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UNIVERSITY OF MOLISE

Department of Medicine and Health Sciences "V. Tiberio"

PhD in

TRANSLATIONAL AND CLINICAL MEDICINE

XXXIV CYCLE

SSD: MED/30 - OPHTHALMOLOGY

PhD Thesis

NEUROPROTECTION IN DEGENERATIVE EYE DISEASES

Tutor:

Prof.

Ciro Costagliola

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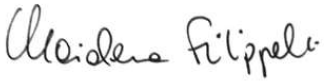

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ABSTRACT

It is estimated that worldwide there are around 285 million people of all ages with visual impairment; among these, 39 million are blind. There is a strong association between increasing age and visual impairment. Diabetic retinopathy (DR) and glaucoma are among the most important causes of vision loss in high-income regions of Central/Eastern Europe. Glaucoma affects more than 70 million people worldwide and it leads to progressive optic nerve degeneration, with a gradual loss of retinal ganglion cells (RGCs). DR is one of the most frequent complications of diabetes mellitus and it is now acknowledged as a neurodegenerative disease of the retina. In the early stages of DR, selective RGCs loss is observed without evident micro-vascular changes. Thus, glaucoma and DR share the development of progressive neurodegeneration. Therefore, a clear knowledge and understanding of the mechanism of RGCs death and how to compensate these events is essential for preserving the sight of those who are affected by these diseases. In recent decades, with the purpose to give an effective answer and solution to this issue, there was a strong boost in the field of neuroprotection. The term neuroprotection refers to any therapy that prevents, retards, or reverses apoptosis associated to neuronal cell death following primary neuronal lesions. The aim of our work was to prove whether cotreatment with citicoline and homotaurine has neuroprotective effects on cell survival in primary retinal cultures under experimental conditions mimicking retinal neurodegeneration similar to those occurring in glaucoma and in DR. In addition, the study aimed to establish the levels of pro-inflammatory cytokines and soluble mediators (TNF-α, IL6, IL2, and PDGF-AB) in 28 vitreous biopsies taken from patients with proliferative diabetic retinopathy and treated with increasing doses of curcumin (0. 5 and 1μM), with or without homotaurine (100μM) and vitamin D3 (50 nM). Primary cultures were derived from the retina of fetal rats and tested with citicoline plus homotaurine (100 μM). Thereafter, neurotoxicity was triggered using excitotoxic levels of glutamate and high glucose concentrations. The effects on retinal cultures were evaluated by cell viability [(3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide -MTT reduction assay] and immunodetection of apoptotic oligonucleosomes (Cell Death Detection ELISAPLUS kit). Then, in order to evaluate the anti-inflammatory effect of curcumin, homotaurine, and vitamin D3, ELISA tests were conducted on the supernatants from the 28 vitreous biopsies that were incubated with the bioactive molecules at 37◦C for 20 h. It was also tested, in a subset of four vitreous, the expression of pro-inflammatory genes and mitogen-activated genes. The concentration of the soluble mediators was calculated from a calibration curve and expressed in pg/mL. Shapiro-Wilk test

was used to verify the normality of distribution of the residuals. Continuous variables among groups were compared using the General Linear Model. Homoscedasticity was verified using Levene and Brown-Forsythe tests. Post- hoc analysis was also performed with the Tukey test. A $p \leq 0.05$ was considered statistically significant. The results proved that a combination of citicoline and homotaurine synergistically decreases proapoptotic effects associated with glutamate- and high glucose-treated retinal cultures. In addition, it was shown that pro-inflammatory cytokines are associated with inflammation and angiogenesis, although there is a discrete variability in the doses of the mediators investigated among the different vitreous samples. Curcumin, homotaurine, and vitaminD3 individually have a slightly appreciable anti-inflammatory effect. However, when used in association, these substances modify the average levels of the soluble mediators of inflammation and retinal damage. Therefore, in the future, a multi-target treatment taking neuroprotective compounds may provide a therapeutic strategy for neurodegenerative eye diseases such as glaucoma and DR.

CHAPTER 1: RETINAL DEGENERATION- POTENTIAL THERAPEUTIC TARGETS AND FACTORS AIMING NEUROPROTECTION

1.1 Introduction

Neuroprotection is defined as the capacity for a treatment to prevent, retard or reverse neuronal cell death by intervening in and inhibiting the pathogenetic cascade that results in cell dysfunction and consequent death [1]. The concept of neuroprotection echoes and recalls that of cytoprotection. In fact, even in cytoprotective therapies, the target is the loss of the affected cell [2]. For several years, neuroprotection has been playing an important role in eye diseases, in particular for retinal degeneration. The composite and elaborate structure of the retina predisposes its physiology to disorders from several pathological factors. Diseases such as diabetic retinopathy and glaucoma impair vision in different ways; however, the neurodegeneration of retinal cells is common to all pathologies [3]. The process of neuronal cell death is due to several and different types of injuries and it involves apoptosis. Blocking the apoptotic cascade leading to cell death may avoid cell death and consequent loss following neuronal injury (neuroprotection). Loss of neural visual cells results in vision loss [4]. It is well known that retinal ganglion cells (RGCs) death occurs in several ocular diseases. Diabetes mellitus and glaucoma are the two major causes of selective RGCs death in the retina. These two diseases are also the most common causes of irreversible blindness worldwide. Blindness due to retina degeneration and optic neuropathies is not reversible since RGCs lack the ability for self-renewal and have a limited capacity for self-repair [5-6]. Therefore, understanding the mechanism of RGCs death and how to counteract these events is crucial for preserving the sight of those who suffer from these diseases [7]. The rationale of neuroprotection is to counteract the main pathways implicated in the RGCs apoptosis, including: the abnormal increase of excitatory neurotransmitters and reactive oxygen species (ROS); the deficiency of neurotrophins resulting from the blockage of the retrograde axonal transport from the lateral geniculate nucleus of the thalamus; the dysregulation of ion channel activities; the loss of intracellular self-repair processes. All these different pathways consistently lead to RCGs loss and glial cells activation [8]. Hence, neuroprotection strategies are needed to maintain neuronal integrity or to keep damaged cells functioning. Therefore, the identification and application of neuroprotective agents is a central focus of modern medicine.

1.2 Retina: hints of functional anatomy

The retina is part of the central nervous system (CNS) and is composed of neurons, glial cells, and blood vessels [9]. Thanks to the retina it is possible to capture and transmit the incoming photons throughout the neuronal pathways as both electrical and chemical signals for the brain to obtain a visual image. The retina is set on the posterior segment and forms the innermost edge among the other layers of the eye which are the choroid (vascular layer) and the sclera (fibrous layer) [10]. The retina originates embryologically from the optic vesicle [11]. The optical part of the retina consists of two sheets, one external (retinal pigmented epithelium) and one internal (neuronal retina). Retinal pigment epithelium (RPE) consists of a single layer of cuboidal epithelial cells. This layer is closest to the choroid and provides nourishment and supportive functions to the neural retina. The neuronal retina consists of nine layers (**Fig.1**), from the outside to the inside are distinguished :

1. Inner segment / outer segment layer: inner segments and outer segments of rods and cones (photoreceptors); the inner segments are rich in mitochondria (essential for high metabolic demands of the photoreceptor cells) [12];
2. External limiting membrane: separates the inner segment portions of the photoreceptors from their cell nuclei;
3. Outer nuclear layer: cell bodies photoreceptors;
4. Outer plexiform layer: rods and cones projections ending in the rod spherule and cone pedicle, respectively. These make synapses with dendrites of bipolar cells and horizontal cells. In the macular region, this is known as the Fiber layer of Henle.
5. Inner nuclear layer: contains the nuclei and surrounding cell bodies of the amacrine cells, bipolar cells, and horizontal cells;
6. Inner plexiform layer: contains the synapse between the bipolar cell axons and the dendrites of the ganglion and amacrine cells;
7. Ganglion cell layer: contains nuclei of RGCs, the axons of which become the optic nerve fibres, and some displaced amacrine cells.
8. Nerve fibre layer: axons of the ganglion cell bodies;
9. Inner limiting membrane: basement membrane developed by Müller cells [13].

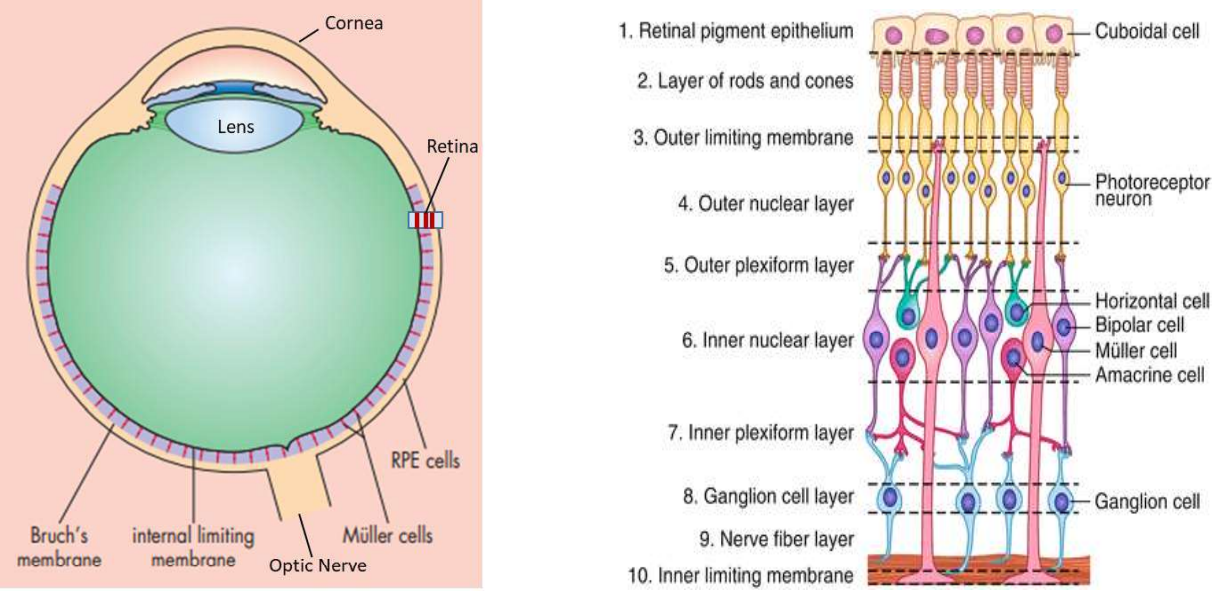

Fig.1 The eye and retinal layers [14-15]

Briefly, the neuronal component of the retina is constituted by six types of neurons: photoreceptors (rods and cones), bipolar cells, horizontal cells, amacrine cells, and retinal ganglion cells. These latter are the output cells of the retina that convey the visual signals to the brain visual targets [6]. With the exception of the fovea, ora serrata, and optic disc, the neural retina is organized in layers, determined by the orientation of the müllerian glia, its organizational backbone. Basically, there is the photoreceptor layer plus the bipolar and ganglion cell layer, which represent the outer first neuron and inner second neuron of the visual pathway (**Fig. 2**).

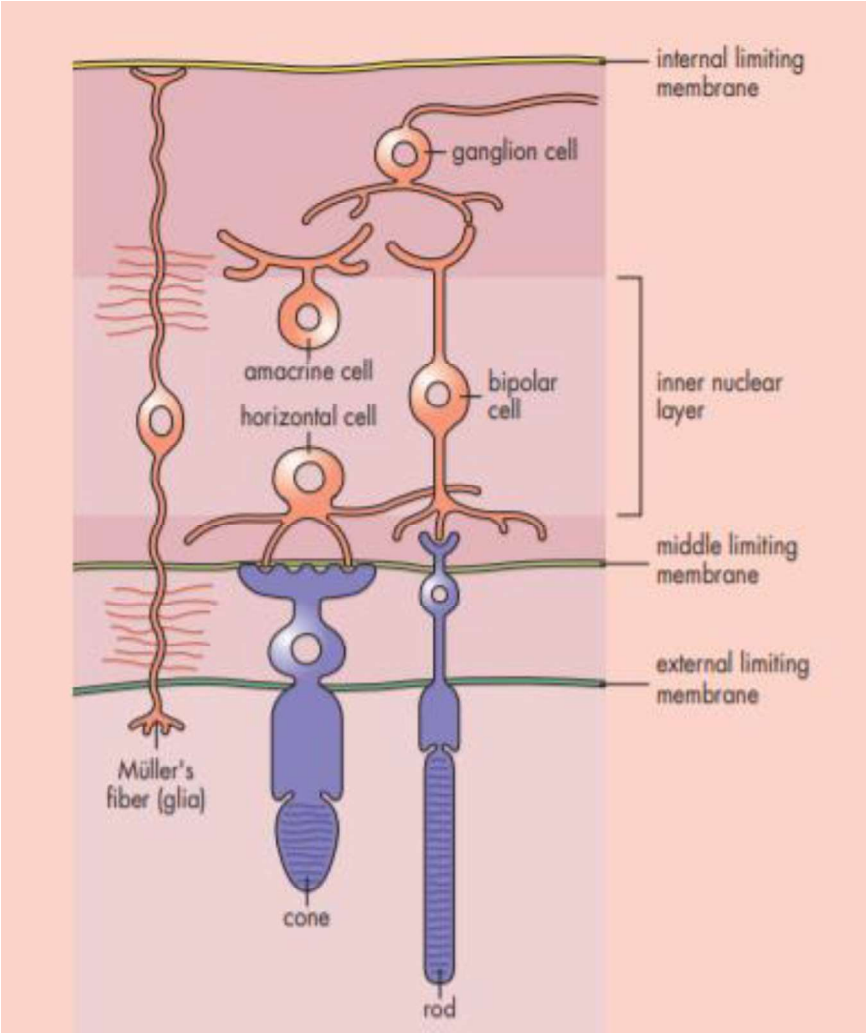

Fig.2 Neuronal connections in the retina and participating cells [15]

1.3 Retinal Ganglion Cells

In the human retina there are about 0.7 to 1.5 million RGCs [16]. These numbers may be different among individuals and as a function of retinal location. RGCs are distinguished by a soma from which the originating axon goes initially in the retinal nerve fiber layer (RNFL). Then, these axons converge turning into the optic disc, cross the lamina cribrosa at the optic nerve head (ONH), and constitute the optic nerve [17].

