UNIVERSITÀ
DI PAVIA

PhD IN BIOMEDICAL SCIENCES
DEPARTMENT OF BRAIN AND BEHAVIORAL SCIENCES
UNIT OF NEUROPHYSIOLOGY

Targeting early stages of diabetic retinopathy: the modulation of VEGF *via* PKCβII/HuR cascade

PhD Tutor: **Prof. Alessia Angela Pascale**

PhD co-Tutors: **Dr. Nicoletta Marchesi**

Prof. Stefano Govoni

PhD dissertation of

Foroogh Fahmideh

XXXV cycle

academic years 2019-2022

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PREFACE

This PhD thesis contains all the results of my research activity undertaken at the Department of Drug Sciences, University of Pavia, Pharmacology section. My work mainly focused on the investigation of Human antigen R (HuR), which acts as a pivotal protein in a variety of pathologies including neurodegenerative and retinal vascular diseases. HuR belongs to the family of the RNA-binding proteins (RBP) ELAV (*E*mbryonic *L*ethal *A*bnormal *V*isual), which at post-transcriptional level play a key role in dictating the fate of newly transcribed mRNAs. ELAV proteins can modulate the cellular response to various stimuli such as proliferation, stress, apoptosis, differentiation, senescence, inflammation, and immune activation especially through post-transcriptional adjustments of the cytoplasmic stability and rate of translation of specific targeted mRNAs. Since the expression of critical proteins that are involved in maintaining cellular homeostasis is regulated by HuR, a derangement in this process can affect various cellular pathways and subsequently contribute to the development of different diseases (Pascale and Govoni, 2012).

Diabetic Retinopathy (DR) is one of the disorders where the implication of HuR has been demonstrated by my group of research (Amadio et al, 2010). DR, among the most common complications of diabetes mellitus, is characterized by degeneration of retinal neurons and neoangiogenesis. More specifically, the disease can be classified into two stages: an early non-proliferative phase (NPDR) and an advanced proliferative (PDR) phase. Typical early changes of this pathology are the thickening of the basement membrane, hyper-permeability, and formation of microaneurysms. These functional alterations are followed by microvascular occlusions leading to a progressive retinal ischemia that induces the production and release of the Vascular Endothelial Growth Factor (VEGF). VEGF is a potent mitogen for endothelial cells that ultimately causes neoangiogenesis (Behl and Kotwani, 2015; Kida et al., 2021), further it has a primary role in promoting vascular hyperpermeability, indeed via phosphorylation of endothelial tight junction proteins modulates their degradation, finally fostering blood-retinal barrier disruption (Hicklin and Ellis, 2016; Moran et al., 2016). Of interest, my group of research previously documented that the diabetes-induced hyperglycemia stimulates protein kinase C βII (PKC βII), which in turn, via activation of HuR, leads to a higher expression

of VEGF (Amadio et al., 2010). Notably, blocking the increase of VEGF via modulation of this novel PKCβII/HuR cascade can represent a new molecular target for a pharmacologic intervention, especially during the early phase of the disease where preventive approaches are strongly needed.

As introduced in chapter 1, with the aim of validating the involvement of the mentioned cascade in DR, we set up two distinct *in vitro* models applying two different stimuli, namely human umbilical vein endothelial cells (HUVEC) were exposed to phorbol 12-myristate 13-acetate (PMA), which mimics diacylglycerol and whose synthesis is triggered by diabetic hyperglycemia, while human retinal endothelial cells (HREC) were treated with a high glucose concentration for different times. Indeed, the identification of proper *in vitro* models is crucial for drug discovery, as they can allow to screen promising effective molecules. To this aim, given that preventive treatments for DR are limited and most of the available treatments are focused on end-stage disease, we also investigated the capability of troxerutin, an antioxidant flavonoid, to impact on this cascade in both the aforementioned *in vitro* models. Based on the obtained results, this study demonstrates that troxerutin can hinder hyperglycemia-induced VEGF rise in both models through the regulation of the PKCβII/HuR pathway. Further, the present findings also suggest the potential use of troxerutin as a preventive treatment during the early phases of DR.

As reported in chapter 2, I was also involved in a collaborative activity with the Department of Cardiovascular, Neural and Metabolic Sciences at the IRCCS Istituto Auxologico Italiano of Milan, in which by using a dual-flow bioreactor, we investigated a simplified model of nervous-cardiovascular systems crosstalk. This system represents a useful tool for setting up, for the first time, a 2-way connected culture of human neuroblastoma cells (SH-SY5Y) and human coronary artery smooth muscle cells (HCASMC) through a dual-flow IVTech Live- Box2 bioreactor. The system was tested by treating the cells with angiotensin II (AngII) in both static and dynamic conditions and exploring the effect on the PKCβII/HuR/VEGF pathway, since both AngII and the PKCβII/HuR/VEGF cascade are relevant in cardiovascular and neuroscience research. We observed that, only when the two cell lines were connected and treated in dynamic conditions, there is a synergic AngII-dependent VEGF production in SH-SY5Y cells coupled to an AngII-dependent activation of the PKCβII/HuR/VEGF pathway in HCASMC. These preliminary observations resemble more closely

what seen *in vivo*, where the increase of VEGF induced by AngII may promote angiogenesis. More importantly, these results underscore that this system can represent a useful tool for studying the crosstalk between cells in dynamic conditions, with the advantageous opportunity of cultivating each cell line in its own medium, thus mimicking, at least in part, a distinct tissue *milieu* (Marchesi et al., 2020).

In chapter 3, two of my review articles regarding another major ocular neurodegenerative disease, glaucoma, are reported. Glaucoma, the leading cause of irreversible blindness in the world, is characterized by progressive retinal ganglion cells (RGC) degeneration and sight loss. The pathophysiology of this disease is not entirely understood; however, it is accepted that apoptosis of RGC and optic nerve fibers give rise to a degenerative and irreversible optic neuropathy resulting in the loss of vision (Gupta and Yücel, 2007) .

The most important risk factor for glaucoma is elevated intra ocular pressure (IOP), nonetheless, it is worth exploring modifiable glaucoma risk factors other than IOP (Coleman and Kodjebacheva, 2009) to better control and lower the burden of the disease. To this aim, as included in part A of chapter 3, we wrote a comprehensive review of the current evidence regarding the effect of lifestyle, dietary habits and supplementation on incidence and progression of glaucoma and their mechanistic correlates. The available data suggest that supplementation with various nutrients/compounds might impact the incidence or progression of glaucoma, even though it should be underscored that nutritional supplements and herbal medicines cannot substitute the traditional anti-glaucoma treatments. However, to determine whether nutritional supplements can become part of the adjuvant treatment of glaucoma, their clinical usefulness and role must be further studied and confirmed in well-designed randomized clinical trials (Fahmideh et al., 2021).

In concert with the previous notion, in section B, the necessity of having a clear frame of regulatory rules, designed for approval and licensing eye drops used in glaucoma is discussed in the other included review. Furthermore, the concept of medical devices made of substances (MDMS), increasingly used in the healthcare system alongside classic medicinal products, is examined. The substantial difference between MDMS and medicinal products lies in their mechanisms of action: the former is based on mechanical, or chemical/physical while the latter is related to pharmacological, metabolic, and immunological mode of action. Among the different substances being administered in adjuvant therapy of

glaucoma, citicoline is a challenging example, worthy of attention. Citicoline has been used in several countries for decades, based on its properties and route of administration, as a drug, food supplement, a food for special medical purposes, or can be dispensed as an MDMS. Its analysis may help to exemplify some of the problems around the sometimes fuzzy border between MDMS and medicinal products suggesting the need for new definitions and regulatory decisions about MDMS (Marchesi et al., 2022).

In chapter 4, through a review paper, the possible interconnection between the eye and the Central Nervous System (CNS), a topic of interest for several years, has been discussed. This strong relation between the eye and the brain is due to the same embryologically origin, so that the retina is considered the "window of the brain" (London et al., 2012). Therefore, studying more deeply the interconnection between neurodegenerative ocular diseases such as glaucoma, age-related macular degeneration, DR, retinitis pigmentosa and neurodegenerative pathologies of CNS like Alzheimer's and Parkinson's disease may help to better define the underlying mechanisms of these heterogeneous disorders as well as the etiology and the correlated risk factors. More importantly, their better comprehension could help to develop new therapies, thus reducing the burden of these diseases and improve the quality of life (Marchesi et al., 2021).

Finally, as discussed in the last chapter of this thesis, I participated in another collaborative project with the IRCCS Mondino Foundation of Pavia. In this study, we propose a new molecular mechanism of action for dimethyl fumarate (DMF), an effective treatment for relapsing remitting Multiple Sclerosis (MS), besides the engagement of the Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2) pathway. My group of research previously documented the potential entanglement of HuR in MS pathogenesis. In the present work, we explored HuR protein levels in peripheral blood mononuclear cells (PBMCs) from MS patients before and after 12 months of DMF treatment compared to healthy controls. Considering that HuR may act on various targets, playing a protective role against oxidative stress, we hypothesized that, according to its primary structure, manganese-dependent superoxide dismutase (*SOD2*) transcript could be a new target of HuR protein. Further, we also examined the potential influence of DMF treatment on HuR/*SOD2* interaction. We were able to demonstrate that DMF induces an increased expression of HuR protein, which ultimately interacts more strongly with *SOD2* transcript promoting the expression of this antioxidant protein, which

possibly contributes to slow down disease progression. Therefore, the activation of this molecular cascade can constitute an additional tool that the cells can exploit to counteract the oxidative stress associated with MS development. Further, the broad-spectrum profile of HuR-mediated effects makes this RNA-binding protein a suitable target that might explain the multifaceted molecular mechanisms underlying the pharmacologic effectiveness of DMF in MS.

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CHAPTER 1

Troxerutin effect on counteracting hyperglycemia-induced VEGF upregulation in endothelial cells

Chapter 1

The following manuscript was published in *Frontiers in Pharmacology* in 2022 as:

Front Pharmacol. 2022; 13: 951833.
Published online 2022 Aug 15. doi: 10.3389/fphar.2022.951833
PMCID: PMC9420903
PMID: 36046820

Effect of troxerutin in counteracting hyperglycemia-induced VEGF upregulation in endothelial cells: a new option to target early stages of diabetic retinopathy?

F. Fahmideh,[1,†] N. Marchesi,[✉1,*,†] L. I. M. Campagnoli,[1] L. Landini,[2] C. Caramella,[1] A. Barbieri,[1] S. Govoni,[1] and A. Pascale[✉1,*]

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[1] Department of Drug Sciences, Pharmacology Section, University of Pavia, Pavia, Italy
[2] Bausch & Lomb—Iom S.p.A, Vimodrone (Milan), Italy
[✉]Corresponding author.

Edited by: Muneeb U. Rehman, King Saud University, Saudi Arabia
Reviewed by: Chiara Bianca Maria Platania, University of Catania, Italy

Effect of troxerutin in counteracting hyperglycemia-induced VEGF upregulation in endothelial cells: a new option to target early stages of diabetic retinopathy?

Foroogh Fahmideh, Nicoletta Marchesi, Lucrezia Irene Maria Campagnoli, Linda Landini, Carla Caramella, Annalisa Barbieri, Stefano Govoni and Alessia Pascale

Abstract

Diabetic retinopathy (DR), one of the most common complications of diabetes mellitus, is characterized by degeneration of retinal neurons and neoangiogenesis. Until today, the pharmacological approaches for DR are limited and focused on counteracting the end-stage of this neurodegenerative disease, therefore efforts should be carried out to discover novel pharmacological targets useful to prevent DR development. Hyperglycemia is a major risk factor for endothelial dysfunction and vascular complication, which subsequently may trigger neurodegeneration. We previously demonstrated that, in the rat retina, hyperglycemia activates a new molecular cascade implicating, up-stream, protein kinase C βII (PKC βII), which in turn leads to a higher expression of vascular endothelial growth factor (VEGF), via the mRNA-binding Hu-antigen R (HuR) protein. VEGF is a pivotal mediator of neovascularization and a well-known vasopermeability factor. Blocking the increase of VEGF via modulation of this cascade can thus represent a new pharmacological option to prevent DR progression. To this aim, proper *in vitro* models are crucial for drug discovery, as they allow to better identify promising effective molecules. Considering that endothelial cells are key elements in DR and that hyperglycemia triggers the PKCβII/HuR/VEGF pathway, we set up two distinct in vitro models applying two different stimuli. Namely, human umbilical vein endothelial cells were exposed to phorbol 12-myristate 13-acetate, which mimics diacylglycerol whose synthesis is triggered by diabetic hyperglycemia, while human retinal endothelial cells were treated with high glucose for different times. After selecting the optimal experimental conditions able to determine an increased VEGF production, in search of molecules useful to prevent DR development, we investigated the capability of troxerutin, an antioxidant flavonoid, to counteract not only the rise of VEGF but also the activation of the PKCβII/HuR cascade in both in vitro models. The results show the capability of troxerutin to hinder the hyperglycemia-induced increase in VEGF in both models through PKCβII/HuR

pathway modulation. Further, these data confirm the key engagement of this cascade as an early event triggered by hyperglycemia to promote VEGF expression. Finally, the present findings also suggest the potential use of troxerutin as a preventive treatment during the early phases of DR.

Keywords: Troxerutin, VEGF, Diabetic Retinopathy, Hyperglycemia, PKC, ELAV proteins, Endothelial Cells

Introduction

Diabetic retinopathy (DR) is characterized by degeneration of retinal neurons and neoangiogenesis (Rossino and Casini, 2019). DR is among the leading causes of blindness, and it is the principal cause of impaired vision in working-age patients (Leasher et al., 2016; Wong and Sabanayagam, 2020). Moreover, roughly 98% of patients with type 1 diabetes and approximately 78% with type 2 diabetes for more than 15 years will face DR (Nicholson and Schachat, 2010).

Clinically, DR is classified into two stages: non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). NPDR represents the early stage of DR, wherein increased vascular permeability and capillary occlusion are two main features in the retinal vasculature. PDR, a more advanced stage of DR, is characterized by neovascularization, which is followed by microvascular occlusions leading to progressive retinal ischemia (Wang and Lo, 2018; Semeraro et al., 2019). Although DR has been traditionally considered a retinal microvasculature disease, a neurodegenerative view of the disease has recently emerged. Notably, retinal neurodegeneration is an early process that cannot be reversed. To this regard, hyperglycemia, together with lipid accumulation, is an important amplifier of oxidative stress, which causes dysregulation of cell metabolism and participates in limiting antioxidant defences during the development of DR. Indeed, diabetes-induced oxidative stress is considered as a key component that dysregulates neurotrophic factors and activates apoptosis, thereby damaging retinal neurons (Ola et al., 2017).

Moreover, the study of DR may have relevance also for other neurodegenerative conditions. Indeed, recent literature data (Wang et al., 2021; Yang et al., 2022) underscore the contribution of vascular cellular elements in neurodegenerative diseases such as Alzheimer's dementia, making the cellular processes involving

them a pivotal element in the study of the mechanisms triggering and sustaining neurodegeneration and in the discovery of new druggable targets. Within this context DR offers a model to study the events taking place at the vascular/neuronal interface (Lynch and Abràmoff, 2017).

Accordingly, these processes are also regulated by numerous mediators, including vascular endothelial growth factor (VEGF, also referred as VEGF-A), which is involved in various events underlying DR progression (Duh et al., 2017). Notably, VEGF is physiologically required for regulating the proliferation and growth of endothelial cells during vasculogenesis. However, several pathological conditions occurring in the course of diabetes upregulate the expression of VEGF (Behl and Kotwani, 2015; Kida et al., 2021), which therefore may lead to increased endothelium permeability, decreased inhibition of proapoptotic proteins, activation of various inflammatory mediators, and ultimately neoangiogenesis. Of note, VEGF has a primary role in promoting vascular hyperpermeability, indeed by inducing phosphorylation of endothelial tight junction proteins modulates their degradation, finally fostering bloodretinal barrier disruption (Hicklin and Ellis, 2005; Moran et al., 2016). Moreover, in the retina, hyperglycemia-associated diabetes leads to the generation of diacylglycerol (DAG) that activates protein kinase C (PKC), especially the beta isoform, which is involved in the positive control of VEGF expression (Amadio et al., 2010; Geraldes and King, 2010).

In previous studies, we identified a novel molecular cascade involved in the development of diabetic retinopathy. Specifically, we showed that upon PKCβII activation, the mRNA-binding HuR/ELAV protein is phosphorylated and can bind to VEGF transcript, thus contributing to abnormally enhanced VEGF expression in the retinal tissue (Amadio et al., 2008, 2010). Incidentally, ELAV (embryonic lethal abnormal visual) are RNA-binding proteins (RBP) able to affect the post-synthesis fate of the targeted mRNAs, from the nucleus to the cytoplasm, primarily increasing their cytoplasmic stability and/or translation rate. This family includes HuR, the ubiquitously expressed one, and three neuron-specific members, namely HuB, HuC, and HuD (Pascale and Govoni, 2012; Bronicki and Jasmin, 2013).

Concerning the therapeutic approach, it should be emphasized that treatments for DR are limited and they are mainly focused on the end-stage of this

neurodegenerative disease (Duh et al., 2017), therefore efforts should be carried out to discover novel pharmacological targets useful to prevent DR development.

We reasoned that blocking the increase of VEGF through the modulation of the PKCβII/HuR cascade may represent a new pharmacological option to prevent DR progression. This target may also be of interest in other neurodegenerative conditions associated with derangement of the vascular/neuronal interface such as in brain ischemia.

As previously mentioned, hyperglycemia is an important amplifier of oxidative stress, and some studies show that diabetes-induced retinal vascular dysfunction can be indeed prevented by inhibitors of reactive oxygen species (Kowluru et al., 2001). Within this context, it is worth inspecting the beneficial effects of various natural compounds such as flavonoids. As potent antioxidants, flavonoids have been considered useful molecules to protect neurons in the diabetic retina (Al-Dosary et al., 2017; Ola et al., 2017), also by counteracting VEGF production (Chung et al., 2005).

Troxerutin is a flavonoid derived from *Saphora japonica* characterized by a free radical scavenging ability likely responsible for the cytoprotective effect observed in different cell types (Panat et al., 2016).

Troxerutin is a hydroxyethylrutoside; thus, most of its pharmacodynamic and pharmacokinetic properties were evaluated based on this structure (Vinothkumar et al., 2014). There are few direct studies on the pharmacokinetics profile of troxerutin in humans. The oral absorption is not high (it is estimated at approximately 10%). However, it should be noted that the oral absorption of flavonoids is a complex process, and their degree of absorption depends not only on the lipophilicity of the molecules, but also on the influence of transporters and enzymes on the membrane surface (Xin et al., 2018). The maximum reported plasma concentration of hydroxyethylrutosides is 142 μg/L that was reached following a single dose of 900 mg orally administered. This substance has a half-life of 24 h after oral administration and 1 h after intravenous use, suggesting the possibility of once-a-day oral administration (Aziz et al., 2015). Like other flavonoids, it undergoes hepatic metabolism and is eliminated predominantly via the bile system, thus it should be taken with caution in patients with hepatic impairment (Ramelet, 2017). To the best of our knowledge, the maximum daily dose that has ever been used in humans is 7,000 mg orally (Glacet-Bernard et al.,

1994). Troxerutin has a safe profile and can cause few adverse reactions, mostly minor gastrointestinal discomfort (Aronson, 2016).

Interestingly, this flavonoid has been shown to reduce neovascularization and VEGF protein production in the retina of diabetic rats (Chung et al., 2005). Moreover, in humans, one study showed that troxerutin given at high doses effectively counteracts retinal vein occlusion thanks to its rheologic properties (Glacet-Bernard et al., 1994).

Therefore, considering that endothelial cells are key elements in DR and that hyperglycemia is a critical factor for diabetes development, we employed two distinct endothelial cell lines applying two different stimuli with the aim of mimicking a hyperglycemia-induced VEGF upregulation via activation of PKCβII/HuR cascade. Namely, human umbilical vein endothelial cells (HUVEC) were exposed to PMA (phorbol 12-myristate 13-acetate), which mimics DAG whose synthesis is triggered by diabetic hyperglycemia (Dasevcimen and King, 2007), while human retinal endothelial cells (HREC) were treated with a high glucose concentration for different times.

We then identified the optimal experimental conditions able to determine an increased production of VEGF in both cellular models. Subsequently, in search of molecules useful to prevent DR development, we investigated the capability of troxerutin to counteract not only the rise of VEGF but also the activation of the PKCβII/HuR cascade in both *in vitro* models.

Materials and methods

Cell cultures and treatment

HUVEC were obtained from Sigma, plated in 25 cm^2 flasks, and cultured in an all-in-one ready-to-use medium (Endothelial Cell Growth Medium; Sigma-Aldrich, Milan, Italy). The flasks were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells were treated with PMA at 100 nM and/or troxerutin at different concentrations (see results).

HREC were obtained from Innoprot, plated in fibronectin coated 75 cm^2 flasks, and cultured in a specific medium (Endothelial Cell Medium; Innoprot, Bizkaia, Spain) with the addition of 10% Fetal Bovine Serum (FBS), Endothelial Cell

Growth Supplement (ECGS), 100 units/mL penicillin and 100 µg/ml streptomycin solution. The flasks were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells were treated with high glucose concentration (25 mM) and/or troxerutin at different concentrations (see results).

MTT assay

Mitochondrial enzymatic activity was estimated by MTT [3- (4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma-Aldrich). A cell suspension of 3.5 x105 cells/well (for 24 and 48 h) and 5 $x10^4$ cells/well (for 72 h and 1 week) in 200 µL was seeded into 96-well plates. Following each treatment, we performed MTT assay following the protocol published in our previous paper (Marchesi et al., 2020). The absorbance values were measured at 595 nm using a Synergy HT microplate reader (BioTek Instruments, Vermont, United States), and the results were expressed as % with respect to control.

Western blotting

Proteins were measured according to Bradford's method using bovine albumin as an internal standard. Proteins were diluted in 2x SDS (Sodium Dodecyl Sulphate) protein gel loading solution, boiled for 5 min, and separated onto 12% SDS-PAGE. The anti-HuR mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, United States), the anti-PKCβII mouse monoclonal antibody (Santa Cruz Biotechnology) and the anti-VEGF rabbit monoclonal antibody (Abcam, Cambridge, MA, United States) were diluted based on each data sheet instructions. Concerning the specific Western blotting procedure, we followed the protocol published in our previous paper (Marchesi et al., 2020). Densitometric analysis were performed using the ImageJ image-processing program.

ELISA assay for vascular endothelial growth factor

The VEGF protein release into the medium was estimated with the respective ELISA kit (Enzo LifeScience, Farmingdale, NY, United States), according to the relative manufacturer's instructions.

This test is based on quantitative sandwich enzyme immunoassay technique, where a VEGF specific monoclonal antibody is already pre-coated on a microplate. Standards and samples were pipetted into the wells and any VEGF present was bound by the immobilized antibody. According to the manufacturer's

instructions, we performed a specific number of washes to remove unbound substances. After that, an enzyme linked polyclonal antibody specific for VEGF was added to the wells. The plate was then washed to remove any unbound antibody-enzyme reagent, a substrate solution was added, and the color developed in proportion to the amount of VEGF bound in the initial step. The color development was stopped with HCl 1N, and the yellow intensity of the color was measured (450 nm) by means of a Synergy HT microplate reader (BioTek Instruments).

Statistical analysis

The GraphPad Prism statistical package (version 7, San Diego, CA, United States) was used for the statistical analysis. The data were analyzed by analysis of variance (ANOVA) followed, when significant, by an appropriate *post hoc* comparison test, as detailed in the legends. Differences were considered statistically significant when p-value ≤ 0.05. The results are expressed as mean ± SD. The N in the legend figure indicates the number of independent experiments, each with 2–3 replicates.

Results

In the first part of the study, we exposed HUVEC to different concentrations of PMA and evaluated the intracellular protein expression of VEGF. The results obtained by Western blotting technique indicate that PMA challenge induces a significant increase in the intracellular content of VEGF following 48 h of exposure at 100 nM (**Figure 1A**). Using this condition, in parallel, we measured the amount of VEGF released in the HUVEC medium using ELISA technique. As depicted in **Figure 1B**, HUVEC challenged with 100 nM PMA for 48 h show increased extracellular levels of VEGF.

Subsequently, using a direct *in vitro* approach, we assessed in HUVEC whether troxerutin could affect cell viability. For this purpose, we performed a MTT cytotoxicity assay. The effect of troxerutin addition to the culture medium was tested at different concentrations (10 nM–1 mM) for 24 and 48 h. In the adopted experimental conditions, we found that troxerutin was safe at all the tested concentrations and times of exposure (Supplementary **Figure S1**). Indeed, a tested product is considered to have a cytotoxic potential only when the cell

viability decreases to <70% in comparison to the control group (Srivastava et al., 2018). Considering that, in our experimental conditions, troxerutin was safe up to 1 mM, we performed the following experiments using troxerutin at this concentration. However, to be more confident, we performed additional MTT assays to verify the cell viability of HUVEC exposed for 48 h to more elevated concentrations of troxerutin (10 and 30 mM). The results indicate that even these higher concentrations of troxerutin do not affect the mitochondrial activity of HUVEC cells (data not shown).

In light of the previous findings (see **Figure 1**), we evaluated the combined effect of PMA (100 nM) and troxerutin (1 mM) exposure on the PKCβII/HuR/VEGF cascade in HUVEC.

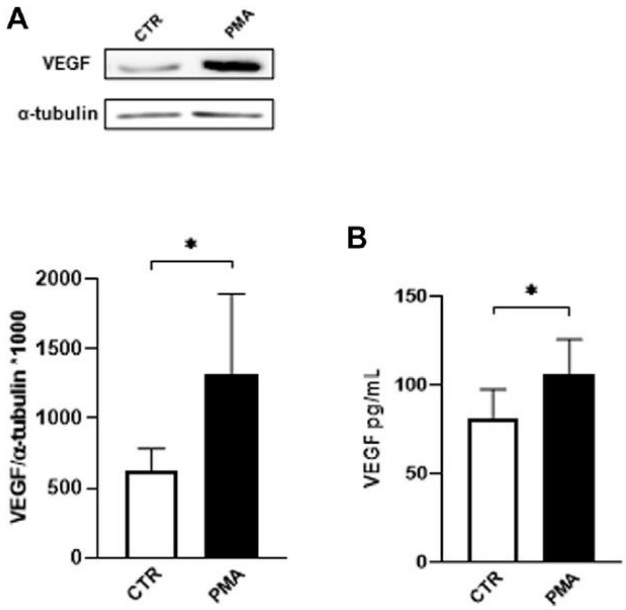

FIGURE 1. (A) Effect of PMA on VEGF intracellular content in HUVEC cells. Cells were exposed to PMA (100 nM) for 48 h. Densitometric analysis of VEGF protein levels. The results are expressed as mean grey levels ratios (mean ± S.D.) of VEGF/α-tubulin immunoreactivities × 1000 measured by Western blotting (upper side: cropped Western blotting images and lower side: densitometric analysis). **(B)** Effect of PMA on VEGF release in the medium of HUVEC cells. Cells were exposed to PMA (100 nM) for 48 h. VEGF protein levels were measured by ELISA. The results are expressed in pg/mL (mean ± S.D.). *p < 0.05, Student's t-test for both intracellular and released VEGF, n = 4 independent experiments. CTR, control; PMA, phorbol 12-myristate 13-acetate.

The data, depicted in **Figure 2**, indicate the capability of troxerutin 1 mM to counteract the increase of both PKCβII and VEGF, the latter both intracellularly (**Figure 2A**) and in the medium (**Figure 2B**), induced by PMA treatment. In

preliminary experiments, we also explored lower concentrations of troxerutin (10 nM, 100 nM and 1 μM), but none of them was able to hinder the VEGF rise induced by PMA, therefore for the following experiments we used troxerutin at 1 mM. Notably, the mM range is in agreement with the effective concentrations of the compound, found in literature, able to produce an antioxidant action when employing cellular models (Panat et al., 2016).

Concerning HuR protein, we observed a trend, although not significant, towards an increased amount of HuR after PMA exposure that was prevented by troxerutin co-exposure. Nevertheless, since the activation of HuR, through its phosphorylation, is an early event, we assessed changes in HuR phosphorylation status following both PMA and the combined treatment (PMA and troxerutin). After 12 h of PMA administration, we found that HuR phosphorylation was significantly increased at serine residue ($p < 0.05$) and that this rise was prevented by the presence of troxerutin (data not shown).

The second part of the study was conducted on HREC, another relevant cell line within the setting of DR, which are cells isolated from the human healthy retina. We firstly investigated the effect of troxerutin on HREC cell viability/proliferation. Based on the results, on HUVEC cells, we decided to focus on the mM range for troxerutin concentrations. Troxerutin's effect on HREC was tested at different concentrations starting from 1 to 30 mM for 72 h (values % ± S.D.: CTR: 100 ± 4.4; Troxo 1 mM: 103 ± 4.1; Troxo 10 mM: 92.2 ± 4.6; Troxo 30 mM: 71.1 ± 6.4). We observed a slight (around 30%) decrease in cell viability only at 30 mM after 72 h of troxerutin incubation, which is still above the "biocompatibility" threshold. As for HUVEC, we decided to perform the following experiments using troxerutin at 1 mM.

