13
SH3BGRL
GALM
NAMPT
LFQ intensity
CTRL
T2D
NPDR
PDR

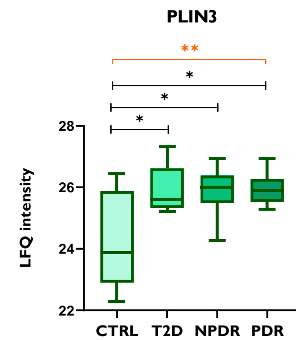

CALML3
**
**
*
*
LFQ intensity
32
30
28
26
24
22
CTRL T2D NPDR PDR


EIF5A / EIF5AL1
**
**
*
LFQ intensity
29
28
27
26
25
24
23
CTRL T2D NPDR PDR


SERPINF2
**
***
#
LFQ intensity
28
26
24
22
20
CTRL T2D NPDR PDR


CSTB
**
#
*
LFQ intensity
32.0
31.5
31.0
30.5
30.0
29.5
29.0
CTRL T2D NPDR PDR


CTSL
**
**
*
*
LFQ intensity
28
26
24
22
20
CTRL T2D NPDR PDR


PLIN3
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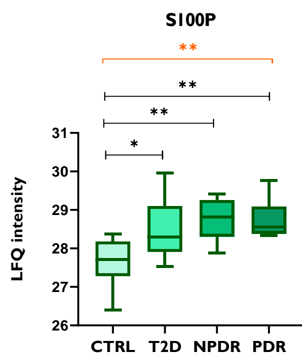
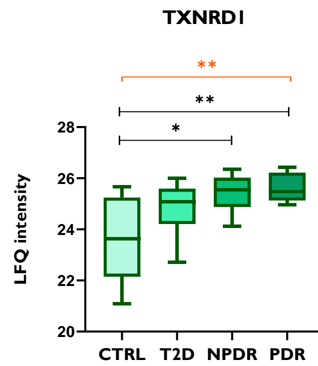
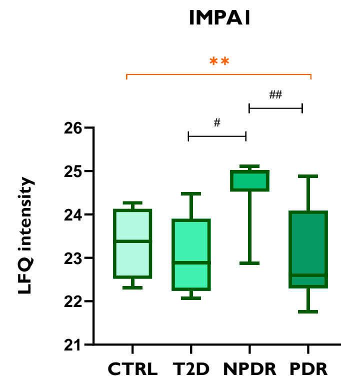
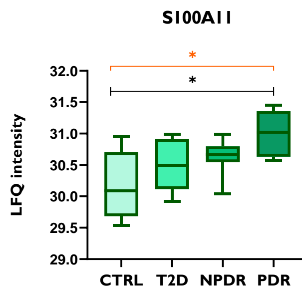
*
*
*
LFQ intensity
28
26
24
22
CTRL T2D NPDR PDR


S100P
**
**
**
*
LFQ intensity
31
30
29
28
27
26
CTRL T2D NPDR PDR


TXNRD1
**
**
*
LFQ intensity
28
26
24
22
20
CTRL T2D NPDR PDR


IMPA1
**
##
#
LFQ intensity
26
25
24
23
22
21
CTRL T2D NPDR PDR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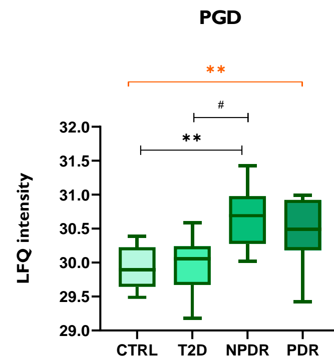
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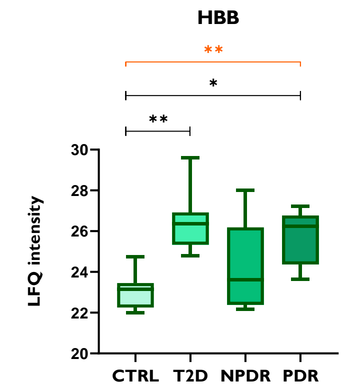
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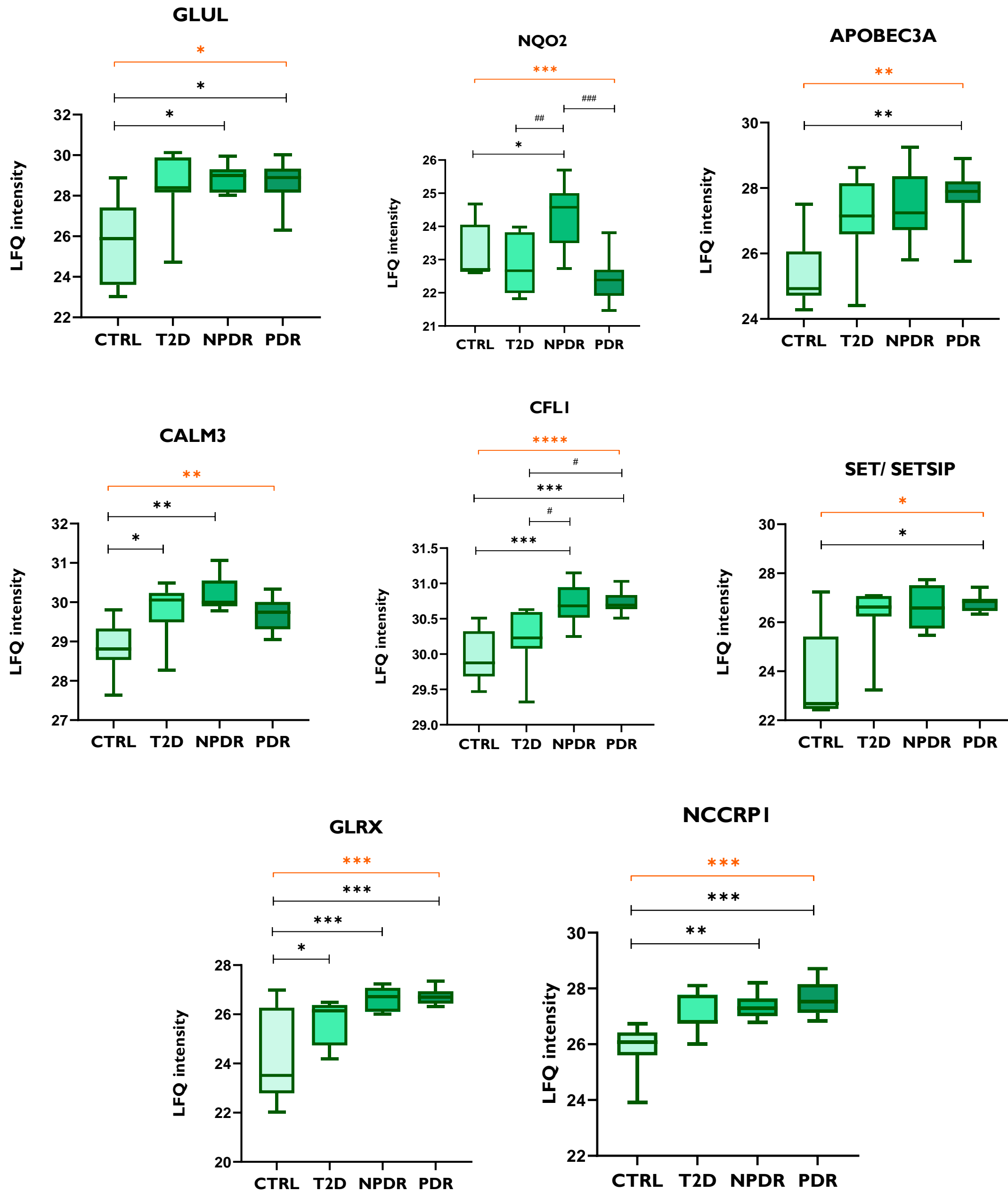