There are three classes of RGCs:

1. W-ganglion: small, 40% of the total, broad fields in the retina, excitation from rods. Detection of direction movement anywhere in the field.
2. X-ganglion: medium diameter, 55% of the total, small field, color vision. Sustained response.
3. Y- ganglion: largest, 5%, very broad dendritic field, respond to rapid eye movement or rapid change in light intensity. Transient response.

RGCs, based on their functions and projections, are divided into five main classes:

1. Midget cell (parvocellular, or P pathway; P cells)
2. Parasol cell (magnocellular, or M pathway; M cells)
3. Bistratified cell (koniocellular, or K pathway)
4. Photosensitive ganglion cells
5. Other ganglion cells project to the superior colliculus for eye movements (saccades) [18].

During gestation, RGCs lengthen their axons to synapse in target areas of the brain [19]. The capacity of RGCs to spread their axons decreases with age and the ability to regenerate their axons is lost early in development [20]. It should also be mentioned that RGCs are sensitive especially to neurodegenerative damage. This is because of the deficient mitochondrial dynamics and axonal transport, as well as oxidative stress and energy depletion, considering the high metabolic demand characteristic of these cells, mainly due to the asymmetric myelination [17]. Additionally, over the past few years, it has been widely deepened the reason why RGCs lose the ability to regenerate after an injury. These studies have shown that several factors are involved in this process of the transition from the rapid axon growth of immature neurons into the poor axon growth of mature neurons in the CNS. Among the factors most involved, there are cyclic adenosine monophosphate (cAMP), phosphatase and tensin homologue (PTEN)/mammalian target of rapamycin (mTOR), and Krüppel-like family (KLF) transcript factors [6]. Actually, several other transcription factors have also been studied for axon growth and regeneration [21]. An important role in the regulation of RGCs apoptosis is played by the tumor suppressor p53. It should be noted that the overstimulation of N-methyl-D-aspartate (NMDA) receptor activates a p53-dependent pathway of cell death [22]. Moreover, an environment defective in growth-promoting trophic factors is held jointly responsible for the failure to regenerate. In this regard, it is well known the importance of trophic factors, such as

nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and ciliary neurotrophic factor (CNTF), in promoting viability and axonal regeneration of RGCs [23-25]. Finally, a crucial way to understand and deepen the signaling pathways involved in the growth and axonal degeneration of the RGCs it has been just through the study of glaucomatous damage.

1.4 Glaucoma: neurodegenerative factors and potential therapeutic targets

Glaucoma is a neurodegenerative disease and it is described as a group of irreversible, progressive optic neuropathies that can lead to severe visual field loss and blindness due to a gradual loss of RGCs [26-28] (**Fig.3**). At present, more than 70 million people worldwide are suffering from glaucoma and it is expected that nearly 112 million people will be affected by glaucoma by 2040 [29]. The risk factors for glaucoma onset are multiple. Among these are increased intraocular pressure (IOP), family history of glaucoma, genetics, age, gender, race (non-white ethnicity), myopia, pseudoexfoliation, disc haemorrhage, vasospasm, systemic hypotension/hypertension, and smoking [30-32]. Although the pathogenesis is not entirely clear (i.e., mechanical/ischemic insult, neuroinflammation, etc.) [33], it is known that an increased IOP promotes collapse and compression of the optic nerve. Not by chance elevated IOP is a principal risk factor and it is the only one on which therapeutic action is currently possible. Management of elevated IOP is typically started with medical therapy. The latter consists of β-blockers, carbonic anhydrase inhibitors, α-agonists, miotics, and prostaglandin analogs. The elevated IOP leads to an altered axonal transport of RGCs and consequent growth factors deficiency, triggering RGCs apoptosis [34-36]. Additionally, neurotrophic factors deprivation [37] , elevated concentrations of excitatory aminoacids such as glutamate[38], and oxidative stress [39] may play an important role in RGCs apoptosis too. Current OCT studies suggest predominant damage of the inferior and superior optic nerve quadrants where M-cells are mainly located, with a relative sparing of the temporal quadrant (P-cells). This distribution of damage is similar to that of other neurodegenerative diseases such as Alzheimer's disease and multiple system atrophy [17]. It should be recalled that RGCs are terminally differentiated neurons and are unable to regenerate by cell division. Hence optic nerve damage is irreversible. Indeed, the main goal of neuroprotection is to keep these cells alive to boost the therapeutic benefit achieved by IOP lowering. However, several studies highlighted that even glaucomatous patients with IOP within the normal limits will progress in losing RGCs [40]. Therefore , since glaucomatous optic neuropathy can occur despite having ocular pressures

within a normal range, neuroprotection is a crucial tool in order to provide the possibility to counteract the irreversible loss of RGCs [41-43].

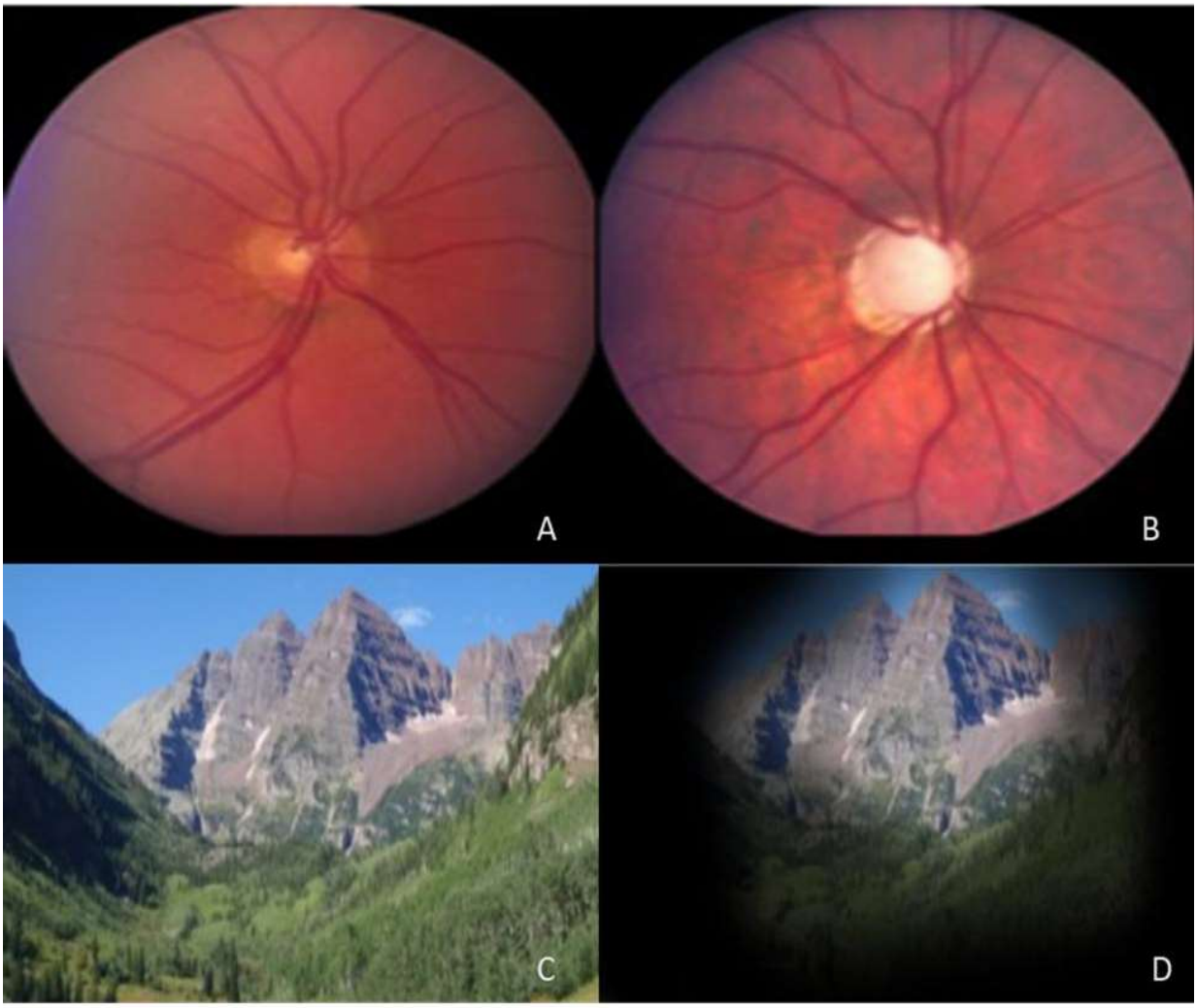

Fig.3 Comparison between a healthy patient and one with glaucoma [44- 45].

A)Healthy optic disk; B) glaucomatous optic disk ; C) normal vision; D) simulated vision in a patient with advanced glaucoma.

In recent decades, in the field of neuroprotection, there has been a great ferment. Becker B. and colleagues conducted one of the first studies to prove pharmacologic modulation of neural damage in glaucoma using oral administration of an antiepileptic agent called phenytoin [46]. This antiepileptic agent stabilizes the inactive state of voltage-gated sodium channels which

could modulate glutamatergic transmission, and it resulted neuroprotective in a rat model with elevated IOP [47]. Phenytoin has several actions on neurons and mainly it is involved in cell excitability, hence the neuroprotective action on RGCs could be achieved by improving excitotoxic N-methyl-D-aspartate (NMDA) action on third-order retinal neurons. However, this drug does not appear to have responded to the endpoints of several clinical trials [4]. Further neuroactive drugs that have antiexcitotoxicity action were tested in order to reach neuroprotection, among these there was an antagonist at NMDA receptors named memantine. Initially this drug was used to treat Parkinson's disease. Memantine binds only to open-state NMDA channels and has therapeutic benefits against excitoneurotoxicity mediated by the action of NMDA receptors. In the physiologic state, memantine does not prevent neuronal synaptic signal transmission, as it is competitive only for high levels of glutamate that result in neuronal damage and death through excitotoxic mechanisms caused by excessive activation of NMDA receptors [48]. Brooks DE and colleagues found that, in a dog glaucoma disease model, memantine decreased the level of free glutamate in the vitreous [49]. Morever, already in 1996, Dreyer EB et al. came to the conclusion that the level of glutamate detected in the vitreous body of the group of patients with glaucoma was much more elevated when compared with that in a control population [50]. These studies provided the basis for assuming NMDA receptor antagonists as a viable neuroprotection strategy for glaucoma. In 2000–2006, it was conducted a phase III randomized, placebo-controlled trial to evaluate the efficacy of memantine in 1179 patients aged 18–82 years old [51]. However, this trial did not meet endpoint significance versus placebo control. This was probably due to the study design. Nevertheless, some data emerged and the memantine demonstrated statistically significant visual field benefit under high dose conditions compared to lower dose [4, 51].

Several studies have shown that the neurotoxic effect of NMDA is mediated by calcium influx into neural cells, causing apoptosis and cell death [52]. Thus, calcium-channel blockers (CCBs) seem to be an effective way to achieve neuroprotection in glaucoma. Conceptually, CCBs rescue RGCs by blocking cell death mediated by calcium influx and by increasing local blood flow in ischemic tissues by inducing vasodilation [53]. On the other hand, a possible drawback of CCBs is that although they may increase blood flow, these agents may affect the autoregulation of blood circulation at the optic nerve head (ONH) during acute IOP elevation [54]. In this regard it should not be forgotten that oral CCBs required for systemic hypertension

may be detrimental for ONH in patients suffering from glaucoma; hypotension seems to diminish ONH blood flow, which is one of the risk factors for the onset of glaucoma [55].

Another molecule with neuroprotective activity appears to be brimonidine (a selective alpha-2 receptor adrenergic agonist). Since RGCs are rich in alpha-adrenergic receptors brimonidine may counteract RGCs death by direct interaction with alpha-2 adrenergic receptors, leading to reduced accumulation of extracellular glutamate and blockade of NMDA receptors [56-57].

Evidence in the literature highlighted a possible role of oxidative stress in RGCs degeneration [58]. Aqueous humor of eyes with glaucoma have: lower levels of antioxidants [59] and elevated oxidative stress markers [60]. Moreover, in the plasma of glaucoma patients have been detected decreased plasma levels of glutathione [61], increased lipid peroxidation products [62], and antibodies against glutathione-S-transferase [63]. In addition, tissue analysis studies comparing cultured human trabecular meshwork (TM) from non-glaucomatous eyes to that with glaucoma demonstrated higher concentrations of reactive oxygen species, reduced cell membrane potentials, and decreased ATP production in the TM of eyes with glaucoma [64]. Presumably, an insufficient reactive oxygen species (ROS)-neutralizing mechanisms underlie the ROS accumulation in the TM [65-67]. Therefore, antioxidants (e.g Coenzyme Q10, vitamin E, polyphenolic flavonoids, anthocyanosides) through the inhibition of ROS and the up-regulation of cell defense systems can improve RGCs survival [68-71].

It has been suggested that Nitric Oxide (NO) is involved in RGCs degeneration [72-74]. In the retinas of rats with induced glaucoma have been detected levels of NO to twice normal values [75]. There are three forms of nitric oxide synthase (NOS): NOS-1 (neuronal NOS) and NOS-3 (constitutive NOS) act as vasodilators or neurotransmitters in normal retinal tissue, while NOS-2 (inducible NOS) concurs to RGCs neurotoxicity [76]. According to some Authors, NOS-2 inhibitor (e.g aminoguanidine, N-nitro-L-arginine) resulted effective in delaying RGCs degeneration [73, 77-79]. However, the data obtained so far on NOS inhibitors are inconclusive and controversial [80-82]. For completeness, it must be said that cannabidiol (the non-psychotropic component of marijuana) and synthetic cannabinoids (tetrahydrocannabinol and HU-211) have been proved to hold neuroprotective actions thanks

to their ability to reduce the formation of lipid peroxides, nitrite/nitrate and nitrotyrosine. [83-86].