We then investigated the effect of troxerutin on HREC cell viability/proliferation in the presence of continuous high glucose challenge for 72 h and 7 days; to be thorough, we also explored shorter times (24 h, 48 h). The glucose concentration was selected according to Giurdanella et al. work (Giurdanella et al., 2017). The results show no changes in the mitochondrial activity following treatment with glucose (25 mM) at any of the investigated times (Supplementary **Figure S2**). Further, we also did not observe any change in the mitochondrial activity following the co-incubation of glucose and troxerutin (1 mM) for 72 h and 7 days (data not shown)

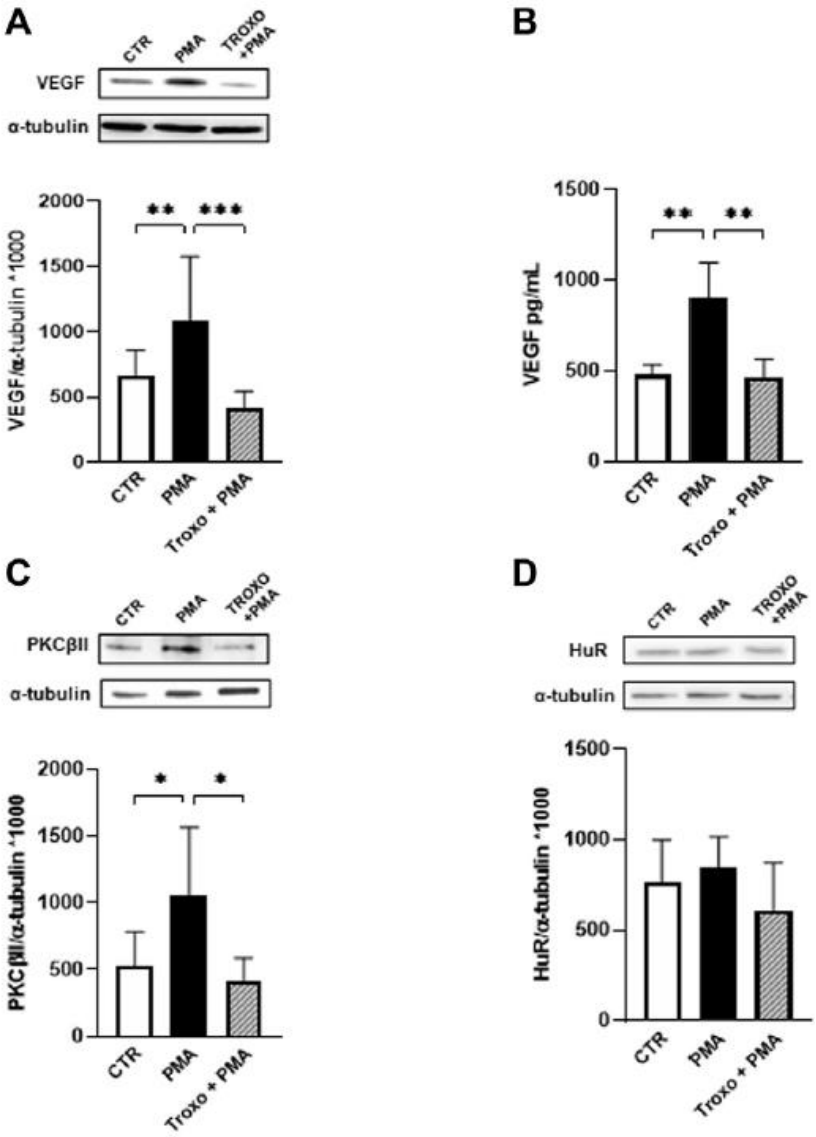

FIGURE 2. Effect of PMA and troxerutin on VEGF intracellular content (**A**) and on its release in the medium (**B**) in HUVEC. HUVEC were co-exposed to PMA (100 nM) and troxerutin (1 mM) for 48 h. The results are expressed as mean grey levels ratios (mean ± S.D.) of VEGF/α-tubulin immunoreactivities × 1000 measured by Western blotting [(**A**); upper side: cropped Western blotting images and lower side: densitometric analysis] and VEGF amount in pg/mL (mean ± S.D.) evaluated by ELISA (**B**). Effect of PMA and troxerutin on PKCβII (**C**) and HuR (**D**) intracellular content in HUVEC. HUVEC were co-exposed to PMA (100 nM) and troxerutin (1 mM) for 48 h. The results are expressed as mean grey levels ratios (mean ± S.D.) of PKCβII/α-tubulin and HuR/α-tubulin immunoreactivities × 1000 measured by Western blotting (upper side a cropped Western blotting image and lower side densitometric analysis). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Dunnett's Multiple Comparisons test, n = 4–8 independent experiments for both intracellular and medium levels. CTR, control; PMA, phorbol 12-myristate 13-acetate; Troxo, troxerutin.

Thereafter, intracellular protein levels of VEGF were evaluated following 72 h (**Figure 3A**) and 7days (**Figure 3B**) of high glucose (25 mM) exposure with and without troxerutin (1 mM). A significant rise in VEGF protein levels was

detected after exposure to high glucose at both times of incubation. Notably, troxerutin 1 mM was able to counteract this increase in VEGF in both the experimental conditions. Moreover, VEGF release was assessed in the medium following 72 h and 7 days of high glucose challenge where we observed a significant increase in VEGF release after 7 days of high glucose exposure. Again, the co-incubation with troxerutin prevented this rise (**Figure 3C**).

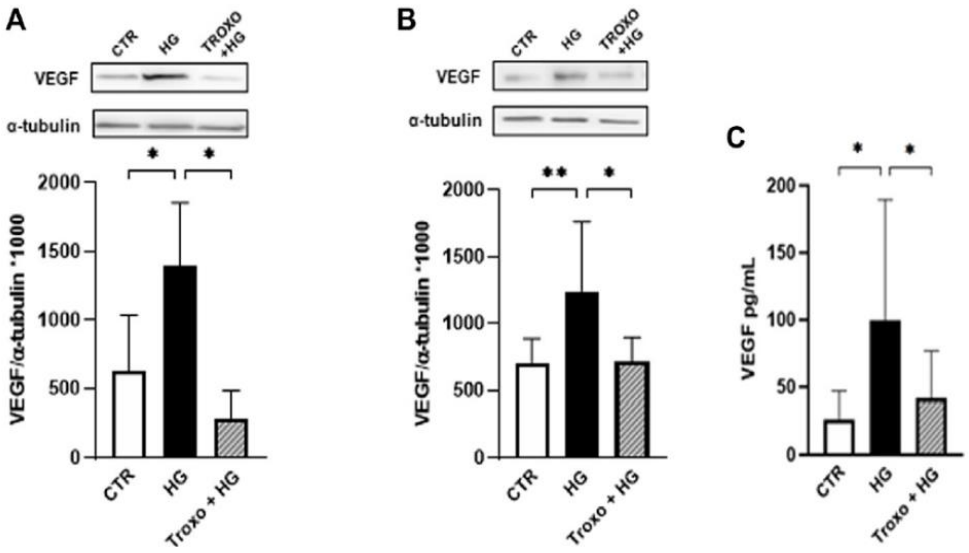

FIGURE 3. Combined effect of high glucose and troxerutin on VEGF in HREC. VEGF was evaluated in the total homogenates of HREC following exposure for 72 h (**A**) and 7 days (**B**) to high glucose levels (25 mM) with or without troxerutin (1 mM). Upper side: cropped representative Western blotting images. Lower side: densitometric analysis. The results are expressed as mean grey levels ratios (mean ± S.D.) of VEGF/α-tubulin immunoreactivities × 1000 measured by Western blotting. (**C**) VEGF release was evaluated by ELISA following 7 days exposure to high glucose levels (25 mM) with or without troxerutin (1 mM). The results are expressed in pg/mL (mean ± S.D.). *$p < 0.05$, **$p < 0.01$, Dunnett's Multiple Comparisons test, n = 4–8 independent experiments. CTR, control; HG, high glucose; Troxo, troxerutin.

As for HUVEC, we then investigated whether the coadministration of continuous high glucose and troxerutin for 72 h and 7 days was able to affect PKCβII and HuR proteins (**Figure 4**). Preliminary experiments were also performed at 24 and 48 h (data not shown); however, 72 h and 7 days were the best conditions to further explore the PKCβII/HuR/VEGF pathway. After 72 h of high glucose exposure, we observed a statistically significant increase in PKCβII protein levels (**Figure 4A**), while no changes were observed in HuR protein (**Figure 4B**). To this last regard, as previously mentioned or HUVEC, we cannot exclude an activation of HuR itself at this time. Of interest, troxerutin was able to counteract

the rise in PKCβII induced by the high glucose stimulus. Following 7 days of continuous glucose exposure, the entire cascade was overexpressed; indeed, we observed an increase in the content of all the examined proteins (**Figures 4C, D**). Again, troxerutin was able to counteract the rise in the intracellular content of PKCβII/HuR proteins induced by such a prolonged high glucose stimulus.

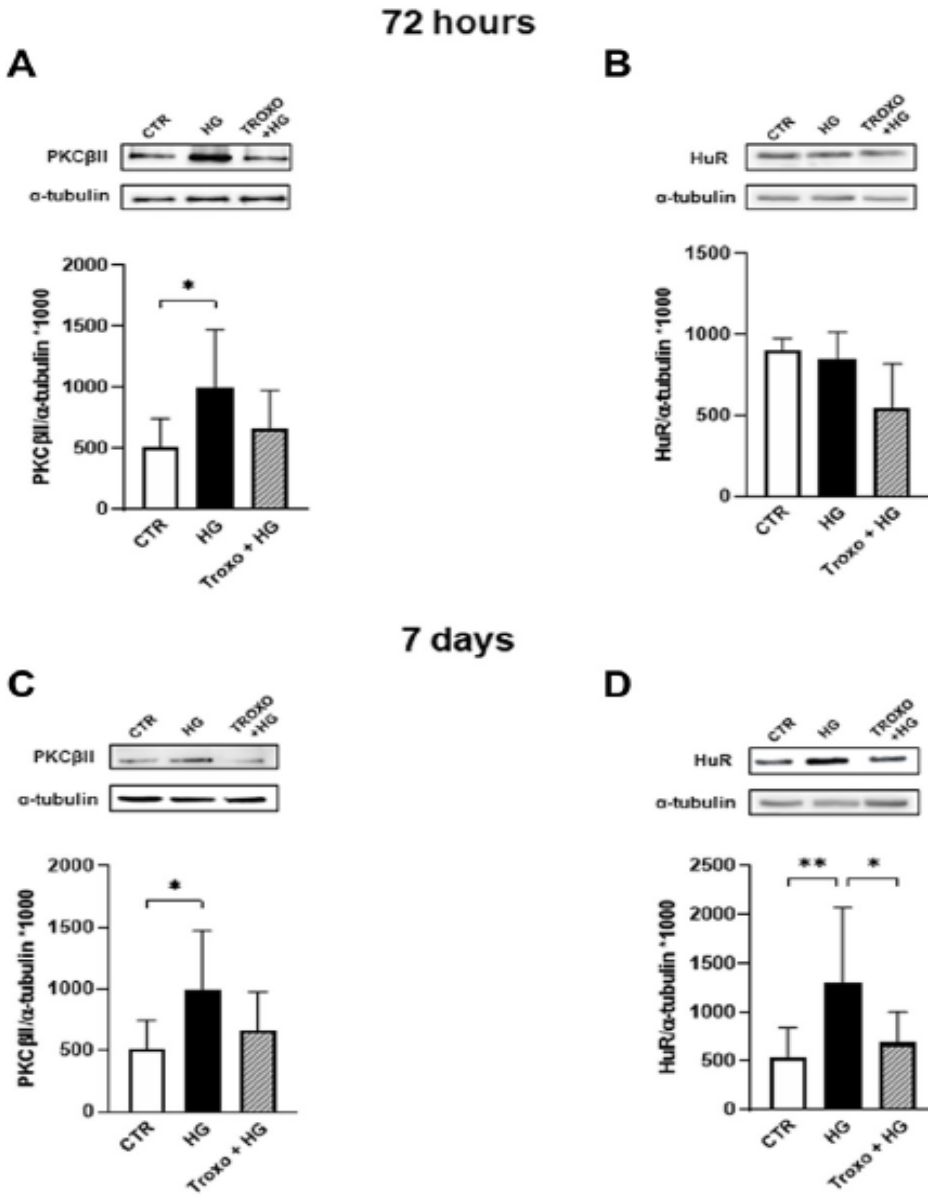

FIGURE 4. Combined effect of high glucose and troxerutin on PKCβII and HuR in HREC. PKCβII and HuR were evaluated in the total homogenates of HREC following exposure for 72 h (**A, B**) and 7 days (**C, D**) to high glucose levels (25 mM) with or without troxerutin (1 mM). Upper side: cropped Western blotting images. Lower side: densitometric analysis. The results are expressed as mean grey levels ratios (mean ± S.D.) of PKCβII/α-tubulin and HuR/ α-tubulin immunoreactivities × 1000 measured by Western blotting. *p < 0.05, **p < 0.01, Dunnett's Multiple Comparisons test, n = 4–8 independent experiments. CTR, control; HG, high glucose; Troxo, troxerutin.

Discussion

The study of the events at the interface of vascular/neuronal cells assumes a critical importance in the investigations of DR progression which is sustained by hyperglicemia (Ceriello, 2000; Madsen-Bouterse and Kowluru, 2008; Sun et al., 2010; Cecilia et al., 2019; Meza et al., 2019). Notably, aberrations in endothelial function often precede many of the abnormalities observed in diabetes and may even precede neurodegeneration. To this last regard, it should be underscored that, besides endothelial cells, the neural retina as well could represent an additional target for drug discovery given that the involvement of neuroretina has been found in an *in vivo* model of early DR (Platania et al., 2019).

Although several molecular mechanisms have been invoked to explain the dysfunctions associated with elevated glucose levels, the primary mechanism underlying the endothelial dysfunction in diabetes mellitus remains largely unknown (Maruhashi and Higashi, 2021). Suggested mechanisms include the polyol pathway flux, oxidative stress, and nonenzymatic glycation (Brownlee, 2005). Within this context, a relevant place is undoubtedly taken by PKC, whose activation is related to many vascular abnormalities (Dasevcimen and King, 2007). PKC comprises at least ten serine-threonine kinases that are widely expressed and engaged in a variety of cellular processes (Battaini and Mochlyrosen, 2007; Govoni et al., 2010). It is worth noting that hyperglycemia causes an increase in DAG, the physiological PKC activator. Although other PKC can also be implicated, the PKCβ appears to be the isoenzyme that is largely activated in the retina among the other isoforms (Bucolo et al., 2021). We previously showed that activated PKCβII is able to stimulate the RBP HuR, via phosphorylation, which in turn targets VEGF mRNA, finally leading to an increased amount of the correspondent VEGF protein (Amadio et al., 2010; Bucolo et al., 2021).

It should be emphasized that, currently, the most commonly used treatments for DR are mainly aimed at patients who are in the more advanced phases of the disease. Although in many cases these treatments slow the progression of DR and present some effectiveness in preventing vision loss, they are not effective in all patients and are invasive therapies, so new, more compliant therapies that are more effective especially in the early stages of DR are needed (Matos et al., 2020). Therefore, taking into account the lack of compounds useful to prevent the development of this neurodegenerative disease, blocking VEGF upregulation,

through the modulation of the PKCβII/ HuR pathway, can constitute a novel pharmacological target that can be exploited in drug discovery in the search of effective molecules against DR.

Within this context, even though numerous studies have sought to identify possible treatments for the prevention and treatment of DR, little attention has been given to natural compounds. Indeed, molecules such as flavonoids have been proven to have significant antioxidant and anti-inflammatory effects (Rossino and Casini, 2019). In many animal models and human studies, it has been shown that flavonoids, a large family of compounds that are extracted from plants, can prevent or attenuate complications associated with DR, as they can modulate lipid and carbohydrate metabolism and insulin resistance, mitigate hyperglycemia, suppress oxidative stress and inflammatory processes (Testa et al., 2016).

In this context, we investigated the capability of troxerutin, an antioxidant flavonoid, to affect the PKCβII/HuR/VEGF molecular pathway. In addition to the described antioxidant action (Panat et al., 2016; Al-Dosary et al., 2017; Ola et al., 2017), troxerutin has been also reported to exert several additional pharmacological effects, including anti-inflammatory, antihyperlipidemic, and nephroprotective. Besides, it is suggested to be endowed with some therapeutic roles against neurodegenerative, cardiovascular diseases and diabetes (Zamanian et al., 2020). To this last regard, in a clinical study, the troxerutin-treated group, as compared with the placebo one, showed significant improvement in visual acuity, retinal circulation times, and macular edema; further, treated subjects exhibit diminished progression of ischemia and decreased red blood cell aggregability (Glacet-Bernard et al., 1994). Moreover, in diabetic rats, oral administration of troxerutin at the early stage of DR has been shown to significantly reduce VEGF protein levels compared to controls (Chung et al., 2005).

As mentioned before, we used two different endothelial cell lines challenged with two distinct stimuli: HUVEC with PMA and HREC with glucose. HUVEC cell line provides a classic model system to study many aspects of endothelial functions and disease-associated alterations, such as normal and abnormal angiogenesis, oxidative stress, and inflammation-related pathways. Indeed, HUVEC have been tested to demonstrate stimulation-dependent angiogenesis and key endothelial cell signaling pathways (Kocherova et al., 2019). As a stimulus,

we selected PMA, a direct activator of PKC since it mimics its physiologic stimulator, DAG. Indeed, in retinal vascular cells, the early biochemical changes associated with diabetic hyperglycemia leads to the generation of DAG (Dasevcimen and King, 2007). Therefore, PMA is an appropriate stimulus to mimic some of the earliest events induced by high glucose on endothelial cells. Our present data show a significantly increased protein content of VEGF, at both intracellular and extracellular levels, after 48 h of PMA challenge in HUVEC cells. This rise in VEGF seems to rely upon the engagement of the PKCβII/HuR cascade, which acting at post-transcriptional level favors the expression of VEGF. These results confirm our previous findings showing, both *in vitro* (Amadio et al., 2008, 2012; Platania et al., 2020) and *in vivo* (Amadio et al., 2010; Bucolo et al., 2021), the key implication of the RNA binding protein ELAV/HuR in modulating VEGF expression. Of interest, we document that troxerutin is able to successfully counteract VEGF increase and to hinder, upstream, the activation of the PKCβII/HuR pathway.

A subsequent approach was the use of HREC cells, which are isolated from the human retina and have become a valuable model to examine the effects of diabetes as a whole on the mechanisms of retinal endothelial cell damage and repair (Malek et al., 2018). In this model, we challenged the cells with a physiologic stimulus, glucose, for 72 h and 7 days. Specifically,we observed an increase in VEGF levels, both intracellularly and extracellularly, following the glucose challenge. Once again, the rise in VEGF is mediated by the activation of PKCβII/HuR pathway. Indeed, we observed an upregulation of this cascade, which was more evident following 7 days of glucose exposure. In this last regard and in agreement with our previous work (Amadio et al., 2010; Bucolo et al., 2021), we can hypothesize that HuR, besides promoting VEGF protein expression, can affect not only the expression of PKCβII but also its own expression. Of note, we also document that troxerutin, again, is able to prevent the hyperglycemia-dependent VEGF increase and PKCβII/HuR upregulation.

It should be stressed that troxerutin counteracts the increase of VEGF in both models without suppressing the physiological production of VEGF, which has instead beneficial functions for endothelial cells (Amadio et al., 2016). Indeed, we did not find any significant difference in the amount of VEGF between controls and troxerutin-treated samples (i.e., controls versus troxerutin + PMA or troxerutin + high glucose). These results confirm previous data (Amadio et al.,

2010) and support the concept that the activation of this cascade is only responsible for the abnormal/detrimental production of VEGF.

Taken together, the present findings emphasize the engagement of the PKCβII/HuR cascade as an early event triggered by hyperglycemia to promote the expression of VEGF, the primary player in vascular hyperpermeability and endothelial proliferation. Indeed, we confirmed the key involvement of this cascade in two different endothelial cell lines challenged with two distinct stimuli able to directly induce hyperglycemia (high glucose itself) or mimic one of the early consequences of glycemic conditions (the synthesis of DAG) (see **Figure 5**). Blocking the activation of this pathway can thus constitute a new pharmacological approach to face DR development. Therefore, both models can be helpful within the drug discovery field to assess the potential effect of different compounds as a preventive therapeutic option such as troxerutin. In this regard, we show here that, remarkably, troxerutin is able to preempt the hyperglycemia induced increase in VEGF in both *in vitro* models, thus suggesting its potential use in DR. It seems that troxerutin ameliorates diabetic retinopathy by downregulating neoangiogenesis factors as well as hindering free radical production, since the latter plays a central role in neuronal degeneration by activating the inflammatory and apoptotic pathways. Indeed, protecting retinal neurons from oxidative stress and hindering VEGF upregulation, together with the consequent vascular damage, through the use of natural substances such as troxerutin may be an efficacious strategy for a preventive treatment during the early phases of DR. However, we cannot exclude the possibility that troxerutin may also act at retinal level also via additional mechanisms. For example, a recent work documented that among the gene pathways networks commonly dysregulated in DR retinas are included those linked to fibrosis, another key hallmark of DR that is presently targeted by pharmacological research (Platania et al., 2018). Indeed, troxerutin has been shown to be endowed with an antifibrogenic action (Geetha et al., 2015), therefore future experiments may explore the capability of troxerutin to also counteract retinal fibrosis. Further, considering the implication of genes associated to platelet activation in DR (Platania et al., 2018) and that troxerutin has been reported to improve retinal viscosity microcirculation (Malinska et al., 2019) future studies may also address the effect of troxerutin on platelets function.

Nevertheless, despite all these beneficial effects, troxerutin is manly present into the market in multi-component supplements, hence the present data may direct the market towards the development of troxerutin-based preparations specifically addressed to ocular diseases. Further, future efforts should also be done to explore whether mechanisms as those here described take place at the doses and times that are used in systemic administration in humans, and also following the use of topical formulations.

Moreover, it will be of interest to investigate whether the described pathway and troxerutin have a role also in events taking place at the level of cerebral vascular endothelial cells exposed to high glucose concentrations.

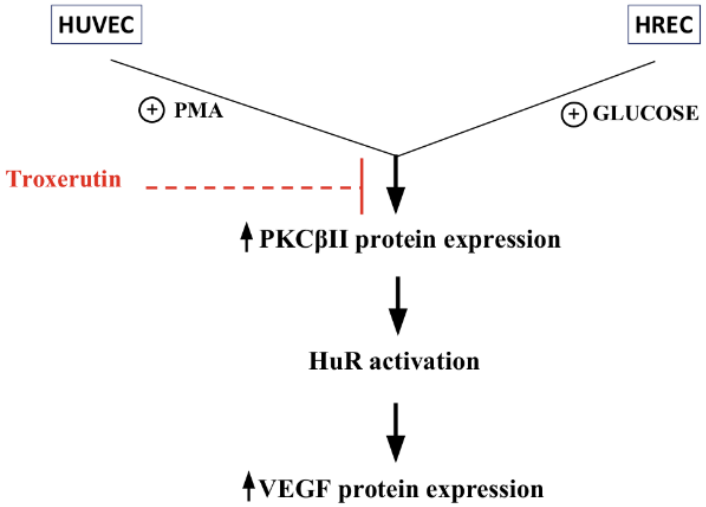

FIGURE 5. Outline of the effect of troxerutin on the PKCβII/HuR/VEGF cascade activated by hyperglycemia in endothelial cells. The figure shows the engagement of the PKCβII/HuR cascade in determining the increase in VEGF expression within the events triggered by hyperglycemia and indicates the upstream effect of troxerutin in counteracting the upregulation of the entire cascade. We did not investigate in deep the mechanisms of troxerutin-induced reduction in VEGF expression, but it can be supposed that troxerutin may inhibit the over-expression of PKC or its targets in these diabetic in vitro models. The analysis of the troxerutin targets through the Swiss TargetPrediction software (http://www.swisstargetprediction.ch) shows that troxerutin may indeed interact with several PKCs, including PKCβII, thus suggesting that additional mechanisms may be studied upstream/downstream its antioxidant actions to better characterize the effect of this substance.

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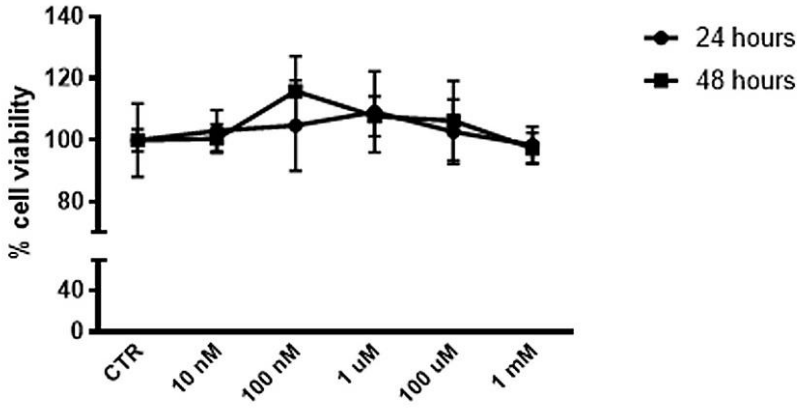
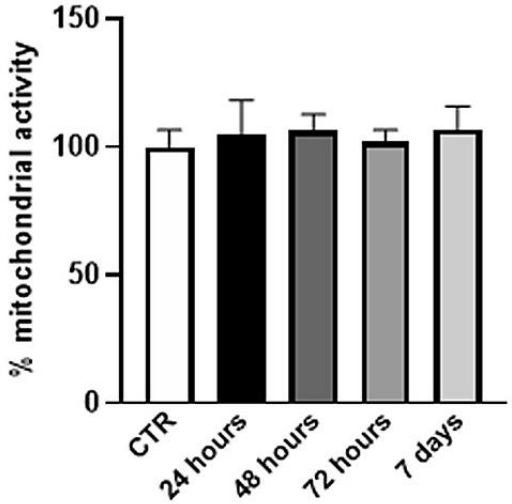
Supplementary Material



Supplementary Figure S1. Effect of troxerutin on HUVEC mitochondrial activity. HUVEC were exposed to different concentrations of troxerutin for 24 and 48 h. Absorbance values obtained after the MTT assay are expressed as mean percentages ± S.D. over controls (CTR, 100%), n=8 independent experiments. CTR, control.



Supplementary Figure S2. Effect of glucose exposure on HREC mitochondrial activity. HREC were exposed to high glucose (25 mM) in continuous contact for 24 h, 48 h, 72 h and 7 days. The results are expressed as mean ± S.D. related to MTT measures as a percentage of controls (CTR, 100%), n=8 independent experiments. CTR, control; HG, continuous high glucose.

CHAPTER 2

Collaborative activity: assessment of a simplified model of nervous-cardiovascular systems crosstalk by PKCβII/HuR/VEGF pathway activation

Chapter 2

The following manuscript was published in *PLOS ONE* in 2020 as:

PLOS ONE

OPEN ACCESS PEER-REVIEWED

RESEARCH ARTICLE

Use of dual-flow bioreactor to develop a simplified model of nervous-cardiovascular systems crosstalk: A preliminary assessment

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Published: November 30, 2020 • https://doi.org/10.1371/journal.pone.0242627

Use of dual-flow bioreactor to develop a simplified model of nervous-cardiovascular systems crosstalk: A preliminary assessment

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Abstract

Chronic conditions requiring long-term rehabilitation therapies, such as hypertension, stroke, or cancer, involve complex interactions between various systems/organs of the body and mutual influences, thus implicating a multiorgan approach. The dual-flow IVTech LiveBox2 bioreactor is a recently developed inter-connected dynamic cell culture model able to mimic organ crosstalk, since cells belonging to different organs can be connected and grown under flow conditions in a more physiological environment. This study aims to setup for the first time a 2-way connected culture of human neuroblastoma cells, SH-SY5Y, and Human Coronary Artery Smooth Muscle Cells, HCASMC through a dual-flow IVTech LiveBox2 bioreactor, in order to represent a simplified model of nervous-cardiovascular systems crosstalk, possibly relevant for the above-mentioned diseases. The system was tested by treating the cells with 10nM angiotensin II (AngII) inducing PKCβII/HuR/VEGF pathway activation, since AngII and PKCβII/HuR/VEGF pathway are relevant in cardiovascular and neuroscience research. Three different conditions were applied: 1- HCASMC and SH-SY5Y separately seeded in petri dishes (static condition); 2- the two cell lines separately seeded under flow (dynamic condition); 3- the two lines, seeded in dynamic conditions, connected, each maintaining its own medium, with a membrane as interface for biohumoral changes between the two mediums, and then treated. We detected that only in condition 3 there was a synergic AngII-dependent VEGF production in SH-SY5Y cells coupled to an AngII-dependent PKCβII/HuR/VEGF pathway activation in HCASMC, consistent with the observed physiological response *in vivo*. HCASMC response to AngII seems therefore to be generated by/derived from the reciprocal cell crosstalk under the dynamic inter-connection ensured by the dual flow LiveBox 2 bioreactor. This

system can represent a useful tool for studying the crosstalk between organs, helpful for instance in rehabilitation research or when investigating chronic diseases; further, it offers the advantageous opportunity of cultivating each cell line in its own medium, thus mimicking, at least in part, distinct tissue *milieu*.