Figure 4.25 Multiple comparison of LFQ intensities of the 32 proteins. The graph above shows the comparisons of LFQ intensities between control subjects and diabetic subjects without and with retinopathy (NPDR and PDR) for the 32 statistically significant proteins in the ANOVA test. *P<0.05; **p<0.01; ***p<0.001 vs CTL, $^{\#}$P<0.05; $^{\#\#}$p<0.01; $^{\#\#\#}$p<0.001 vs T2D. In this analysis the proteins PDIA3, S100P, GLRX and CALML3 are significantly differently expressed in diabetic subjects without and with retinopathy vs control subjects, while ABHD14B, TXNDC17, SH3BGRL, NAMPT, EIF5A/EIF5AL1, TXNRD1 and NCCRP1 are significantly differently expressed only in diabetics with retinopathy vs control subjects, with a tendency to be associated with the disease progression.

For a better understanding of the analyzes carried out with the statistically significant expressed proteins, was performed a comparison of those commonly expressed among diabetics without (T2D), with retinopathy (NPDR and PDR) in relation to the proteins highlighted in the multiple comparison. From this analysis, it was possible to identify that some proteins are present in all analyzes.

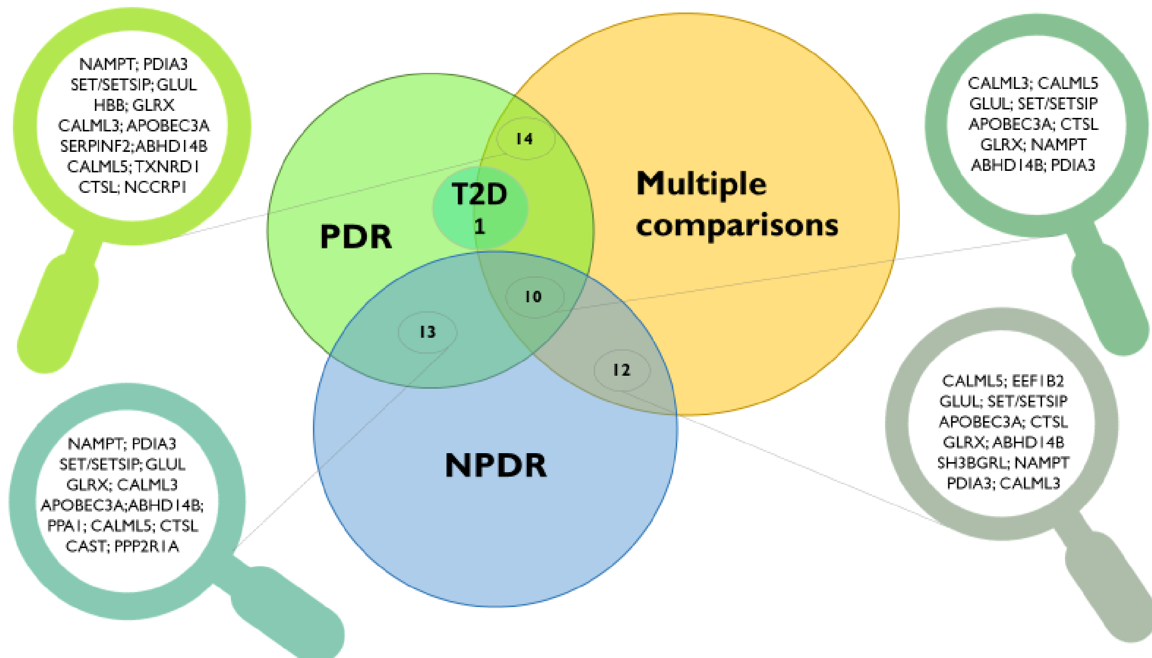

Figure 4.26 Common statistically significant proteins. This image represents the correlation between the statistically significant proteins that were differently expressed in diabetic subjects without and with retinopathy related to controls, as well as the proteins statistically significant highlighted in multiple comparisons. It is shown that 10 proteins (CALML3, CALML5, GLUL, SET/SETSIP, APOBEC3A, CTSL, GLRX, NAMPT, ABHD14B and PDIA3) are differently expressed in common among the two analysis (NPDR and PDR vs controls, as well as multiple comparisons). On other hand, 12 and 14 proteins are commonly statistically significant in NPDR vs control and PDR vs control, respectively related to multiple comparisons proteins. Previously was shown that 1 protein is common to T2D and NPDR vs control, and 13 proteins are common to NPDR and PDR vs control.

After identifying the differently expressed proteins among the groups and make the comparisons between each other, a protein interaction analysis of the significantly expressed proteins was performed using STRING database version 11.0.

From the 13 common proteins between diabetics without and with different stages of retinopathy (NPDR and PDR), the database identified 9 proteins with just one interaction between 2 of them (Fig. 4.27)