In recent years, citicoline (cytidine 5′-diphosphocholine)—a naturally occurring (endogenous) compound—has been studied in neurology and in ophthalmology. Citicoline, which is metabolized in the body to cytidine and choline, is active in the biosynthetic pathway of cell membrane phospholipids and it is able to increase levels of neurotransmitters in the central nervous system (CNS) producing neuroprotective effects in different CNS injury models [87-90]. In particular, because it is a precursor of the neurotransmitter acetylcholine, it is a basic component in the synthesis of the major neuronal membrane phospholipid, phosphatidylcholine. Therefore it is critical to guarantee structural integrity and facilitate both cell signaling and transport across the cell membrane. Citicoline has been reported as a neuroprotective molecule acting through mechanisms relevant to glaucoma and diabetic retinopathy. The effects proposed to explain the neuroprotective activity of citicoline have been completely reviewed and include reduction of glutamate excitotoxicity and oxidative stress, antiapoptotic effects, neurotrophic properties, the elevation of neutrofins levels, improvement of axonal transport, effects on nonglutamatergic neurotransmitter systems, enhancement of the release of neurotransmitters such as norepinephrine (noradrenaline) and dopamine, effects on remyelination, improvement of mitochondrial function including cardiolipin synthesis, restoration of membrane integrity, and modulation of insulin signaling [89-93]. Nuclear magnetic resonance studies have also highlighted that malfunction of the cholinergic system in the visual pathway may be a relevant component of the pathophysiological mechanisms involved in glaucoma. Thus, the dysfunction of the cholinergic system supports the significance of choline supplementation [94-97]. Current studies have correlated the neuroprotective activity of citicoline to its ability in activating sirtuin-1 (SIRT1) which is crucial for neuronal plasticity, cognitive functions, as well as protection against aging-associated neuronal degeneration, and cognitive impairment [91,92]. Recently, it has been demonstrated that a daily intake of a fixed combination of citicoline, homotaurine, and vitamin E in addition to the topical medical treatment significantly increased the total score of the contrast sensitivity test and the quality of life in patients with open angle glaucoma [98].

Homotaurine (3-amino-1-propane sulphonic acid, tramiprosate) is an analogue of 4-aminobutyrate (γ-aminobutyric acid, GABA). It is a small natural aminosulfonate compound identified in various species of marine red algae and then chemically synthesized and introduced into clinical use under the name of tramiprosate [99]. Homotaurine has

neuromodulatory and neuroprotective effects, with the capacity to interfere with the course of amyloid-related diseases by binding to the soluble amyloid protein. This latter capacity is important in glaucoma as it has been seen that amyloid plaques tend to accumulate also at the level of the RGCs inducing their apoptosis [100]. Moreover, thanks to its affinity to GABA A receptors, homotaurine modulates cortical inhibitory activity by lowering the response of neurons to excitatory stimuli of glutamate [101-102]. A current pilot study highlighted that oral administration of homotaurine, forskolin, carnosine, and folic acid improves IOP in patients with primary open-angle glaucoma [103].

Vitamin E is a member of the family that contains lipid-soluble tocopherols and tocotriols and essential micronutrients with strong antioxidant activities. In patients affected by glaucoma, a daily intake of Vitamin E was effective in reducing the progression of the disease [104].

Finally, another class of molecule extensively studied for neuroprotection in glaucoma is represented by Neurotrophic Factors (NTFs). To NTFs belong: nerve growth factor (NGF), ciliary neurotrophic factors (CNTF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), neurotrophin 4/5 (NT4/5), glial cell-derived neurotrophic factor (GDNF) family, and the neuropoietic cytokine family. Several preclinical studies have proved that topical or intravitreal NTFs may inhibit, slow, or reverse RGCs death in animal models of experimental glaucoma [8, 42, 105]. However, NTFs use for the treatment of retinal and/or optic nerve diseases are restricted because of the short half-life in vivo and low bioavailability. Therefore, in order to achieve therapeutic effects in the posterior ocular segment multiple topical or intravitreal administrations of these compounds are required. This was the major limitation of this neuroprotective approach. Currently, to exceed this limit and in order to gain therapeutic levels of neuroprotective agents in the target sites are developing different drug delivery systems [8]. It is therefore clear that there are many pathways on which action should be taken. Therefore a multi-target approach is the most valid one for the purpose of substantial neuroprotection (**Fig.4**).

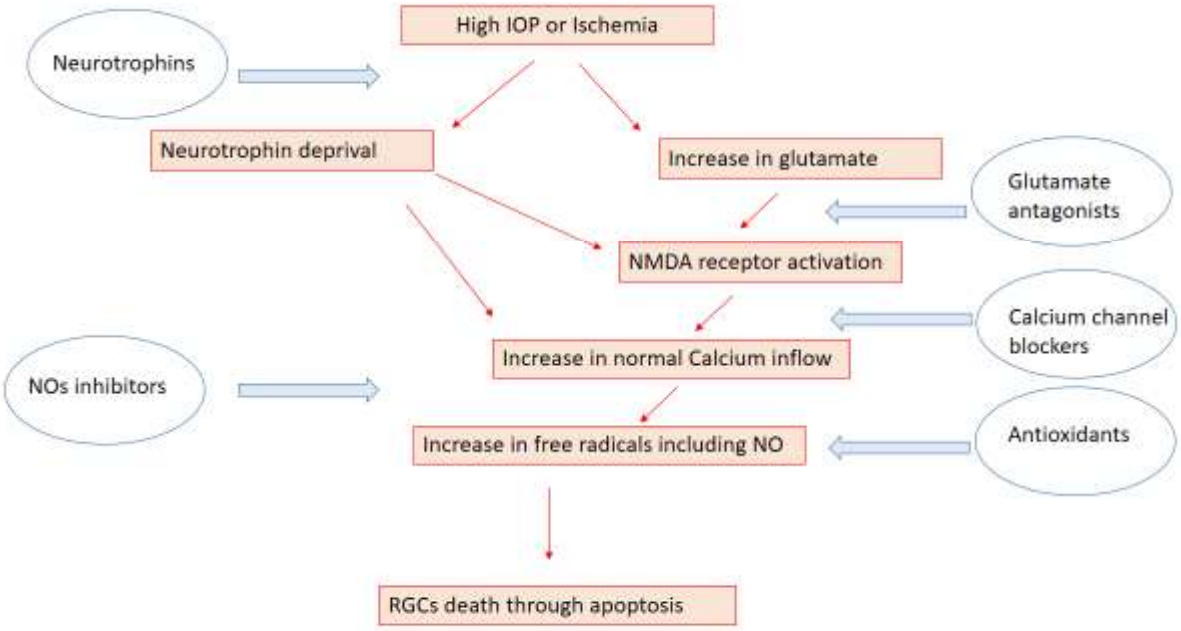

Fig.4 Simplified pathway of RGCs death and assumed mechanism of neuroprotective agents [86].

IOP, intraocular pressure; NMDA, n-methyl-D-aspartate; NOS, nitric oxide synthase; RGCs, retinal ganglion cells.

1.5 Diabetic retinopathy: neurodegenerative factors and potential therapeutic targets

Diabetic retinopathy (DR) is one of the most common complications of diabetes mellitus and is one of the leading causes of preventable vision loss and blindness in the adult working-age population worldwide. Progressive blindness results from the long-term accumulation of pathological abnormalities in the retina of diabetic patients (**Fig.5**).

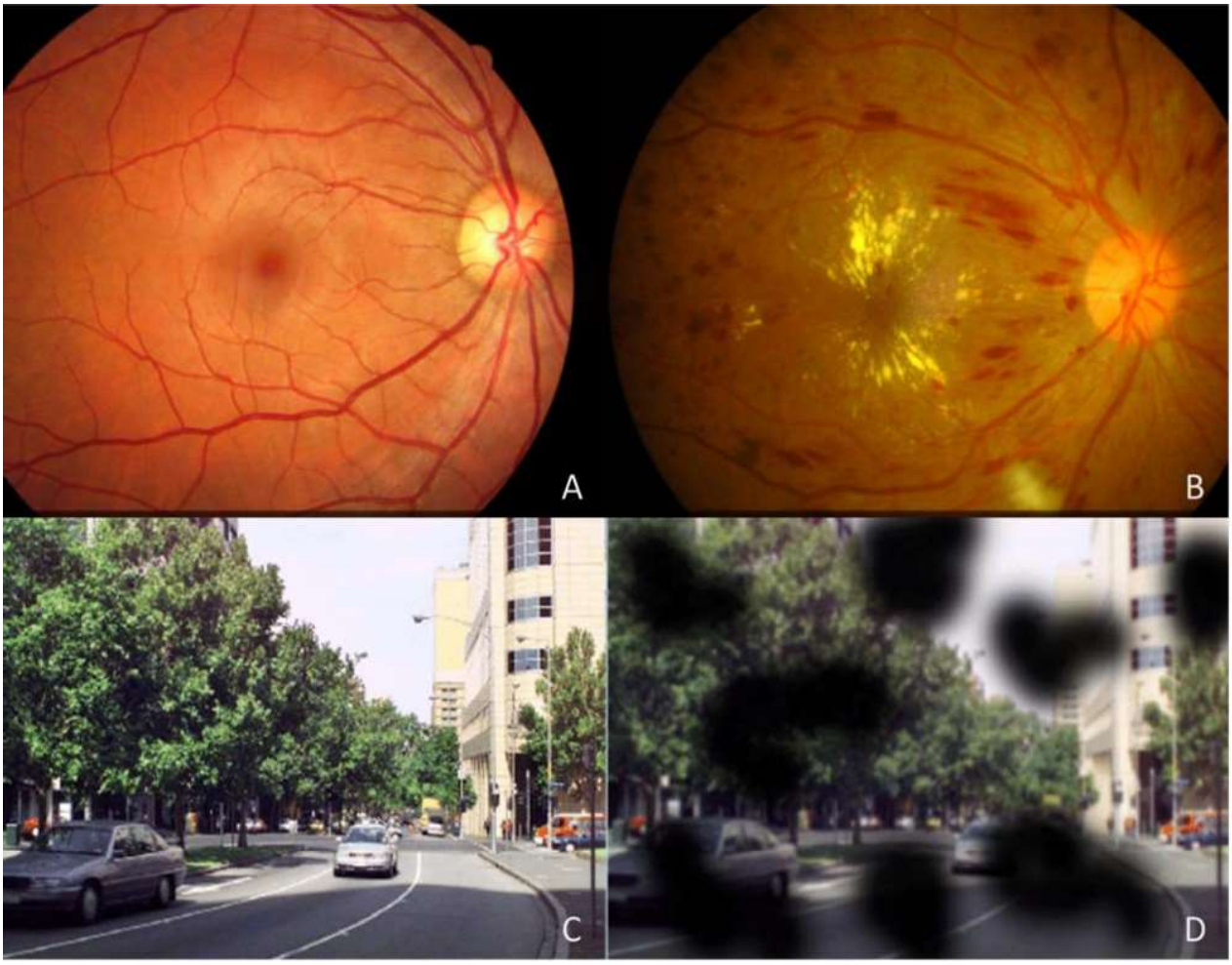

Fig.5 Comparison between a healthy patient and one with diabetic retinopathy [106-107].

a)Normal retina; B) Diabetic retina; C) Normal vision; D) simulated vision in a patient with advanced diabetic retinopathy.

The International Diabetes Federation (IDF) estimated the global population with diabetes mellitus (DM) to be 463 million in 2019 and 700 million in 2045. According to a systematic

review and Meta-analysis published a few months ago, nearly 1 in 5 persons with diabetes worldwide have diabetic retinopathy [108] (**Fig.6**).

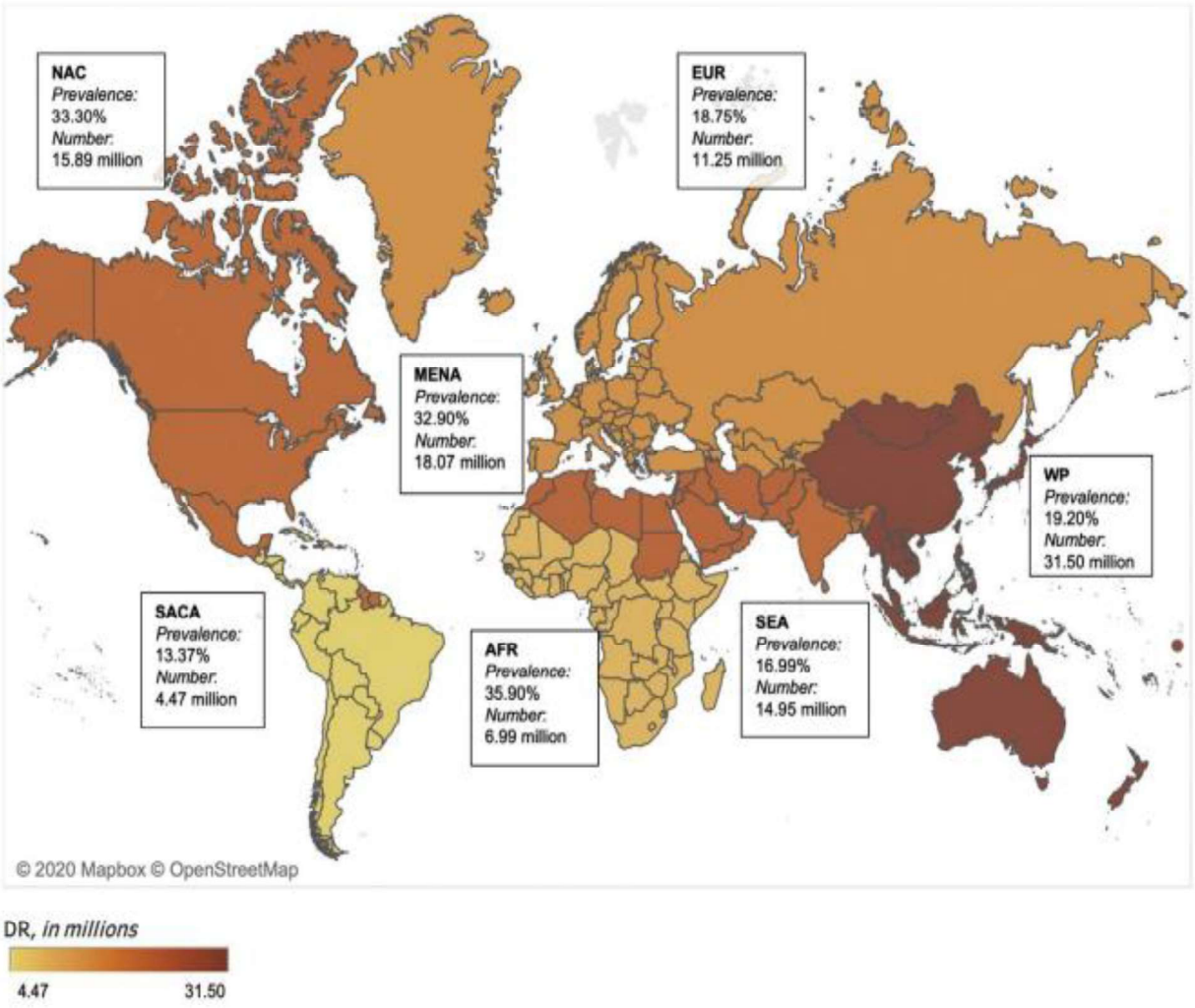

Fig.6 Global map showing diabetic retinopathy (DR) prevalence and numbers by International Diabetes Foundation world regions in 2020 [108].