Introduction

Biomedical molecular research aimed to the study of complex relationships between various tissues, as it happens in chronic diseases or when investigating resilience from a traumatic event, such as for example in rehabilitation or during aging, needs translationally relevant experimental models. *In vivo/ex vivo* models using laboratory animals or classic *in vitro* cultures on petri dishes have been extensively employed so far allowing researchers to achieve many important findings. However, cells growing in a petri dish do not behave like the original cells belonging to organs in living organisms. They are not connected with the whole complex environment and, furthermore, they are seeded on a hard matrix without being subjected to flow conditions [1]. Overall, this setup is far from properly reproducing the physio-mechanical characteristics of the organ of interest, and cells do not communicate with other different cell types neither bioelectrically nor through biohumoral exchange. Of note, though, a static system is useful to study reactions to specific stimuli, at both biochemical and molecular level. On the other side, such a system is not representative of the exchange of information through various mechanisms occurring physiologically between the various cell types located in different organs. These complex interactions are better modelled in *in vivo* models that, on the other hand, may be hard to study at molecular level. The recent development of Next-Generation In Vitro Testing Tools, engineered within the 3Rs research, opens a new scenario to explore living systems providing a bridge between the use of cultured cells on a petri dish and in vivo/ex vivo experiments on small laboratory animals. An example of such a tool are IVTech Bioreactors [1], where, within the tissue engineering field, a bioreactor is defined as a device able to simulate a physiological environment allowing cell or tissue growth. Inside IVTech Bioreactors, cells are subjected to the physiological shear stress and nutrient absorption typical of the blood stream [1, 2], and two cell types can be connected to study their reciprocal crosstalk under physiological or pathological conditions. This system is modular, with culture chambers designed to be added sequentially or in parallel, thus simulating

a multiple organ system [1, 3–5]. Further, it is designed to be consistent with plates or transwells, thus allowing the use of standard protocols for *in vitro* procedures.

The primary goal of the present study was to create for the first time an *in vitro* model able to connect two distinct cell types, namely human neuroblastoma cells (SH-SY5Y; widely used as a neuronal-like cellular model [6]) and Human Coronary Artery Smooth Muscle Cells (HCASMC), in a dual-flow IVTech LiveBox2, thus representing a simplified model of the nervous-cardiovascular systems crosstalk. Since bioreactor technology is relatively recent, information on a number of methodological aspects is still lacking (i.e. culture conditions, growing condition for several types of cells, flow parameters etc.). Moreover, with respect to the classic *in vitro* models, extensively used within the last decades and for which several procedures are available, only a few tried-and-tested experimental protocols are available for researchers to work under dynamic conditions, especially when considering specific interconnected cell lines, as SH-SY5Y cells and HCASMC. Therefore, our primary aim was to develop and share with the scientific community a new co-culture set-up involving specifically SH-SY5Y cells and HCASMC, where each cell type is seeded in its own medium, to avoid a forced adaptation to a different culture medium, and under flow conditions, thus mimicking a more physiological environment.

Secondly, we aimed to explore some aspects of the nervous-cardiovascular systems crosstalk. The dynamic reciprocal relationship between brain and heart is important both in acute, second by second regulation, and in chronic derailment situations when either organ is suffering because of a disease or of an improper drug use (see [7] for an extensive analysis of these relationships). In particular, within this context, we chose to explore some of the relationships involving AngII treatment effects on the two cell types, seeded inside IVTech Bioreactor Live-Box 2 and connected under flow conditions, and to study AngII-dependent PKCβII/HuR/VEGF (vascular endothelial growth factor) cascade activation in different experimental settings.

Materials and methods

IVTech LiveFlow1and LiveBox2

The system (IVTech Srl., Massarosa, LU, Italy) consisted of a peristaltic pump (IVTech Live-Flow1), which creates the flow, connected with modular and transparent double flow bioreactors named LiveBox2 (LB2), where cells are seeded. Cell medium, in the supplied 25 mL plastic bottle, is connected to the IVTech LiveFlow1and to the LB2 by silicon tubes. LB2 is a dual-flow IVTech bioreactor formed by two chambers, upper and lower, developed to model physiological barriers *in vitro* (**Fig 1**). In particular, LB2 consists of three parts (**Fig 1C**):

1. an apical chamber with a wet volume of 1.5 mL, equipped with an inlet and an outlet tube;
2. a basal chamber with a wet volume of 1 mL, equipped with an inlet and an outlet tube;
3. a membrane holder, placed between the two chambers.

All the components of the IVTech bioreactor were autoclaved and the entire experiment was performed under a laminar flow hood (**Fig 1B**). Membranes were conditioned keeping them in ethanol 70% for two hours and exposed to UV light for 15 min, before cell seeding.

The hood surface was cleaned with the same detergents normally used to sterilize materials employed in cell cultures. The removable transparent glass bottom allows live imaging during culture and enables sample processing (**Fig 2**).

The possibility of having two independent circuits, one for the apical and one for the basal chamber, is an advantage that allows solving the problem of having two different media. Chambers are made in a biocompatible silicone polymer with self-sealing properties [4]. The chambers were connected by tubes, and the circuit dimensions were calculated using allometric laws [3]. The membrane is a polyester 0.45 μm-pore membrane, optically transparent and treated to permit cell adhesion; the pores allow the passage of cell metabolism products, preventing the translocation of the cells between the two environments of the bioreactor. Modules can be configured in different ways to obtain in series or in parallel

circuits. The whole system is designed to be compatible with the most common laboratory instruments, like microscope and incubators, having the connected bioreactors the typical size of a multi-well plate (**Fig 1A, 1B and 1D**).

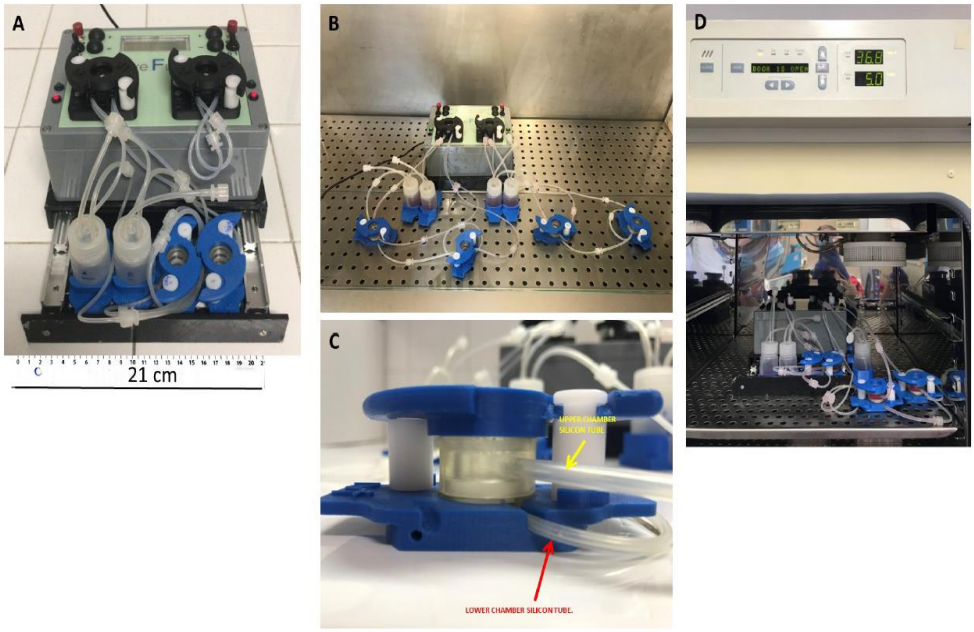

Fig 1. The IVTech system settings. A) Example of basic system size with two LB2, measuring stick in cm. B) The complete setting with four LB2 (for AngII vs control treatments) under laminar flow hood. C) Example of LB2 with outlet silicon tubes, in the figure the lower chamber is hidden by the blue support. D) The complete setting with four LB2 inside humidified incubator at 37°C with 5% CO_2.

Static cell cultures

Human neuroblastoma SH-SY5Y cells were obtained from ATCC (Manassas, VA) and cultured in T75 flasks in a humidified incubator at 37°C with 5% CO_2. SH-SY5Y cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin, L-glutamine (2 mM), non-essential amino acids (1 mM), and sodium pyruvate (1 mM). HCASMC were obtained from Gibco and were cultured in a humidified incubator as SH-SY5Y cells (at 37°C with 5% CO_2). HCASMC were grown in Medium 231 supplemented with Smooth Muscle Growth Supplement and 1% penicillin–streptomycin. In MTT experiments, the cells were exposed to 1, 10 and 100 nM AngII (Sigma, A925) for 6, 24 and 48 hours. For Western blotting experiments, the cells were exposed to 10 nM AngII for 6 hours. The entire experiment was performed under a laminar flow hood.

Dynamic cell cultures

HCASMC and SH-SY5Y cell types were seeded in two different LB2. The LB2 allows to monitor what happens in the first and second compartment independently, both for observations under the microscope (**Fig 2**), and for any sampling. In the connected setting, HCASMC were in contact with mediators eventually released by SH-SY5Y cells, thus simulating the crosstalk between tissues (**Fig 3**). SH-SY5Y cells were seeded at $2x10^5$ cells/mL, on the glass in the bottom of LB2, with a tangential configuration, whereas HCASMC were seeded at $8x10^4$ cells/mL onto the membrane within the other LB2 (**Figs 2 and 3**). LiveFlow1system together with the two LB2 were placed in the cell culture incubator at 37˚C with 5% CO_2, to keep an aseptic condition (**Fig 1D**). Both cells lines were stimulated with 10 nM AngII. The entire experiment was performed under a laminar flow hood.

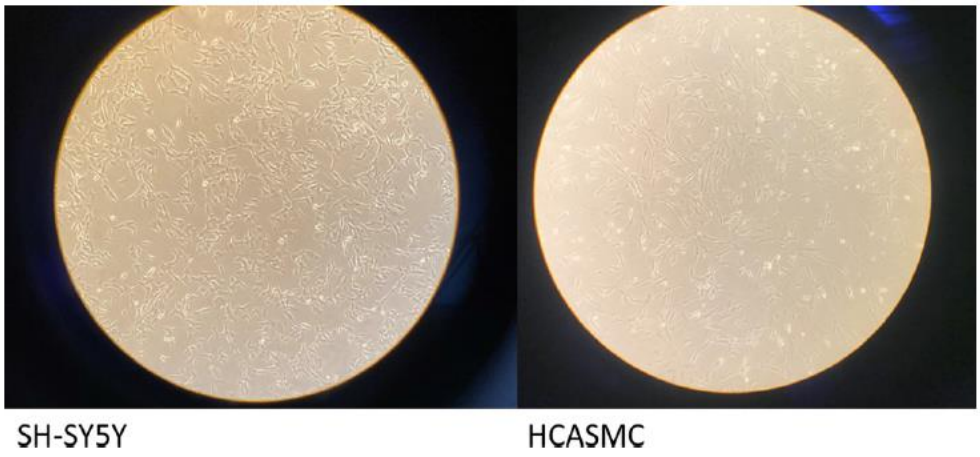

Fig 2. SH-SY5Y cells (left) and HCASMCs (right) seeded under flow condition in two different LB2. SH-SY5Y were seeded on the glass in the bottom of LB2, HCASMC were seeded onto the membrane within the other LB2.

Experimental design

The experimental design was developed according to the following steps:

1. Assessment of SH-SY5Y and HCASMC viability after AngII treatment in static conditions performed by MTT, after exposing for 6, 24 and 48 hours both cell types, seeded in petri dishes, to increasing concentrations of AngII (1-10-100nM).

2. Assessment of AngII-dependent PKCβII/HuR/VEGF activation in static conditions, in SH-SY5Y and HCASMC cell types, performed by Western blot.

3. Assessment of AngII-dependent PKCβII/HuR/VEGF activation evaluated, under dynamic conditions, in separately seeded SH-SY5Y and HCASMC cell types without any biohumoral exchange between them and performed by Western blot.

4. Assessment of: a) AngII-dependent PKCβII/HuR/VEGF activation investigated, under dynamic conditions, in connected SH-SY5Y and HCASMC cell types, and performed by Western blot; b) VEGF release examined in SH-SY5Y and HCASMC respective medium evaluated by ELISA. Biohumoral exchange was possible through the porous membrane.

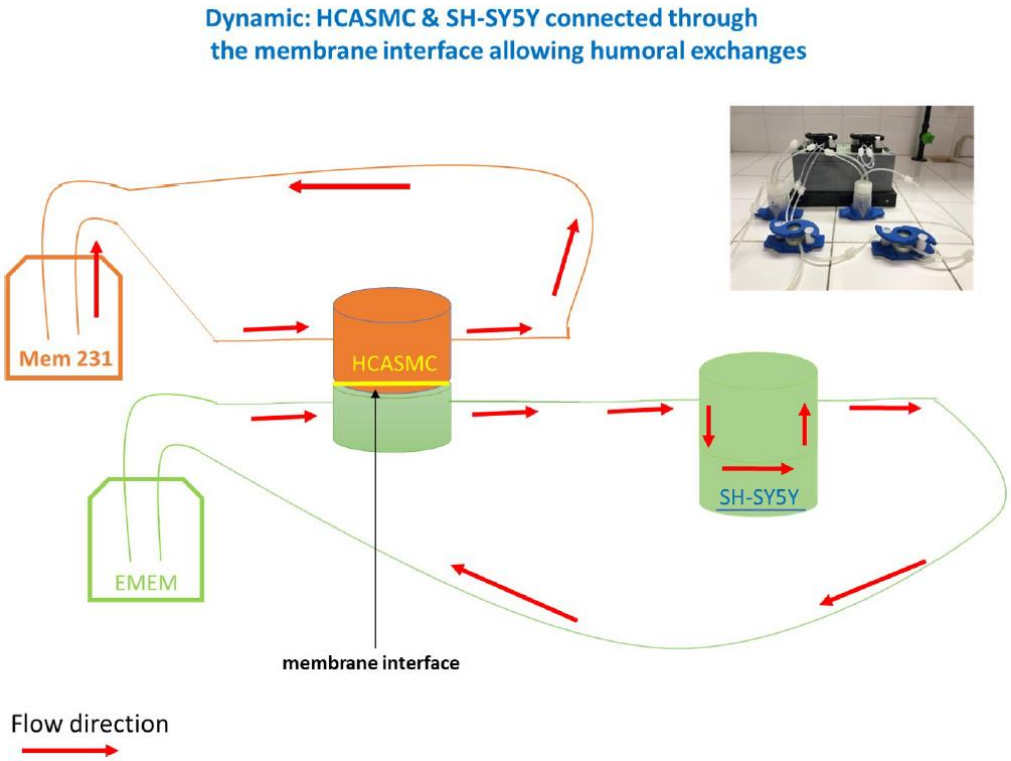

Fig 3. The experimental setting enables to connect, under flow conditions, the two cell lines exposed to their own medium (orange: HCASMC; green: SH-SY5Y). The mediators released from SH-SY5Y cells can interact with HCASMC cells through the membrane interface. This setting avoids the potential confounding effect of a common culture media, thus allowing the identification of specific factors released by each cell type. As indicated in **Fig 1B**, the bioreactor consists of two modules, one setting used for control and the other for treatment with AngII (10nM).

MTT assay

Mitochondrial enzymatic activity was estimated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma). A cell suspension of $5x10^3$ cells/mL (for HCASMC cell line) and $2x10^4$ cells/mL (for SH-SY5Y cell line) was seeded into 96-well plates. Following each treatment of 6, 24 and 48

hours, 50 µL of MTT (concentration equal to 2.5 mg/mL) were added to each well. After incubation at 37° C for 3 hours, the purple formazan crystals were formed. The formed crystals were solubilized in dimethylsulfoxide (DMSO; Sigma-Aldrich). Specifically, after removing the MTT from the wells, 100 µL of DMSO were added in order to lyse the cellular and mitochondrial membranes and solubilize the formazan crystals. After 10 minutes, absorbance values were measured at 595 nm using a Synergy HT microplate reader (BioTek Instruments) and the results expressed as % with respect to control.

Western blotting

Proteins were measured according to Bradford's method, using bovine albumin as internal standard. Proteins were diluted in 2xSDS protein gel loading solution, boiled for 5 min and separated on 12% SDS-PAGE. The anti-PKCβII rabbit polyclonal antibody (Santa Cruz), anti-HuR mouse monoclonal antibody (Santa Cruz) and the anti-VEGF rabbit monoclonal antibody (Abcam) were diluted based on each datasheet instructions. The nitrocellulose membrane signals were detected by chemiluminescence. The same membranes were re-probed with α-tubulin antibody and used to normalize the data. Statistical analysis of Western blot data was performed on the densitometric values obtained with the ImageJ image-processing program (https://imagej.nih.gov/ij).

ELISA assay

The VEGF protein levels in SH-SY5Y and HCASMC cells were estimated in the respective medium with a specific ELISA kit (R&D Systems Inc.), according to the manufacturer's instructions.

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF is already pre-coated onto a microplate. Standards and samples were pipetted into the wells and any VEGF present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for VEGF was added to the wells. Following a wash to remove any unbound antibody enzyme reagent, a substrate solution was added and the colour developed in proportion to the amount of VEGF bound in the initial step. The colour development was stopped and the intensity of the colour measured (570/450 nm).

Statistics

For statistical analysis the GraphPad Instat statistical package (GraphPad software, San Diego, CA, USA) was used. The data were analysed by analysis of variance (ANOVA) followed, when significant, by an appropriate *post hoc* comparison test, as detailed in the legends. Differences were considered statistically significant when p values ≤ 0.05.

Results

Static conditions

Cell viability after AngII treatment. The cell viability was studied after exposing both SH-SY5Y and HCASMC cell types for 6, 24 and 48 hours to increasing concentrations of AngII (1nM, 10nM and 100nM). SH-SY5Y cell viability was unaffected after 6 and 24 hours of AngII exposure (at all the tested concentrations). Instead, after 48 hours treatment, consistent with previously published data [8], a significant decrease in SH-SY5Y viability was observed at all AngII concentrations (85.5% ±2.1, 78.1% ±2.1 and 74.9% ±1.9 at 1nM, 10nM and 100nM respectively). On the contrary, and as expected [9], MTT assay carried out on HCASMC did not show a significant decrease in cell viability following 6, 24 or 48 hours, at any AngII concentration (**Fig 4**).

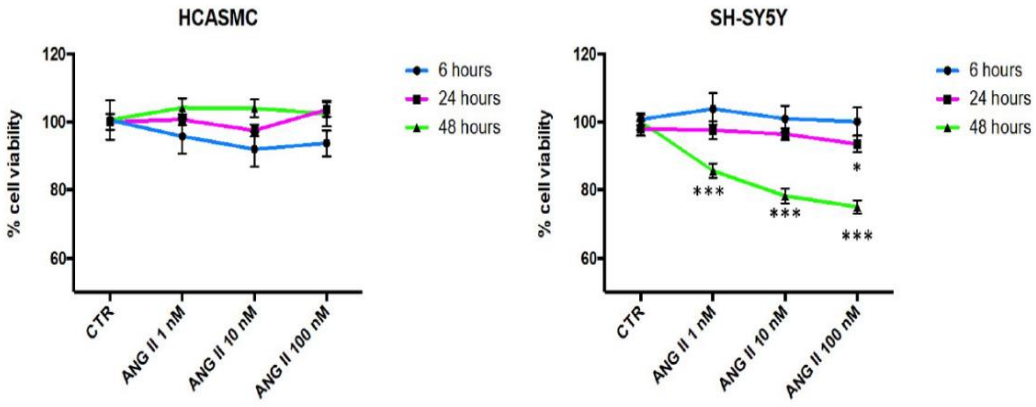

Fig 4. Cell viability of SH-SY5Y cells after 6, 24- and 48-hours following Angiotensin II (ANG II) exposure at 1 nM, 10 nM, 100 nM. The values are expressed as mean MTT [in %±S.E.M.]. ***p< 0.001, *p<0.05 vs control (CTR), Dunnett's multiple comparisons test (n = 16).

Based on this evidence, the 10 nM AngII treatment for 6 hours was chosen, as it did not affect viability and it was well tolerated by both cell lines, and specifically by SH-SY5Y.

AngII treatment and PKCβII/HuR/VEGF cascade. There was no significant difference in PKCβII/HuR/VEGF cascade activation after 10 nM AngII treatments in both cellular types when seeded in static conditions (**Fig 5**).

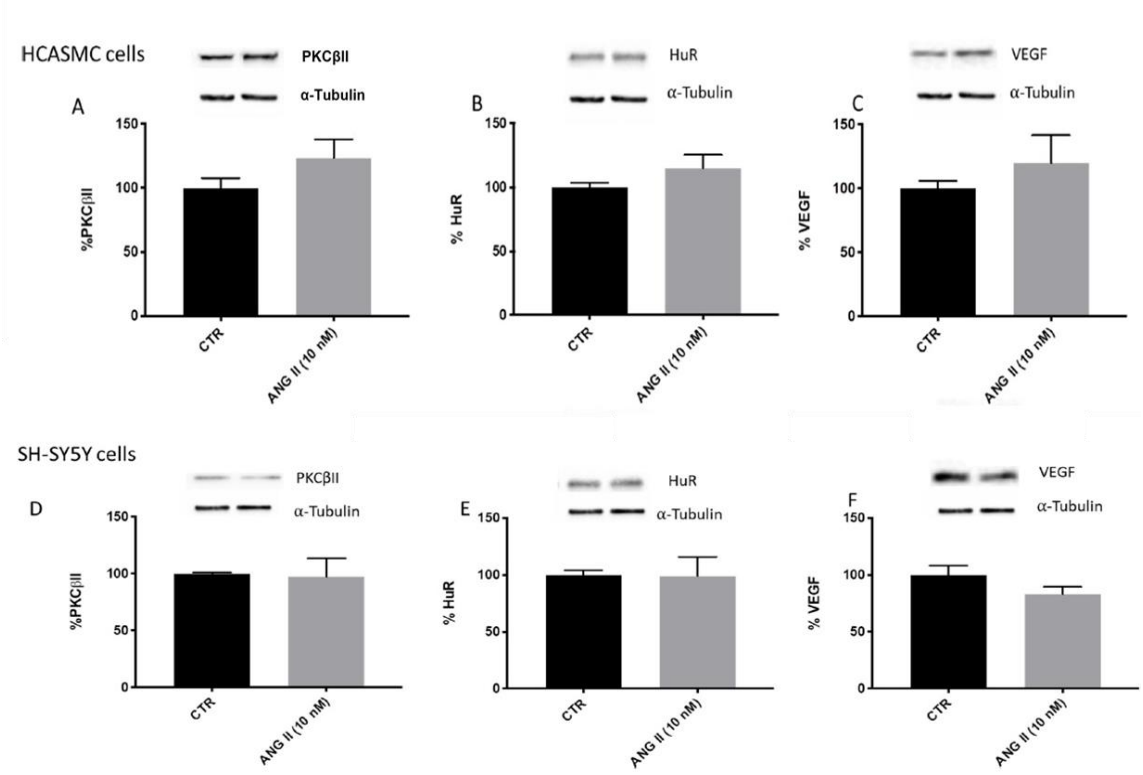

Fig 5. Densitometric analysis and representative Western blotting of PKCβII, HuR and VEGF protein levels in the total homogenate of HCASMC (upper) and SH-SY5Y cells (lower) exposed to solvent (CTR) or Angiotensin II (ANG II) at 10 nM for 6 hours, in static condition. PKCβII, HuR and VEGF bands were normalized to α-tubulin, and the results are expressed in percentage ± S.E.M. with respect to the control value (100%), n = 8–10.

Dynamic conditions

Parameters. The following parameters have resulted to be the most suitable for cell growth in LB2:

a. Cell density: $8x10^4$ cells/mL for HCASMC and $2x10^5$ cells/mL for SH-SY5Y.

b. Flow Rate: 200 µL per minute in both circuits.

AngII treatment and PKCβII/HuR/VEGF cascade in non-connected cells. When cell types were separately seeded in dynamic conditions, the results confirmed what seen in static cultures, with no difference in PKCβII/HuR/VEGF cascade activation after AngII treatments in both cellular types (**Fig 6**).

Cell lines in connection: AngII treatment and PKCβII/HuR/VEGF cascade. Once connected each other as shown in **Fig 3**, following AngII exposure at 10 nM for 6 hours under flow conditions, there was a statistically significant increase of PKCβII/HuR/VEGF pathway activation in HCASMC (VEGF: +215.2% ±58.3 vs control, PKCβII: +85.8% ±34.5 vs control, HuR: +100.4% ±32.3 vs control), while no change was observed in SH-SY5Y cells (**Fig 7**). Nevertheless, we observed an AngII-dependent increase in VEGF protein release in SH-SY5Y medium (control: 80.5 pg/mL ± 8.8, AngII: 138.0 pg/mL ± 6.7, control vs treatment p = 0.002) while no changes were found in VEGF protein release in HCASMC medium (control: 93.5 pg/mL ± 33.5, AngII: 80.5 pg/mL ± 15.5, control vs treatment p = N.S.) (**Fig 8**)

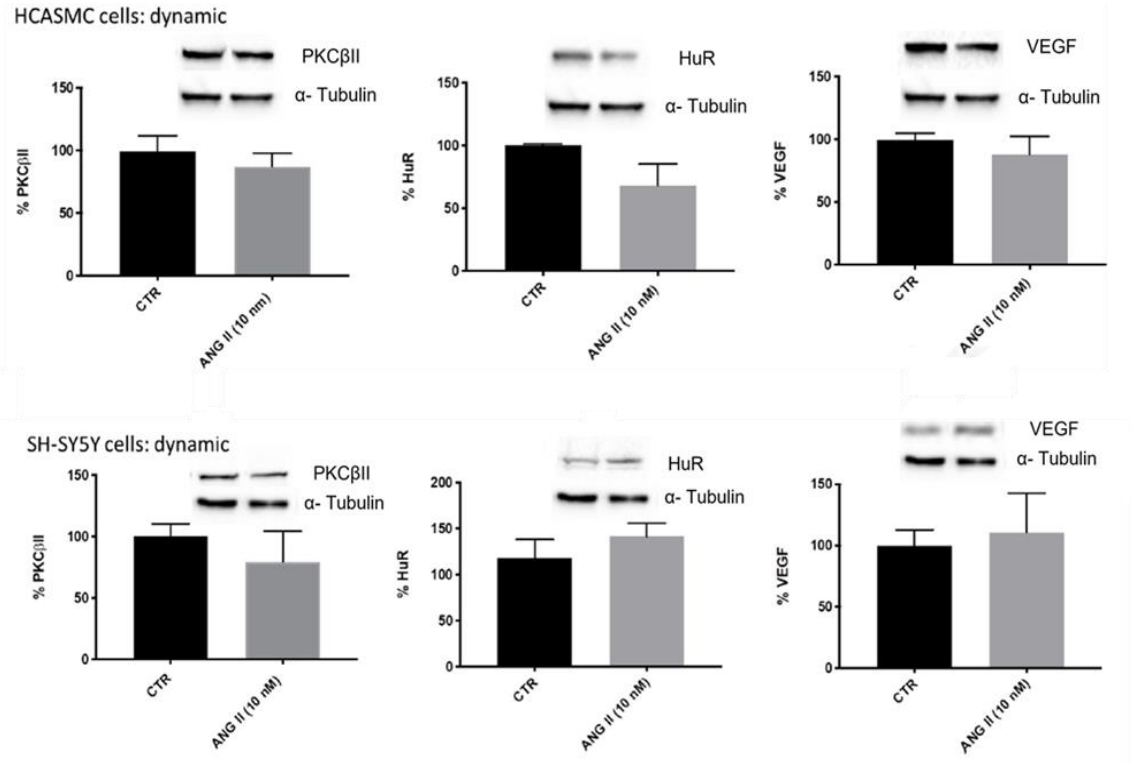

Fig 6. Densitometric analysis and representative Western blotting of PKCβII, HuR and VEGF protein levels in the total homogenate of HCASMC (upper) and SH-SY5Y cells (lower) exposed to solvent (CTR) or Angiotensin II (ANG II) at 10 nM for 6 hours under flow conditions, not connected. PKCβII, HuR and VEGF bands were normalized to α-tubulin, and the results are expressed in percentage ± S.E.M. with respect to the control value (100%), n=3.

Discussion

Our group has been involved for years in studying the interactions between the nervous and cardiovascular systems [10–13], and the evidence that stress [11], pain [14] or peptides acting at central level [10, 15] might affect cardiovascular functions has pushed us to deepen the study of the crosstalk between the two systems. The main novelty of this study was to create an *in vitro* model able to explore the in vitro dialogue between two distinct human cell lines (SH-SY5Y; HCASMC), grown and connected in a bioreactor, representing a simplified model of the nervous-cardiovascular systems crosstalk.