From the 32 significant differently expressed proteins in multiple comparison, STRING database identified all proteins with 18 interactions. According to the same database analysis, this network has more interactions than expected (Fig. 4.28) and the description of these proteins are presented in Table 5.

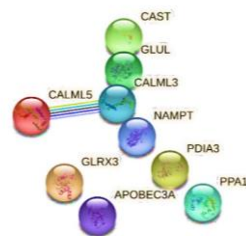

Figure 4.27 Interactome of common proteins between NPDR and PDR. Each node of this image represents the statistically significant proteins that were differently expressed among NPDR and PDR subjects vs control subjects. In this network, CALML5 and CALML3 are the only proteins that interact with each other.

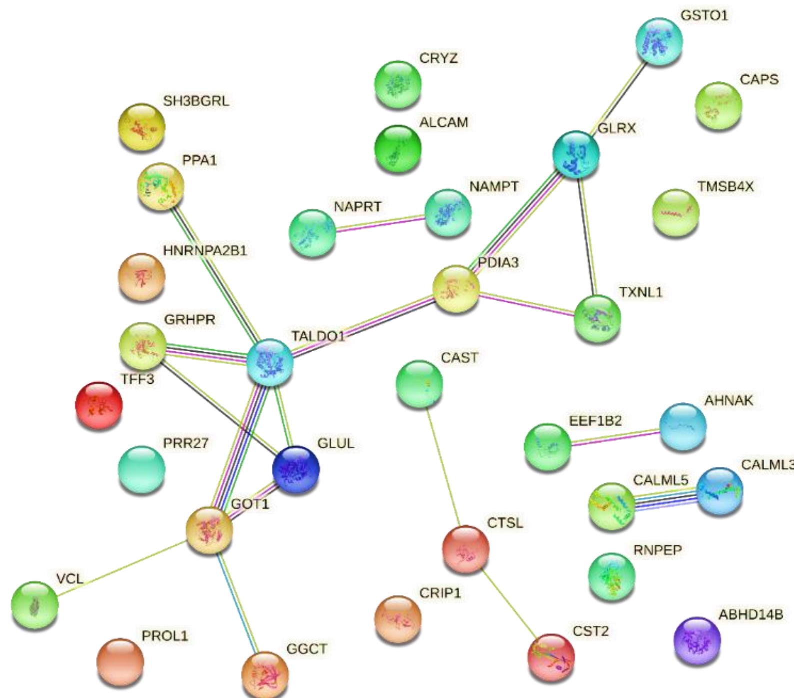

Figure 4.28 Interactome of common proteins between NPDR and PDR. Each node of this image represents the statistically significant proteins that were differently expressed in multiple analysis.

The image shows that although some proteins don't interact with anyone, others interact with many of them in a cluster manner or in a isolated manner.

Table 5- Description of proteins with interactions between each other among health subjects and diabetic subjects without and with retinopathy.

Gene name	Protein name	Function and biological process
AHNAK	Neuroblast differentiation-associated protein AHNAK	Neuronal cell differentiation
EEF1B2	Elongation factor 1-beta	Stimulate the exchange of GDP bound to EF-1-alpha to GTP
CALML3	Calmodulin-like protein 3	Binding activity; regulation of neurotransmitter levels and transport; phosphatidylinositol signaling system
CALML5	Calmodulin-like protein 5	Binding activity; may be involved in terminal differentiation of keratinocytes
CAST	Calpastatin	Protease inhibitor
CTSL	Cathepsin L1	Important for the overall degradation of proteins in lysosomes
CST2	Cystatin-SA	Thiol protease inhibitor

GGCT	Gamma-glutamyl cyclotransferase	Catalytic activity; play a significant role in glutathione homeostasis; induces release of cytochrome c from mitochondria with resultant induction of apoptosis
GOT1	Aspartate aminotransferase	Biosynthesis of L-glutamate and regulation of glutamate levels.
GLRX	Glutaredoxin-1	Cell redox homeostasis
TXNL1	Thioredoxin-like protein 1	Cell redox homeostasis
GLUL	Glutamine synthetase	Regulation of neurotransmitter levels and transport
GRHPR	Glyoxylate reductase/hydroxy pyruvate reductase	Enzyme with hydroxy-pyruvate reductase, glyoxylate reductase and D-glycerate dehydrogenase enzymatic activities
TALDO1	Transaldolase	Balance of metabolites in the pentose-phosphate pathway
VCL	Vinculin	Actin filament (F-actin)-binding protein; regulates cell- surface E-cadherin expression; play important roles in cell morphology and locomotion