AFR= Africa; EUR= Europe; MENA= Middle East and North Africa; NAC= North America and Caribbean; SACA= Souuth and Central America; SEA= South East Asia: WP= Western Pacific

DR results in microvascular changes, which are linked to RGCs loss, reactive gliosis, and inner retinal thinning. [7, 109-110]. The thinning of the ganglion cell complex, (retinal nerve fiber

layer + ganglion cell layer+inner plexiform layer), is 0.54 μm/year which is just like to the reduction observed in advanced glaucoma [111]. In the early stages of diabetes in the retina, selective RGCs loss is observed without overt micro-vascular changes [112 -114]. The role of inadequate energy control and metabolic status in RGCs death in the diabetic retina are under extensive investigation. Therefore, although originally diabetic retinopathy was assumed entirely as a microvascular disease, currently DR has been recognized as a neurodegenerative disease of the retina [115-116]. In the retina, glia and neurons intimately interact with retinal vasculature to preserve the homeostasis necessary for normal retinal function [117]. Diabetes disrupts the interaction between these cells leading to retinal neurodegeneration which in turn causes early microvascular changes (breakdown of the blood-retinal barrier -BRB, vasoregression, and impairment of neurovascular interaction) [118 -120]. Additionally, since the retina is a neuronal tissue, it produces neurotrophic factors, such as NGF, BDNF, NT-3, and NT-4. However, diabetes progressively alters the level of multiple trophic factors/signaling pathways in the retina, reducing the presence of survival signals and increasing apoptosis [117]. During the initial stage, non-proliferative diabetic retinopathy (NPDR) is mostly asymptomatic, with the onset of microhemorrhagic and microischemic events and an increase in vascular permeability. Thereafter, the progression of the disease is coupled with the onset of a chronic inflammatory state and neovascularization (Proliferative Diabetic Retinopathy-PDR) in a vicious spiral that induces and feeds the accumulation of damage to the retina through hypoxia, oxidative stress, and widespread neurodegeneration. As a matter of fact several clinical tools such as multifocal electroretinography (ERG), flash ERG, contrast sensitivity, color vision, and short-wavelength automated perimetry, shown functional deficits in the neuronal component of retinas during the early stages of diabetes [117]. Among the metabolites, hyperglycemia is known to be the main factor activating several metabolic pathways that are source of damage for the retina [115]. Two clinical trials, Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS), have proved that tight control of hyperglycemia in both type 1 and type 2 diabetes patients significantly reduced the risk of DR development and progression. A characteristic of diabetic retinopathy is the High Glucose (HG) concentrations induced neurotoxicity and one of the first retinal responses to hyperglycemic stress is the upregulation of vascular endothelial growth factor (VEGF), a central target of present therapies [99, 121]. In addition to this, an increased level of glutamate has been detected in both the vitreous and the retina of diabetic patients, suggesting a neurotoxic role of glutamate, which may be harmful for retinal neurons, especially retinal ganglion cells, because of excitotoxicity [115-116, 122- 123]. The principal cause of neuronal cell death following

glutamate-induced activation of N-methyl D-aspartate (NMDA) receptors is the influx of calcium and sodium into cells leading to the development of free radicals and apoptosis [117]. Likewise, several studies have highlighted that during diabetes in the retina and in the vitreous there is an overexpression of excitatory proteins, such as glutamate and NMDA [124-125]. Elevated levels of glutamate, increased oxidative stress, the overexpression of the renin-angiotensin system, and the upregulation of receptors for advanced glycation-end products (RAGE) play a basic role in the retinal neurodegeneration induced by diabetes. It is thus clear that the balance between the neuroprotective and the neurotoxic factors is decisive for retinal neurons survival [126]. It is therefore evident that diabetic retinopathy shares with glaucoma the development of a progressive neurodegeneration. Moreover, in proliferative diabetic retinopathy (PDR), vitreous humor goes through structural and molecular changes as well as changes in its composition, which play a key role in sustaining the progression of the disease [127]. The vitreous humor is a clear, colourless gel-like structure of 4 mL in volume, which occupies the space between the lens and the retina, filling 80% of the eye's volume [128] (**Fig.7**). It is composed of 98-99% of water, then there are a small amount of ions /cations, proteins (predominantly collagen), and polysaccharides such as hyaluronic acid [129]. Studies conducted on vitreous patients affected by PDR showed that in this category of patients there are abnormal levels of bioactive molecules with pro-angiogenic, pro-inflammatory, and neuromodulatory activities [128]. Therefore, the vitreous serves as a reservoir of soluble signaling mediators that could worsen retinal damage. Thus, the vitreous obtained from patients with PDR can be used to measure the anti-angiogenic/anti-inflammatory activity of new biomolecules that could be possible candidates for the treatment of diabetic vitreoretinopathy [130].

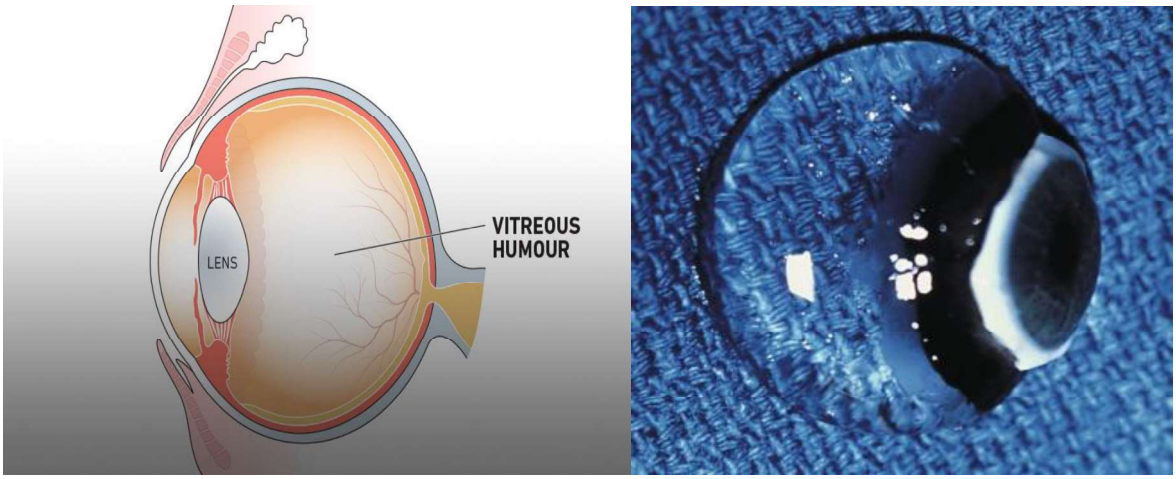

Fig.7 Vitreous body: schematic representation and *in vivo* image [131-132].

It has also been remarked that Vitamin D3 levels appear to be lower in diabetes mellitus type 2 patients, and this could have therapeutic implications. Vitamin D may be involved in the pathogenesis of diabetic retinopathy through its effects on the immune system and on angiogenesis. Vitamin D has an anti-inflammatory effect by decreasing the proliferation of several pro-inflammatory cytokines, lymphocytes, and natural killer cells [133]. In addition, it has been found that the active metabolite of vitamin D, calcitriol, is an effective inhibitor of retinal neovascularization in a mouse oxygen-induced ischemic retinopathy model [134-135]. At the early stages of RD there are also diminished levels of folic acid and vitamin-B12, and it is potentially harmful for neurons [136]. Satyanarayana and colleagues [137] noted in DR a link between vitamin-B12 deficiency and hyperhomocysteinemia. The effects of elevated homocysteine levels on retinal function in both *in vitro* and *in vivo* models have proved that homocysteine promotes apoptosis in RGCs [138-139]. Folic acid / vitamin-B12 supplementation are known to diminish homocysteine levels, thus they may be a potential treatment strategy to ameliorate neurodegeneration keeping under control the levels of homocysteine [140]. There is numerous evidence that indicates increased levels of excitotoxic metabolites, including glutamate, branched-chain amino acids, and homocysteine in cases of diabetic retinopathy. The altered levels of metabolites lie to an activation of several metabolic pathways (e.g polyol, hexosamine, diacylglycerol [DAG] protein kinase C [PKC] pathway) leading to increases in oxidative stress and decreases in the level of neurotrophic factors [117] (**Fig.8**).

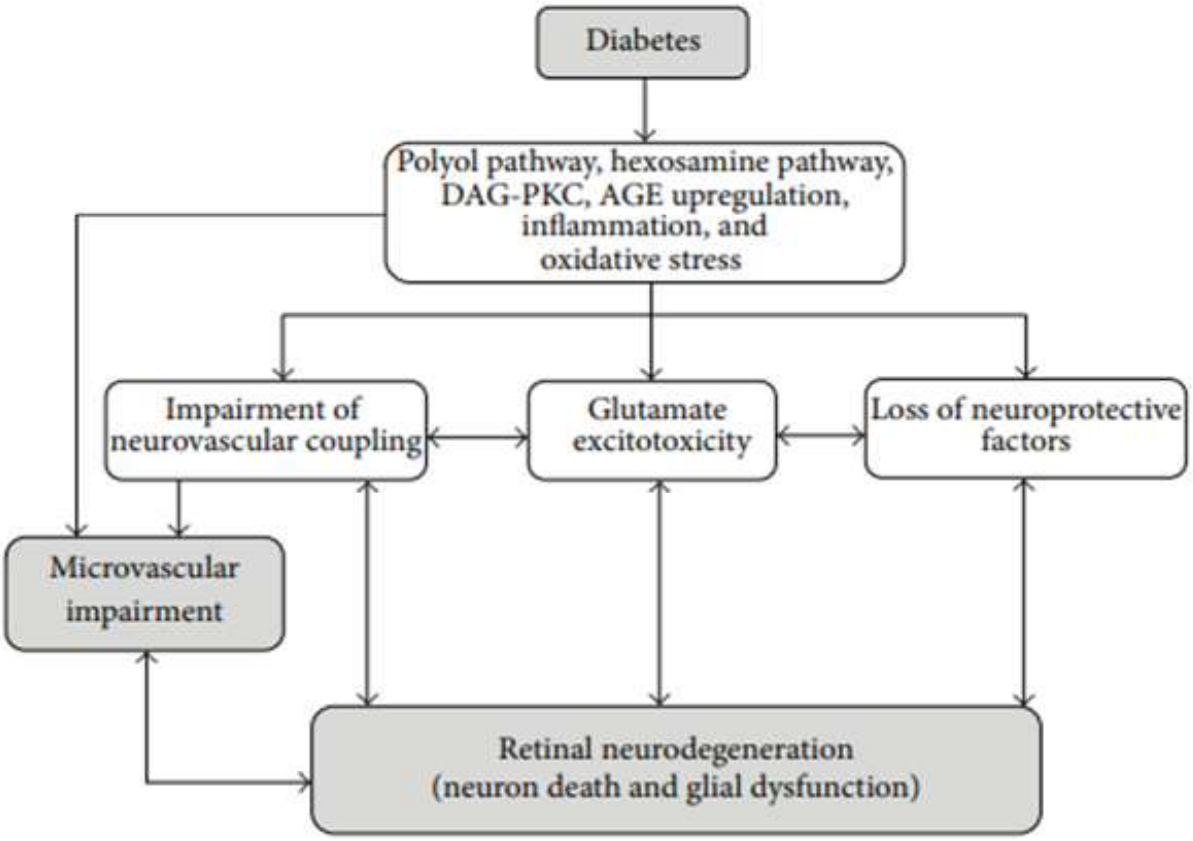

Fig.8 Potential therapeutic targets based on pathogenic machanisms involved in retinal neurodegeneration induced by diabetes [141].

As regards therapeutic alternatives for PDR, they currently are: laser photocoagulation, intravitreal injection of drugs targeting the VEGF and steroid agents and for more severe cases vitreoretinal surgery [142]. Nevertheless, although often these therapeutic approaches are effective in the short term, they cause side effects and are indicated substantially for the advanced stages of the disease and moreover, to date, no drugs are available that prevent the incidence or progression of DR. In addition, it is not sustainable for many patients to be able to go to the hospital several times a month to undergo intravitreal injections or laser sessions and check-ups. All this represents a strong economic and organizational burden (staff, outpatients, operating theatres, etc.) for health care facilities that, as the number of affected patients increases and therefore the number of patients under treatment rises, can no longer cope with all the requests.