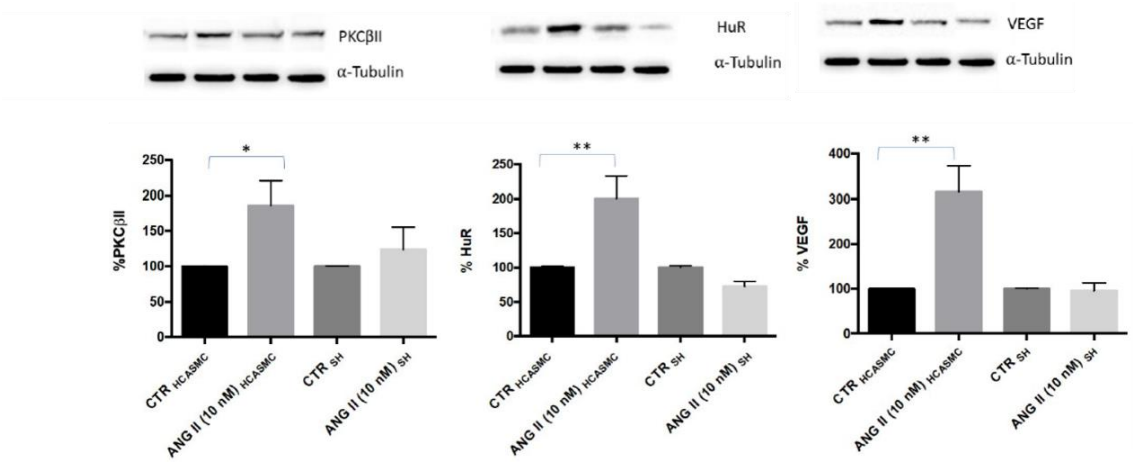

Fig 7. Densitometric analysis and representative Western blotting of PKCβII, HuR and VEGF protein levels in the total homogenate from HCASMC and SH-SY5Y (SH) cells connected and exposed to solvent (CTR) or Angiotensin II (ANG II) at 10 nM under flow conditions for 6 hours. Optical densities of PKCβII, HuR and VEGF bands were normalized to α-tubulin, and the results are expressed in percentage ± SEM with respect to the relative control value (100%). *p<0.05; **p<0.01; Unpaired t test, n = 5.

The innovative feature of this work is represented by the possibility to have two different environments in LiveBox2, where the conditions can be set up by the user. In particular, two different environmental conditions were used: the apical side of the LiveBox2 chamber was filled with the SH-SY5Y medium, whereas the basal compartment was filled with the HCASMC medium. This is an innovative feature since, in general, a multi-organ approach needs to previously characterize the potential common medium, which has to be compatible with all the tissues within the pathway [16]. This process forces the cells to adapt to a novel environmental condition that is not the best option for them; therefore, this could cause a change in their behaviour. In this work, we solved the problem by using the native media developed for that specific cell type. Another innovative contribution consists in providing an experimental set up useful to culture cells

belonging to different organs, connected under flow conditions and seeded in a dual flow bioreactor. Specific guidelines on flow rate and speed to use, or volume and type of culture medium were lacking and, in this regard, we have improved technical knowledge on bioreactors use for connecting cells growing in a different medium.

This also represented an improvement within the 3Rs research (Replacement, Reduction, Refinement of animal models), which develops new methods to enhance the quality standards of preclinical experimental models. To test the system, we explored AngII treatment effects on the two cell types, seeded inside IVTech Bioreactor LiveBox 2 and connected under flow conditions, and we investigated AngII-dependent PKCβII/HuR/VEGF cascade activation in different experimental settings.

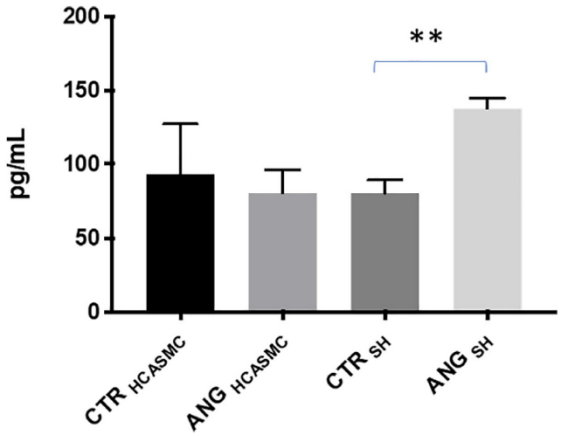

Fig 8. Released VEGFA protein in the medium of cells, following treatment with Angiotensin II for 6 hours under flow conditions when cells were connected. The release of VEGFA (pg/mL) was measured by ELISA and expressed as the means ± S.E.M. **p < 0.01, Student's t-test, n = 5.

In mammal organisms, AngII has important roles in both cardiovascular and nervous systems: the renin-AngII system (RAS) constitutes one of the most important systems in the physiological regulation of blood pressure, and an inappropriate level of AngII is considered as a major risk factor in the development of cardiovascular diseases. Furthermore, several studies have demonstrated that an altered RAS may cause both neurodegeneration [17] and cardiovascular complications, often affecting each other's [18–21]. Moreover, although controversial [22], the existence of a so-called brain renin–angiotensin system [23–28], which might play a role in the regulation of neuroinflammation and progression to potential rehabilitation problems, is gaining interest, with concerns on societal and individual patient costs [11, 29–32]. Since Ang II is a

potent stimulator of VEGF [33, 34] and its stimulus is able to favour the shuttling of HuR protein from the nucleus to the cytoplasm [35–37], a possible AngII-dependent activation of PKCβII/HuR/VEGF pathway was investigated in HCASMC and in SH-SY5Y, both in static and under flow conditions. The PKCβII/HuR/VEGF pathway is a molecular cascade, first described by our group in retinal bovine pericytes, which controls VEGF expression also under hypoxic conditions. In particular, PKCβII is able to increase VEGF protein expression through the RNA-binding protein ELAV/HuR [37–39]. This *in vitro* model was settled through four main steps: first, in preliminary experiments, the cells under flow with respect to static conditions were observed, also selecting the more appropriate parameters for the dynamic setting. Second, AngII-dependent PKCβII/HuR/VEGF pathway activation in SH-SY5Y and HCASMC, both under static and dynamic conditions, were separately studied. Third, the best growing conditions to put the two cell lines in connection were identified. Finally, the two cell lines subjected to flow were connected and exposed to AngII treatment, evaluating the PKCβII/HuR/VEGF pathway in a condition of potential crosstalk.

We think we have reached the goal to culture and study different cell types in connection, mainly thanks to the use of a dual flow IVTech LiveBox 2 bioreactor system, which allows cells to grow in their own medium, enabling physio-pathological phenomena to be simulated *in vitro*. This system represents an innovative model, developed according to the 3Rs research objectives, that meets the necessary requirements of a bioreactor apparatus [40]. Finally, unlike the classical co-culture, dual-flow bioreactor made possible communication among different cell types, without the potential confounding effect of a common culture medium, thus allowing the identification of specific factors released by each cell type.

From the data collected during the setting of the system, and following the AngII treatment, we could make some preliminary considerations on cell response: according to literature [8], data on SH-SY5Y showed a weak decrease in viability (93.5%±1.8) after 24 hours of treatment with AngII 100 nM. MTT assay carried out on HCASMC did not show a significant decrease in cell viability following 6, 24 or 48 hours, at any AngII tested concentration. This is consistent with literature data showing that HCASMC treated at the same concentrations of angiotensin used in this work do not lose their ability to divide and migrate [9]. After the set-up of the system, we verified the feasibility of seeding and connecting HCASMC and SH-SY5Y cell types inside the dual flow model of

IVTech LiveBox2 bioreactor under flow conditions, thus allowing biochemical communication between the two cell types, each grown in its culture medium. According to literature and to our preliminary experiments, a flow of 200 µL/min was applied in both circuits. In fact, in this model, higher flow rates caused cells detachment from the membranes and some evidence [41–43] suggested possible DNA damage following excessive shear stress. The behaviour displayed by the cells, once put in communication, has generated some critical information not otherwise collectable with previous methodologies:

1) In HCASMC connected to SH-SY5Y cells, AngII treatment caused an increased intracellular expression of VEGF through the activation of the PKCβII/HuR cascade, with no change in VEGF release in HCASMC medium. The absence of a change in VEGF in HCASMC medium, despite its increase in SH-SY5Y medium (**Fig 8**), indicates the good separation of the two media ensured by the membrane interface. However, the results suggest that VEGF released by SH-SY5Y was able to stimulate HCASMC laying onto the membrane, thus possibly activating the PKCβII/HuR cascade. Nevertheless, we cannot exclude that the duration of the experiment (6 hours) was not enough to allow HCASMC producing a measurable VEGF amount in the medium.

While VEGF is a pivotal factor for vascular development and angiogenesis [44] and its production is a well-known cell response to hypoxic conditions [45–50], HuR belongs to a small family of evolutionarily conserved RNA-binding proteins, named ELAV, which act at posttranscriptional level and are able to influence virtually any aspect of the post-synthesis fate of the targeted mRNAs [51]. Of interest, VEGF is a target of HuR and we previously demonstrated that, in the rat retina, diabetes-activated PKCβII/HuR cascade induces VEGF overexpression [37]. Further, the upstream inhibition of this cascade blunts these effects, thus supporting the concept that the PKCβII/HuR cascade can modulate, post-transcriptionally, VEGF expression through a route that is independent from the classic VEGF transcriptional control [37]. Notably, in hypoxic conditions, such as those also observed within the context of diabetic retinopathy, both the transcriptional and post-transcriptional pathways may be operant in controlling VEGF expression.

2) A significant increase of VEGF protein was observed in SH-SY5Y medium after AngII cell exposure and connection of the two cell lines.

The release of VEGF in the medium by SH-SY5Y cells may be interpreted as a reaction to hypoxia, this is in accordance with previously published data showing that oxygen deprivation caused VEGF production in SH-SY5Y at both mRNA and protein level, and with the evidence of a correlation between AngII and hypoxic conditions [47, 48, 52–55]. SH-SY5Y cells have AngII type 1 receptor localized in the membrane [56], and when treated with AngII they displayed a stressed behaviour with subsequent upregulation of factors responsible for VEGF-mediated angiogenesis [8, 57]. Considering that we observed in HCASMC an increased intracellular VEGF expression only when HCASMC were connected with SH-SY5Y, it is tempting to speculate that a factor released by SH-SY5Y, perhaps VEGF itself [48], might have given to HCASMC a signal for a risk of hypoxia, thus promoting the activation of PKCβII/HuR/VEGF cascade in HCASMC.

Interestingly, the preliminary observations obtained after the connection of the two cell types through the bioreactor resemble more closely what seen in vivo. In particular, AngII administration in several in vivo models was reported to increase VEGF production, also via HuR activation, and to promote angiogenesis. In mice treated with Ang II infusion there was an increased VEGF expression inside the cells of the aortic wall [58]. In a model of cardiac hypertrophy induced by AngII administration, a significant increase in HuR cytoplasmic translocation, indicative of its activation, was observed in neonatal rat hypertrophic cardiomyocytes [59], thus emphasizing the involvement of HuR in the AngII-mediated increase of VEGF protein expression, as observed in our model.

AngII was also reported to enhance angiogenesis associated with tissue ischemia, via VEGF production. Indeed, in a murine model of myocardial ischemia [60], Ang II-pretreated rat mesenchymal stem cells showed enhanced VEGF synthesis, tube formation and angiogenesis *in vivo*, and in a model of femoral artery occlusion, AngII significantly increased VEGF protein content in ischemic hindlimb [61]. The same group described an AngII-dependent VEGF expression within the neovascular stromal interface in the Matrigel model in mice [62].

Study limitation

This project was designed as a preliminary exploration of a system to culture cells belonging to different organs, connected under flow conditions and seeded

in a dual flow bioreactor. The novelty of this work relies on the use of dual flow bioreactor system to culture and study the crosstalk between HCASMC and SH-SY5Y cell lines in vitro, each maintaining its own medium. Specific guidelines on flow rate and speed to use, or volume and type of culture medium were lacking [63] and, in this regard, we have improved technical knowledge on bioreactors use for connecting cells growing in a different medium. Further applications by other investigators should validate the reliability and robustness of the system. Biochemical studies were used to test the system and not as main goal of our paper. Although we reported and discussed some preliminary observations, a complete biochemical assessment of mediators released by each of the connected cells and a comprehensive study on their eventual proliferation, migration and differentiation were not made. Nevertheless, considering the scope of this project, which was to test new technologies potentially useful in experimental medical research, we feel to have reached the goal of giving useful information on an innovative tool with a great potential in preclinical studies.

Perspectives

Chronic disorders requiring rehabilitation therapies are difficult to investigate due to the extreme complexity of the interaction between the involved systems, e.g. nervous, cardiovascular and/or immune. A comprehensive understanding of the crosstalk among them is still lacking and studies with appropriate methodological approaches are needed to explore in detail the reciprocal role of mediators and cell communication. In this context, this new model is able to be alongside the existing *in vivo*, *ex-vivo* and *in vitro* tools given its unique property of being simultaneously enough simple to allow specific observations and reasonably complex to more closely reflect the physiological conditions of the cell's environment. This work does open new perspectives in future investigation on biochemical crosstalk between cells belonging to different organs and different systems, and describes a new model that can be combined with *in vivo* and classic *in vitro* models, supporting a global approach to 3R's in preclinical research.

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CHAPTER 3

Critical reviews: Evaluation of pharmacological and non-pharmacological approaches in glaucoma, a silent neurodegenerative disease

Part A

The following manuscript was published in *Survey of Ophthalmology* in 2021 as:

ELSEVIER

Survey of Ophthalmology

Volume 67, Issue 3, May–June 2022, Pages 675-696

Review article

Non-drug interventions in glaucoma: Putative roles for lifestyle, diet and nutritional supplements

Foroogh Fahmideh PhD Candidate, PharmD [#] ✉, Nicoletta Marchesi PhD [#] ✉, Annalisa Barbieri PhD ✉, Stefano Govoni PhD ✉, Alessia Pascale PhD ✉

Department of Drug Sciences, Pharmacology Section, University of Pavia, Pavia, Italy

Received 1 April 2021, Revised 15 September 2021, Accepted 20 September 2021, Available online 23 September 2021, Version of Record 21 May 2022.

Non-drug interventions in glaucoma: Putative roles for lifestyle, diet and nutritional supplements

Foroogh Fahmideh, Nicoletta Marchesi, Annalisa Barbieri, Stefano Govoni and Alessia Pascale

Abstract

Glaucoma is a major ocular neurodegenerative disease characterized by progressive retinal ganglion cells degeneration and sight loss. Current treatment options have been limited to reducing intraocular pressure (IOP), known as the leading risk factor for this disease; how- ever, glaucoma can develop even with low or normal IOP and progress despite controlling IOP values. Lifestyle, dietary habits, and supplementation may influence some of the risk factors and pathophysiological mechanisms underlying glaucoma development and progression; thus, the role of this complementary and alternative medicine in glaucoma has received great interest from both patients and ophthalmologists. We provide a summary of the current evidence concerning the relationship between lifestyle, dietary habits, and effects of supplements on the incidence and progression of glaucoma and their targets and associated mechanisms. The data suggest the existence of a therapeutic potential that needs to be further explored with both preclinical and rigorous clinical studies models.

Keywords: Glaucoma, Lifestyle, Dietary Supplements, Nutrients, Vitamins, Smoking, Exercise, Alcohol Drinking, Intraocular Pressure, Optic Nerve

Introduction

Retinal diseases are known causes of visual impairment and blindness that affect economic and educational opportunities, reduce the quality of life, and increase the risk of death [16]. Among them, glaucoma, a major ocular neurodegenerative disease, is the leading cause of irreversible blindness in the world, [71] with an increasing prevalence estimated to affect 76 million people worldwide [6, 195]. Glaucoma is usually classified into two main categories: open-angle glaucoma and angle-closure glaucoma [212]. Elevated intraocular pressure (IOP), aging,

family history, epigenetic and environmental factors are recognized risk factors for glaucoma [135, 212].

The pathophysiology of glaucoma is not entirely understood; however, it is accepted that apoptosis of retinal ganglion cells (RGCs) and optic nerve fibers give rise to a de- generative and irreversible optic neuropathy resulting in the loss of vision [48, 192]. Several mechanisms may play a role in RGC damage, including vascular dysregulation, oxidative stress, immune-inflammatory response, excitotoxicity, and mitochondrial impairment [151, 156].

The most important risk factor for glaucoma is elevated IOP [60]; therefore, the current standard therapy is to lower eye pressure using a combination of topical antiglaucoma medications, laser therapy, and surgical intervention. Although all approved treatments for this disease involve IOP reduction, glaucoma may develop even in the presence of low or normal IOP and progress despite having a controlled IOP. This can occur because of apoptosis and subsequent death of RGCs and adjacent cells, including astrocytes and oligodendrocytes that initiate an apoptosis cascade, leading to further damage of the neural cells [60, 106, 212]. Another probable cause contributing to glaucoma progression despite normal IOP is impaired ocular blood flow. Reduced ocular blood flow may lead to oxidative stress and consequently glaucomatous damage. Furthermore, some patients continue to lose sight notwithstanding well- controlled IOP, suggesting that IOP-independent mechanisms contribute to disease progression. Such mechanisms may resemble those mentioned above, which are also observed in neurodegenerative diseases [45, 60, 106, 156, 212]. Indeed, the literature is consistent in indicating that the control of IOP is not sufficient to arrest the neurodegenerative process [52, 180].

The latter notion has guided the development of studies exploring the possibility of developing novel pharmacological interventions directed to hinder the neurodegenerative process. In this context, both mechanistic and clinical studies have been redirected, although so far with limited success, to examine the possibility of counteracting the degenerative process and controlling IOP. It is increasingly discussed that a neurodegenerative cascade occurs in glaucomatous patients, even in those with normal IOPs [26]. Therefore, independently or complementarily to IOP-based treatments, neuroprotection can be considered an additional therapeutic strategy that aims at preventing or attenuating the degeneration of RGC and other neuronal components [138, 211].

Lifestyle may influence some of the risk factors and pathophysiological mechanisms underlying glaucoma development and progression. Within this context three are the main field of interest: a) general aspects of lifestyle, including physical exercise and habits such as smoking and alcohol consumption; b) diet aspects and attention similarly to the approaches followed in cardiovascular disease prevention; c) the use of specific nutrients as dietary supplements.

Nutritional supplementation comprises a broad array of products intended for ingestion to meet essential nutritional requirements. Because of the possibility that nutritional intake may positively influence some of the risk factors and pathophysiological mechanisms underlying glaucoma development and progression [9, 169], it is of interest to review the subject. These products can be categorized as vitamins, minerals, herbals, botanicals, amino acids, fatty acids, and other dietary supplements used individually or in combination.

We provide a summary of the current evidence regarding the effect of lifestyle, dietary habits and supplementation on incidence and progression of glaucoma and their mechanistic correlates (see **Table 1**).

Lifestyle

The first part of this review will discuss various modifiable factors, including physical activity, body mass index (BMI), cigarette smoking, alcohol consumption, and diet, that have been investigated in relation to their influence on IOP and on the risk of developing primary open-angle glaucoma (POAG) (See **Fig. 1**).

Exercise and physical activity

Exercise facilitates IOP reduction in the immediate post- activity period; however, the activity's type and intensity affect this lowering [100, 228]. Although the exact mechanism is not elucidated, it is suggested to be related to nitric oxide re- lease, as well as shifting the blood flow to the muscles and the raising of plasma osmolality [62, 171]. Nevertheless, exercise may be harmful in one specific glaucoma type, namely pigment dispersion glaucoma [181].

IOP does not have a fixed value and can vary over time. In a crossover study on healthy subjects, IOP was reduced after aerobic activity [133]. Also, data suggest that the current fitness profile affects the intensity of IOP reduction after activity;

further decrease was seen in sedentary individuals compared to active ones [30, 173]. IOP fluctuation should be monitored closely, especially in different lifestyle-related situations, to understand better how the disease can be managed effectively.

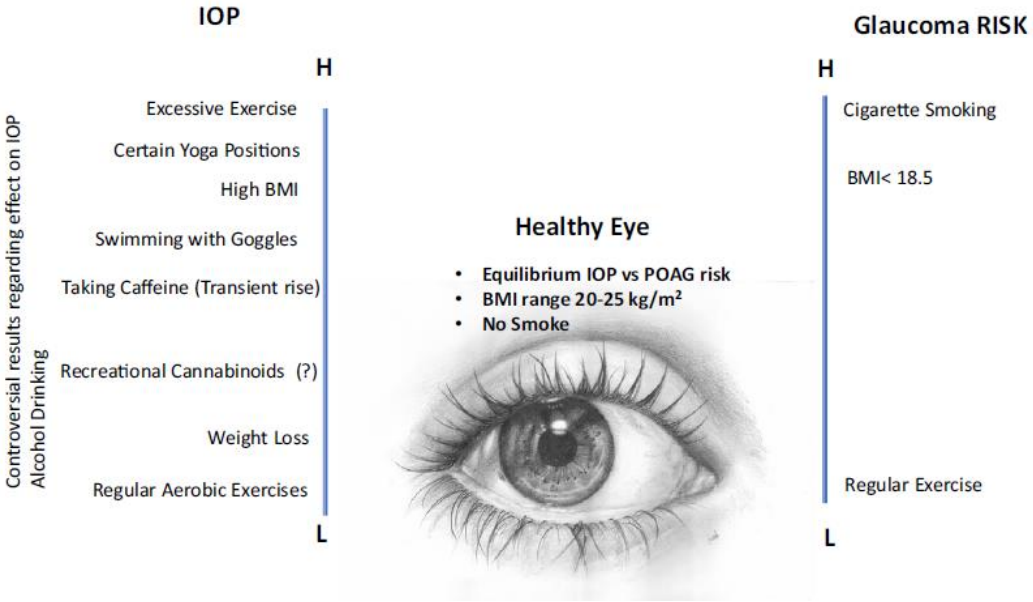

Figure 1. Summary of lifestyle factors affected IOP and glaucoma risk. In this figure we summarized the effects of various lifestyle factors on glaucoma risk and IOP. This figure named "Self Eye"is derived from creative commons section in PowerPoint, which is drawn by Sarickbanana on Deviant art.

The elevated hydrostatic pressure can lead to a substantial transient IOP increase during yoga exercises, especially in headstand posture, so-called Sirsasana, by about 2 times [10] as indicated by 3 case reports showing the worsening of existing glaucoma after practicing head down posture leading to a deterioration of the visual field [8, 13, 49]. Besides, Jasien and coworkers [77] reported that IOP could quickly and rapidly increase in other head-down yoga positions in normal sub- jects and in glaucoma patients. Certain yoga positions with the head placed down, as well as using wind instruments and performing activities with sudden head movements, have potential risk for raising IOP; thus, they should be avoided in glaucomatous patients [49, 133, 157].

Table 1 – Association between nutrients intake and glaucoma.

References	Nutrients examined	Number, type of participants	Observations/Outcomes	Type of study
Yoserizal *et al.*, 2019 [221]	Associations of nutrients intake on glaucoma	581 participants 61 of which were diagnosed with glaucoma	High intake of iron or low intake of vitamin A, and vegetable fat were associated with an increased risk of glaucoma	Cross-sectional
Wang *et al.*, 2018 [210]	Daily dietary intake of polyunsaturated fatty acid (PUFA*), including ω-3 fatty acids	3865 participants in NHANE	Increased daily dietary consumption levels of eicosapentaenoic acid and docosahexaenoic acid were associated with lower likelihood of glaucomatous optic neuropathy. However, consumption levels of total PUFAs in the higher quartiles were associated with a higher risk of glaucoma, which may have resulted from relative intakes of ω-6 and ω-3 fatty acids and other confounding comorbidities. In general, increasing level of dietary ω-3 while controlling consumption of total daily PUFA intake may be protective against glaucoma.	Cross-sectional
Kang *et al.*, 2016 [84]	Dietary nitrate intake, derived from green leafy vegetables	104,987 subjects (≥40 years)	Higher dietary nitrate and green leafy vegetables intake inversely associated with glaucoma risk. Incident self-reported open-angle glaucoma confirmed by medical record review	16-year prospective cohort
Giaconi *et al.*, 2012 [53]	Dietary fruits and vegetables containing different vitamins	584 female subjects	Higher intake of vegetables and fruits rich in vitamin A, vitamin C were associated with a lower likelihood of having glaucoma	Cross-sectional
Coleman *et al.*, 2008 [25]	Fruits and vegetables	1155 female subjects	Higher intake of kale, carrots, and peaches were associated with a lower likelihood of having glaucoma while subjects with higher intake of spinach or orange juice were more likely to have glaucoma	Cross-sectional
Wang *et al.*, 2013 [208]	Vitamins A, C, E intake and serum level	2912 participants (≥40 years)	Only supplementation of vitamin C was found to be associated with decreased odds of glaucoma prevalence	Cross-sectional
Ramdas, *et al.*, 2012 [168]	Dietary antioxidants (carotenoids, vitamins, and flavonoids) and nutrients influence the blood flow (omega fatty acids and magnesium)	3502 adults (≥55 years)	Incident POAG*, detected by visual field testing. People intake equivalents of vitamin A ($P = 0.01$) or vitamin B1 ($P = 0.03$) is associated with an about twofold lower risk of OAG* compared to those with a low intake of these nutrients, and people with a high intake of magnesium have an about threefold increased risk of OAG compared to those with a low intake	9.7-year prospective cohort
Kang *et al.*, 2003 [87]	Dietary antioxidant intake	116,505 female subjects (≥40 years)	Using data from two prospective cohorts, showed little evidence that intakes of dietary carotenoids, vitamins C and E reduces the risk of POAG glaucoma. Confirmation of glaucoma was self-reported or by medical record review	Prospective cohort 9.3 years (average)

OAG = open angle glaucoma; POAG = primary open-angle glaucoma; PUFA = polyunsaturated fatty acid.

Indeed, both being fit and physically active reduces glaucoma risk, and the combined effects of the two factors provide greater protection against glaucoma incidence [132]. Moreover, the risk of glaucoma incidence was decreased in a dose- response relationship among male runners. In other words, the men who ran more and faster were less likely to develop glaucoma [215]. Concerning the progression of glaucoma, prospective studies are rare. Agrawal reported that exercise, along with one of the antiglaucoma treatments, provides a better reduction in IOP than that medication alone [2].

Body mass index (BMI)

Although body mass index is positively correlated with IOP [34, 101, 134, 167], BMI and glaucoma's association remain in- conclusive due to conflicting findings [51, 167]; however, in two population-based studies BMI was inversely associated with prevalence of glaucoma [122, 159]. Once more these observations suggest that IOP and glaucoma are not always necessarily linked. Although obesity has been associated with higher IOP, a lower BMI has been related to a higher incidence of normal-tension glaucoma, especially in women in the 40 −49 age range [122]. Moreover, in 2 Korean observational studies, the influence of other anthropometric parameters, such as fat mass and muscle mass, correlated with IOP and the incidence of POAG [97, 117]. After adjusting for weight and BMI, elevated IOP, as well as lower POAG prevalence, was related to greater fat mass in women, whereas reduced IOP and higher POAG prevalence was associated with greater muscle mass in men [97, 117]. The exact mechanisms of how the different body compositions may affect the prevalence of POAG remain un- clear. Finally, among women with POAG, a prospective study showed that patients with lower BMI developed more para- central visual defects compared to patients with higher BMI [85].

The impact of sudden weight loss on IOP, especially through bariatric surgery, was investigated in some studies [18, 111, 203]. Viljanen and coworkers [203] observed that obese patients who have higher IOP values 6-months after under-going bariatric surgery had post-operative IOP values similar to that of nonobese controls ($P > 0.05$). Furthermore, Lam and coworkers [111], besides confirming the result of the previous study, reported that each 10 unit decrease in BMI correlates with a 2.4 mm Hg decrease in IOP ($r = 0.46$). These observations can be attributed to weight loss or other changes in the body such as fat and protein

distribution, insulin resistance or blood hemostasis Further investigations are needed to fully understand the exact contributor of IOP decreasing.

Smoking

Smoking tobacco is associated with developing ocular diseases such as cataracts and age-related macular degeneration [37]. Tobacco is potentially detrimental to health, and nicotine, the main active component of cigarettes, increases blood flow, but its effect on optic nerve blood flow remains elusive. Observational studies mostly documented conflicting results towards the relation between cigarette smoking and POAG. To our knowledge, there is not any firm evidence that suggests smoking cigarettes leads to increased IOP.

Furthermore, Cigarette smoking was not associated with the increasing incidence of POAG in a large cohort study of more than 100,000 participants [86]. In a case-control study, smoking habit was protective against normal-tension glaucoma [22]. In contrast, smoking was found to be correlated with higher odds of developing glaucoma in a meta-analysis and 2 epidemiological studies [14, 217, 226]. The conflicting results may be related to the multifaceted nicotine mechanisms of action that may have multiple effects. Pharmacological studies indeed propose that nicotine present in cigarettes may have neuroprotective effects in the retina, preventing glutamate-induced excitotoxicity in RGC [75]. On the other hand, nicotine has vasospastic properties that may cause glaucomatous nerve damage by decreasing blood flow [22].