NAMPT	Nicotinamide phosphoribosyltransferase	Catalytic activity; NAD biosynthesis pathway with the resultant component acting as a cytokine with immunomodulating properties and as an adipokine with anti-diabetic properties; neuron death
NAPRT	Nicotinate phosphoribosyltransferase	Catalytic activity
PDIA3	Protein disulfide-isomerase A3	Cell redox homeostasis
PPA1	Pyrophosphatase 1	Catalytic activity

4.3 MULTIPLEX

We assessed the levels of MMP2, 3 and 9 in the tears with the multiplex bead array assay and compared them between the control, T2D, NPDR and PDR groups (Fig. 4.29).

For MMP-2, the levels given in mean ± SEM were12.87 ± 2.67 pg/mL, 12.17 ± 1.87 pg/mL, 16.84 ± 2.91 pg/mL, and 9.13 ±1.2 pg/mL for the CTRL, T2D, NPDR and PDR groups, respectively. Although not statistically significant, MMP-2 levels have a decrease tendency correlated to the disease progression, except for the NPDR group whose levels appear more dispersed among the samples, with a slight tendency to increase (Fig. 4.29). The MMP-3 levels in the tears was significantly increased in the group of T2D patients without ($p<0.01$) or with retinopathy (NPDR,$p<0.001$ and PDR,$p<0.0001$) compared to the control (Fig. 4.29).

MMP-9 levels in tears of T2D with DR (NPDR ($p<0.05$) and PDR ($p<0.01$)) were also significantly higher than those in control group (Fig. 4.29), but no significant differences were found between control and T2D groups.

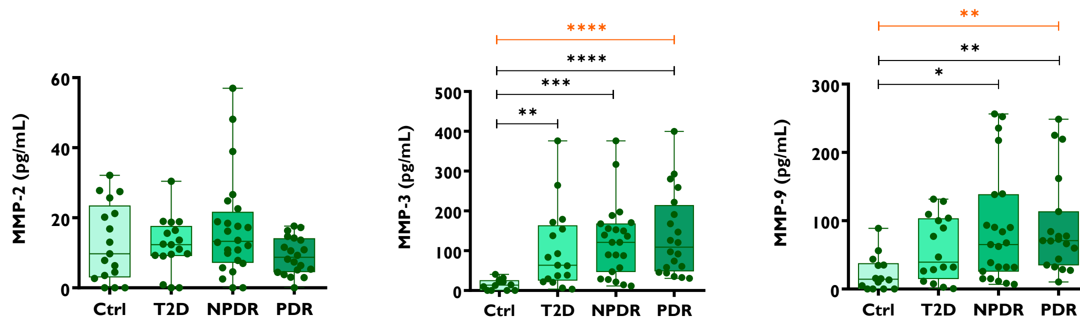

Figure 4.29 The levels of MMP2, 3 and 9 of control, T2D without ad with NPDR or PDR. The figure indicates that, in general, levels of MMP's can be related to the stages of the disease, with statistically significant differences for MMP-3 and MMP-9 levels between groups according to D´Agostino and Pearson normality test, and Kruskal-Wallis test (*$P<0.05$; **$p<0.01$; ***$p<0.001$ vs Ctrl). MMP-2 levels were not statistically significant but there is a tendency towards a decreasing profile in relation to disease progression. A greater dispersion of MMP-2 levels is observed in control and NPDR groups, while PDR group shows higher consistency of levels. On the other hand, levels of MMP-3 and MMP-9 are only consistent in the control group.

4.4 Zymography

Since we found significant changes in MMPs levels, we next investigated whether their levels were correlated with their activities. We assessed the activities of MMP-2 and MMP-9 by gelatin zymography. We did not find significant differences in the activity of MMP-2 or MMP-9 between groups, although a tendency toward increase was observed.

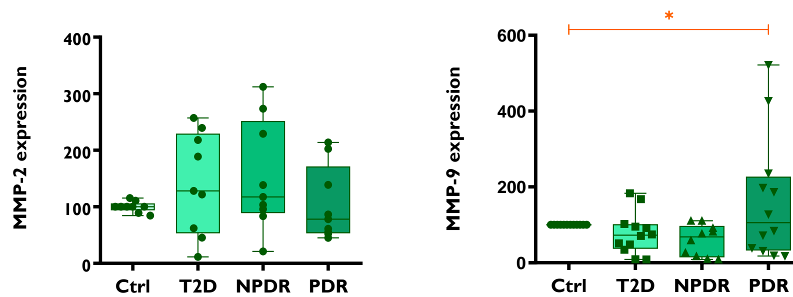

Figure 4.30 MMP-2 and -9 activity in tear samples from Control, T2D without and with NPDR or PDR. n=8-12 per group

4.5 EXTRACELLULAR VESICLES

As MMPs can be enriched in exosomes, we performed a pilot study, in which the vesicles from tears were isolated and characterized. The size and concentration of the exosomes were determined by NTA and visualization was performed by TEM. The concentration mean and modal of exosomes are presented in Table 6.