Currently, neuroprotective compounds have been extensively studied to meet the need for less invasive and longer-lasting treatments in order to prevent visual field loss and preserve visual function. Nutraceuticals are trying to respond to this need with a promising and encouraging alternative for the treatment of NPDR at the early-stage. Several studies (*in vitro* and *in vivo*) demonstrated that a number of different nutraceuticals have relevant antioxidant and anti-inflammatory properties; certain nutraceuticals can prevent the early driven molecular events that induce vitreoretinopathy, acting as upstream regulators of DR [143]. However, what emerges from the studies is that a single compound that strikes only one target has limited efficacy in avoiding the progression of multifactorial diseases. This is true for RD as for other multifactorial diseases. Actually, is now established the hypothesis that, even in retinal neurodegenerations, the use of a combination of substances with synergistic multitarget effects may provide a more effective approach for the prevention of the disease [144-147].

Flavonoids have antioxidant, antiangiogenic, and anti-inflammatory properties. Therefore, selected flavonoids (e.g quercetin) may be effective in the prevention or treatment of ocular diseases such as DR . Six-month treatment with quercetin re-establish levels of glutathione and improves the activities of antioxidant enzymes, reducing the levels of inflammatory cytokines, and protecting RGCs from apoptosis [148]. In diabetic rats also resveratrol decreases retinal expression of genes involved in angiogenesis, inflammation, and oxidative stress [149].

Curcumin, a yellowish non-flavonoid polyphenol that constitutes the principal active compound of *Curcuma longa*, is commonly known for its antioxidant and anti-inflammatory properties [150-152]. Different studies have also underlined its pronounced protective effect on retinal cells against inflammation and oxidative stress [150-154]. Some studies have shown that, in the retina of diabetic rats, a four-month treatment with curcumin induces significant hypoglycemic activity, counteracts the reduction of glutathione levels and promotes the activity of antioxidant enzymes, decreases inflammatory factor levels, and prevents the structural degeneration and increase in capillary basement membrane thickness [155-156].

Carotenoids (lutein and zeaxanthin), widely used for age-related macular degeneration, are also powerful antioxidants capable of inhibiting diabetes-induced retinal oxidative damage [157- 158].

In patients with diabetic retinopathy, the mitochondria- and caspase-dependent cell death pathways are linked to neurodegeneration. Therefore citicoline, a mitochondrial stabilizer

(citicoline indirectly inhibits phospholipase A2 and stabilizes the mitochondrial membrane), is an ideal candidate for neuroprotection [11]. In addition to diabetic retinopathy, the neuroprotective role of citicoline has been discussed previously also with regard to glaucoma.

Kusari et al pointed out that in animal models of diabetes both memantine and brimonidine treatments revealed neuroprotection activity in addition to reduced VEGF protein levels and reduced blood retinal barrier breakdown [159-160]. It is therefore self-evident that are numerous compounds that could be exploited as a valid support for the therapy of diabetic retinopathy.

CHAPTER 2: AIM OF THE STUDY

The aim of this PhD thesis, carried out in collaboration with the FB Vision srl, Castelfidardo, Italy, was to define a potential role of the association of citicoline and homotaurine in performing neuroprotective activity towards the RGCs (primary retinal cultures) under experimental conditions simulating retinal neurodegeneration similar to those occurring in glaucoma and in diabetic retinopathy. In addition, the study aimed to evaluate the anti-inflammatory effect of curcumin, homotaurine, and vitamin D3 on the expression of inflammatory cytokines in human vitreous samples of patients with PDR.

CHAPTER 3: MATERIALS AND METHODS

The study was conducted at the Department of Medicine and Health Sciences "V. Tiberio" of Molise University, Campobasso (Italy) and at the laboratories of FB Vision srl , according to the planned time schedule. The study was admitted to funding by decree R.0002983.05-11-2018 from the resources PON (National Operational Programme) Research and Innovation 2014-2020. Then, it was approved by the CTS (technical-scientific committee) of the Department and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. The experimental part of the study was carried out in the period from June 2019 to June 2021. As regards the experimental part conducted on the vitreous of patients with PDR all the study participants provided written informed consent.

3.1 Study on primary cultures of RGCs

The experiments were conducted on primary cultures of RGCs, to evaluate their viability and apoptotic rate, following pharmacological treatments capable of mimicking pathological conditions such as glaucoma and diabetes.

Primary cultures were derived from the retinas of fetal Wistar rats (18-19 days' gestation). Retinal tissues were mechanically dissociated, and the cell suspensions were plated into 60 mm dish (0.8–1.0 × 106 cells/mL) (Corning, Acton, MA). Retinal cultures were incubated in Eagle's minimal essential medium (MEM) under an atmosphere of 5% CO2 in the air and 10 μM cytosine arabinoside (Sigma, St. Louis, MO) was added to the culture in order to wipe out nonneuronal cells. In this study were used only isolated cells. In agreement with previous studies glutamate neurotoxicity (to simulate glaucomatous damage) was assessed using a 25 min exposure to 100 μM glutamate followed by a 24-hour incubation in the glutamate-free medium [99;161,162]. For the purpose of mimicking the diabetic condition and reproducing a hyperglycemic insult, in the second series of experiments, the cells were treated with high glucose concentrations (HG). When the confluence of the cells reached 80%, the culture medium was supplemented with glucose, achieving a final concentration of 30 mM. Retinal cells were exposed to HG for 96 hours (**Fig.9**). The concentration of glucose in control conditions was 5 mM. Cell viability was assessed using the (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay modified from that of Mosmann [163].

With the aim to assess the effect of citicoline and homotaurine on cell survival, the cells were subdivided into three groups and treated for 24 hours with 1 µM, 10 µM, and 100 µM of citicoline (Kyowa Hakko Bio Co. Ltd., Tokyo, Japan) and with 1 µM, 10 µM, and 100 µM of homotaurine (Truffini e Reggè Farmaceutici, Milan, Italy). In order to assess the neuroprotective effects of citicoline and homotaurine, cells were treated with citicoline100 µM, homotaurine 100 µM, or citicoline + homotaurine 100 µM, 24 hours before glutamate treatment and 30 min before HG treatment. Apoptosis was estimated by using a Cell Death Detection ELISAPLUS kit (Roche Applied Science, Indianapolis, IN). Through this photometric enzyme immunoassay it was possible to obtain the quantitative determination of oligonucleosomes generated from the apoptotic cells.

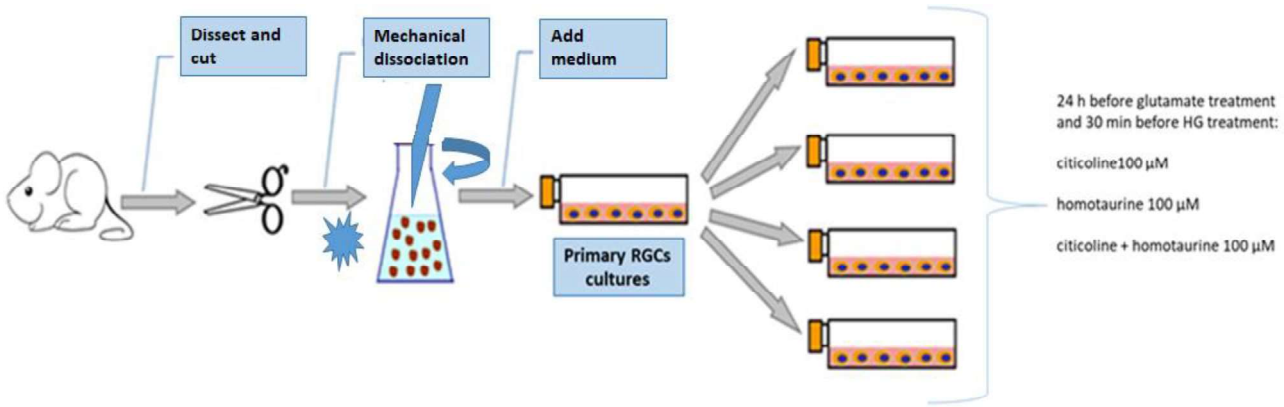

Fig 9 schematic representation of the study on primary cultures of RGCs [164].

3.2 Study on the vitreous of PDR patients

This part of the study was conducted on the vitreous of 28 patients suffering from PDR. It was a prospective study. The enrolled patients were scheduled to undergo a 23-gauge, three-port pars plana vitrectomy for retinal detachment, and all the patients completed the study. Inclusion criteria were age ≥18 years, patients with proliferative diabetic retinopathy requiring vitrectomy, and consent to participate in the study following the indications provided. The exclusion criteria were previous vitrectomy in the study eye, previous buckle surgery, previous intravitreal injection, concurrent retinovascular or other ocular inflammatory diseases, history of ocular trauma, and concomitant intake of any topical or systemic NSAID or corticosteroid therapy, and presence of systemic inflammations. All phakic patients were operated with phacoemulsification of the crystalline lens plus intraocular lens (IOL) implant at the time of vitrectomy to allow a careful cleaning of the vitreous base. Vitrectomy surgery was performed using a 23-gauge transconjunctival system; no triamcinolone was used during any step of the surgery. At the beginning of the surgery, 0.5–1.0 mL of undiluted vitreous was removed, and samples were immediately frozen and stored at −80◦C until analysis. This procedure was used to prevent the vitrectomy intervention itself from generating or altering the expression of cytokines and endothelial growth factors or the BSS (balanced salt solution) from diluting the vitreous. After the removal of the posterior hyaloids, the vitreous base was carefully eliminated. All the visible proliferative vitreoretinopathy (PVR) membranes were dissected, and relaxing retinotomies were done. The retinal periphery was examined for any retinal breaks that were marked with endodiathermy, after which the retina was reattached using perfluorocarbon liquid and air. Three rows of endolaser treatment were applied behind the posterior vitreous base in all the patients (200 spots, 200–250 mW according to retinal pigmentation). All the patients in both groups (treaded group and control group) were prescribed topical dexamethasone (six times per day) and homatropine (two times per day).

Treated Group: Twenty-eight portions of vitreous samples from 28 eyes of patients undergoing vitrectomy for proliferative diabetic retinopathy complications, incubated with curcumin (Cureit®), homotaurine, and cholecalciferol (vitamin D3). Vitreous biopsies were thawed and centrifuged. Thereafter, 50 µL of vitreal fluid from each patient were aliquoted into 96-well plates and incubated for 24 h at 37◦C in 100 µL HBSS (Hank's Balanced Salt Solution) with

increasing doses of curcumin (Cureit®) (0.5µM and 1µM), with or without homotaurine (100µM) and vitamin D3 (50 nM), to assess a possible synergistic effect on the expression of inflammatory cytokines. Vitamin D3 and homotaurine were acquired from Sigma-Aldrich whereas Cureit ® curcumin was supplied by Fisher Chemicals Aurea Biolab. Curcumin and vitamin D3 were first dissolved in DMSO (dimethyl sulfoxide) to final concentrations of 250 and 25 mM, respectively. Homotaurine was resuspended in phosphate buffered saline (PBS) to a final concentration of 500 mM.

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Control Group: The same fractions of vitreous samples (n = 28) were evaluated for the expression of oxidative biomarkers, inflammatory cytokines, and metalloproteinases, without any treatment. Controls were exposed to HBSS containing DMSO.

The day after, all the samples were diluted twice in sample diluent and cytokines were measured by ELISA assay. Quantitative detection of soluble mediators in vitreal biopsies was performed using sandwich ELISA kits with High Sensitivity. IL2 and TNF-α were measured by Human Pre-coated ELISA Kit (BIOGEMS-PEPROTECH), whereas IL6 and PDGF-AB were detected using PicoKine ELISA Kits (Boster Biological Technology). All kit reagents, samples, and standards were prepared according to the manufacturer's instructions. The measured optical density was read at 450 nm and was directly proportional to the concentration of human recombinant proteins in the standards or samples. The concentration of soluble mediators was calculated from a calibration curve and expressed as pg/mL. Each experimental point was replicated three times, and absolute levels of IL6, IL2, TNF-α, and PDGF-AB were measured by ELISA. Afterwards, average levels of soluble mediators measured in treated vitreous were expressed as a percentage of the baseline level, considering the control aliquot from the same patient as the baseline.

Moreover, in order to assess whether curcumin together with vitamin D3 and homotaurine in the vitreal fluid could promote an anti-inflammatory and anti-angiogenic effect, it was checked in a subset of four vitreous the expression of pro-inflammatory genes and mitogen-activated genes in an immortalized cell line exposed for 24 h to vitreal biopsies from patients with PDR together with curcumin, vitamin D3, and homotaurine or not.

3.2.1 Sample size

The sample size was calculated with a suitable macro developed in the SAS language. It was conducted a pilot study from which it was derived the Mean Square Error (MSE). For the purpose of making the data less variable, it was applied the logarithmic transformation that made the residuals normal. The General Linear Model (GLM) provided the standard deviations (square root of MSE) necessary to perform the calculation of the Sample Size (±0.29, ±0.18, ±0.18, ±0.32, respectively, for PDGF-AB, IL2, IL6, and TNF-α). Considering the following differences, on a logarithmic scale, d = 0.32, 0.13, 0.11, and 0.29, which correspond to a reduction of 52.1, 32.4, 22.4, and 48.7% relative to PDGF-AB, IL2, IL6, and TNF-α, it were obtained the following sample sizes with a power of 80% and $\alpha = 0.05$: n = 19, 26, 61, and 28 subjects, taking into account the correction for multiple comparisons. For clinical purposes, we considered n = 28 as the final sample size.

3.3 Statistical analysis

Continuous variables were expressed as mean ± SD. Statistical significance was determined using a one-way analysis of variance (ANOVA). Shapiro-Wilk test was used to verify the normality of distribution of the residuals. To make the residuals normal, we applied suitable mathematical functions in order to respect the Gauss condition. Homoscedasticity was verified by Levene and Brown-Forsythe tests. Post-hoc analysis was performed by the Tukey test. A $p \leq 0.05$ was considered statistically significant. The statistical analysis was performed using SAS v. 9.4 and JMP v. 15 (SAS Institute Inc., Cary, NC, USA).