Marijuana (dried leaves, flowers and stems of *Cannabis sativa* or *Cannabis indica*) is the most used addictive drug after tobacco and alcohol. More recently the smoking of this substance has been legalized both for medical and recreational use in several countries worldwide, and this trend is increasing. Therefore, marijuana's effect on glaucoma progression require attention. Cannabinoids are a large class of chemical compounds derived from the leaves of Cannabis plants [107, 160]. Both increases and decreases of IOP have been reported as a function of the mode of administration and of the precise cannabinoids used. As far as the reduction in IOP, the suggested mechanism is and interaction with the ciliary muscle and Schlemm canal, as well as a modulation of cyclooxygenase-II [189]. Of interest are also the putative neuroprotective properties of cannabinoids that may be modulated by both central and peripheral nervous system. Also, the inhibition of glutamate [38], endothelin-1 [182], and nitric oxide release [114] may play a role.

Table 2 – Ocular effects and eye disease risk associated with caffeine and other methylxanthines use.

References	Nutrients examined	Number, type of participants	Mean follow-up	Observations/ Outcomes	Type of Study
Vera *et al.* 2018 [201]	Caffeine (4 mg/kg) or placebo	40 healthy individuals	30, 60, and 90 minutes after ingesting caffeine or placebo	Caffeine induced an acute IOP* rise (P<0.001) in caffeine group, high-caffeine consumers had a less accentuated (+1.2 mmHg) IOP rise in comparison to low-caffeine consumers (+3.4 mmHg).	Placebo-controlled, double-blind, and balanced crossover study
Wu *et al.*, 2017; NHANES [219]	Caffeinated and decaffeinated coffee, hot tea, iced tea, and soft drinks	1678 subjects	—-	Daily consumers of hot tea are less likely to have glaucoma compared to non-consumers. No significant associations were found between the consumption of coffee, iced tea, decaffeinated tea and soft drinks, and glaucoma risk	Cross-sectional
Pasquale *et al.*, 2012 [158]	Caffeine and caffeinated beverage consumption in relation to the risk of exfoliation glaucoma or exfoliation glaucoma suspect	120,179 subjects	—-	A positive association between heavy coffee consumption and exfoliation glaucoma or exfoliation glaucoma suspect was noted	Prospective cohort
Jiwani *et al.* 2012 [79]	237 mL of caffeinated (182 mg caffeine) and or decaffeinated (4 mg caffeine) coffee	22 POAG*, 18 NTG*, 20 OHT*, 21 POAG suspect, 25 healthy individuals	60 and 90 minutes after coffee ingestion	182 mg caffein statistically increase but likely does not a statistically significant elevation in IOP was noted following acute ingestion of caffeinated coffee when pooling all groups together (+0.99 ± 1.52 P < 0.0001, +1.06 ± 1.67 P < 0.0001 respectively after 60 and 90 minutes)	Randomized controlled trial
Kang *et al.*, 2008 [90]	Caffeine beverage consumption	124,172 healthy subjects	—-	Caffeine intake did not influence POAG risk	Prospective cohort Cross-sectional
Chandrasekaran *et al.*, 2005 [20]	Coffee and caffeine intakes	3654 subjects	30, 60 and 90 minutes after coffee ingestion	POAG patients reporting a high intake of caffeine had a significantly higher IOP compared to those reporting no intake	Randomized controlled trial
Avisar *et al.*, 2002 [7]	180 mg caffeine in 200 mL beverage and decaffeinated coffee 3.6 mg caffeine in 200 mL beverage was compared in patients with NTG or OHT	6 NTG patients and 22 OHT patients	30, 60 and 90 minutes after coffee ingestion	A statistically significant elevation in IOP was noted following acute ingestion of regular caffeinated coffee in both groups after 60 and 90 minutes of drinking.	Randomized controlled trial
Ajaei *et al.*, 2001 [4]	100 mL of coffee prepared from 400 mg of caffeinated coffee	40 healthy subjects		A statistically significant	

IOP = intraocular pressure; NTG = normal tension glaucoma; OAG = open angle glaucoma; OHT = ocular hypertension; POAG = primary open angle glaucoma.

To sum up, whether a positive association exists between cannabinoids taking and IOP lowering or RGC neuroprotection in glaucoma, it should be noted that most glaucoma patients need long-term therapy and loss of effect through tolerance might happen after administration of derivates of cannabinoids. Moreover, increasing clinical evidence focusing more on distinguishing different routes of administration, as well as longer duration of treatment, will help us to broaden our knowledge on effectiveness, adverse effects, and mechanisms of action of this agent. While there has been attention to the potential therapeutic use of marijuana in glaucoma no clear- cut observations are present in literature on the effect of marijuana's recreational use on IOP or glaucoma risk.

Alcohol consumption

The relation between alcohol consumption and risk of glaucoma development is unclear. Several observational studies found no association between alcohol drinking and glaucoma prevalence [35, 91, 102, 220]. On the other hand, three prospective studies showed an increased risk of glaucoma prevalence [83, 217, 226] in alcohol consumers. Although alcohol in a dose- dependent way can lower IOP, some studies showed a positive relationship between alcohol intake and increasing IOP [17, 54, 121, 187, 223]. Notably, these mixed findings may be attributed to individual differences in alcohol metabolism that can affect IOP and the ganglion cell complex. In this regard, in 3 cross-sectional studies, alcohol intake was associated with a thinner macular retinal nerve fiber layer [63, 94, 112].

Caffeine

Caffeine, a methylxanthine alkaloid, is a ubiquitous ingredient in several popular beverages consumed worldwide [29]. Ingestion of caffeine in an animal model of glaucoma led to a reduction in neuroinflammation and a diminishing in retinal ganglion cell loss [130]. Notably, caffeine affects IOP and ocular perfusion pressure (OPP). As shown in **Table 2.** the experimental administration of caffeine either as standardized amounts of coffee or as the pure substance induced acutely an increase in IOP. Indeed, caffeine consumption seems to cause a transient rise in the IOP and OPP levels in both healthy individuals and glaucoma patients [7, 79, 119, 198], though negligible changes have also been reported in healthy individuals [33]. The possible differences between low- and high-caffeine consumers remain unknown (see **Table 2.**). The suggested mechanisms for

elevating IOP by caffeine, which acts as adenosine receptor antagonist and phosphodiesterase inhibitor, are primarily from increased intracellular levels of cyclic AMP, which may cause aqueous humor overproduction [119]. Secondly, cyclic AMP may inhibit aqueous humor outflow by decreasing smooth muscle tone in the filtration apparatus, leading to trabecular fenestrae closure [4]. Thirdly, by affecting blood pressure, caffeine may increase hydrostatic pressure for aqueous humor formation [67, 79]. Even though caffeine increased IOP in one large prospective cohort study [90], overall caffeine consumption was not associated with the risk of developing POAG. One explanation for this may be related to the ability of caffeine to inhibit lipid peroxidation and reduce reactive oxygen species [124]. Lastly, the lack of a link between caffeine and POAG may be because OPP and IOP were both increased, leading to no net effect on overall optic nerve perfusion.

Diabetes and glaucoma

Both retinopathy due to diabetes mellitus (DM) and glaucoma are among the leading causes of blindness and a major public health issue, especially in the elderly population, worldwide [46]. Mixed results were obtained from studies concerning the relation between glaucoma risk and DM [81, 104, 106, 113, 183, 204, 226]. Variations in race, age, study sample, different diagnosis criteria, and possible detection bias may be the cause of the observed inconsistencies; however, in 4 large epidemiological studies, including a meta-analysis and genome-wide association study, having DM was significantly associated with prevalence as well as developing glaucoma [81, 104, 183, 226]. Furthermore, Khatri and coworkers found that patients with DM are more prone to have severe POAG [93]. Recently Choi and coworkers [23] in a large cohort study reported that increased fasting glucose was positively associated with an increased risk of POAG. To conclude it seems that those patients who develop diabetes may require special attention when screening for glaucoma since DM and glaucoma share some common risk factors as well as pathophysiological characteristics similarities like nitric oxide upregulation, ganglion cell loss, and elevated oxidative stress [186, 197, 218].

Diet

Data derived from epidemiological and clinical studies regarding the impact of diet on developing glaucoma odds are limited. Findings from 3 United States

cohorts indicate that there was overall no association between low-carbohydrate intake and POAG risk; however, a diet low in carbohydrates and high in fat and protein from vegetable sources was a possible lower risk of POAG [64]. Kang and colleagues also showed that higher dietary nitrate and green leafy vegetable intake were associated with a lower POAG risk [89]. Higher consumption of fruit, especially fresh oranges and peaches, and vegetables like collard greens/kale may be associated with decreased odds of glaucoma in healthy subjects [53]. Furthermore, in a case-control study, it is suggested that drinking pure fruit juice, consuming more meat with less visible fat, and modest salt during cooking may be a practical solution for patients who are at risk or already suffering from POAG [139]. In general, restricting saturated fat and added sugar and sodium, a habit that is encouraged by many dietary guidelines to prevent cardiovascular disease, is similar to advisable diet habits in glaucomatous patients. Interestingly a recent study implied that healthy lifestyle necessary for ideal cardiovascular health was associated with lower odds for ocular diseases. Thus, it seems that interventions to prevent cardiovascular diseases may also prevent ocular diseases [110].

Another interesting point to consider is the gut-eye axis and its potential impact on ocular disease, especially glaucoma. Recently, Gong and coworkers [57] found that gut microbiome composition and serum metabolic phenotype differs between glaucomatous and healthy individuals. Indeed, for elucidating the association between microbiota and glaucoma development, both more clinical and experimental studies are needed.

Table 1 reports literature observations based on large numbers of people examining their dietary habits and, in some cases, the consumption of specific supplements or a fortified diet. The results support the idea that some dietary nutrients may affect the risk of developing glaucoma; however, the data are not always consistent and, while suggesting the presence of measurable effects in a population, do not allow prediction of the effect in individuals.

Dietary supplements

A body of research has focused on elucidating the role of nutritional supplementation in glaucoma development. It is worth recalling some regulatory definitions related to using of these compounds that fit non-drug categories such as food supplements, integrators, nutraceuticals, dietary supplements, some of

which are common and web denominations, others used by regulatory agencies. In particular, the US Food and Drug Administration (FDA) [213] uses the terminology “dietary supplements” to refer to extracts, concentrates, or combinations of vitamins, minerals, botanicals, herbs, or dietary substances for use by man to supplement the diet by increasing the total dietary intake. One of the primary differences between dietary supplements and drugs relates to health claims. Whereas a dietary supplement is meant to provide nutrients, a drug is designed to treat illness or disease. Food supplements are concentrated sources of nutrients (i.e., minerals and vitamins) or other substances with a nutritional or physiological effect that are marketed in dose form (e.g., pills, tablets, capsules, liquids in measured doses). A wide range of nutrients and other ingredients might be present in food supplements, including, but not limited to, vitamins, minerals, amino acids, essential fatty acids, and various plants and herbal extracts.

In particular, food supplements are intended to correct nutritional deficiencies, maintain an adequate intake of certain nutrients, or to support specific physiological functions. They are not medicinal products and as such cannot exert a pharmacological, immunological, or metabolic action. Therefore, their use is not intended to treat or prevent diseases in humans or modify physiological functions. These regulatory definitions implicate that, no matter the name adopted for commercial purposes, the producers cannot claim clinical effects on glaucoma unless the substance has been thoroughly investigated and registered for use as a drug. On the other hand, several clinical studies (though not registration studies) explored the effect of various food supplements in this domain.

Vitamins and minerals

Vitamins A, C, and E are well-known antioxidants that may prevent or retard age-related eye disorders such as cataract and age-related macular degeneration [95]. These vitamins hinder oxidative stress by both direct and indirect antioxidant activities and protect, at least in *in vitro* experimental models, against free radicals [41].

Based upon the underlying theory about the association between oxidative stress and ganglion cell death without IOP elevation in primary open-angle glaucoma [74, 137, 177, 194], and because of the antioxidant properties of vitamins, it has been hypothesized that these common dietary supplements may be of interest as potential neuroprotective agents by protecting RGC injury against oxidative

stress in the pathogenesis of glaucoma [76, 172]. Although several epidemiologic studies and small randomized clinical trials have been conducted on the association of vitamins with glaucoma, the results are often conflicting, leaving physicians and patients in doubt about the effect of vitamins on glaucoma, as detailed in the following paragraphs.

The high lipid content of nerve cells underscores the importance of lipid-soluble vitamin E, especially α-tocopherol, which has hormone-like regulatory mechanisms with its probable transporter proteins and receptors (like scavenger receptor class B, type I), exerting neuromodulatory effects on the eye and other tissues. Neuroprotective effects of vitamin E-based pharmaceutical products in retinal diseases and glaucoma have been clinically investigated [39, 191]. In particular, Engin and coworkers [40] examined the neuroprotective effect of α-tocopherol against glaucomatous damage. Their results show statistically significant improvements in visual fields and the pulsatility and resistivity indexes of both ophthalmic and posterior ciliary arteries after 6- and 12-months supplementation with either 300 mg/day or 600 mg/day of α- tocopherol. In this study, ocular blood flow improvement was time- and dose-dependent. On the other hand, Goldblum and coworkers investigated the outcome of filtering surgery with postoperative dietary α-tocopherol supplementation. In this randomized controlled trial IOP reduction and success rate (defined as IOP ≤18 mm Hg with no medication, no needling revision, and no subconjunctival injection) were not statistically different between α-tocopherol-supplemented patients and the placebo group [55]. Notably, the role of antioxidants has also been questioned in other ocular diseases such as age-related macular degeneration [42].

Vitamin D has been associated with several neurodegenerative and psychiatric diseases such as Alzheimer dementia, Parkinson disease, depression, and schizophrenia [32]. Several studies have shown that these diseases may aggravate the progression or elevate the incidence of glaucoma [11, 146, 178]. Furthermore, some studies have found that the serum vitamin D level decreases in glaucoma patients compared with controls, albeit the difference in vitamin D levels between them is limited though significant [56, 127]. On the other hand, a meta-analysis of the available data found no significant difference in serum vitamin D levels between POAG patients and controls, underscoring the great heterogeneity of the published material [120].

Vitamin D administration was shown to regulate IOP in non-human primates. This may suggest a potential role of this vitamin on regulating IOP also in humans [109]. To assess this assumption the relation between serum level of 25-hydroxy- vitamin D [25(OH)D] and IOP was directly examined by Krefting and collaborators in a small, randomized control trial. In this study performed in healthy participants, there were no associations between serum 25(OH)D levels and IOP, and ad- ministration of vitamin D3 to participants with low levels of 25(OH)D did not affect IOP. Hence, these results do not support the role of vitamin D in IOP regulation [105].

Vitamin C is a well-known dietary antioxidant vitamin that neutralizes oxygen radicals and is a reductant of oxidized vitamin E [125]. It plays a role in preventing the onset or progression of age-related visual impairment, such as age-related cataract [224]. A clinical study in Japan reported by Yuki and coworkers [225] showed that serum vitamin C levels were lower in 47 patients with normal-tension glaucoma than 41 control subjects with no eye disease while, in the same group, no differences in serum vitamin A or E levels were found.

Among the American national health and nutrition examination survey participants, Wang and coworkers [208] found neither supplementary consumption nor serum levels of vitamins A and E to be associated with glaucoma prevalence. On the other hand, supplementary consumption of vitamin C was associated with a decreased odds ratio of glaucoma; however, serum levels of vitamin C did not correlate with glaucoma prevalence.

Another large prospective cohort study with more than 100,000 participants showed little evidence of an association between total dietary intake of vitamin C, vitamin E, or vitamin A and a reduced risk of primary open-angle glaucoma [87]. Consumption of specific carotenoids derivatives such as lutein/zeaxanthin present at high concentrations in specific ocular tissues, however, may be related to decreased POAG risk [12]. The use of multivitamins or supplements of vitamins C, E, and A, analyzed by either dose or duration, was unrelated to POAG risk [87]. Conversely, evidence in another cross-sectional study among African-American participants showed that a higher intake of dietary vitamin A and vitamin C was associated with a lower likelihood of having glaucoma [53]. Williams and coworkers [214] reported that nicotinamide (the amide of nicotinic acid, one of the vitamins B3 forms) dietary supplementation in mice significantly reduces mitochondrial vulnerability and prevents glaucoma by supporting

mitochondrial health and metabolism. The relation between serum vitamin B levels and glaucoma in humans remains to be investigated in detail, although several studies found a reduction in serum levels between healthy and glaucomatous individuals [120, 174, 199]. Furthermore, in one cross-sectional study on Asian people in whom normal-tension glaucoma is more prevalent, lower nutrient intake of niacin (nicotinic acid) was associated, while lower intake of other vitamins or minerals did not correlate [80].

A prospective cohort comprising over 3500 participants, studied the impacts of nutrients with either antioxidant properties or blood flow influence on OAG. They found an association between low intake of retinol equivalents, vitamin B1 and high magnesium consumption with an increased risk of OAG, and these effects were IOP-independent [168].

Some studies suggested the role of calcium- and metal- related oxidative stress in the pathogenesis of glaucoma [26, 44]. In this context, some studies showed that high consumption of iron and calcium increases the risk of glaucoma [19, 209]. Moreover, a systematic review found that some minerals like selenium and iron may be associated with risk of glaucoma [166].

Summing up, the data present in the literature are insufficient to support vitamin administration as a tool that can be used specifically to decrease the risk or treat glaucoma. A balanced diet and vitamin intake within the boundaries of a healthy lifestyle appears to be sufficient, as indirectly emerges from the analysis of Ramdas and coworkers [166], in the case of dietary vitamins A and C intake. Specific situations must be studied in depth, as well as situations associated with over- doses of one of these nutrients.

Herbals

A variety of herbal medications is generally used for glaucoma. Among them, Ginkgo biloba, commonly used as an herbal remedy for peripheral vascular, cardiovascular, and cerebrovascular diseases. Ginkgo extracts contain many different flavonoids, including polyphenolic ones, exerting antioxidant properties at mitochondrial level [27, 142].

Besides stabilization of the mitochondria [1] and antioxidant properties, Ginkgo has been shown to have neuroprotective, anti-inflammatory effects as well as to be able to in- crease blood flow through vasodilation and reduce blood viscosity [196]. Some studies have shown the potential neuroprotective effect of this

plant's extract in lowering memory loss and symptoms of cognitive disorders, including Alzheimer and other dementias [68, 123]. Further, various studies have demonstrated the effects of Ginkgo biloba extract (GBE) on visual function. Specifically, it seems useful in treating visual field damage associated with chronic cerebrovascular insufficiency, glaucomatous damage, macular degeneration, and diabetic retinopathy. It should be mentioned that a relationship exists between visual field damage and cerebrovascular insufficiency which may take place within the brain in addition to involve the ocular level [66, 78]. It also alleviates retinal impairment caused by ischemic-reperfusion injury [99].

The effect of Ginkgo on changes of the optic nerve head and RGC has primarily been investigated in animal models [128, 153]. GBE is proposed to be a natural therapeutic agent for glaucoma, particularly the non-pressure-dependent type [136]. One study found that, in a mouse model for unilateral chronic moderately elevated IOP, GBE treatment for five months did not change IOP but did show significantly reduced RGC loss [70].

Based on the role of GBE in reducing blood viscosity and increasing ocular blood flow, being the latter possibly involved in the pathogenesis of NTG, Park and coworkers [155] conducted a randomized clinical trial reporting that GBE significantly increases peripapillary blood flow in patients with NTG compared to placebo. On the other hand, published human studies report conflicting results regarding the effect of GBE in glaucoma. Quaranta and coworkers [164], in a randomized, placebo-controlled, crossover study, found that patients who had received GBE experienced statistically significant improvements in visual field indices compared to the placebo group; however, IOP was similar in the two groups. Guo and colleagues [59] investigated the effects of GBE on 28 newly diagnosed NTG patients using a similar model. This study's outcomes indicate neither improvements in visual field in- dices nor changes in contrast sensitivity in GBE-treated groups compared to placebo. A possible explanation for the difference in the results from these two studies is the differences in the studied population. Conversely, in a cohort study, GBE administration slowed the progression of visual field damage in NTG patients [116]. Moreover, in a randomized controlled trial, plasma oxidative stress markers, visual field, and retinal nerve fiber layer damage in POAG patients who received a 6-month GBE treatment were improved without any significant changes in IOP [179]; however, GBE seems to be well tolerated with low adverse effects, and because of its neuroprotective profile can be used as adjuvant therapy for normal tension

glaucoma [72]. As in many other cases, the lack of rigorous registration studies does not allow to draw firm conclusions on the use of GBE (**Table 3.** Represent a summary regarding the effects of GBE on ocular blood flow/ visual field).

Neuroprotection-based therapies in controlling glaucoma are important due to the destructive effect exerted by glaucoma on retinal ganglion cells [118]. Within this context, several herbal extracts were investigated, among which *Erigeron breviscapus hand-mazz* (EBHM), a traditional Chinese herb containing several kinds of flavones and flavonoids [144]. Animal studies have shown that EBHM could stimulate the recovery of axoplasmic transport of injured RGC and improve the activity of cytochrome oxidase in RGC, especially against N-methyl-D-aspartate induced damage in rat models with acute elevated IOP [229]. In a clinical study, 6-month EBHM administration in POAG patients led to significant improvement in their visual fields [227]. In another randomized controlled trial conducted by Ning [144], visual fields defect scoring in the treatment group improved significantly compared to the placebo group after 2, 4 and 6 months using EBHM, although no changes in IOP were observed in either group.

The only published clinical study on the relation between saffron and glaucoma observed a lowering IOP in patients; the authors suggested that this protective effect against glaucoma may rely on the antioxidant properties of saffron flavonoids [15]. Indeed, flavonoids, polyphenolic compounds present in many plant-based foods, have several biological activities such as reducing damage from oxidative stress [69], improving ocular blood flow [96], decreasing inflammation [188] as well as having neuroprotective effects [200]. Several studies have investigated the association between flavonoid intake and glaucoma because of the properties mentioned earlier. Nevertheless, there is conflicting evidence concerning flavonoids and improvement in visual field. A meta-analysis of 6 clinical trials on various flavonoid supplements in glaucoma or ocular hypertension patients suggested that these agents slow visual field loss progression and improve ocular blood flow [161]. In contrast, a long-term prospective study conducted by Kang and collaborators reported that total flavonoid intake was not associated with a reduced POAG risk; however, higher intakes of flavanols and monomeric flavanols showed a modest association with a lower risk of POAG [84].

Table 3 – Intervention studies examining the effects of *Gingko biloba* extract on ocular blood flow/ visual field.

Reference	Nutrients examined	Number, type of participants	Mean follow-up	Observations/ Outcomes	Type of study
Guo *et al.*, 2014 [59]	*Gingko biloba* extract or placebo, 40 mg 3 times/day	35 patients with NTG* (in 2 groups: treated vs placebo)	4 weeks–8 weeks washout – 4 weeks (prospective)	No effect on the VF* mean defect or contrast sensitivity	Randomized controlled trial
Shim *et al.*, 2012 [184]	*Gingko biloba* extract (80 mg twice a day) or *Vaccinium myrtillus* antocyanosid extract (60 mg twice a day)	332 patients with NTG (in 3 supplement groups including anthocyanins *Gingko biloba* extract, or no medication	Almost 2 years (retrospective)	Both anthocyanins and *Gingko biloba* extract were associated with improved visual function in patients with NTG	Case–control
Park *et al.*, 2011[155]	*Gingko biloba* extract (80 mg twice a day) or placebo	30 patients with NTG	4 weeks	Statistically significant increase in blood flow at almost all points was observed (except for the superior nasal peripapillary area), in comparison to the placebo, without noticeable changes in VF indices	Randomized controlled trial
Wimpissinger *et al.*, 2007 [216]	*Gingko biloba* extract (240 mg)	15 healthy volunteers in Gingko group or placebo	single administration	Retinal blood flow did not differ in both groups. Optic nerve head blood flow significantly increased (+17.29 ± 17.3%, in response to *Ginkgo biloba* versus baseline $P<0.002$)	Randomized controlled trial
Quaranta *et al.*, 2003 [164]	*Ginkgo biloba* extract 40 mg, 3 times/day or placebo	27 patients with NTG (with or without treatment)	4 weeks–8 weeks washout – 4 weeks (prospective)	VF indices improve significantly without changes in IOP*	Randomized controlled trial

IOP = intraocular pressure; NTG = normal tension glaucoma; VF = visual field.

In a randomized clinical study, epigallocatechin-gallate (EGCG), a catechin-based flavonoid present in green tea, favourably influenced inner retinal function in OAG patients who experience moderately to advanced glaucomatous damage [43]. These effects might be from the anti-inflammatory [193], antioxidant [150] and blood flow increasing [126] properties of this agent.

Anthocyanins, another kind of polyphenols, are abundant in berries such as black currant. It is suggested that their consumption provides several health benefits, such as antioxidant and anti-inflammatory effects [108, 140]. Black cur- rant anthocyanin (BCAC) has been shown to improve visual functions [73, 131]. Ohguro and coworkers conducted a series of clinical trials to assess the possible beneficial effect of BCAC in glaucoma progression. Initially, in a pilot clinical trial, they found that the administration of BCAC to patients with normal-tension glaucoma for 6 months caused a significant increase in the blood flow at the optic nerve head as well as at peripapillary retina levels; however, no significant IOP or visual field changes were detected [149]. In a subsequent randomized, placebo-controlled trial, the same authors demonstrated that 2-year BCAC supplementation significantly decreased deterioration of visual field mean deviation and increased ocular blood flow of OAG patients compared to placebo-treated ones. Nevertheless, IOP did not change in either group [147]. Finally, in their last study based on their previous clinical trials, Ohguro and coworkers, [148] analyzed the effect of BCAC on IOP in healthy subjects and glaucomatous patients who also took a prostaglandin analogue. Their findings showed that in these conditions, BCAC might induce IOP de- crease in both groups [148]. To investigate the probable mechanism of this result, Yoshida and coworkers [222] conducted a clinical study in which they concluded that BCAC normalizes serum endothelin-1 levels. Notably, endothelin-1 is a vasoconstrictor that is believed to play a role in ocular perfusion and perhaps in the overall pathogenesis of glaucoma [58 ,61].

Fatty acids

In recent years, there has been growing interest in the health benefits of polyunsaturated fatty acids, particularly omega-3 long-chain polyunsaturated fatty acids (ω-3 PUFAs), in the pathogenesis of ocular diseases [207]. It has been shown that patients with POAG have an abnormal blood fatty acid composition that is characterized by a reduction in eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and omega-3 fatty acids [170]. According to the

Nurses' Health Study [88], a high ratio of omega-3 and omega-6 polyunsaturated fat increases the risk of POAG, particularly high tension. The rationale of using these agents is to improve retinal function through the inhibition of NF-κB (a transcription factor) activation and the subsequent synthesis of inflammatory cytokines via antiangiogenic effects of ω-3 PUFAs in human endothelial cells and improvement of glaucomatous optic neuropathy; [21, 143, 176] however, the number of clinical studies with PUFAs in glaucoma is small. In the National Health and Nutrition Examination survey, increased daily dietary intake levels of EPA and DHA were associated with a lower likelihood of glaucomatous optic neuropathy; however, consumption levels of total PUFAs in the higher quartiles were associated with a higher risk of glaucoma. This study also hypothesizes that increasing the proportion of dietary ω-3 consumption levels while controlling overall daily PUFAs intake may be protective against glaucoma [210]. Finally, oral omega-3 supplementation has been associated with reducing IOP in normotensive adults [175] and pseudoexfoliation glaucoma patients [36].

Commercially available supplement mixtures

Different pre-clinical and clinical studies imply that supplements solely or in combination may have notable clinical potentials. They are generally recognized as harmless products with a good safety profile and, because there is no cure for glaucoma, the administration of supplement mixtures may possess preventive or treatment properties worth exploring. On the other hand, this is a field of intense commercial activity, and, based on the assumed lack of harmful effects, several preparations are proposed, through the web, based on few not always properly controlled studies. Meta-analyses do not provide conclusive evidence. Professionals should be able at to use peer reviewed literature to take personal decisions tailoring the risk/benefit/cost balance in individual patients. Having this in mind, the following paragraphs examine examples of the available preparations.

Coenzyme Q10 (CoQ10) is a mitochondrially targeted antioxidant that has neuroprotective activity in different disorders [3, 103]. Indeed, it has been shown to protect retinal cells against oxidative stress in different *in vitro* and *in vivo* experiments [115, 141, 145]. Topical CoQ10 also prevents RGC apoptosis and loss in a glaucoma-related animal model [31]. Vitamin E, as mentioned before, is an antioxidant, and its combination with CoQ10 in a mouse model of glaucoma inhibits glutamate excitotoxicity and oxidative stress [115] The effects of topical

CoQ10 therapy in combination with vitamin E (CoQun®) were assessed in 22 open-angle glaucoma patients also receiving a β-blocker in comparison with 21 patients receiving a β-blocker monotherapy alone. This study reported a beneficial effect of CoQun® therapy on RGC's visual function in OAG patients after 12 months [154]. More recently, Ozates and coworkers [152] randomly divided their pseudoexfoliation glaucoma cases into two groups and then used topical Co-Qun® in one group in addition to a prostaglandin agent for one month. They investigated the effect of the treatment on the levels of an oxidative stress marker, malondialdehyde, in aqueous humor. They observed significantly lower superoxide dismutase levels in the group subjected to the combined therapy versus patients without CoQun® treatment, whereas the malondialdehyde level was not different in the two mentioned groups.