Table 6- Concentration, mean and mode of exosomes isolated from tears from control, T2D, NPDR and PDR groups

	Concentration (particles/mL)	Size (nm)	
	Mean ± SEM	**Mean ± SEM**	**Mode ± SEM**
CTRL	$4.65 \times 10^9 \pm 2.52 \times 10^8$	233.2 ± 6.7	114.2 ± 1.1
CTRL	$1.96 \times 10^9 \pm 9.98 \times 10^7$	204.2 ± 8.0	201.5 ± 23.5
T2D	$1.94 \times 10^9 \pm 1.00 \times 10^8$	185.5 ± 7.5	164.6 ± 15.9
NPDR	$5.44 \times 10^9 \pm 3.47 \times 10^8$	280.2 ± 5.2	135.2 ± 7.2
NPDR	$1.70 \times 10^9 \pm 7.91 \times 10^7$	188.2 ± 5.3	142.1 ± 16.3
PDR	$7.52 \times 10^8 \pm 4.50 \times 10^7$	194.7 ± 8.5	132.9 ± 14.5
PDR	$3.01 \times 10^9 \pm 1.62 \times 10^8$	178.1 ± 1.8	109.4 ± 5.1

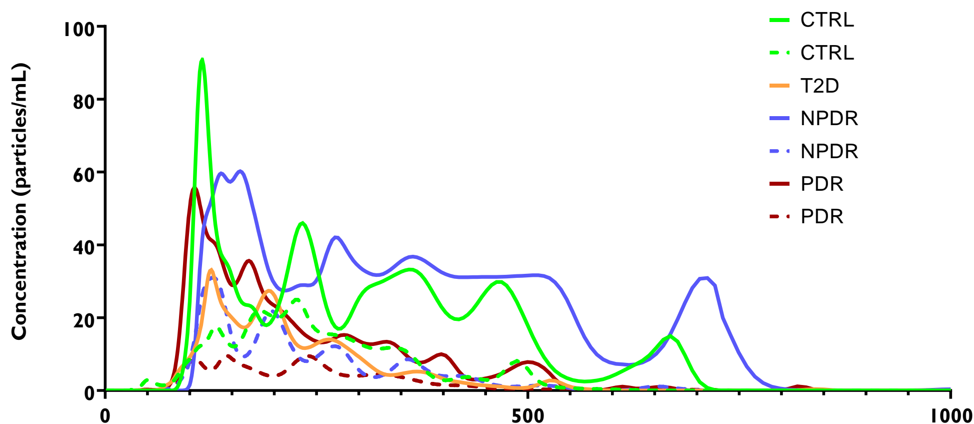

Figure 4.31 Nanoparticle tracking analysis showing the concentration and size of exosomes from human tears using Total Exosome Kit

Western blotting of harvested exosomes detected the presence of the cd63 exosome marker, approximately between 50-60 kDa (Fig. 4.32).

Figure 4.32 Western blot detection of CD63 (exosome marker) in exosomes isolated from human tears.

Tear samples and vesicles isolated from tears exhibit a spherical shape when observed by transmission electron microscopy (Fig. 4.33). In general, there was no significant differences between tear samples and exosomes isolated from them. Regarding the observed characteristics, the vesicles vary in sizes and density.

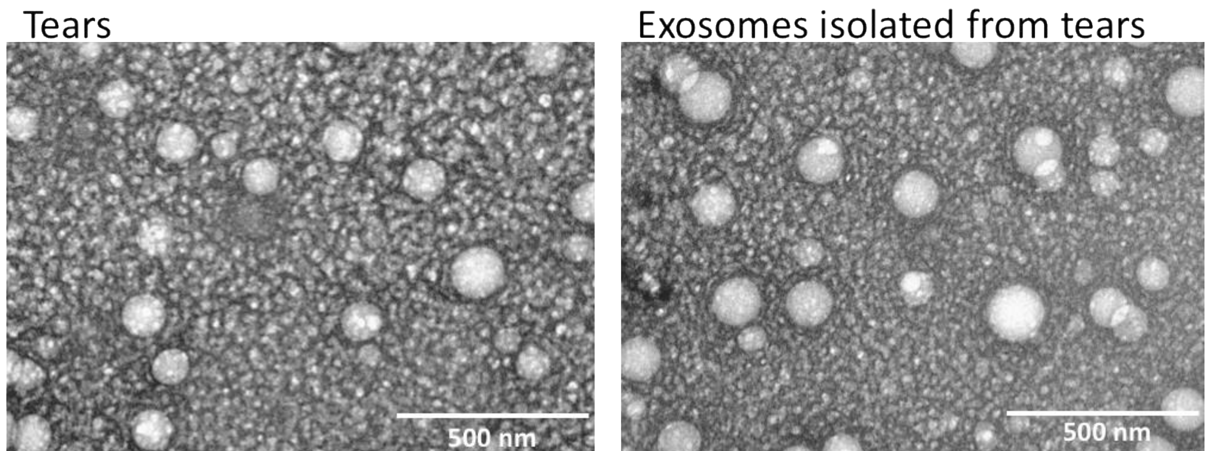

Figure 4.33 Submicroscopic structures in a tear samples of T2D patient.

Due to the very small number of samples analyzed (1-2 samples/ group). We did not perform a detailed characterization of the vesicles. However, it is possible to observe the presence of dense aggregates of macromolecules in the vesicles (Fig. 4.33). Some of the vesicles had morphological characteristics of exosomes, mainly cup-shaped, with sizes between 110-200 nm (Fig. 4.33). Inside of some of the vesicles surrounded by a well-defined membrane, some electrodense granules were detected.