CHAPTER 4 : RESULTS

4.1 Study on primary cultures of RGCs

Firstly, in order to assess the potential neuroprotective activity of citicoline and homotaurine, we treated retinal cells with increasing concentrations (1 μM, 10 μM, and 100 μM) of citicoline or homotaurine for 24 hours. MTT assay demonstrated that retinal cells were well preserved in citicoline- or homotaurine-treated cultures, with no evidence of toxicity after treatment at 1, 10, or 100 μM (**Fig10**). Therefore, the RGCs viability was not hit by the treatment with citicoline or homotaurine. This result is consistent with that of previous studies [99,165-166]. Secondly, with the aim to assess if the cotreatment with citicoline and homotaurine was able to induce a synergistic neuroprotective effect against glutamate excitotoxicity, RGCs were exposed to citicoline 100 μM, homotaurine 100 μM, and citicoline + homotaurine 100 μM, 24 hours before glutamate treatment. When RGCs were pretreated with citicoline or homotaurine alone a significant increase ($p < 0.001$) in cell viability was detected (citicoline more than homotaurine). However, when citicoline and homotaurine were used in combination the effect on viability increased further and in a statistically significant way ($p < 0.001$). So, The combined administration of citicoline and homotaurine proved a significant synergistic cytoprotective effect. The same kind of effect occurred when we evaluated the apoptosis, measured by the number of oligonucleosomes released. Apoptosis (oligonucleosomes released), was significantly reduced in cells incubated with 100 μM citicoline 24 hours before treatment with glutamate ($p < 0.001$) (**Fig.11 A**). A similar result was obtained by treating the cells with 100 μM homotaurine ($p < 0.001$). The combination of citicoline and homotaurine reduced the apoptotic rate to a greater extent than the single compounds ($p < 0.001$). The same experiments were then conducted to assess the neuroprotective effect of citicoline and homotaurine on RGCs against damage from HG. The results obtained were comparable to those achieved for excitotoxic damage by glutamate (**Fig.11 B**). Actually, both citicoline and homotaurine have a cytoprotective effect on RGCs and when used together a synergistic effect is obtained.

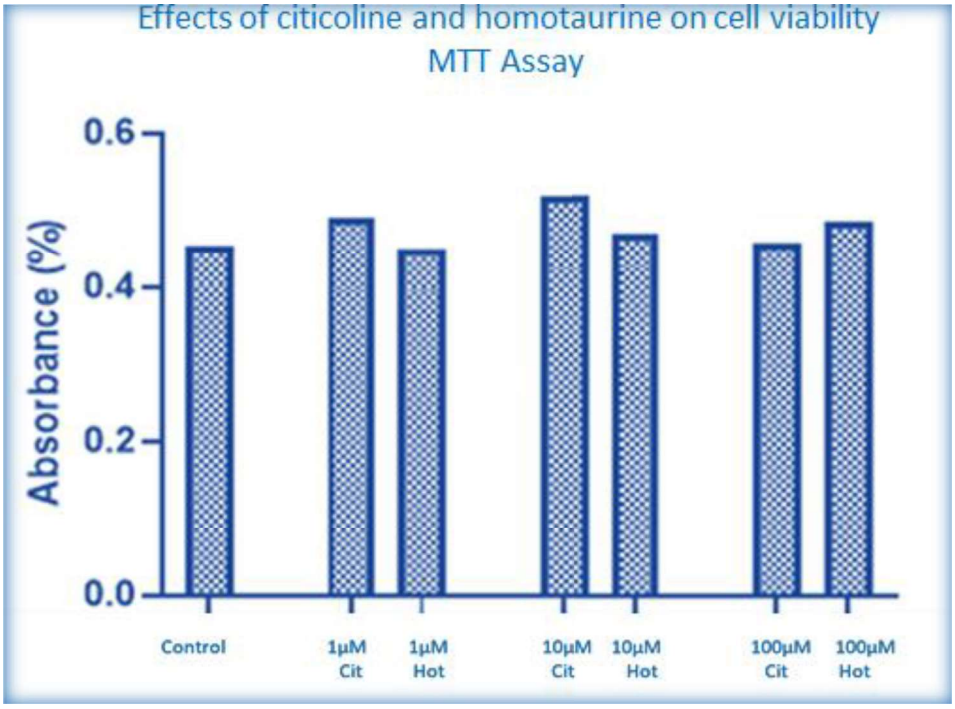

Fig 10 MTT assay shows that primary culteres of RGCs were well preserved when treated with citicoline (Cit) or homotaurine (Hot)-treated. No evidence of citotoxicity after treatment at 1, 10, or 100 μM was observed.

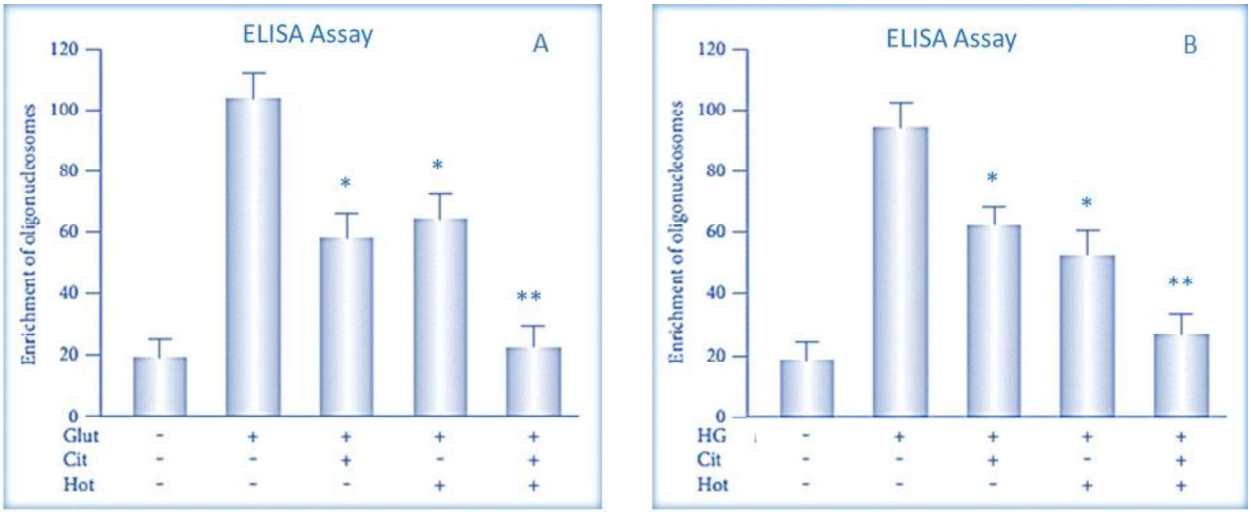

Fig. 11 Cotreatment of citicoline and homotaurine significantly reduces the apoptotic rate in glutamate-treated cells (A) and in high glucose-treated cells (B).

Cit = citicoline; Hot= homotaurine; Glut= glutamate; HG= High Glucose

Apoptosis (oligonucleosomes released), is significantly reduced in cells incubated with 100 μM citicoline 24 hours before treatment with glutamate or HG. A similar result is obtained by treating the cells with 100 μM homotaurine. The combination of citicoline and homotaurine reduces the apoptotic rate more than treatment with individual compounds.

*p value $< 0{,}001$ versus glutamate (A) or High Glucose (B)

** p value $< 0{,}001$ versus citicoline and homotaurine alone

4.2 Study on the vitreous of PDR patients

Vitreous biopsies from twenty-eight patients with PDR were analyzed. The mean age a the time of the vitrectomy (± standard deviation) was 68.9 ± 7.8 years. Of the 28 included patients, 16 (57.1%) were males and 12 (42.9%) were females. Mean time (± standard deviation) since diagnosis of diabetes mellitus in these patients was 31.4 ± 8.7 years. In vitreous samples, the pro-inflammatory cytokines IL6, TNF-α, and IL2 and the angiogenic factor PDGF-AB were all detectable in the conditions of the sample. Mean IL6, IL2, TNF-α, and PDGF-AB levels in the vitreous of the patients are reported in **Table 1**.The post-hoc analysis proved statistically detectable changes in the concentration of TNF-α, IL2, and PDGF-AB in response to treatment with curcumin, homotaurine, and vitamin D3. Precisely, the p-values for between group comparisons were as follows: TNF-α: (untreated vs. curcumin 0.5µM + homotaurine 100µM + vitamin D3 50 nM) p = 0.008, (curcumin 0.5µM vs. curcumin 0.5µM + homotaurine 100µM + vitamin D3 50 nM) p = 0.0004, (curcumin 0.5µM vs. curcumin 1µM +homotaurine 100µM + vitamin D3 50 nM) p = 0.02, (curcumin 1µM vs. curcumin 0.5µM + homotaurine 100µM + vitamin D3 50 nM) p = 0.025, and (homotaurine 100µM + vitamin D3 50 nM vs. curcumin 0.5µM + homotaurine 100µM + vitamin D3 50 nM) p = 0.009 (**Fig. 12**); IL2: (untreated vs. curcumin 0.5µM + homotaurine 100µM + vitamin D3 50 nM) p = 0.0023 and (curcumin 0.5µM vs. curcumin 0.5µM + homotaurine 100µM + vitamin D3 50 nM) p = 0.0028 (**Fig. 13**); PDGFAB: (untreated vs. curcumin 0.5µM + homotaurine 100µM+ vitamin D3 50 nM) p = 0.04, (untreated vs. curcumin 1µM + homotaurine 100µM + vitamin D3 50 nM) p = 0.0006, (curcumin 0.5µM vs. curcumin 1µM + homotaurine 100µM+ vitamin D3 50 nM) p = 0.006, and (homotaurine 100µM +vitamin D3 50 nM vs. curcumin 1µM + homotaurine 100µM + vitamin D3 50 nM) p = 0.022 (**Fig.14**). IL6 levels were not really influenced by any of the treatment (**Fig.15**). Gene expression performed on 4 vitreous biopsies revealed that vitreal fluids have an up-regulation of the cyclinD1 gene and the pro-inflammatory cytokine genes TNFα and IL6 on human HEK293 cells; by contrast when vitreal fluids were taken in combination with curcumin, vitamin D3, and homotaurine such levels were down-regulated (**Fig.16 A-B-C**).

Table 1 - Levels of soluble mediators in vitreal biopsies from patients with diabetic retinopathy.

Parameter	**Untreated**	**Curcumin 0.5 μM**	**Curcumin 1 μM**	**Homotaurine 100 μM + Vitamin D3 50 nM**	**Curcumin 0.5μM+ Homotaurine 100 μM + Vitamin D3 50 nM**	**Curcumin 1μM + Homotaurine 100 μM + Vitamin D3 50 nM**	P
	mean±SD (95% CI)	mean±SD (95% CI)	mean±SD (95% CI)	mean±SD (95% CI)	mean±SD (95% CI)	mean±SD (95% CI)	
PDGF-AB*[a]*(pg/ml)	842.68±459.61 (664.50 to 1020.86)	780.43±466.58 (599.51 to 961.35)	657.58±311.24 (536.89 to 778.26)	735.94±466.40 (555.09 to 916.79)	538.32±345.39 (404.40 to 672.25)	406.41±213.85 (323.48 to 489.33)	**0.0003**
IL2*[b]*(pg/ml)	85.17±47.33 (66.81 to 103.52)	81.95±44.33 (64.76 to 99.14)	63.00±30.51 (51.17 to 74.83)	71.81±41.05 (55.89 to 87.73)	55.93±26.73 (45.56 to 66.30)	60.21±26.84 (49.80 to 70.62)	**0.0005**
IL6*[c]*(pg/ml)	16.71±7.02 (13.99 to 19.43)	15.81±6.11 (13.44 to 18.18)	15.28±5.88 (13.00 to 17.56)	15.96±7.63 (13.00 to 18.92)	13.78±8.03 (10.67 to 16.90)	15.52±8.39 (12.27 to 18.78)	0.32
TNF-α*[c]*(pg/ml)	112.56±72.85 (84.32 to 140.81)	113.27±50.20 (93.81 to 132.74)	110.68±82.95 (78.52 to 142.85)	108.61±74.37 (79.78 to 137.45)	59.31±42.09 (42.99 to 75.63)	66.52±43.59 (49.62 to 83.43)	**0.0001**

[a]sqrt transformed, [b]inverse transformed, [c]$\log_{10}$ transformed

Post-hoc Analysis:

PDGF-AB - (untreated vs. curcumin 0.5 μM + homotaurine 100 μM + vitamin D3 50 nM) p=**0.04**; (untreated vs. curcumin 1 μM + homotaurine 100 μM + vitamin D3 50 nM) p=**0.0006**; (curcumin 0.5 μM *vs.* curcumin 1 μM + homotaurine 100 μM + vitamin D3 50 nM) p=**0.006**; (homotaurine 100 μM + vitamin D3 50 nM *vs.* curcumin 1 μM + homotaurine 100 μM + vitamin D3 50 nM) p=**0.022**

IL2 - (untreated *vs.* curcumin 0.5 μM + homotaurine 100 μM + vitamin D3 50 nM) p=**0.0023**; (curcumin 0.5 μM *vs.* curcumin 0.5 μM + homotaurine 100 μM + vitamin D3 50 nM) p=**0.0028**

TNF-α - (untreated vs. curcumin 0.5 μM + homotaurine 100 μM + vitamin D3 50 nM) p=**0.008**; (curcumin 0.5 μM vs. curcumin 0.5 μM + homotaurine 100 μM + vitamin D3 50 nM) p=**0.0004**; (curcumin 0.5 μM *vs.* curcumin 1 μM + homotaurine 100 μM + vitamin D3 50 nM) p=**0.02**; (curcumin 1 μM *vs.* curcumin 0.5 μM + homotaurine 100 μM + vitamin D3 50 nM) p=**0.025**; (homotaurine 100 μM + vitamin D3 50 nM *vs.* curcumin 0.5 μM + homotaurine 100 μM + vitamin D3 50 nM) p=**0.009**.