Other studies on the correlation between antioxidants and glaucoma have reported controversial results. To evaluate the effect of oral antioxidants supplementation on POAG patients, Garcia and coworkers [50] conducted a study with two commercially available products, ICAPS R® and OFTAN MACULA®, similar in composition multivitamins and minerals, except for ω3 fatty acids present in the former one. One hundred seventeen POAG patients under topical antiglaucoma medications were divided into three groups, including ICAPS R®, OFTAN MACULA® and control groups. Visual field global indices, peripapillary retinal nerve fiber layer thick- ness, and macular ganglion cell complex thickness showed no differences among the groups at the beginning and the end of the 2-year follow-up. On the other hand, in a recent clinical trial, one-month oral administration of antioxidants in Optic Nerve Formula®, which is composed of essential vitamins and minerals, ω3 polyunsaturated fatty acids and dietary polyphenolic nutrients, showed an increase in biomarkers of ocular blood flow within retinal and retrobulbar vascular beds in patients with OAG [65]. Within this context, Galbis-Estrada and coworkers [48] looked at the relationship between consumption of a mix of dietary supplements containing different antioxidants and essential polyunsaturated fatty acids, named Brudysec 1.5®, on the expression of cytokines and chemokines in tears from patients with POAG or a dry eye disorder. The findings of this study showed that inflammation biomarkers such as tumor necrosis factor αand interleukin 6 increased significantly in glaucomatous patients' tears and were diminished upon antioxidants/ essential polyunsaturated fatty acids supplementation.

The effect of flavonoids presents in Mirtogenol® on IOP in asymptomatic subjects with intraocular hypertension was evaluated in another study. Mirtogenol® is a commercial product with a combination of pine bark and bilberry extract. Two- month consumption of this product contributed to reduced IOP and improved ocular blood flow compared to the control group [190].

Forskolin is a natural compound, rapid and reversible activator of adenylyl cyclase that decreases IOP by reducing aqueous humor production in animals [205]. It has also been shown to stimulate neuronal survival by stimulating neurotrophin activity in models of RGC death [82]. This agent, which is available in different commercial supplements, was evaluated in different clinical studies. A product mainly containing forskolin and rutin (Kronek®) produced an improvement in the electroretinogram amplitude in POAG patients after 6-month of supplementation, suggesting the probable neuroactive effect of the combination of these molecules. [185]. Furthermore, in another pilot study, the administration of this product for 30 days led to significant IOP reduction in POAG patients [163]. Gangliolife® is another commercially available food supplement that mainly contains forskolin, homotaurine, and l-carnosine. The rationale of the investigation was to study the potential neuroprotective properties as well as the synergic effects of these compounds on RGC both in vitro and in vivo models of hypertensive retinal ischemia in mice. The results showed that the RGC survival was increasing following the administration of combination compounds. Further, Mutolo and coworkers [138] investigated whether a one-year consumption of this product has beneficial effects on different ocular indices and evidence of neuroactivity in RGC on POAG patients. They observed that this supplement improved pattern electroretinography (PERG) amplitude and foveal sensitivity in treated patients.

Agomelatine, an atypical antidepressant, is used to treat major depressive disorder. Melatoninergic compounds, in particular agomelatine, exert antiapoptotic and antioxidant activities and decrease IOP, thus suggesting that these agents can be used to prevent glaucoma progression [5, 28]. In addition, short-term administration of 25 mg/day agomelatine (Valdoxan®) on POAG patients taking different glaucoma- controlling medications leads to a further decrease of IOP [162].

Tables 4 and **5** summarize the effect of the various substances and nutrients connected in the text focusing on IOP (**Table 4.**) and other ocular and biochemical parameters (**Table 5.**)

Table 4 – Interventional studies analyzing the effects of various nutrients on IOP.

Reference	Nutrients examined	Number, type of participants	Mean follow-up	Observations/Outcomes	Type of study
Pescosolido *et al.*, 2015 [162]	25 mg per day of agomelatine	10 patients with hypertensive POAG*	30 days	The oral systemic agomelatine 30% decreased IOP* in both eyes of all POAG patients who went multiple drug treatment with anti-glaucoma eye drops ($P<0.001$).	Pilot prospective study
Krefting *et al.*, 2014 [105]	Capsules of vitamin D3 20,000 IU twice per week or placebo	78 participants with low serum 25(OH)D	6 months	This study in healthy participants revealed no associations between serum 25(OH)D levels and IOP, and administration of vitamin D3 to participants with low levels of 25(OH)D did not affect IOP	Randomized controlled trial
Jabbarpoor Bonyadi *et al.*, 2014 [15]	Saffron extract 30 mg/day or placebo	34 POAG patients in 2 groups	4 weeks (treatment + 4 weeks washout)	A statistically significant IOP reduction was noted after 3 (10.9 ± 3.3 mmHg in the saffron group as compared to 13.5 ± 2.3 mmHg in the control group) ($P = 0.013$) and 4 weeks (10.6 ± 3.0 versus 13.8 ± 2.2 mmHg) ($P = 0.001$) of treatment	Randomized controlled trial
Sisto *et al.*, 2014 [185]	A commercially available product containing forskolin extract, rutin and vitamins B1 and B2. One tablet, twice daily or control group	45 patients	6 months	IOP significantly decreased in treated group after 6-month treatment to14.6 mmHg ($P<0.05$)	Open randomized case-control trial
Vetrugno *et al.*, 2012 [202]	2 tablets per day of Kronek® containing rutin and forskolin in addition to their usual topical drug treatment	97 (52 in the treatment group, and 45 in the reference group)	1 month	oral administration of this supplement could allow a further 10% reduction of IOP values ($P<0.01$).	Open case-control trial
Goldblum *et al.*, 2009 [55]	Daily oral intake of 300 mg alpha-tocopherol-acetate	39 POAG, PXF* post-trabeculectomy patients	2 months	No significant difference in Trabeculectomy failure risk, IOP or success rates between the groups.	Randomized controlled trial
Chung *et al.*, 1999 [24]	*Ginkgo biloba* extract	11 healthy volunteers	2 days	*Ginkgo biloba* extract did not alter IOP	Randomized controlled trial

IOP = intraocular pressure; POAG = primary open-angle glaucoma; PXF = pseudoexfoliative glaucoma.

Table 5 – Studies of various nutrients intake on eye and other eye health-related parameters.

Reference	Nutrients examined	Number, type of participants	Mean follow-up	Observations/Outcomes	Type of study
Villadoniga *et al.*, 2018 [175]	DHA* 1g	47 patients (DHA group 23, controls 24	6 months	Plasma total antioxidant capacity increased significantly, while plasma malondialdehyde (P=0.02), and plasma IL-6* levels (P=0.006) were decreased in treated group. Also, IOP* in both eyes decreased significantly (right eye 17.5% P=0.01 and 19.2% left eye P=0.007)	Open label randomized controlled trial
Harris *et al.*, 2018 [65]	A commercially supplement that is a mixture of essential vitamins and minerals, omega-3 polyunsaturated fatty acids, dietary polyphenolic nutrients, a modified essential amino acid, botanical extracts, and flax seed oil or placebo	45 OAG patients in 2 groups	1 month	One-month oral administration of supplement increases biomarkers of ocular blood flow within retinal and retrobulbar vascular beds in patients with OAG*	Randomized controlled trial
Dewi Sari *et al.*, 2016 [179]	40 mg *Ginkgo biloba* extract, 2 times daily or placebo	40 POAG patients in 2 groups	6 months	Significant improvement in plasma oxidative stress markers (malondialdehyde, glutathione peroxidase, P= 0.001 for both), VF* mean defect (P= 0.011), VF* pattern standard deviation (P= 0.02) as well as superior and inferior retinal nerve fiber layer thickness (P= 0.001 and P= 0.035 respectively) compared to placebo group. No significant change in IOP in either group.	Randomized controlled trial
Mutolo *et al.*, 2016 [138]	A commercially supplement that is a mixture of forskolin, homotaurine, carnosine, B vitamins, magnesium or without treatment	22 patients with POAG patients (in 2 groups: treated or not)	1 year	A significant decrease of IOP and an improvement of PERG* amplitude and foveal sensitivity	Randomized controlled trial
Garcia-Medina *et al.*, 2015 [50]	Two different antioxidants supplementation including vitamins and minerals (one with omega-3 fatty acids and one without)	117 patients with POAG in 3 groups with 2 different supplements and no treatment	2 years	No effect of treatment was observed for VF mean deviation, VF pattern standard deviation, retinal nerve fiber layer thickness, ganglion cell complex thickness between groups	Randomized controlled trial

Table 5 (continued)

Reference	Nutrients examined	Number, type of participants	Mean follow-up	Observations/Outcomes	Type of study
Lee *et al.*, 2013 [116]	*Gingko biloba* extract, 80 mg twice a day	42 patients with NTG[a]	12.3 years (4 years observation + 8 treatment) (retrospective)	Decelerated VF progression mean deviation, pattern standard deviation and visual field index but not IOP	Cohort
Ohguro *et al.*, 2012 [147]	Black currant antocyanoside extract 50 mg/day or placebo	38 patients with OAG (in 2 groups: treated and placebo)	2 years (prospective)	Less VF mean deviation deterioration and increased ocular blood flow was seen in treated group	Randomized controlled trial
Park *et al.*, 2011 [155]	*Gingko biloba* extract 80 mg twice a day or placebo	30 patients with NTG (treatment or placebo)	4 weeks	Increased temporal peripapillary retina flow ($P<0.01$) not in optic cup area or nasal neuroretina rim	Randomized controlled trial
Zhong *et al.*, 2010 [227]	*Erigeron breviscapus* (vant.) *handmazz* (EBHM); a Chinese herbal drug,	40 POAG patients	6 months	Visual field indices improved significantly in the treatment group for the entire group after 6 months, and in patients with moderate and late glaucoma after 2, 4 and 6 months, no significant differences were found in the placebo group. No significant difference found for visual acuity, IOP, cup-to-disk ratio in either group	Randomized controlled trial
Kim *et al.*, 2010 [98]	1.5 g Korean Red Ginseng, administered orally 3 times daily (Ginsenoside)	36 OAG patients	12 weeks+8 weeks washout + 12 weeks placebo	Ocular blood flow improved significantly during treatment phases when compared to baseline ($P=0.005$) but no change during placebo phases. IOP and VF did not change significantly.	Randomized controlled trial
Falsini *et al.*, 2009 [43]	Epigallocatechin gallate	18 OAG patients and 18 ocular hypertension patients	3 months	PERG[a] significantly improved only in OAG patients compared either to baseline ($P<0.05$) or to PERG amplitude in the same patients after placebo ($P<0.05$); Visual fields did not change significantly	Randomized controlled trial
Wang *et al.*, 2008 [144]	A Chinese herbal drug, *Erigeron breviscapus* (vant.) *handmazz* or placebo	99, POAG or chronic angle-closure glaucoma (post-surgery)	6 months	Visual fields defect scoring in treatment group improved significantly compared to the placebo group at 2 and 4 months and 6 months. IOP and visual acuity did not change significantly in either group.	Randomized controlled trial
Steigerwalt et. al, 2008 [190]	A supplement containing 40 mg of French maritime pine bark extract, and 80 mg of standardized bilberry extract twice daily or no treatment	38 asymptomatic subjects with intraocular hypertension were either given supplement (20 subjects) or were not treated (18 subjects)	6 months	IOP significantly decreased, in Mirtogenol® group (IOP=22.0 mmHg ± 2.3) and in control group (IOP=24.7±2.2 mmHg) ($P<0.05$) as well as ocular improvement in blood flow ($P<0.05$).	Randomized controlled trial

Table 5 (*continued*)

Reference	Nutrients examined	Number, type of participants	Mean follow-up	Observations/Outcomes	Type of study
Ohguro *et al.*, 2007 [149]	Anthocyanins extracted from Black currant in tablet form once a day	30 NTG patients	6 months	Ocular blood flow of the optic nerve head and its surrounding retina and plasma endothelin-1 increased significantly ($P<0.05$ for both). No significant changes in IOP or visual field.	Pilot uncontrolled study

DHA = docosahexaenoic acid; IOP = intraocular pressure; NTG = normal tension glaucoma; OAG = open angle glaucoma; PERG = pattern electroretinography; POAG = primary open angle glaucoma; VF = visual field.

Conclusions

It is worth exploring modifiable glaucoma risk factors other than IOP to control better and lower the burden of this disease; however, the current weight of evidence is not sufficient to make a strong recommendation regarding lifestyle behaviors and, in particular, the use of dietary supplements. Furthermore, any study showing an impact on IOP, particularly uncontrolled studies, may be just regression to the mean that has been shown in many other IOP-related studies. Regarding visual function, for tests such as those on the Humphrey Visual Field Analyzer that are subjective, there is also a learning curve that may explain improvements in testing over time. This notion may be extended to any uncontrolled studies mentioned in this paper. In addition, there is a lack of structural endpoints analysis such as optical coherence tomography (OCT) that is used for measuring nerve fiber layer (OCT RNFL) and optic nerve parameters, which would provide more objective data compared to the functional outcomes more of- ten used in these studies.

The current data suggest that supplementation with various nutrients/compounds might impact the incidence or progression of glaucoma, even though it should be underscored that nutritional supplements and herbal medicines cannot substitute for routine antiglaucoma treatment. Specifically, we highlight the influence of different nutrients with various properties and mechanisms of action on glaucoma; however, it should be emphasized that the clinical usefulness and role must be further studied and confirmed in well-designed randomized clinical trials to determine whether nutritional supplements can become part of the adjuvant treatment of glaucoma. This development will require investments in clinical studies and at the regulatory level. Presently it will be difficult to frame any of the

mentioned substances within the regulatory rules designed for drug approval and licensing since most of them act through multiple mechanisms that cannot be reconciled with a single principal pharmacological mechanism; however, the relevant information is whether or not they have a therapeutic effect that, once demonstrated, can be recognized through an appropriate regulatory approval processes [165] (for a discussion see Racchi and coworkers, 2020).

Method of literature search

We used a retrospective analysis of published articles in PubMed as well as Google Scholar to identify and select papers for this review. Search keywords included glaucoma and - diet, - glaucoma review, - vitamins, - minerals, - nutrients, - dietary supplement, - herbals, - fatty acids, - caffeine, - Gingko biloba, - lifestyle, - smoking, - cigarette, - marijuana, - alcohol, - BMI, - weight loss, - bariatric surgery, - swimming, - physical activity, - diabetes. Additionally, we reviewed the references concluding in the literature and selected them to provide further information on the area of interest. To be more comprehensive, we did not limit publication year; however, only papers in English were evaluated. The focus of our searching was on human epidemiological and clinical human studies.

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Part B

The following manuscript was published in *Frontiers in Drug Safety and Regulation* in 2022 as:

MINI REVIEW article

Front. Drug Saf. Regul., 27 June 2022
Sec. Substance-Based Medical Devices
https://doi.org/10.3389/fdsfr.2022.933471

This article is part of the Research Topic
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Pharmacological Versus Non-Pharmacological and Ancillary Mechanisms in Eye Drops Used in the Treatment of Glaucoma

Nicoletta Marchesi†, Foroogh Fahmideh†, Annalisa Barbieri*, Marco Racchi, Alessia Pascale and Stefano Govoni

Department of Drug Sciences, Section of Pharmacology, University of Pavia, Pavia, Italy

Pharmacological versus non-pharmacological and ancillary mechanisms in eye drops used in the treatment of glaucoma

Nicoletta Marchesi, **Foroogh Fahmideh (co-First Author)**, Annalisa Barbieri, Marco Racchi, Alessia Pascale and Stefano Govoni

Abstract

Medical Devices Made of Substances (MDMS) are increasingly used in the healthcare system alongside classic medicinal products and constitute an important field of experimentation and innovation in the biomedical field. In fact, these products are rapidly establishing themselves as a valuable therapeutic resource and are available in various forms including, but not limited to, creams, syrups, nasal or oropharyngeal sprays, and eye drops. MDMS are marketed to treat different diseases and the advantages and benefits of the use of these products can be claimed, once proven their clinical activity. What are the differences between medicinal products and MDMS? The substantial difference lies in the mechanism of action: the first case is based on pharmacological, metabolic, and immunological actions while the second one is based on mechanical, or chemical/physical action. Sometimes the boundaries are not well defined and there is a need for a reassessment and a consensus on the underlying concepts and definitions, also in the light of the increasing ability to recognize molecular mechanisms underneath the action of several substances not acting through an easy recognizable unique target (as a receptor, for example). In the present paper, we discuss the role of eye drops as an example of MDMS used in glaucoma, a widely diffused eye disease. The choice is due to the fact that some products used in this field of application and containing similar substances are marketed either as medicinal products or as medical devices or, using other dosage forms, as food supplements. Accordingly, it is important to underscore in the various cases what may be the principal mode of action and the contribution of additional mechanisms as derived, for example, from system pharmacology data. Their analysis may help to exemplify some of the problems around the sometimes fuzzy border between MDMS and medicinal products suggesting the need for new definitions and regulatory decisions about MDMS.

Keywords: MDMS, Glaucoma, Mechanism of Action (MOA), System Pharmacology, Eye Drops, Food Supplements, Medicinal Product, Citicoline

Introduction

Medical devices made of substances (MDMS) are health products used to cure or prevent an illness. From the patient's point of view are hardly distinguishable from conventional medicinal products and they should comply with strict regulatory definitions involving in their therapeutic action only physicochemical and not pharmacological, immunological, or metabolic mechanisms as, usually, medicinal products do.

Indeed, medical devices are vastly used in the healthcare system alongside conventional medicinal products. The EU Medical Devices Regulation (Regulation (EU) 2017/745) recently placed several devices under new classification rules (EUR-Lex- 02017R0745-20200424 - EN - EUR-Lex, 2022).

MDMS are designed to treat various diseases, and patients are expected to obtain advantages and benefits with the use of these products. Clinical data are required to claim any therapeutic activity and the safety of the device should be corroborated by a post-market clinical follow-up. It should also be stressed that medical devices may be composed of substances or combination of substances which may give origin to whole body exposure (see Annex VIII of EUR-Lex - 02017R0745-20200424-EN-EUR-Lex, 2022), going beyond the physicochemical mean thus requiring further refinement of the studies on the mechanism of action.

Even if the European regulatory constraint defines precise boundaries between MDMS and medicinal products in terms of mode of action (physical-chemical versus pharmacological, immunological, metabolic), this definition appears to be limited and insufficient to describe the increased complexity of the biological mechanisms elicited by several substances included some of those present in the MDMS. The regulatory setting is founded on the classical thinking of a pharmacological (immunological, metabolic) action, based on an easy and traceable primary hierarchically organized target and a lock-key interaction between a substance and its target. This definition is not incorrect, but it is presently insufficient to explain the biological and therapeutic effects of several substances and there is the need to expand and overcome this dated setting while defining the mode of action of a medicinal preparation, an MDMS, or, in general, a natural substance. The definition of pharmacological mechanism of action has been recently amended and further detailed in the non-binding guideline proposed by the Medical Device Coordination Group Document (MDCG 2022-5,2022)

substantially expanding the possibility to recognize a mechanism as belonging to pharmacological, immunological, metabolic domain (as in the case, for example of substances of herbal origin) when the principal mode of action is complex and difficult to define or to tribute to a specific substance. On the other hand, the same document is open to the possibility that a product containing such substances having pharmacological action could be qualified as a MDMS if the pharmacological action is ancillary to the principal intended action of the device. In order to discuss these points, we decided to deal with a pathology, glaucoma, and a substance, citicoline, both topics on which we accumulated some direct experience in the past years.

GLAUCOMA: A relevant clinical problem and an unmet medical need

It is estimated that 67 million people worldwide have glaucoma and glaucoma is the second leading cause of irreversible blindness. Glaucoma is a disease in which increased intraocular pressure is the leading cause of a subsequent degeneration of the axons of retinal ganglion cells (RGCs), which make up the optic nerve. The neurodegenerative process can progress in spite of intraocular pressure control (Davis et al., 2016). The loss of RGCs leads to loss of vision, and if untreated, to blindness (Lavik et al., 2011; Fahmideh et al., 2021). Drugs commonly used for glaucoma treatment aim to decrease intraocular pressure, mostly in form of eye drops, which, according to the clinician intention, should slow the rate of disease progression sufficiently to avoid functional impairment from the disease. Eye drops used in managing glaucoma decrease eye pressure by helping the eye's fluid to drain better and/or decreasing the amount of fluid made by the eye. Drugs to treat glaucoma are classified by their active ingredient. These include prostaglandin analogs, beta-blockers, alpha agonists, carbonic anhydrase inhibitors, and rho kinase inhibitors. In addition, combination drugs are available for patients who require more than one type of medication. An older class of medications, the cholinergic agonists (such as pilocarpine) are not commonly used nowadays due to their side effects (Weinreb et al., 2014). Considerable efforts have been made to develop neuroprotective glaucoma treatments that prevent optic nerve damage. With the development of neurotrophic, antioxidant, anti-ischemic, anti-inflammatory, antiapoptotic, and immunomodulatory therapeutic approaches, the broad field of neuroprotection in glaucoma shows progress in reducing neurodegeneration and thus stabilizing visual function in experimental studies. Unfortunately, no firm evidence exists

that these agents can prevent long-term disease progression in patients with glaucoma, and still, there is a long way from basic research to the clinic (Weinreb et al., 2014; Jünemann et al., 2021). Complementary and alternative medicine is meant to be used as adjuncts to traditional therapy, including oral food supplements and MDMS (usually in the form of eye drops). It is estimated that 5–15% of glaucoma patients, reportedly spending billions of dollars annually, take some form of alternative medicine based only on their impression that it will help treat their glaucoma (John Hetherington, 2013). Nutritional supplementation comprises a broad array of products intended for ingestion to meet essential nutritional requirements. These products can be categorized as vitamins, minerals, herbals, botanicals, amino acids, fatty acids, and other dietary supplements used individually or in combination (Fahmideh et al., 2021). Regarding MDMS used in glaucoma, some of the claims lay on neuroprotection (by restoring the integrity of retinal ganglion cell membrane) and antioxidant activities including topical coenzyme Q10, citicoline, hyaluronic acid, mannitol, and vitamins B12 and E alone or in combinations. It should be mentioned that, so far, no neuroprotective drugs have been approved by the FDA and the clinical studies behind these substances are few and the majority limited to non-randomized ones.

CITICOLINE at the border of various regulatory domains

Among the different substances, citicoline is a challenging example, worthy of attention. Indeed, citicoline has been used in several countries for several decades and, based on its properties and route of administration, this substance can be used as a drug, as a food supplement, as a food for special medical purposes, or can be dispensed as an MDMS.

Citicoline, the generic name of the International Non-proprietary Name of cytidine-5′-diphosphocholine (CDP- choline, CDPCho), is a particular molecule with psychic stimulating and nootropic activity (Adibhatla and Hatcher, 2002). In Japan and Europe, citicoline was originally used as a prescription injectable drug for the treatment of cerebrovascular and cognition disorders in people who are healing from a stroke. Nowadays, it is world widely used as an over counter dietary supplement.

Citicoline plays a vital role in the biosynthesis of phospholipids and their precursors and in maintaining the phospholipid components in the cell

membranes. Its mechanism of action as well as its biological effects are multifactorial and include, but are not limited to, 1) preservation of cardiolipin and sphingomyelin; 2) restoration of phosphatidylcholine; 3) stimulation of glutathione synthesis; 4) reduction of glutamate concentration; 5) rescue of mitochondrial function, preventing neural apoptosis; 6) synthesis of myelin; 7) improvements of acetylcholine synthesis. These actions (see also Oddone et al., 2021 for a review) may lead to the prevention of endothelial dysfunction and exert a neuroprotective role of the retina (Pascale et al., 2012; Parisi et al., 2018). Thus, the neurotherapeutic effect of citicoline could be multifarious, mainly by improving neuronal membrane integrity, maintaining cellular communications with its environment, reducing oxidative stress, and improving the synthesis of neurotransmitters such as acetylcholine and dopamine. There is, in fact, evidence of a clinical effect of citicoline on several neurodegenerative diseases such as Parkinson's disease, senile and vascular dementia, and stroke (Vale, 2008; Alvarez-Sabín et al., 2013; Gareri et al., 2017; Mehta et al., 2019).

Indeed, the route of administration, dosage form, and consumption do affect its indication. When this molecule is given, it is metabolized, resulting in the production of choline. The latter is a precursor of acetylcholine, one of the most important neurotransmitters of our nervous system, involved in numerous cognitive functions, such as, for example, memory and attention (Grieb et al., 2015). In fact, nootropic substances generally carry out their actions by promoting the production of neurotransmitters, providing the body with the molecules necessary for their synthesis (Gandolfi et al., 2020).

Use of citicoline in glaucoma

Recently, due to its neuroprotective properties, citicoline has been proposed and studied as a complementary treatment of glaucoma (both as special food for medical purposes and as MDMS available as eye drops, in the latter case in association with hyaluronic acid, based on its activities in preserving the cell membrane, see below for further comments) (See **Table 1**).

Systemic administration (as a food supplement or food for special medical purposes)

A recent review extensively summarized the relationship between the cholinergic nervous system and visual function and the potential implications for glaucoma neuroprotection and/or neuroenhancement (Faiq et al., 2019).

Nevertheless, in 2014, EFSA (European Food Security Authority) pronounced on the scientific substantiation of a health claim related to the new food cytidine 5-diphosphocholine and maintenance of normal vision in elderly subjects since middle age. EFSA concluded that a cause-and-effect relationship has not been established between the consumption of CDP-choline and the maintenance of normal vision; therefore, the previously mentioned health claim cannot be supported (Agostoni et al., 2014).

In the aforementioned examples the use of citicoline in symptomatic disease (glaucoma), is proposed whereas its intake by the asymptomatic general population for possible prophylaxis of this disease is not considered as supported.

Eye drop administration

Citicoline in eye drops can counteract the visual impairment of glaucoma (see also **Table 1** summarizing the main characteristics of clinical studies with citicoline in patients with glaucoma). Notably citicoline (2%) eye drop administration can give origin to substantial intravitreal concentrations of the compound (Carnevale et al., 2019). A study highlighted that the combination of citicoline in eye drops reduces eye pressure and slows down both anatomical and functional glaucomatous damage (Rossetti et al., 2020a). The study results showed that if glaucoma patients are accompanied by eye drops containing citicoline, in addition to ocular hypotensive therapy, the glaucomatous damage slows down significantly. Literature data show the positive effects of citicoline in glaucoma and more general in neurodegenerative diseases (Parisi et al., 1999, 2008, 2015; Ottobelli et al., 2013). It is interesting to note that in some studies (Roberti et al., 2014; Parisi et al., 2015, 2019), when glaucomatous patients were treated with citicoline eye drops, the improvement of retinal ganglion cell function (detected by pattern electroretinogram) and neural conduction along the visual pathways (detectable by shortening of visual evoked potentials) were observed. These outcomes demonstrate that citicoline not only prevents the progression of glaucoma but may assist the functional recovery of injured retinal ganglion cells as shown by recovery in the nerve signal conduction in treated patients possibly due to the RGC membrane stabilization (Parisi et al., 2015, 2019).

Once again, all the eye drops preparations containing citicoline used in these studies were medical devices and, as highlighted before, a medical device should

not base its activity on pharmacological, immunological, or enzymatic properties. The view is not easy to be reconciled with the observed actions unless played exclusively at the plasma membrane level preserving its integrity.

From a more general point of view, it will be important to thoroughly compare the doses used in the various studies following the different ways of administration to gain information on the citicoline eye levels when given topically and systemically.

Some additional notes involving the mechanism of the described actions and their relevance to the regulatory classification of the product

According to the literature data, there is the possibility that citicoline, given systemically, as a food supplement or food for special medical purposes, may act through its multiple interactions (described in the previous sections and also depicted in **Figure 1**) which do not fit the definition of pharmacological (immunologic, metabolic) mechanism because the final effect derives from complex interactions that bring about changes in a way that cannot be pinpointed at the single target/ receptor level (Bilia et al., 2021).

As suggested by some of the nodes in **Figure 1** there are elements indicating citicoline involvement in glaucoma in pathways known to have an important role in neurodegeneration and apoptosis. Neurodegenerative pathways are of interest in the development of glaucoma since this condition is recognized not only as an ocular disease but also as a neurodegenerative disorder. In these years, many experimental and clinical studies have shown that in glaucoma, neuronal degeneration occurs not only at the level of the retina and optic nerve but also along the entire visual pathway and the brain.

A pathway that acts on glaucoma pathogenesis is caspase-3 (CASP3), citicoline has a potential neuroprotective effect by being involved in apoptosis through the CASP3 target and therefore management of neurodegenerative disorders. The citicoline effect is attributed to the control of neuronal apoptosis and to the induction of the regeneration of newborn RGCs neurites in experimental models including retinal explants and rat optic nerve crush model (Oshitari et al., 2010; Kitamura et al., 2019). In an *in vitro* study, citicoline administration to rat primary retinal cell cultures protected from apoptosis, by means of a reduced frequency of caspases activation and accumulation of apoptosis markers, in the

presence of glutamate-induced excitotoxicity and high glucose challenge (Matteucci et al., 2014). In addition, in a recent study on a methanol-intoxicated retina model in rats, it is hypothesized that citicoline is able to minimize the loss of retinal ganglion cells and the disruption of photoreceptors, to suppress ganglion layer edema, to increase the expression of the antiapoptotic BCL-2 protein, and finally to decrease the expression of the proapoptotic caspase-3 protein (Laksmita et al., 2021).