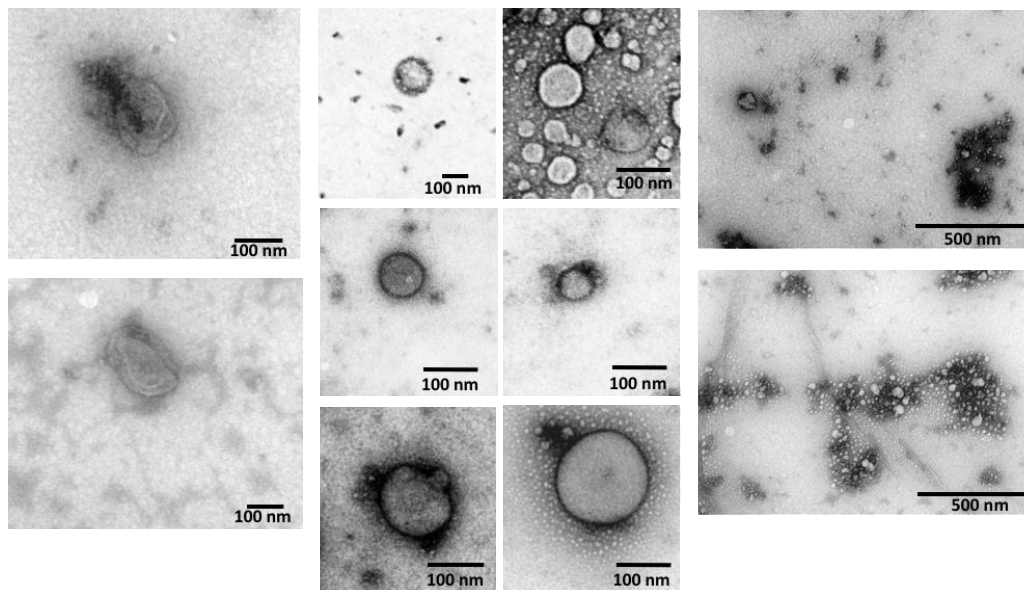

Figure 4.34 Morphological structures found in tears or isolated from tears using the Total Exo kit.

There were other rounded structures, of low-density, without surrounding membrane, of different sizes, arranged in an isolated or grouped way and eventually inserted into each other. Rounded structures were also observed, arranged in isolation, with a high-density outer membrane, with sizes ranging from 55-140 nm. Some structures have a spherical and / or irregular shape of high-density with an enclosing capsule, while other structures of medium density have round shape, arranged in a grouped manner, with some low-density aggregates inside, sizing between 70-230 nm. There are also other structures of size between 24-44 nm of low density and external membrane.

In order to evaluate whether extracellular vesicles contained MMPs, we performed a Western Blotting. We found that the active form of MMP9 is present in extracellular vesicles (Fig. 4.35). No band was detected for MMP2 (data not shown).

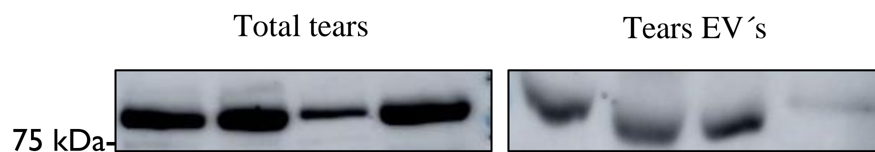

Figure 4.35 Western blot detection of MMP-9. Active form of MMP-9 was detected (~ 82 kDa) in total tears and tear EVs

DISCUSSION

5. DISCUSSION

In the recent years, the interest on tears as a potential source of biomarkers and for diagnosis of several diseases has been increasing, due to its relatively simple composition compared to other body fluids, such as blood and serum. Besides that, a gentle collection of tears, such as the one that is carried out with Schirmer test strips, is considered a noninvasive method that enables the assessment of tear components (proteins/AMPs and inflammatory mediators), giving us a better insight into the pathophysiology of diabetic retinopathy. In this study, we used the Schirmer test, that is used in clinical practice, for proteomics analysis and assessment of proteins of interest by a bead -based multiplexed immunoassay. We identified a panel of 34 proteins/AMPs/inflammatory mediators differentially expressed in tear fluid when we performed a multiple comparison with the four groups included in this study: control healthy controls, T2D patients without retinopathy, T2D patients with NPDR and T2D with PDR.

We found decreased total protein concentrations in tears from T2D without DR and with NPDR compared to the control group. Although no significant differences in total protein concentrations between controls and diabetics without retinopathy were previously described, our data is in agreement with previous reports showing lower total protein concentrations in tears of patients with NPDR compared to healthy controls (Éva Csősz 2012, Nurul Hafizah Amil-Bangsa 2019). However, contrary to what was previously stated (Éva Csősz 2012), we did not observe any decrease in total protein concentration in tears from PDR group compared to NPDR. The tear collection method used in this study was different from the ones used in the studies above mentioned. The type of tears under analysis was also distinct (basal vs reflex), since it depends on the approach used for collection. When comparing to other studies, another and important issue is related with the samples processing, namely the need to extract the proteins from Schirmer strips, which is not required when tear collection is performed with glass capillaries.

Although it was not performed a detailed study, including a McMonnies´s dry eye questionnaire or other questionnaires, to confirm some of the symptoms associated with dry eye, mainly, foreign body sensation or itching, we performed a diagnosis as having dry eye based on Schirmer´s test. In addition, we performed another tear function assay, the

TBUT. Schirmer and TBUT values were significantly reduced in NPDR and PDR groups, meaning an impairment of tear fluid production and stability in DR. These results are corroborated by some reports (David L. DeMill 2016, Garzón P. Sandra Johanna 2019) which indicate that diabetic individuals are prone to dry eye, compared to age-matched healthy individuals, and that with the progression of DM and DR, the risk is even greater. Chronic hyperglycemia, peripheral autonomic neuropathy, reduced insulin levels and microvascular dysfunction, are risk factors which in diabetic subjects cause the decreased density of neuronal fibers of lacrimal glands and cornea, modifications of cornea and conjunctiva epithelium, increased osmolarity of the tear film (Xinyuan Zhang 2016), which altogether contribute to an inflammatory environment. These results suggest that dysfunction of the lacrimal functional unit may be related to the progression to dry eye disease in T2D patients.