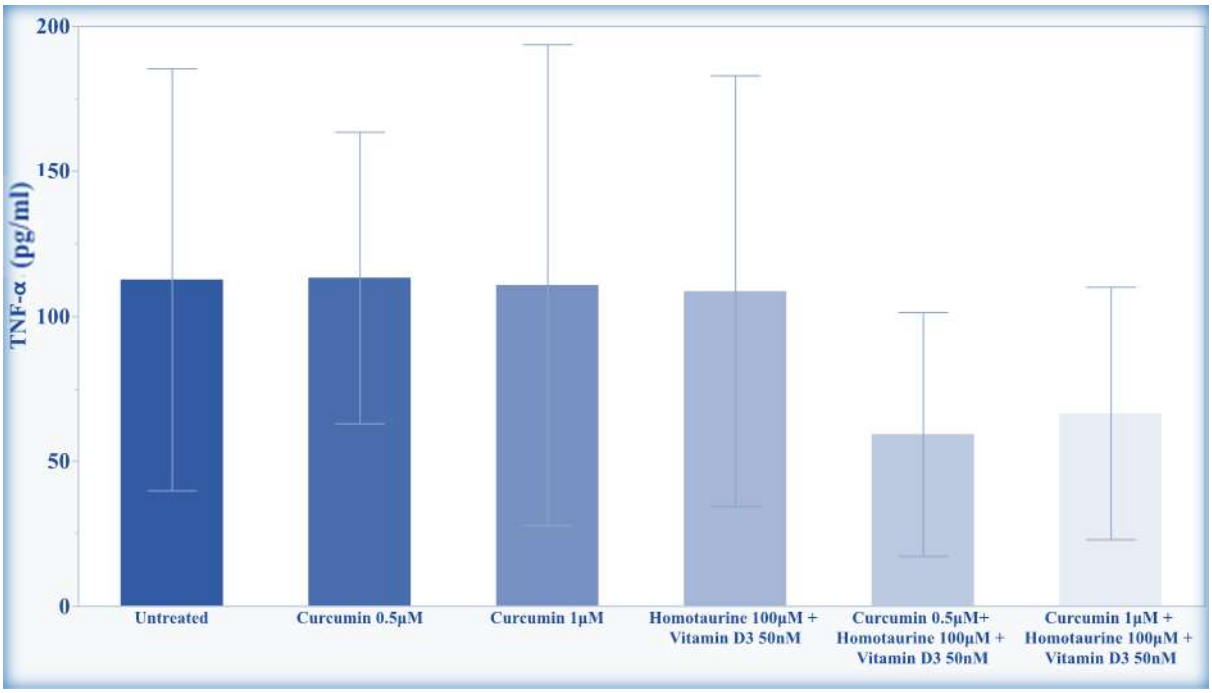

Fig. 12 – Histogram of the mean and standard deviations of TNF-α by experimental groups

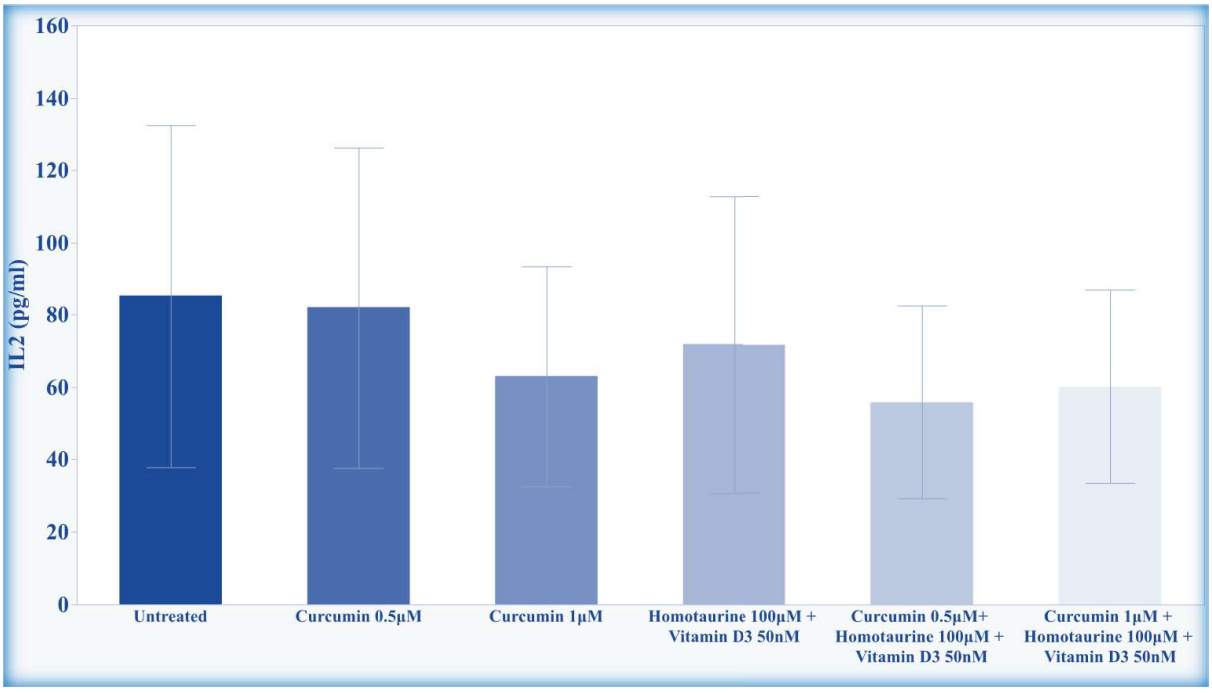

Fig.13 - Histogram of the mean and standard deviations of IL2 by experimental groups

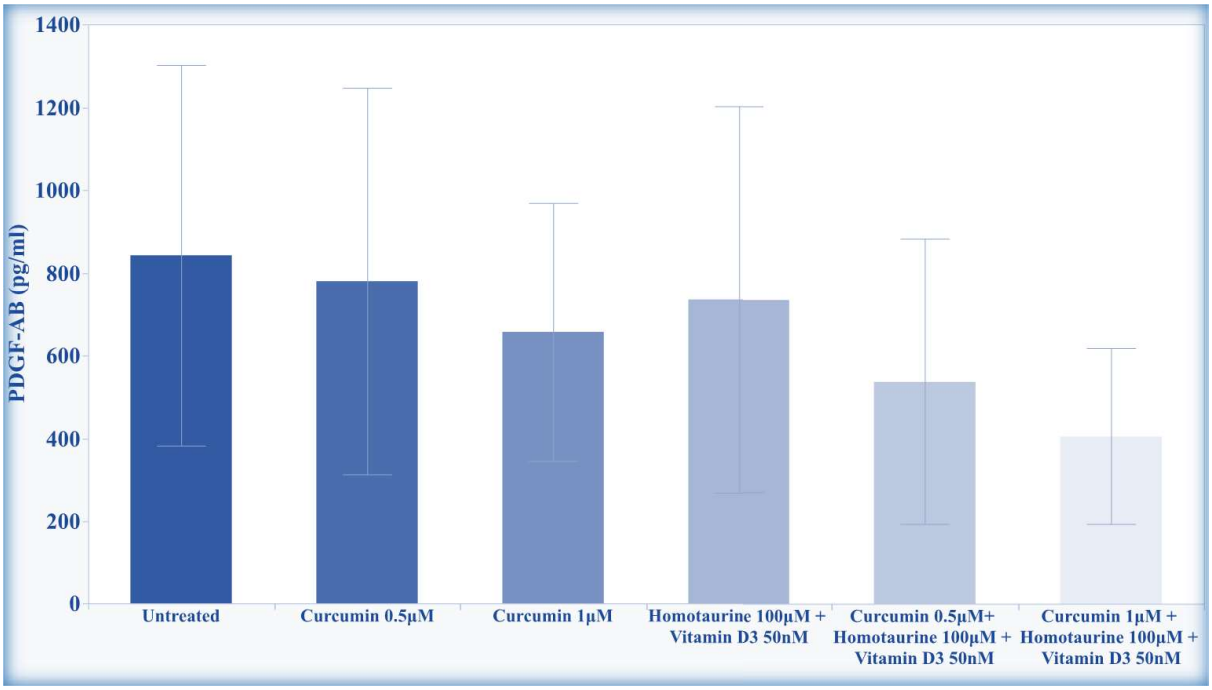

Fig. 14 - Histogram of the mean and standard deviations of PDGF-AB by experimental groups

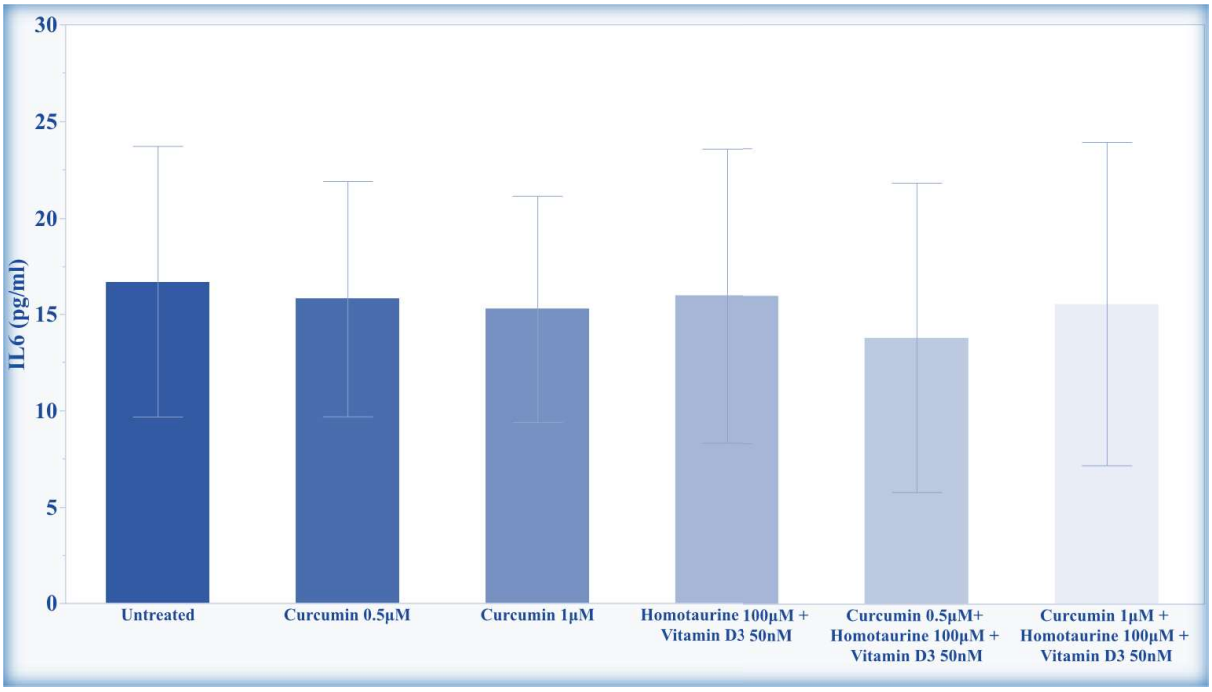

Fig. 15 - Histogram of the mean and standard deviations of IL6 by experimental groups

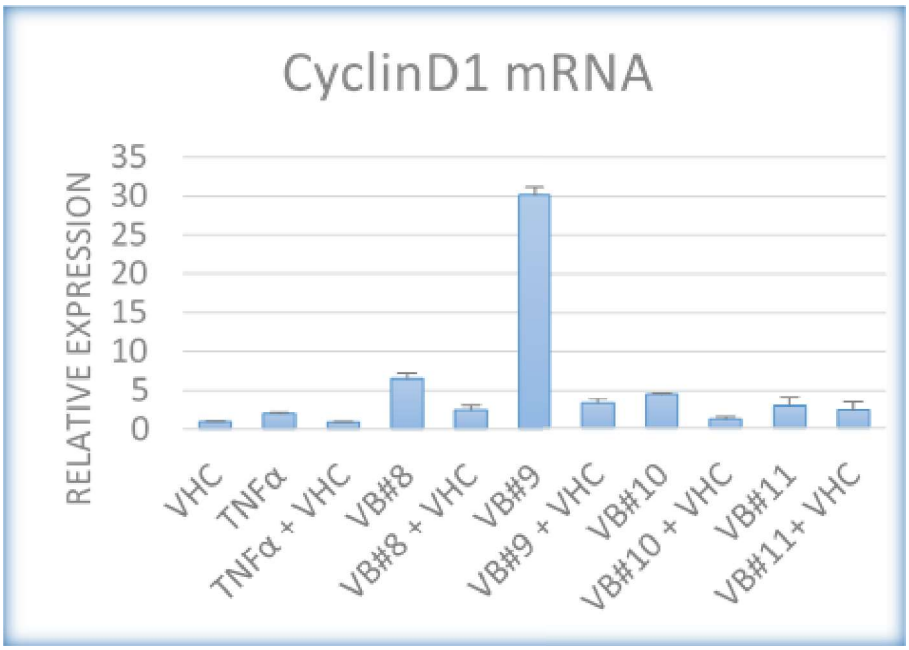

Fig 16 A Gene expression (CyclinD1 mRNA) in the vitreous of diabetic retinopathy patients