The treatment with eye drops containing citicoline may be effective in suppressing oxidative stress and controlling inflammation in UVB corneal injury. Not only CASP3 was evaluated but also other targets of the protein-protein interactions as Matrix metalloproteinase (MMP)−2 and −9. In particular, after immunofluorescent staining and Western blot analysis, an increased MMP-2, -9, and caspase-3 in the UVB-only group compared with the UVB/citicoline group have been shown. Citicoline treatment may be effective in suppressing oxidative stress and consequently controlling inflammation in UVB corneal injury. Citicoline exerts this effect by inhibiting lipid peroxidation and increasing antioxidant defense mechanisms (Tokuc et al., 2021). AKT1 also is an interesting "crowded" node emerging from **Figure 1.** Indeed, the PI3K/AKT pathway plays a role in neurodegeneration and in glaucoma, being involved in retinal ganglion cells and trabecular meshwork cell apoptosis, and in autophagy (He et al., 2018). However, as far as to our knowledge, there are only indirect data showing in animal studies that citicoline may act regulating such pathway in a radiation-induced brain injury rodent model (Abdel-Aziz, Moustafa and Saada., 2021).

As it emerges from the previous paragraphs citicoline has many biological interactions with cellular mechanisms. As depicted in **Figure 1** a bioinformatic analysis based on the interaction between targets of citicoline and glaucoma involved genes provides a conspicuous list of plausible targets, including some major crossroad intersections with neurodegeneration, apoptotic processes, vascular and metabolic pathways linked to oxidative stress. The questions arising are: 1) can all these nodes and putative pathways be confirmed by direct experimental data? 2) are these pathways engaged at the doses used clinically and through which route of administration? And, further, do these mechanisms fit exactly any of the present regulatory constraints to distinguish whether the product is an MDMS or a medicinal product?

TABLE 1. Clinical studies with citicoline in patients with glaucoma

Product Dispensed as*	Indicated Dose and Time of Treatment	Other Active Substances Present in the Product	Study Design and Number of subjects	Parameter Measured, Comparator, and Observed Results	References
Medicinal product	1 g IM injection per day for 10 days	—	Non-randomized clinical study 30 patients (47 eyes) suffering from open-angle glaucoma	Improvement of visual fields was observed in the patients who had already taken beta-blocker eye drops. The authors suggested decreased glaucomatous optic nerve damage	Giraldi et al. (1989)
Medicinal product	1 g IM injection per day for 2 cycles of 60 days with 120 days of washout period	—	Randomized placebo-controlled clinical study. Citicoline group (n = 25) and placebo group (n = 15)	The placebo group was treated with a physiologic solution. Visual evoked potentials and pattern-electroretinograms improved in the citicoline group at different timelines	Parisi et al. (1999)
Medicinal product	1 g IM injection per day for 15 days repeated every 6 months lasted for 10 years	—	Placebo-controlled clinical study. 11 patients were treated with citicoline, while 12 patients received no treatment at all	They all had an ocular pressure normalized by topical pharmacological treatment. Citicoline administration seems to prevent the progression of perimetric deficits in glaucomatous patients	Virno et al. (2000)
Food supplement	1 g orally taken per day for 2 weeks, 2 days of washout, and repeated another 14 days of treatment	—	Non-randomized clinical study. 21 glaucomatous eyes	Improvement of visual evoked potentials in glaucomatous patients	Rejdak et al. (2003)
Food supplement	500 mg orally taken citicoline per day for 4 months, followed by a 2-months of washout, after which the therapy cycle was repeated again for another 6 months	—	Randomized clinical study. Citicoline group (n = 30) and control group (n = 30), the latter did not receive any treatment	Increased retinal nerve fiber layer thickness and ganglion cell complex thickness were observed in the citicoline group compared to the control (without citicoline) group after 12 months. The authors suggested that citicoline therapy seems to be effective in slowing POAG progression	Lanza et al. (2019)
Food supplement	500 mg orally taken citicoline per day in 2 groups: Group 1 topical IOP lowering therapy alone for the first 4 months, after which they received treatment in addition to the topical therapy for the next 4 months. Group 2 received treatment in addition to the topical IOP lowering therapy for 4 months and then continued with the topical therapy alone for the next 4 months	homotaurine 50 mg, and vitamin E 12 mg	Observational, cross over study. 41 glaucomatous patients in group 1 and 63 glaucomatous patients in group 2	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 POAG.	Marino et al. (2020)
Food supplement	250 mg orally taken citicoline per day for 3 months and 1 month washout period	—	Randomized clinical study. 27 glaucomatous patients were in the treatment group while 27 patients were assigned to the control group	Increased inferior quadrant retinal nerve fiber layer thickness in the citicoline group at 3 months was significantly greater than in the control group. Study data show that citicoline may have a significant impact on slowing glaucoma progression, which could have a potential neuroprotective effect	Sahin et al. (2022)
MDMS Eye drops	200 mg citicoline eye drops 3 times daily for 4 months followed by 2 months washout period	Hyaluronic acid 20 mg	Randomized clinical study 24 glaucomatous eyes were treated with topical citicoline, and another 23 glaucomatous eyes were only treated with IOP lowering treatment	Topical treatment with citicoline in POAG eyes induces an enhancement of the retinal bioelectrical responses (an increase of pattern electroretinogram amplitude) with a consequent improvement of the bioelectrical activity of the visual cortex	Parisi et al. (2015)
MDMS Eye drops	200 mg citicoline eye drops 3 times daily for 3 years	Hyaluronic acid 20 mg	Randomized, double-masked, placebo-controlled, clinical study. 40 patients were in the citicoline group whereas 38 patients were in the placebo group	Patients receiving citicoline eye drops lost lesser retinal nerve fiber layer thickness in 3 years, versus the placebo group. The authors suggest that citicoline could be a complementary treatment in the management of patients with progressing glaucoma	Rossetti et al. (2020b)

**Different commercially available forms of citicoline are present in Europe, The reported classification was done by the authors based on the route of administration and does not necessarily reflects regulatory boundaries. In the case of the Medicinal Product category it was considered that the injectable form of citicoline is approved in Europe and Japan for use in stroke, head trauma, and other neurological disorders. The use of the injectable preparation for glaucoma is experimental. Several oral preparations of citicoline alone or in combination are used as a dietary supplements with no claims allowed but their use was experimental in the quoted studies. However citicoline is also available in oral formulations as food for special medical purposes for the dietary management of patients with glaucoma pharmacologically stabilized and with progressive loss of visual field. The drop dosage form of citicoline in European countries is available and considered a medical device indicated in glaucomatous patients as coadjutant to hypotensive therapy.*

AbbreviationsIM: intramuscular; MMDS, medical devices made of substances; POAG, primary open-angle glaucoma; IOP, intraocular pressure.

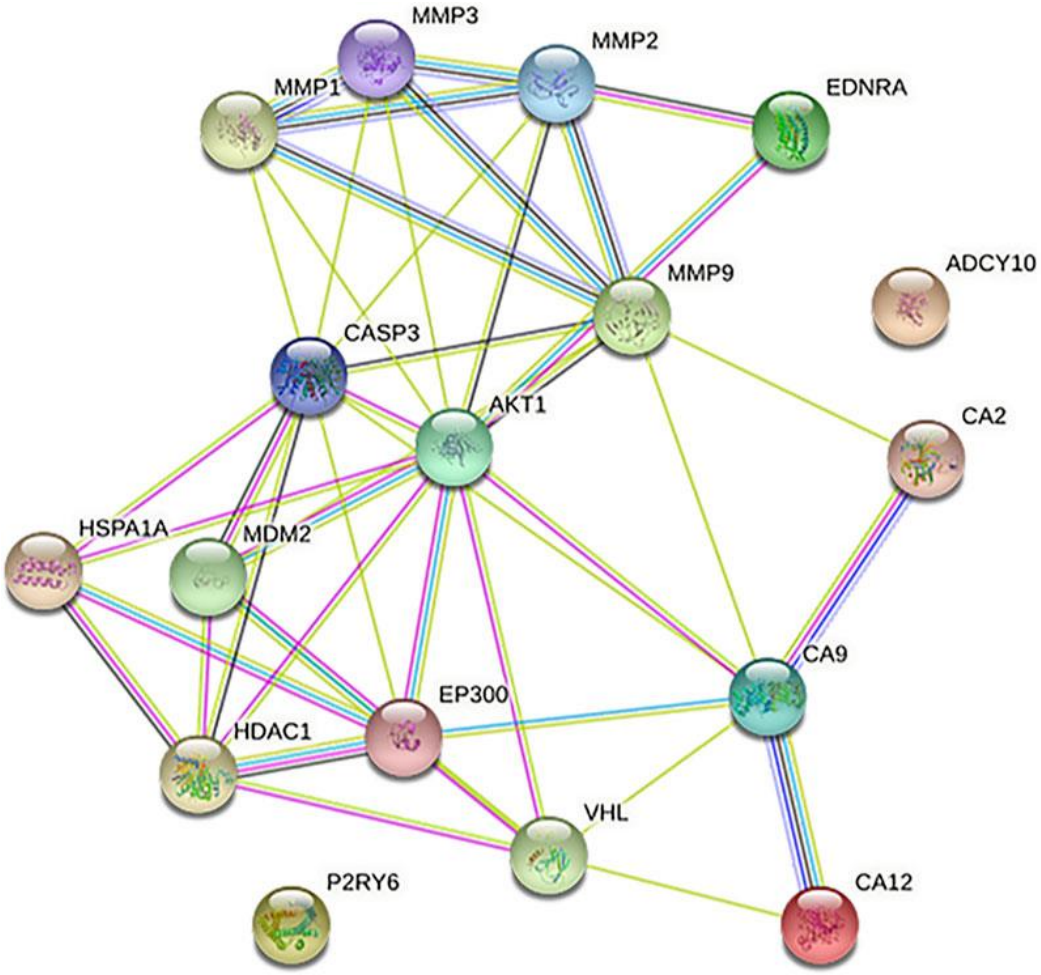

Figure 1. PPI (protein-protein interaction) Network. The chemical structure of Citicoline was imported into SwissTargetPrediction data library (www. swisstargetprediction.ch) and the target of citicoline was predicted (Gfeller et al., 2014). In DisGenNet (https://disgenet.org/) the keyword of "Glaucoma" was selected to collect the targets of this disease. In order to analyze the interaction between target proteins, the targets of citicoline and glaucoma were selected and imported into the STRING database (https://string-db.org); the interactions were analyzed by selecting "Homo sapiens" as organism and setting the confidence basis to 0.4 as done by Lian and Zheng (Liang et al., 2016; Zheng et al., 2021). The image uses connectivity strength as the driving force for the layout, posing strongly connected nodes closely together, at the same time the more edge enters each node, the more involved each enzyme is in the pathogenesis of glaucoma (edges represent functional associations) (ACDY10: adenylate cyclase type 10; AKT1: protein kinase B (serine-threonine specific protein kinases); CA12: carbonic anhydrase 12; CA2: carbonic anhydrase 2; CA9: carbonic anhydrase 9; CASP3: caspase-3; EDNRA: endothelin receptor type A; EP300: histone acetyltransferase p300; HDAC1: histone deacetylase 1; HSPA1A: heat shock protein family A; MDM2: mouse double minute 2; MMP1: matrix metallo proteinase-1; MMP2: matrix metallo proteinase-2; MMP3: matrix metallo proteinase-3; MMP9: matrix metallopeptidase 9; P2RY6: pyrimidinic receptor P2Y6; VHL: Von Hippel-Lindau tumor suppressor).

The findings of the studies briefly summarized above confirm the suggestion stemming out from the bioinformatic model involving multiple targets and a

network of various correlators. The relevance of some of these targets were already suggested by clinical studies or literature searching whereas others are presently based only on bioinformatics data and are waiting for an experimental confirmation.

Conclusion

The case of the medical applications of citicoline and in particular of its use of glaucoma is emblematic of the difficulties of classifying some substances neatly and unambiguously as belonging to a regulatory class (medicinal product, medical device based on substances, food supplement, food for special medical purposes) (as also summarized in **Figure 2**). There are several variables including commercial choices (development investments, market access easiness, price/reimbursement) and the difficulties emerging when examining the mechanism of action of a given substance. As far as the latter point, the new investigational and bioinformatic techniques have opened to the concept of system pharmacology, which may apply to a single substance having multiple targets (as in the citicoline example) as well as to complex mix of substances, as derived from natural sources, for which is not possible to tribute the effect to a an easy and traceable primary hierarchically organized target and a lock-key interaction between a substance and its target, but rather to the resultant balanced action on multiple targets (see also Racchi et al., 2016; Bilia et al., 2021). In some cases these substances have already an history of either food or medical (albeit not registered) use in humans and a known profile of safety. Presently their development either as drugs or as MDMS is paved by difficulties and one is the discrimination between pharmacological and non-pharmacological mode of action that, as shown in the case of citicoline, may not be easy to discriminate and may differ according to associated variables such as the way of administration, the dose, the selection of a target among many possible/available. Provided the demonstration of a clinical activity consistent with the intended proposed use and the compliance to safety standards it may be proposed that such active substances, not fully complying with the present regulatory definitions concerning the mode of action, may follow either the MDMS or the medicinal product registration procedure rather than be confined to the fuzzy domain of food supplements which does not prevent their use but does not impose clinical demonstration of their efficacy and therefore, correctly, does not allow specific claims.

The way of reasoning here used for citicoline as a case study may be applied also to a mix of substances or to complex substances of natural origin at the cost of exponentially increasing difficulties because of the need to: 1) identify all the potential targets for each single molecule in study and match them with critical pathogenic biological targets/ pathways underlying the disease; 2) verify to what extent each target is critical in the development/control of the disease and their hierarchical organization; 3) verify to what extent the activation and/or inhibition of the biological target takes place at the doses/routes of administration used for the intended purposes. It has then to be decided whether these mechanisms involve specific interactions, at the molecular level, with the biological targets and whether their engagement orientates toward the classification as medicinal product or allows also the MDMS classification depending upon the use, the dose, the mode of administration. On the other hand, these aspects dealing with system biology and network pharmacology are further blurring the boundaries between drugs and MDs underscoring the need to further develop the scientific debate.

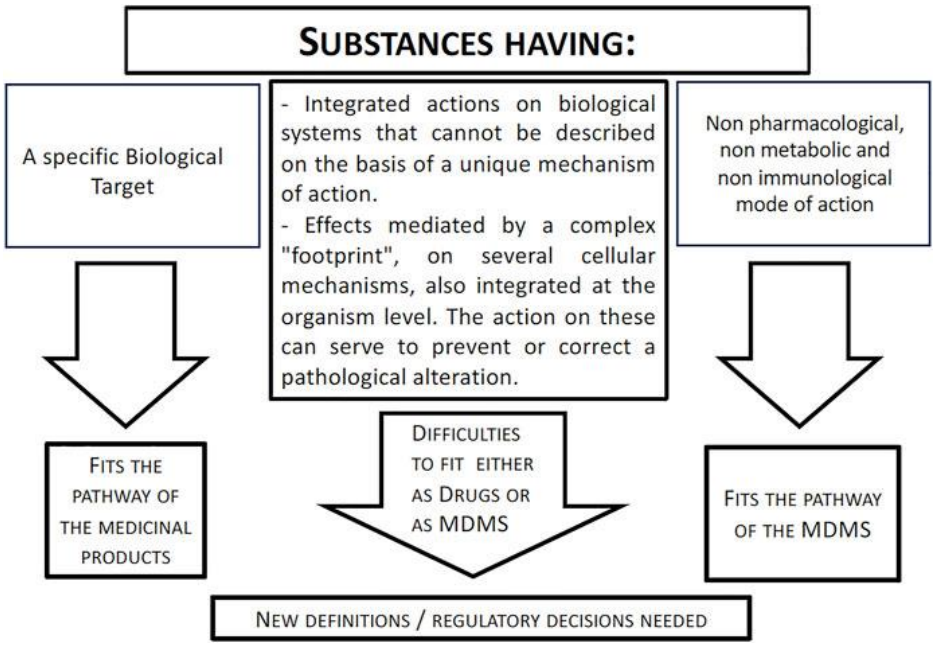

Figure 2. Mode of action of a substance and relationships with regulatory pathways. The figure shows that the advancements in the knowledge of cell signalling pathways and in bioinformatics and system pharmacology have led to the understanding of novel mechanisms underlying the activity of several substances. These mechanisms do not fit the usual definitions used to classify a substance as belonging to a medicinal product or a MDMS domain. A revision of the present criteria when evaluating a mode of action of a substance integrating these new aspects may be needed. See text for further comments.

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CHAPTER 4

The possible interconnection between the eye and the Central Nervous System

Chapter 4

The following manuscript was published in *Cells* in 2021 as:

Cells. 2021 Sep; 10(9): 2394.
Published online 2021 Sep 12. doi: 10.3390/cells10092394
PMCID: PMC8469605
PMID: 34572041

Ocular Neurodegenerative Diseases: Interconnection between Retina and Cortical Areas

Nicoletta Marchesi, Foroogh Fahmideh, Federica Boschi, Alessia Pascale, and Annalisa Barbieri*

Alessandro Attanzio, Academic Editor, Erica Buoso, Academic Editor, and Fabrizio Biundo, Academic Editor

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Department of Drug Sciences, Pharmacology Section, University of Pavia, Viale Taramelli 14, 27100 Pavia, Italy; nicoletta.marchesi@unipv.it (N.M.); foroogh.fahmidehtavako01@universitadipavia.it (F.F.); federica.boschi@unipv.it (F.B.); alessia.pascale@unipv.it (A.P.)
*Correspondence: annalisa.barbieri@unipv.it; Tel.: +39-0382987778

Ocular neurodegenerative diseases: Interconnection between retina and cortical areas

Nicoletta Marchesi, **Foroogh Fahmideh**, Federica Boschi, Alessia Pascale and Annalisa Barbieri

Abstract

The possible interconnection between the eye and central nervous system (CNS) has been a topic of discussion for several years just based on fact that the eye is properly considered an extension of the brain. Both organs consist of neurons and derived from a neural tube. The visual process involves photoreceptors that receive light stimulus from the external environment and send it to retinal ganglionic cells (RGC), one of the cell types of which the retina is composed. The retina, the internal visual membrane of the eye, processes the visual stimuli in electric stimuli to transfer it to the brain, through the optic nerve. Retinal chronic progressive neurodegeneration, which may occur among the elderly, can lead to different disorders of the eye such as glaucoma, age-related macular degeneration (AMD), and diabetic retinopathy (DR). Mainly in the elderly population, but also among younger people, such ocular pathologies are the cause of irreversible blindness or impaired, reduced vision. Typical neurodegenerative diseases of the CSN are a group of pathologies with common characteristics and etiology not fully understood; some risk factors have been identified, but they are not enough to justify all the cases observed. Furthermore, several studies have shown that also ocular disorders present characteristics of neurodegenerative diseases and, on the other hand, CNS pathologies, i.e., Alzheimer disease (AD) and Parkinson disease (PD), which are causes of morbidity and mortality worldwide, show peculiar alterations at the ocular level. The knowledge of possible correlations could help to understand the mechanisms of onset. Moreover, the underlying mechanisms of these heterogeneous disorders are still debated. This review discusses the characteristics of the ocular illnesses, focusing on the relationship between the eye and the brain. A better comprehension could help in future new therapies, thus reducing or avoiding loss of vision and improve quality of life.

Keywords: Eye, CNS, Retinal Neurodegeneration, Neurodegenerative Diseases, Age-Related Diseases

Introduction

The brain is linked to particular sense organs. We see from the eyes, and the information collected reaches specific neurons of visual cortex in the forebrain. The eye provides a unique window of the brain due to its special connection; the retina, photosensitive nerve tissue that covers the inner surface of the eye, is properly considered part of the brain [1–3]. The typical retina of a mammal contains several different types of neurons (**Figure 1**), each of which has its own morphology and specific function: The signals of the photoreceptors (rods and cones) are processed in cascade by groups of amacrine, bipolar, and horizontal cells that are in connection with the ganglion cells, responsible for transmitting information to the brain [4]. In detail, the light passes through the cornea, pupil, lens, and vitreous, and arrives to the retina, generating visual stimuli, which are transformed into electrical impulses and transported through the optic nerve to the brain. The brain interprets them by giving shape to images. Therefore, the retina is a functional unit of the central nervous system that converts a light signal into a nerve impulse, being physically connected to the brain via axons of the optic nerve. The complex synaptic connections that underlie the visual system have long been known [5–7]. Despite their different morphology, retinal ganglionic cells (RGCs) and neurons are anatomically similar; both are made up of a cell body, dendrites, and axons. RGCs' axons form the optic nerve (**Figure 1**) that is covered with the myelin and with meninges, like all other nerve fiber tracts. The central nervous system and the eye are protected sites. The eye has the blood–retinal barrier (BRB) and the CNS has the blood–brain barrier (BBB). These barriers are quite similar, being both composed of non-fenestrated endothelial cells connected by tight junctions [3]. The endothelial cells that form the BBB and BRB are able to provide oxygen and glucose in adequate concentrations for neuronal function, while they prevent the flux of other molecules and cells in order to protect the neuronal environment [8]. Both the eye and the brain have limited capacities for regeneration. Apart from the presence of a limited number of progenitors, nerve cells do not replicate. Hence, degeneration as well as immune-mediated inflammation can induce irreversible damage to neurons, atrophic alterations like those present in neurodegenerative diseases, typical of the central nervous system, or blindness.

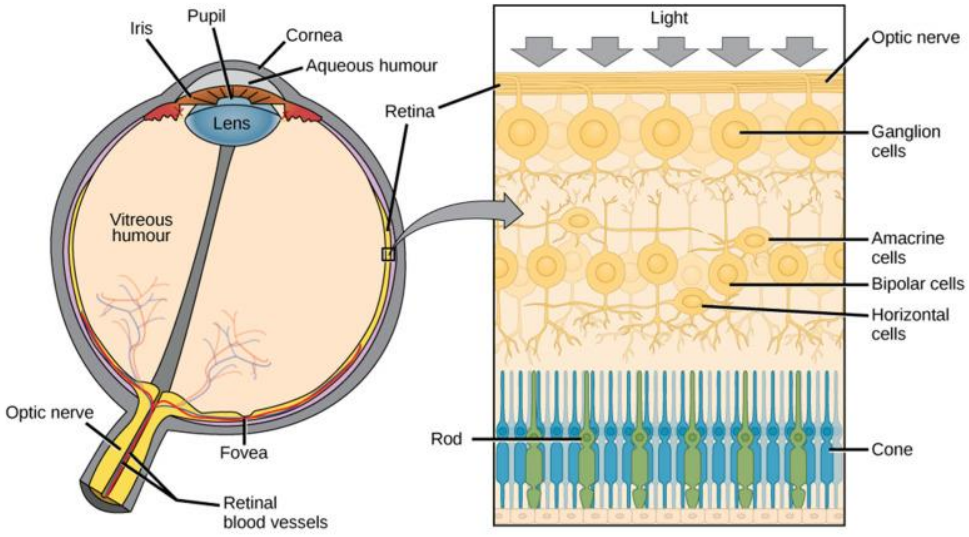

Figure 1. Anatomy of eye and retina. The different components and structures of the eye with the detail (in the right panel) of the composition of the retina.

The retinal tissues consist of neurons, vascular tissue, and glial cells, which interact each other, resulting in a neurovascular system. In particular, the retina is structurally made up of two overlapping sheets: one external in contact with the choroid, pigment epithelium, and the other one internal in relation to the vitreous body, sensory retina. The pigment epithelium maintains the external blood–ocular barrier. Retinal blood vessels maintain the internal blood–ocular barrier.

The breakdown of the blood–retinal barrier (BRB) is considered pathognomonic for the development of retinopathy [9]. Moreover, degeneration of specific retinal neurons in several ocular diseases (i.e., glaucoma, age-related macular degeneration (AMD), diabetic retinopathy (DR), and retinitis pigmentosa (RP)) is the leading cause of irreversible blindness [3, 10]. Indeed, in the industrialized world, the most frequent causes of blindness are eye neuropathology diseases. Prevalence varies with age. While age-related macular degeneration is the most frequent cause in old age, younger people are more often affected by diabetic retinopathy.

Briefly, glaucoma is characterized by progressive optic nerve degeneration and can be considered a neurodegenerative disorder of both the eye and the brain [11, 12]. Age-related macular degeneration is a common eye disease in the macula, the part of retina responsible for sharp, central vision. It causes visual recognition difficulties. It is also associated with higher rates of cognitive decline in late life and higher risk of dementia [13, 14]. Neurodegeneration plays a significant role

in the complex pathology of diabetic retinopathy. Evidence suggests the onset of neurodegeneration occurs early on in such disease [15, 16]. Retinitis pigmentosa is a retinal dystrophy. It is characterized by the progressive degeneration and death of photoreceptors, resulting in an initial loss of night vision and a progressive constriction of the visual field [17, 18]. Retinitis pigmentosa does not have similarities with a particular disease of the CNS but there is evidence that it is correlated with significant reductions in gray matter volume, mainly in the occipital cortex of RP patients [19].

Nowadays, few drugs are approved for the treatment of the abovementioned eye diseases [20]. Generally, the therapies are still limited to symptomatic actions. A summary of the main drugs currently in use in therapy is reported in **Table 1**.

In order to achieve therapeutic benefits in ocular impairments, focused on tissue repair or regeneration, several strategies such as gene therapy, stem cell therapy, and target discovery through genomic research represent significant promise [21]. Currently, there is no therapy to modify the disease-associated degenerative changes and no effective treatment to reverse the loss of vision when photoreceptors degenerate or lose their ability to transmit the visual stimuli to the brain or when retinal ganglionic cells die. In recent decades, experimental evidence underlines the approach to treat blinding diseases through regenerative medicine [10, 22]. Some innovative ocular therapies, based on a variety of transplantable products and novel drug-delivery technologies including nanoparticles, nanomicelles, and liposomes, should be able to revolutionize treatment of numerous blinding disorders [23].

However, the strong link between the eye and the central nervous system is supported by evidence that the ocular alterations existing in various neurodegenerative pathologies of the CNS and visual manifestations sometimes precede central symptoms. Moreover, the retina is a CNS compartment that can be easily analyzed with optical techniques, such as, for example, the optical coherence tomography (OCT), so retinal changes may reflect the pathological features in the brain early in the disease processes [24, 25].

Moreover, several etiological factors such as oxidative stress, neuroinflammation, proteolytic degradation, dysregulation of ocular hemodynamic parameters, transsynaptic degenerative changes, genetic causes, and aberrant cellular signaling are involved in neurodegeneration and cell loss associated with both CNS and retina disorders [26].

Table 1. Some drugs currently used in major ocular diseases

Diseases	Therapeutic Category	Drugs	Route of Drug Administration	Effect/Mechanism of Action	References
Glaucoma	Prostaglandin analogue	Latanoprost Bimatoprost Travoprost	Topical instillation (drops)	Reduction of intraocular pressure (IOP)/Increase uveoscleral outflow	[27,28]
	Protaglandin/Rho-kinase transporter inhibitor association	Latanoprost/Netarsudil	Topical instillation (drops)	Increase of the trabecular outflow/Decreasing the aqueous production/Decrease the episcleral venous pressure	[28–31]
	Rho-kinase transporter inhibitor	Netarsudil	Topical instillation (drops)	Reduction of intraocular pressure (IOP)/Increase of the trabecular outflow	[32]
	β-receptor antagonists	Timolol Betaxolol Levobunolol Carteolol Metipranolol	Topical instillation (drops)	Reduction of intraocular pressure (IOP)/Decrease aqueous humor production	[28,33,34]
	α2-receptor agonists	Brimonidine Apraclonidine	Topical instillation (drops)	Reduction of intraocular pressure (IOP)/Decrease aqueous humor production	[28,33–35]
	Carbonic anhydrase inhibitors	Brinzolamide Dorzolamide	Topical instillation (drops)	Reduction of intraocular pressure (IOP)/Decrease aqueous humor production and increase uveoscleral outflow	[28,33,34,36]
	Cholinergic receptor agonists	Pilocarpine Carbachol	Topical instillation (drops)	Reduction of intraocular pressure (IOP)/Increase trabecular outflow	[28,34]
AMD	Anti-VEGF	Ranibizumab Aflibercept Pegaptanib Conbercept Brolucizumab	Intravitreal injection	vessel growth/Inhibition of the biological activity of VEGF	[31,37–39]
	Photodynamic therapy	Verteporfin	Intravenous	Elimination of the abnormal blood vessels in wet-form macular degeneration	[39,40]
DR	Anti-VEGF	Ranibizumab Aflibercept Bevacizumab	Intravitreal injection	Reduction of new blood vessel growth/Inhibition of the biological activity of VEGF	[16,41]
RP	Supplements/Vitamin	Vitamin A Omega 3 (DHA) Lutein	Topical instillation/oral	Improve photoreceptor metabolism, slowing its death by apoptosis	[42–45]

AMD: Age-Related Macular Degeneration; DR: Diabetic Retinopathy; RP: Retinitis Pigmentosa; IOP: Intraocular Pressure; VEGF: Vascular Endothelium Grown Factor; DHA: Docosahexaenoic Acid.