Studies indicate that the inflammatory environment created by dysfunction of LFU, and the pathophysiological changes of diabetes/ diabetic retinopathy, can be associated to changes with inflammatory mediators, including the metalloproteinases. These are an important class of endopeptidases that have a variety of functions in physiological and pathological processes (Jones 2014). Of particular interest are the gelatinases (MMP-2 and MMP-9) and the stromelysin (MMP-3). It is described that the metalloproteinase have a possible dual role in development and progression of DR (Kowluru 2010, Kowluru 2012). In the pre-neovascularization phase, MMP-2 and -9 play a critical role in mitochondrial damage, causing apoptosis of endothelial cells in the retina, while in the following stages, facilitate neovascularization (Renu A Kowluru 2012). Increased levels of MMP-2 and -9 are reported to be increased in diabetic subjects and in individuals with dry eye (Stephen C Pflugfelder 2017). Here, the results indicate a trend towards a positive correlation as much as between levels and activity of MMPs in tears, and the stages of DR.

Thus, our results demonstrate that the routine use of tests to lacrimal function unit and ocular surface can be valuable for the routine follow-up of diabetic patients, as an approach of screening, preventing and early treating other eye complications of diabetes. On other hand, the results corroborate that in addition to the underlying

pathology, diabetic retinopathy can somehow have an impact and/ or be reflected in the function of the lacrimal functional unit and in the tears composition.

In a first approach when we compared the control group with each group while just one protein was changed in diabetic subjects without retinopathy, 29 and 12 proteins were exclusively changed among NPDR and PDR subjects, respectively. Besides that, 13 proteins were commonly changed among diabetic subjects with different stages of retinopathy.

32 proteins were differently expressed in the multiple comparison between the 4 groups, which 10 (CALML5, GLUL, SET/SETSIP, APOBEC3A, CTSL, GLRX, NAMPT, ABHD14B, PDIA3 and CALML3) of them were common with the first analysis performed.

These proteins are not known to be abundant in tears and, generally are proteins described as being related to stress oxidative response, immunomodulatory properties, relevant in angiogenesis and healing process (Éva Csősz 2017). For example, the proteins TYMP, GLUL, SERPINF2 and WARS1 form a cluster related to blood vessel morphogenesis and development, and these proteins were found to be significantly changed in PDR group compared to the control group. With this information, we can infer a probable implication in the pathogenesis of retinopathy. CTSL, TYMP, APOBEC3A and NCCRP1 form a cluster related to carbohydrate derivative catabolic process, while CTSL, RAB1A and NCCRP1 a cluster related to to glycoprotein metabolic process. These findings are relatively attractive because the levels of these proteins were shown to be significantly changed when a multiple comparison test was performed between the 4 groups, implying a relation with diabetes progression and microvascular complications such as diabetic retinopathy. IGKV3D-11, IGKV2D-24 and CTSL are involved in the production of molecular mediator of immune response, immunoglobulin production and adaptive immune response processes, with the first 2 proteins being significantly changed only in the PDR group compared to the control group.

In general, many of these proteins have already been described as present in tear fluid in previous proteomics studies. However, a complex analysis involving healthy,

diabetic individuals without retinopathy and with the various stages of retinopathy, to our knowledge, had not yet been performed.

A previous study reported 20 proteins differently expressed in tears from diabetic individuals compared to health individuals by the ESI-Q-TOF MS/MS analysis. Among these, 2 were up-regulated (beta-2 microglobulin and DJ-1 protein) and the others were down-regulated (S100A4/A8/A9, adenine phosphoribosyl transferase isoform, envelop protein, keratin 31, SAP1 protein, lipocalin 1-like-1, lipocalin, cytokeratin 4, lipocalin 1 precursor, HSP27, beta globin phosphohistidine phosphatase, phosphohistidine phosphatase). In other studies, NPDR patients had reduced levels of lipocalin-1, HSP27, beta- microglobulin in tears and increased levels of endothelin and neuron-specific enolase, while PDR subjects had increased levels of nerve growth factor, APOA1, lipocalin 1, lactotransferrin, lacritin, lipophilin A and Immunoglobulin lambda chain (Hyun-Jung Kim 2012) . Some of these proteins were also identified in our study, although without statistically significant difference, probably due to the small sample size. Nevertheless, these proteins are described as being involved in immune processes, inflammatory responses and oxidative stress, in general, they are described as abundant proteins in the tear fluid, it is not well known if they have a direct implication in diabetic retinopathy pathophysiology. However, certain aspects such as the type of study and groups involved, as well as, the type of tears collected, the extraction procedures, and the proteomics techniques chosen for each type of study, must be considered in this comparative analysis between the present study and those previous studies.

One of the enrichments analyzes, the GO cellular component, showed that most of the quantified proteins are associated with the extracellular space and are present in extracellular vesicles, including exosomes. This data corroborates with a study that states that proteins from the tear fluid (from principal and accessories lacrimal glands, as well as from cells of ocular surface) are mostly from the extracellular region, whereas proteins from the lacrimal fluid (exclusively from lacrimal gland) are mostly from the cytosol, followed by the extracellular region. Considering that the tears were collected using the Schirmer test, the samples contain proteins secreted not only by the tear glands, but also by the epithelial cells of the ocular surface, stromal immune cells and meibomian

gland acinar cells, justifying the results obtained. On the other hand, a study of isolation of exosomes in the tears of healthy individuals revealed results that indicate that exosomes in tears are produced by epithelial cells (A. E. Grigor'eva 2016).