VHC = Vitamin D3 50nM + Homotaurine 100µM + Curcumin 1µM

TNFα = Tumor Necrosis Factor alpha 50ng/ml ; VB# = Vitreal Biopsy #

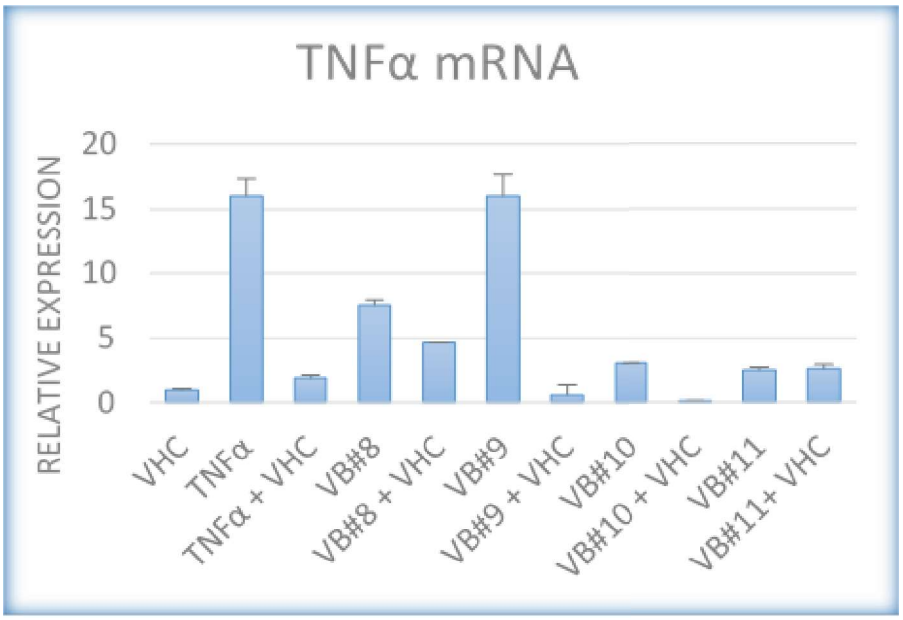

Fig16 B Gene expression (TNFα mRNA) in the vitreous of diabetic retinopathy patients

VHC = Vitamin D3 50nM + Homotaurine 100µM + Curcumin 1µM

TNFα = Tumor Necrosis Factor alpha 50ng/ml; VB# = Vitreal Biopsy

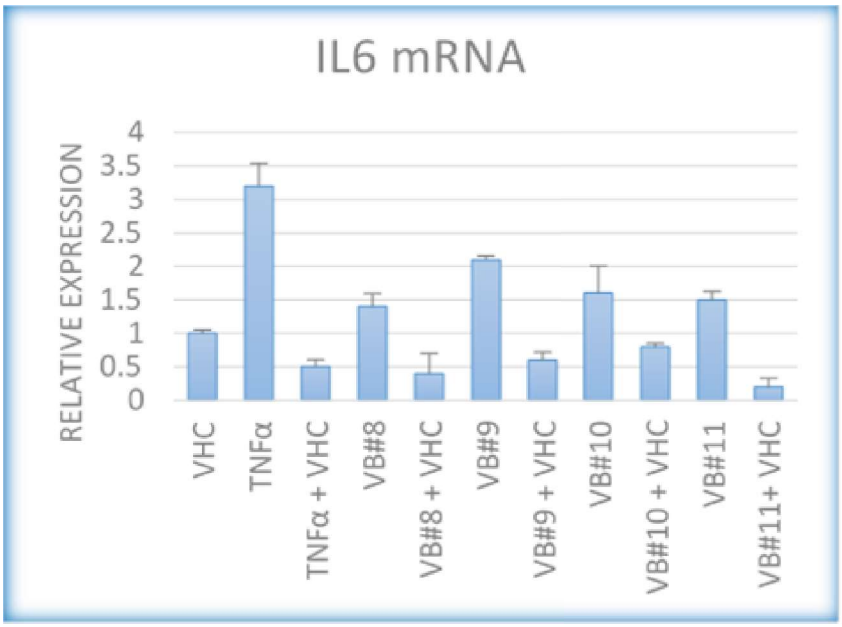

Fig16 C Gene expression (Il-6 mRNA) in the vitreous of diabetic retinopathy patients

VHC = Vitamin D3 50nM + Homotaurine 100μM + Curcumin 1μM

TNFα = Tumor Necrosis Factor alpha 50ng/ml ; VB# = Vitreal Biopsy #

CHAPTER 5: DISCUSSION

Neuroprotection is proposed to preserve function despite an injury. Over the past 30 years, several studies of potential neuroprotective agents have yielded promising results. However, not always and not all compounds have proven effective as neuroprotective agents in humans. Clinical trials have probably not always been able to respond to primary endpoints because of how study design was done. Glaucoma is a chronic and progressive optic neuropathy, characterized by degeneration of RGCs. Currently, the only available treatments for glaucoma are effective in lowering IOP and therefore in arresting or slowing the disease progression. However, glaucomatous optic neuropathy can occur despite having IOP within a normal range. Often, patients affected by glaucoma continue to undergo progressive glaucomatous optic nerve damage and vision impairment despite appropriate regulation of IOP. It is these cases, in particular, that would benefit from an alternative strategy of neuroprotection, aimed at preventing RGCs death. At the moment, treatments able to recover retinal and neural function are not available for clinical use. In the last decades, encouraging perspectives in glaucoma treatment have emerged, and ongoing research is focusing on the study of novel molecules with neuroprotective and/or neuroregenerative activity [8]. Likewise, it is well established that diabetic retinopathy is a neurovascular disease. Several studies asserted neurodegeneration as an early event in the DR. Current studies have also investigated mechanisms of neurodegeneration with the purpose of revealing a promising target for successful neuroprotection. However, the exact molecular mechanism of neuronal damage in the diabetic retina is still under study. Diabetes-induced dysregulated levels of excitotoxic metabolites, altered neurotrophic support/signaling, and oxidative stress are among the potential causes of neurodegeneration. Increasing interest has been shown in protecting retinal neurons, especially RGCs, which are vulnerable to being damaged due to diabetes. An ongoing work must be done to better comprehend the mechanism of diabetes-induced neurodegeneration. That effort may be useful in detecting a better target and compounds to preserve RGCs in order to mitigate the progression of diabetic retinopathy [117]. This doctoral project was proposed in response to this cognitive and therapeutic gap. Through this PhD study, it was possible to determine the neuroprotective activity of citicoline and homotaurine.

The study showed the synergistic neuroprotective effects of citicoline and homotaurine used in combination on primary RGCs exposed to glutamate toxicity and HG levels. The data proved that cotreatment with citicoline and homotaurine has a direct neuroprotective effect in an experimental model of retinal neurodegeneration. The latter through the use of glutamate

and HG allowed to reproducing two models of retinal degeneration of strong impact. Actually excitotoxicity from glutamate, although implicated in the pathophysiology of several retinal degenerations, has recreated the characteristic environment of glaucomatous damage, while HG has recreated the typical diabetic environment. Moreover, in ophthalmology, these two diseases are among the most important in terms of health and socio-economic burden. As a matter of fact, the precise mechanism by which the citicoline and the homotaurine exert their neuroprotective action is not yet fully known. Nevertheless, some authors have proposed that the citicoline is able to counteract the neuronal apoptosis induced by HG throughout the downregulation of caspase -3 and caspase-9 active forms expression and throughout the upregulation of neurotrophic factors expressions such as BDNF and CNTF. Probably the latter are temporarily being adjusted in response to retinal damage [167-168]. The ability to counter apoptosis has also been demonstrated with regard to homotaurine. Therefore, it was seen that homotaurine was able to counteract apoptosis in the RGCs of rats with induced diabetes [169-170]. Moreover, in consideration of the considerable increase in reactive oxygen species and more generally oxidative stress induced by glutamate excitotoxicity and HG neurotoxicity, the antioxidant activity of citicoline and homotaurine is crucial to obtain an effective neuroprotection [171-172]. In addition, it has been assumed that homotaurine opposes glutamate excitotoxicity by enhancing glutamate transport expression. In this way, glutamate levels are reduced. In diabetic patients, taurine (homotaurine analogue) is also involved in the pathogenesis of glaucomatous damage since taurine is crucial for RGCs survival [173-174]. As a matter of fact, from literature data and from our study it is clear that citicoline and homotaurine in combination could be a potential new approach to prevent and treat eye neurodegenerative diseases, such as glaucomatous retinopathy and diabetic retinopathy. Moreover, based also on our *in vitro* results, the first few data on the *in vivo* use of citicoline and homotaurine have also recently been published. This study was conducted by our research group; the fixed combination of citicoline 500 mg, homotaurine 50 mg, and vitamin E 12 mg (Neuprozin®) was provided by FB-Vision (Via Piceno Aprutina, 47 63,100 Ascoli Piceno, Italy). It was a multicenter, observational, cross-over, short-term, pilot study on primary open-angle glaucoma patients with stable controlled IOP. From this study resulted that a daily intake of a fixed combination of citicoline (500 mg), homotaurine (50mg), and vitamin E (12mg) in addition to the topical medical treatment significantly increased the total score of the contrast sensitivity test and the quality of life in patients affected by primary open angle glaucoma [98].

Additionally, this doctoral study assessed also the anti-inflammatory effect of curcumin, homotaurine, and vitamin D3 on the expression of inflammatory cytokines in human vitreous samples of patients with PDR. Our study underlined the ability of curcumin to lower cytokine levels in the vitreous of patients suffering from diabetes. As in the study conducted on primary cultures of RGCs also from the study conducted on PDR vitreous emerged that more than the single compound is the synergistic effect that should be exploited. In this regard we detected an additional anti-inflammatory effect when curcumin was combined with homotaurine and vitamin D3, suggesting that these compounds can adjust the inflammatory network between the vitreous and retina at various times and levels. This effect is corroborated by the gene expression experiment which proved that the association of curcumin, vitamin D3, and homotaurine down-regulate the cyclinD1 gene and the pro-inflammatory cytokine genes TNFα and IL6 expression. As evidenced in previous studies the synergism of curcumin with other bioactive molecules like those in turmeric makes a beneficial impact by producing a high concentration of "free curcuminoids" in the blood plasma [175]. Furthermore, homotaurine has been proven effective not only in counteracting glutamate excitotoxicity, as we have seen in our study on primary culture of RGCs, but also in reducing proinflammatory cytokines in synergy with other compounds. This result contributes to the growing body of literature showing neuroprotective effects of homotaurine [99]. Likewise, the association of curcumin and homotaurine with vitamin D3 has also been confirmed to be successful, supporting the results of several studies that have determined vitamin D as having a key role in diabetes. Therefore, vitamin D deficiency is linked to impaired insulin synthesis and secretion in animal models of diabetes [176-177]. Vitamin D3 reduces ROS induced by diabetes and has protective effects against retinal vascular damage and cell apoptosis in association with the inhibition of the ROS/TXNIP/NLRP3 inflammasome pathway [178-179]. In our study, only IL6 levels have not changed significantly in response to treatment with the different compounds tested. This result could be a consequence of the cross-talk between IL1β and IL6 signaling, more specifically, as has been reported by Shen et al., it could be due to the inhibitory action of IL1β on IL6 signaling. [180]. With this study, therefore, it is confirmed that natural anti-inflammatory compounds play a major role through their capacity to decrease cytokine levels and control the inflammatory network [181]. These data also indicate that the use of these compounds could lead to a possible reduction of the rate of administration of anti-neovascularization agents, being beneficial for the quality of life of these patients [130]. The study made on the vitreous of patients with PDR we intend to conduct it also on the vitreous of patients with glaucoma. Already in 1996 E B Dreyer et al. performed amino acid analyses on

vitreous specimens that were obtained from patients who were undergoing cataract extraction. Samples were collected prospectively from those patients who sustained an inadvertent rupture of the posterior capsule between 1988 and 1993. After the study, the Authors concluded that "the excitatory amino acid glutamate is found in the vitreous body of glaucomatous eyes at concentrations that are potentially toxic to retinal ganglion cells. The increased level of this known neurotoxin is consistent with an excitotoxic mechanism for the retinal ganglion cell and optic nerve damage in glaucoma. Therapies to protect neurons against glutamate toxic effects may prove to be useful in the management of this blinding disease" [50].

Another crucial point is the best timing for the use of neuroprotective compounds. Neuroprotection in the setting of glaucoma and DR should be contemplated as an early treatment before it occurs an advanced, non-recoverable damage. The "timing to intervene" is one of the conceptual and methodological issues that hinder the translation of experimental results to clinical practice. Actually, a basic difference between animal and human clinical trials in the neuroprotection field is the time of the intervention. In most experimental studies, the neuroprotective agent is given at the time or even prior to the injury, unlike human studies, in which the patient is eligible for enrollment after the disease is well established. Therefore, neuroprotective agents, as a new type of therapeutic approach should be given early or even in "prevention" in patients at risk of developing the disease and should then be prolonged over time to complement and be supportive of conventional therapies. In addition, the advances in both retinal imaging and functional assessment will empower detecting early changes and will expedite drug discovery and delivery strategies to improve visual prognosis in patients suffering from glaucoma and diabetes. Moreover, this new procedure will promote the implementation of personalized treatments [90]. In this perspective, the detection of further biomarkers might lead to potential therapeutic targets and additional treatment options to improve neuroprotection effectiveness in the context of individually customized therapy. This would maximize the patient's outcomes, with fewer collateral effects, leading to a reduction in the number of treatments, and improving the control of side effects [181]. Therefore, this type of therapeutic approach could have consequences not only on the health and quality of life of the individual patient but also on the health-care and socio-economic sectors. Finally, although further studies are needed, data of this PhD thesis provided further explanations on neuroprotective compounds and are widely inserted in the dense tissue of neuroprotection in the field of degenerative retinal diseases.

CHAPTER 6: CONCLUSIONS

The present study proved that, on well-known experimental conditions of retinal neurodegeneration, the cotreatment with citicoline and homotaurine has synergistic neuroprotective effects. Moreover, the findings of this PhD thesis confirm that pro-inflammatory cytokines and angiogenetic factors are linked to inflammation and angiogenesis, which synergistically contribute to the pathogenesis of DR. Curcumin, homotaurine, and vitamin D3 individually have a slightly appreciable anti-inflammatory effect. However, when used in combination, these substances are able to modify the average levels of the soluble mediators of inflammation and retinal damage. The obtained results emphasise that a multi-target approach may provide a therapeutic strategy for glaucoma and DR treatment in the future. In conclusion, retinal neuroprotection is the next frontier in ophthalmic disease and it could fill the gap of a critical unmet need.

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