Since most of the proteins reliably quantified are associated with extracellular vesicles and that MMPs can be enriched in exosomes, we performed a pilot study, in which exosomes from tear fluid were isolated and characterized. We found the presence of structures with typical characteristics of exosomes both in the total tear fluid or in samples of isolated exosomes, However, it was also possible to visualize other distinct structures. Studies indicate that exosomes-isolation using a precipitation-based method, like that one that was used in this study, can result in the presence of contaminating structures named "non vesicles", microparticles, cell debris and macroaggregates (A E Grigor'eva 2017). In this work, the isolation method consists in the use of a polymer, usually polyethylene glycol, to dehydrate and precipitate the vesicles. However, not only them are precipitated but there is also coprecipitation of other extracellular vesicles, protein aggregates and extracellular proteins. As expected, we detected MMP-9 in the exosomes from tears of the control group. A larger sample size with all the remaining groups is necessary for a better insight of exosomes from tear fluid in DR. It would be interesting to carefully evaluate and characterize the isolated vesicles, not only from the physical point of view but mainly from the point of view of composition, in order to understand what information these vesicles may be carrying and from which cells are derived, eventually using omics methodology to study tears vesicles.

In this study we identified several proteins that are changed in the context of DR. Due to the small sample size, a study with a larger population should be performed in order to validate our results. Due to the ease of tear collection method, using Schirmer test strips, and well patient adherence to it, a more detailed analysis of the tears is worth. The identification of a set of biomarkers can improve early diagnosis of DR and ensure prompt treatment for this vision-threatening disease.

CONCLUSIONS AND FUTURE PERSPECTIVES

6. CONCLUSIONS AND FUTURE PERSPECTIVES

Tears proteomic analysis can provide valuable information that can infer a panoramic and holistic image of a health eye, both anterior and posterior segment. This may be useful in clinical practice regarding stratification of patients at risk for ocular complications of diabetes as well as for screening, diagnosing, prevention of disease progression and eventually for a better selection of individualized treatment. Several previous studies have already shown some potential biomarkers from tears for some diseases, be they ocular or systemic. Therefore, research for new information or little clarified about physiology, pathophysiology and/or with diagnostic importance, can be provided with studies of same nature. According to Willcox et al (2017) and Zhou et al (2012) the lacrimal proteome has about 1800 and 1500 proteins, respectively, of which very few have been identified as altered in pathological processes. Proteins commonly found altered in tears have been described to be essentially associated with cell adhesion, cell growth, immune response, antimicrobial response anti-apoptotic process and protein binding.

Concerning DR, much remains to be investigated so that robust conclusions can be reached. The question of standardizing the techniques of tears sampling, extraction and processing is crucial for validating the results. Our initial hypothesis was that tears could be a source of useful biomarkers in the for diagnosing, staging and monitoring the progression of DR. Herein, this study showed that there is a need to be increasingly aware about ocular surface of diabetic patients. Our results indicate that even before the diagnosis of DR, there are changes in tear production and stability, with a tendency to worsen in a situation of long-term diabetes. Also, that certain proteins associated with inflammatory processes, oxidative stress and angiogenesis are found differently expressed in the tears of patients with NPDR. Therefore, special attention to the ocular surface may be a special and urgent component in the follow-up and management of both diabetic patients and diabetic patients with DR, which can be extremely important before serious complications arise. Wherefore, study of other functions of the ocular surface and tears are necessary for more robust conclusions about the effect of diabetes and DR at this level and so any other useful conclusions for the diagnosis can be drawn.

It is known that the duration of diabetes is one of the risk factors, if not one of the most important for the development and progression of DR. However, there is considerable

variability in the evolution and progression of retinopathy among diabetic individuals. Some diabetic individuals with long-standing diabetes, older than 15-20 years, do not develop DR. On other hand, some diabetic individuals in a few years of illness and with good metabolic control develop retinopathy, some of which evolve rapidly. In addition, there are diabetics with retinopathy who do not show good therapeutic outcomes for the options currently available. In this context, a similar longitudinal study would be important to ascertain the different phenotypes of DR and relate them to the possible biomarkers present in the tears, and to monitor them throughout the course of the disease.

With the evidence that MMPs are implicated in the pathophysiology of DR, it would be essential to make an analysis of the tissue inhibitors of MMPs (TIMPs) which are responsible for regulating the proteolytic activity of MMPs. Thus, an investigation would be made to access the association that may exist with DR. On other hand, it would also be useful to evaluate levels and activity of MMPs in a longitudinal approach in order to better understand the role of these proteins in the progression of the disease and their relevance for diagnosis.

In this study, aspects such as sampling at different times of the day (morning vs afternoon), use of different eyes, age-match in certain assays, the predominance of a certain gender, the medication and lifestyle that individuals have and previous therapeutic interventions can presuppose a high variation between individuals and, therefore, the results should be analyzed with some caution. On other hand, most of the proteins found as differently expressed were highlighted in the group of diabetics with NPDR, which can be particularly interesting and lead us to interrogate the reason behind a greater number of changes occur in that group compared to the others. Despite these factors, with a small sample, it was possible to obtain interesting and statistically significant results, relatively new in relation to the previously described on this matter. Even so, the fact that they are not representative and reflect only a small part of the study that can be carried out cannot be ruled out. Therefore, a larger number of samples, as well as an in-depth and individualized analysis of the proteins identified as differently expressed in the various groups and a better study of the association between extracellular vesicles and DR stages are aspects that are undoubtedly should be taken into account in a near future.

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7 REFERENCES

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