CNS and Eye Neurodegeneration

Neurodegenerative CNS pathologies are debilitating and quite untreatable diseases that involve morphologic alterations and progressive loss of function of neurons, thus causing progressive degeneration and/or death of nerve cells. They include both movement disorders (so-called ataxias) or mental disfunction (so-called dementias). CNS diseases involve neurons, glial cells, and the vascular system, even if only the neurons suffer progressive damage. The communication between the cells occurs early; then the entire cellular structure is compromised up to death. Genetic factors are also involved in the etiopathogenesis of some illnesses; genetic influence increases the chances of developing neurodegenerative diseases [46, 47] (**Figure 2**). The most known, widespread, and common illnesses among the elderly population are Alzheimer disease and Parkinson disease, affecting worldwide more than 30 million and 5 million subjects, respectively [48, 49]. Some evidence highlights that neurodegenerative processes involve both the central nervous system and the retina, being the anterograde (postsynaptic neurons) and retrograde (presynaptic neurons) trans-synaptic neurodegeneration, caused by retinal ganglion cells' death, the main mechanism involved [50]. Such atrophic neural alterations involve neurons and axons as a result of injury of the cells with which they are in communication, causing an interruption of the synaptic stimulus [51]. Therefore, trans-synaptic degeneration is a process that spreads damage from the initial site to neuronal projections. Such a trans- synaptic degeneration has been long proven in the motor system and cerebellar pathways; only over the last decades, the presence of retrograde trans-synaptic degeneration has been highlighted in the human visual system, with particular relation to glaucoma [52–54]. It contributes to visual impairment observed in association with other various diseases, including, for example, multiple sclerosis [55]. Moreover, CNS pathologies display ocular manifestations due to direct degeneration of the visual pathways [56], often related to a direct injury to the optic nerve and/or retinal ganglion cells [57]. Alzheimer disease and glaucoma, both being diseases of the elderly, have several epidemiological and histological overlaps in pathogenesis [58]. Among the etiological factors, the neuroinflammatory response is considered of crucial importance in the major neurodegenerative diseases of the CNS related to age; such inflammatory response in the brain can occur in the retina as it represents an extension of the brain. Nevertheless, microglial cells, the immunocompetent cells of the CNS, are key factors in these neurodegenerative lesions as they respond to

injury and degeneration with morphological changes, proliferation, migration, and inflammatory cytokine production [59]. Furthermore, a number of microglia can rapidly increase under pathological conditions, such as inflammation/neuroinflammation, and, therefore, they are implicated in the initiation and progression of several neurological disorders; they represent a common hallmark of various retinal degenerative and inflammatory diseases [60, 61].

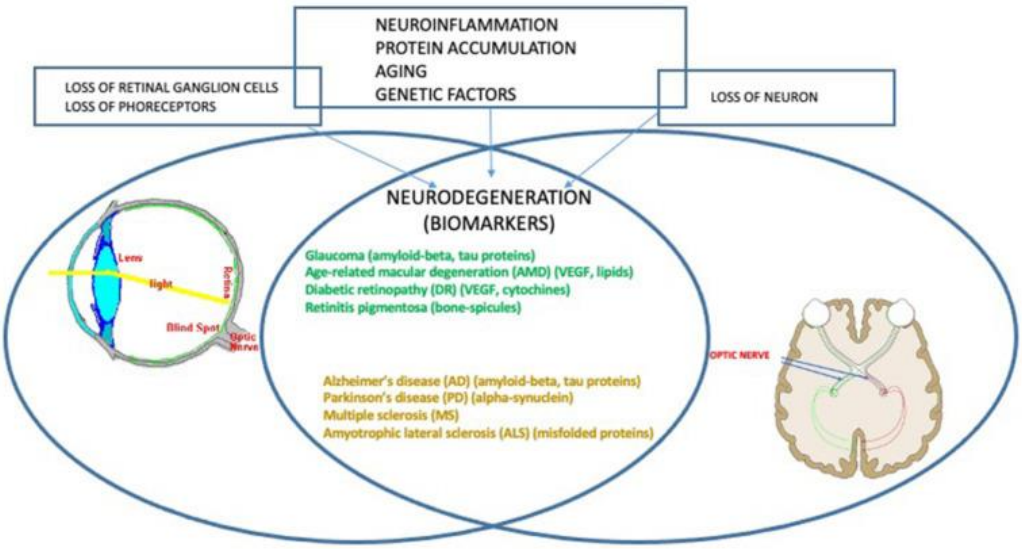

Figure 2. Central nervous system and ocular neuropathologies. The CNS and the eye share the same factors in the etiopathology of disease. Neurodegeneration is common, as are some biomarkers, reported in parentheses for each pathology.

For some time, science has been studying if retinal neurodegeneration is predictive for Alzheimer disease (AD) and Parkinson disease (PD). In patients with AD or PD, several studies have shown changes in the nerve fibers of the retina [2]. It was noted that subjects with preclinical AD showed retinal microvascular and structural alterations. Venous narrowing and reduced blood flow have been especially determined [62]. PD is also associated with retinal thinning [63, 64], which, as a consequence, is associated with reduced retinal blood flow. Visual abnormalities are prominent in AD, and most of them are due to RGC loss and are believed to develop before cognitive impairment [14]. In particular, Alzheimer disease is characterized by the accumulation in the brain parenchyma of extracellular amyloid-beta (Aβ) peptide aggregates, by intracellular deposits of hyper- phosphorylated tau protein, by neurodegeneration, and glial activation). However, these changes occur in the brain long before

cognitive deficits. The challenge is to be able to recognize these disorders before clinical symptoms [65]. Recent evidence from human samples and mouse models indicates the possibility of detecting protein aggregates and other distinctive pathological hallmarks in the retina, providing the way for rapid non-invasive detection of Alzheimer disease biomarkers [66]. Generally, these biomarkers are detected either through cerebrospinal fluid analysis, brain imaging, or post-mortem. Given that the eye possesses neural and vascular similarities to the brain, it is now strongly underlined that the retina is a direct window through which it is easier to possibly monitor the neurodegeneration processes linked to Alzheimer disease [14, 48, 67]. Interestingly, retinal Aβ levels, which reflect those of the brain, appear to become a promising opportunity for early detection of AD-related cerebral changes and cognitive decline [48]. Currently, all the ocular biomarkers (i.e., studied with detection of Aβ-related retina changes, PET (positron emission tomography) imaging, OCT (optical coherence tomography) and OTC angiography, and cerebral spinal fluid molecules) are considered in a promising way as a means to improve, understand, and monitor adequate AD and other neurodegenerative diseases' therapies [68].

It is well known that Parkinson disease is characterized by the loss of dopaminergic neurons in the substantia nigra. Furthermore, it is also established that, before degeneration, in dopaminergic neurons it is evident that proteins accumulate, one of which in particular, alpha synuclein, seems to play a fundamental role. In fact, as the disease progresses, a great amount of this protein (known as Lewy bodies) are found increasingly widespread [69]. Parkinson disease patients show very early vision defects and alpha- synuclein accumulations also in the retina [70]. Furthermore, mutations that induce an increase in the expression of this protein lead to Parkinson disease, demonstrating that this protein seems to play a crucial role in the pathogenesis. PD is usually diagnosed on the presence of several motor symptoms, such as tremors, muscle stiffness, and balance problems. PD is also associated with several non-motor symptoms including disorders of mood, such as apathy, anhedonia and depression, cognitive dysfunction in the form of working memory deficits, and complex behavioral disorders [71]. However, motor symptoms develop after prolonged progression, with significant damage to the dopaminergic neurons. Interestingly, it was demonstrated that in PD there is also a thinning of the retinal walls and retinal microvasculature alterations [64], so that, this ocular damage could represent an early non-motor symptom of the disease. Therefore, Parkinson

disease progression is also associated with the structural changes of the retinal nerve fiber layer; in fact, greater changes at this level as well as macular thickness were found in patients with PD compared to controls [72]. These axonal alterations caused by PD can be detected using optical coherence tomography, an imaging technique developed to evaluate retinal disease, and these special measurements are usefully considered as biomarkers of PD progression [73]. However, not only AD and PD, but also other neurodegenerative CNS diseases are related to ocular damages.

Optic neuritis can be an early sign of multiple sclerosis (MS), an autoimmune demyelinating and neurodegenerative disease of the central nervous system. Pathogenic mechanisms include inflammation by T- and B-lymphocytes and cells of innate immunity as well as oxidative stress; several other factors that lead to neurodegeneration include microglia activation, chronic oxidative injury, and accumulation of mitochondrial damage in axons [74, 75]. Inflammation of the optic nerve, a condition known as optic neuritis, is one of the most frequent clinical manifestations and it can be an early sign of MS. The con- sequences are different vision disturbances such as a decrease in monocular visual acuity, often associated with pain, oscillopsia, linked to the presence of nystagmus, and diplopia, i.e., double vision caused by imperfect alignment of the eyeballs usually due to an injury to the oculomotor system. Patients with multiple sclerosis have also shown a reduction in the optic nerve perfusion and in the thickness of the retinal nerve fiber layer compared to healthy subjects. Recent evidence demonstrated that OCT and OCT-angiography images reflect the loss of retinal ganglion cells and axonal damage due to MS [76].

Additionally, amyotrophic lateral sclerosis (ALS) affects the neurons of the central nervous system, in particular, the spinal and cortical motor neurons. It is a fatal, progressive, degenerative pathology involving loss of the first motor neurons located in the brain and the second motor neurons located in the brain stem and spinal cord. These events lead to the loss of control of the muscles responsible for movement. In up to 50% of the affected population, there are other extra-motor manifestations such as changes in behavior, executive dysfunction, and language disturbances, and these problems are so severe to meet the clinical requirements of frontotemporal dementia in 10%–15% of patients [77]. Underlying the pathology, several molecular mechanisms are involved, such as excitotoxicity, mitochondrial disorders, alterations in axonal transport, oxidative stress, accumulation of misfolded proteins, and neuroinflammation, in

addition to genetic factors [78]. Recently, it has been shown that it also affects the visual system; although at present ophthalmic complications are not considered as a classic symptom of ALS, recent evidence underlines those retinal changes such as thinning, axonal degeneration, and protein inclusion have been found in many patients [79]. Therefore, even in these circumstances, the retinal conditions are being proposed as a possible biomarker of ALS.

Glaucoma

Glaucoma is currently one of the most common causes of irreversible visual impairment and blindness in the world [80]; it includes a group of heterogeneous eye diseases, with closed-angle glaucoma and open-angle glaucoma the two main types. Generally, glaucoma is due to the increase in the internal pressure of the eye, that is, the intraocular pressure (IOP), which irreparably damages neurons; in some cases, the reduction of the blood supply to the optic nerve, which cause loss of visual field, is involved [54, 81]. In recent years, the literature argues in favor of the fact that glaucoma is a widespread neurodegenerative disease involving the CNS, as the correlation is strong between the dysfunction and death of CNS neurons with retinal ones. Moreover, neurodegenerative pathways that contribute to transynaptic neurodegeneration in AD, as well as in other CNS diseases, might also be similar to those in neurodegeneration correlated to glaucoma [11, 82]. Retinal ganglion cell damage is a characteristic of both glaucoma and AD, along with discovery of amyloid-beta and tau protein deposition, known to be pathognomonic of AD, in the retina and aqueous humor of the eye [58]. In particular, primary open-angle glaucoma (POAG), the most common type, is characterized by slow, progressive, degeneration of retinal ganglion cells and their axons in the optic nerve, leading to visual field defects [83]. Intraocular pressure (IOP) is considered a major risk factor for the development of POAG, and the modified optic nerve head is the site of initial damage. However, elevated IOP is not present in all types of POAG, and in normal-tension glaucoma IOP is not elevated, so other risk factors are likely involved in the optic neuropathy. Literature evidence provides that the pressure and composition of the cerebrospinal fluid (CSF) surrounding the optic nerve may have critical involvement in the pathogenesis of glaucoma [83]. In this regard, the presence of the glymphatic system was described. This particular system is a brain-wide paravascular pathway for CSF–interstitial fluid exchange that facilitates clearance of interstitial solutes, including amyloid-beta, from the brain. If the glymphatic

system does not operate properly, amyloid-beta brain accumulation occurs in AD. In the same way, the glymphatic system may also have potential clinical relevance for the understanding of glaucoma. Aβ accumulation may be implicated in the development of retinal ganglionic cells' apoptosis. Recent studies indicated that accumulation of amyloid-beta, which is associated with the progression of Alzheimer disease, may also be responsible for retinal ganglion cell death in glaucoma, so the neurodegenerative processes in glaucoma could share, at least in part, a common mechanism with Alzheimer disease [83]. Interestingly, literature data, although derived from animal studies, found time-dependent expressions and localization of Aβ in the retina as well as in the optic nerve head after chronic IOP increase seen in glaucoma [84]. Moreover, nowadays, it is well established that glaucoma leads to ganglion cell death through several other mechanisms including oxidative stress, neuroinflammation, and mitochondrial dysfunction [85, 86]. Retinal ganglion cells and optic nerve fibers are particularly rich in mitochondria, necessary organelles to produce energy for nerve conduction. The reduction in energy production and the increase in the production of free radicals at the mitochondrial level are to be considered as potential additional mechanisms in the etiopathogenesis of glaucoma. Definitely, the identification of cellular mechanisms and molecular pathways related to retinal ganglion cell death is the first step toward the discovery of new therapeutic strategies to control glaucoma [87, 88].

Age-Related Macular Degeneration

Age-related Macular Degeneration (AMD) is an ocular pathology that involves the central area of the retina, the so-called macula, causing an irreversible reduction in distinct vision, and it is one of the leading causes of blindness in developed countries. AMD is classified into a dry form, with about 80% of incidence in the population affected, and a wet form or neovascular form, with about 20% of incidence. In particular, in dry age-related macular degeneration, characteristic lesions, called drusen, appear. These are accumulations of cellular waste that can be reabsorbed or calcified. In wet macular degeneration, in addition to drusen, there is the anomalous formation of new vessels under the retina, responsible for the exudative evolution of macular degeneration [89, 90]. Therefore, localized sclerosis under the retina, the accumulation of lipids, and alterations in the metabolism of the retinal pigment epithelium (RPE) contribute to the macular degenerative process [91]. Under these conditions, the

physiological metabolism of the retina is prevented. Moreover, retinal hypoxia may induce an upregulation of VEGF by the RPE and thus promote the growth of abnormal vessels from choroid, with VEGF being the main factor related to ocular neovascularization [92, 93]. Furthermore, the RPE is crucial for the maintenance of photoreceptor cells as it promotes a physiological vascular environment. In particular, RPE keeps retinal nerve tissue healthy by secreting hormones, transporting molecules, eliminating dead cells, and modulating immune factors. The RPE is responsible for the transport of nutrients, ions, and water. It absorbs light and protects the retina from photooxidation; in addition, it is responsible for stabilizing the concentration of ions in the subretinal space to keep the photoreceptors excitable. To maintain RPE homeostasis and function, a particular molecular network is necessary, with microRNAs being indispensable components [94].

With aging, several modifications occur in the RPE cells as a result of their altered capacity for removing residual substances, leading to a further damage in the pathogenesis of AMD. The main risk factor for AMD is age, but family history, female sex, smoking, and high blood pressure can somehow contribute; among these, several studies suggest that smoking is the main oxidative stress factor [20]. Nevertheless, different oxidative damage, such as light exposure or inflammation that affect the retina, has been strongly linked with AMD [37]. Globally considered together, oxidative stress and mitochondrial damage in the retinal pigment epithelium may play an important role in the pathogenesis of age-related macular degeneration [95]. It is well known that mitochondrial dysfunction has been associated with aging, as well as with several age-related diseases, such as Alzheimer and Parkinson diseases, suggesting that ocular and CNS neuropathologies share more than one biochemical mechanism. Moreover, AMD also is associated with non-visual impairment such as phonemic verbal fluency, verbal memory, establishment of cognitive decline during life, and higher risk of dementia [13,96]. Once again, it is emphasized how cognitive impairment, as well as visual impairment, is common and superimposed among older adults.

Diabetic Retinopathy

Diabetic retinopathy (DR) is a complication of diabetes that affects the eyes, causing severe visual impairment. It is induced by damage to the blood vessels in the light-sensitive part of the eye, the retina, with the vasculopathy being the

main involved pathophysiologic mechanism [15]. It can develop in subjects affected by both type 1 and type 2 diabetes. There are two types of retinopathies. The first is the early diabetic retinopathy, also known as nonproliferative diabetic retinopathy (NPDR). As the disease progresses, the walls of the blood vessels weaken and are subject to microaneurysms, small swellings that, when damaged, lead to bleeding. Then there is the risk of an accumulation of fluids, i.e., formation of edema, in the macula, which cause reduced vision. The second type is proliferative or advanced diabetic retinopathy (PDR). It is the most serious type because it coincides with the abnormal growth of new blood vessels damaging the retina. Diabetes is, in fact, associated with a growth of weak blood vessels, more prone to rupture, or smaller vessels, and this leads to a lower oxygen transport capacity to the retinal tissues. As a result, new vessels are stimulated by the formation of ischemic areas in the retina. In fact, retinal microvascular disease is an early compromission, induced by low-grade, persistent leukocyte activation, which causes repeated episodes of capillary occlusion and progressive retinal ischemia [97]. This situation can induce detachment of the retina or an abnormal flow of fluid into the eye, causing glaucoma. The underlying molecular mechanisms associated with vascular dysfunction, especially endothelial dysfunction, in DR are multi- factorial. Chronic inflammation, oxidative stress, leukocytosis, dysregulated growth factors and cytokines, and disruption of peroxisome proliferator-activated receptor-γ are mainly involved [98]. Diabetic retinopathy is prevalent in around 35% of patients with diabetes. The disease progresses slowly, causing damages that become progressively irreversible. Unfortunately, treatment options are limited. As therapeutic approaches, photocoagulation of the ischemic areas of the retina to stabilized blood vessels, intravitreal injections with VEGF-inhibitory agents or corticosteroids, and ocular surgery can be applied [99]. It is worth noting that anti-VEGF agents used in clinical practice, such as ranibizumab, bevacizumab, and aflibercept, are considerably different in terms of molecular interactions when they bind with VEGF [100]; therefore, characterization of such features can improve the design of novel biological drugs potentially useful in clinical practice. Recent findings hypothesize that retinal neurodegeneration represents a critical, early component of DR. It occurs prior to the vascular changes classically associated with DR and contributes to disease pathogenesis [101, 102]. In the retina, neurons, glia, and vasculature form the blood–retinal barrier (BRB), which functions as the maintenance of energy, homeostasis, and neurotransmitter regulation. In the

progression of diabetes, the BRB is damaged early and its breakdown is sustained by RPE secretion of different factors, among which the main ones are vascular endothelial growth factor (VEGF) and proinflammatory cytokines (i.e., TNF-α, IL-6, IL-1β) [103]. It is interesting to note that VEGF may act as a negative regulator of pericyte function, with these cells being involved in early BRB abnormalities in diabetic retinopathy [104]. During progression of DR, the retina is infiltrated by the above-mentioned secreted factors' cells and serum proteins, further damaging blood vessels and neurons. Moreover, in addition to vascular damage and the loss of BRB integrity, other neurodegenerative changes occur in the retina such as apoptosis, glial cell reactivity, microglial activation, and altered glutamate metabolism that could prove some of the functional deficits in vision [101, 105]. Additionally, to point out the neurodegeneration, clinical evidence indicates CNS lesions in patients with diabetic retinopathy; detection of small punctate white matter lesions in the brain and cortical atrophy in some regions suggests that there is an association between retinopathy and brain tissue damage [106]. Other studies highlight that diabetes-induced retinal neurodegeneration and brain neurodegenerative diseases share common pathogenic pathways. Indeed, DR patients might exhibit abnormalities in the central nervous system, often showing impaired cognition and increased risks of dementia as well as Alzheimer disease [107].

Retinitis Pigmentosa

Retinitis pigmentosa (RP) is an inherited retinal dystrophy leading to progressive loss of the photoreceptors and retinal pigment epithelium and resulting in blindness usually after several decades [17, 108, 109]. Usually, it is bilateral, but some evidence reports of unilateral eye involvement with RP. It affects approximately one subject in 5000 worldwide, making RP one of the most common inherited diseases of the retina [110]. Generally, degeneration of rod photoreceptors, the cells controlling night vision, precedes and exceeds cone degeneration, as a majority of RP genetic mutations affect rods selectively. Early symptoms of retinitis pigmentosa include impaired night vision and peripheral vision [109]. The main clinical hallmarks consist of bone-spicule deposits, waxy optic disc, and shrinked retinal vessels. As previously pointed out, retinitis pigmentosa does not share alterations with neurodegenerative diseases of the CNS; it is linked with reduction of white matter volume in the brain, as seen in RP patients [19].

There is no definitive therapy. In some cases, it is possible to slow down the degenerative process with strategies such as the administration of vitamins, and protection from sunlight and combined approaches, such as gene-replacement therapy, may be useful to slow photoreceptor cell death [111, 112]. Different RP gene mutations are the basis of alterations in molecular mechanisms such as phototransduction cascade, vitamin A metabolism, interactive cell–cell signaling or synaptic interaction, and intron splicing of RNA [113]. Moreover, previous studies revealed that an insufficiency of the ubiquitin-proteasome system (UPS) to process misfolded proteins in affected photoreceptor cells could be involved [114]. Impairments of UPS function in the central nervous system underlie an increasing number of genetic diseases, many of which affect the retina [115].

Conclusions

As the prevalence of neurological diseases increases dramatically with age and the aging population increases, neurodegenerative diseases could have an ever-increasing impact on people's quality of life. Early diagnosis and optimal follow-up are critical for better disease management and for delaying progression and disability. Growing evidence suggests that the eye is like the brain: Both organs can suffer the effects of time and they could be affected by neurodegeneration. While brain damage occurs mainly in the form of cognitive diseases, such as Alzheimer or Parkinson, neurodegeneration can present itself in the form of glaucoma in the eyes. The retina and optic nerve are an embryological extension of the brain tissue, and the retina provides a unique opportunity to evaluate the alterations caused by neurological diseases, showing a cellular composition similar to that of brain tissue. Through optical coherence tomography (OCT), a high-resolution technology, it is possible to detect alterations in clinical conditions. Therefore, a measurement of changes in intraretinal layer thickness is a reliable signal linked to axonal loss or related neuroinflammation of neurodegenerative pathologies. Early signs of retinal damage are present in Parkinson and Alzheimer diseases as well as in multiple sclerosis. There is increasing evidence that beta-amyloid is a factor involved in the development of ganglion cell apoptosis in glaucoma; preclinical studies demonstrate that retinal ganglion cells, subjected to a chronic increase in intraocular pressure, show abnormal processing of a precursor protein of beta-amyloid, suggesting a correlation between AD and glaucoma [11]. Accumulations of alpha-synuclein in

the brain present in Parkinson disease affect dopaminergic neurons, leading to the development of motor symptoms. If high concentrations of this protein are present in the retina, it leads to the death of amacrine cells that contain dopamine, leading to a reduction in visual acuity [116]. Moreover, cerebro-spinal fluid circulatory failure contributes to the development of glaucoma. AMD is a reduction of visual function related to the aging process of the eye: The macula, containing numerous photoreceptors, alters until it loses its characteristics. This phenomenon is due to retinal cell degeneration and death. Moreover, AMD and AD share the same biomarkers; in AMD there is also evidence of protein misfolding disease similar to Alzheimer disease. Recent studies show that differential expression of miRNAs (miR-9, miR-23a, miR-27a, miR-34a, miR-146a, miR-155) has been found to be dysregulated both in AMD and AD [117]. Current evidence suggests that neurodegeneration of the retina is a critical component of diabetic retinopathy, in addition to the damage to the blood vessels of the ocular tissue. Retinitis pigmentosa is a retinal dystrophy characterized by the gradual loss of photoreceptors and dysfunction of the pigment epithelium. This pathological context put into evidence that the retina progressively reduces its ability to transmit visual information to the brain via the optic nerve. A great importance lies in the studies of those measurable substances within the body, called biomarkers, which monitor the development of the disease and the effectiveness of potential drugs. Since the neurodegenerative process is already biologically advanced by the time the symptoms appear, having biomarkers available in the preclinical phase, to signal the pathological process in progress, is essential to obtain effective therapies, even more if the biomarkers are sensitive to therapeutic treatments.

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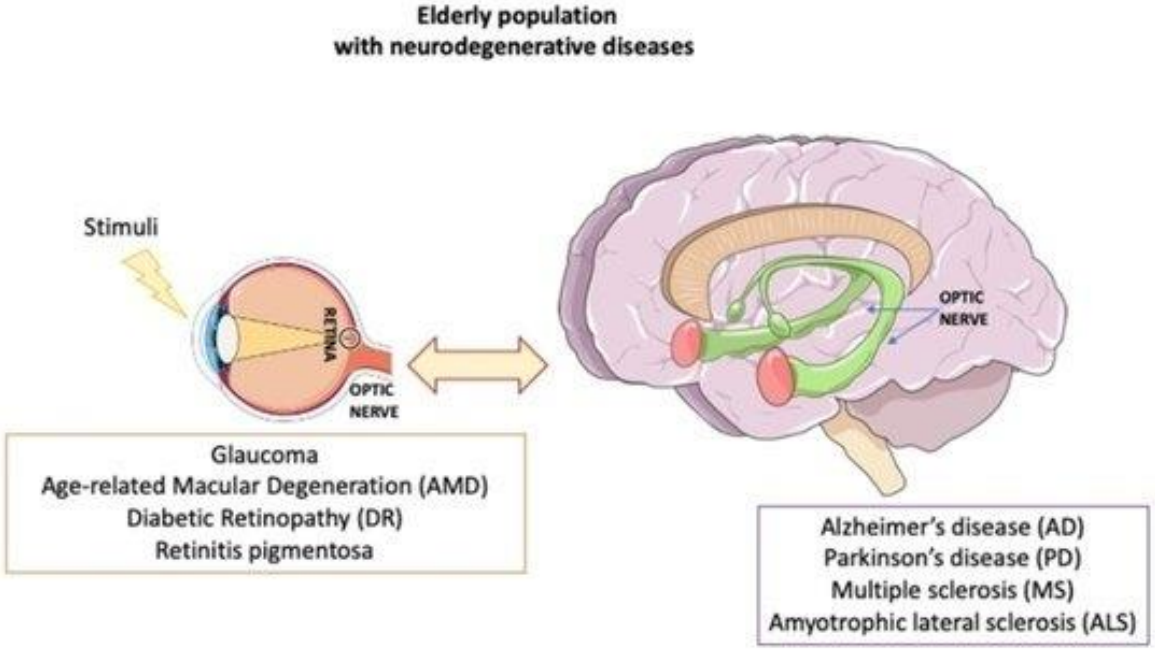
Supplementary Material (Graphical Abstract)



CHAPTER 5

Collaborative activity: A new mechanism of action for dimethyl fumarate in Multiple Sclerosis

Chapter 5

The following manuscript has just been published in *Multiple Sclerosis and Related Disorders* in 2022 as:

ELSEVIER

Multiple Sclerosis and Related Disorders

Available online 29 September 2022, 104197

In Press, Journal Pre-proof

EVIDENCE FOR NOVEL CELL DEFENCE MECHANISMS SUSTAINED BY DIMETHYL FUMARATE IN MULTIPLE SCLEROSIS PATIENTS: THE HuR/*SOD2* CASCADE

Giulia Mallucci [a, †], Nicoletta Marchesi [b, †] ✉, Lucrezia Irene Maria Campagnoli [b], Federica Boschi [b], Foroogh Fahmideh [b], Sara Fusco [a], Eleonora Tavazzi [a], Stefano Govoni [b], Roberto Bergamaschi [a], Alessia Pascale [b] ✉

[a] IRCCS Mondino Foundation, Pavia, Italy

[b] Department of Drug Sciences, Section of Pharmacology, University of Pavia, Pavia, Italy

Received 20 April 2022, Revised 22 September 2022, Accepted 27 September 2022, Available online 29 September 2022.

Evidence for novel cell defence mechanisms sustained by dimethyl fumarate in Multiple Sclerosis patients: the HUR/SOD2 cascade

Giulia Mallucci, Nicoletta Marchesi, Lucrezia Campagnoli, Federica Boschi, **Foroogh Fahmideh**, Sara Fusco, Tavazzi Eleonora, Stefano Govoni, Roberto Bergamaschi and Alessia Pascale

Abstract

Background: Dimethyl fumarate (DMF) is an effective treatment for relapsing remitting Multiple Sclerosis (MS) and its mechanisms of action encompass immunomodulatory and cytoprotective effects. Despite DMF is known to activate the Nrf2 pathway, Nrf2-independent mechanisms have been also reported and new insights on the underlying molecular mechanisms are still emerging including transcriptional and post-transcriptional events.

At this regard, we focused on a small family of RNA-binding proteins, the ELAV-like proteins, that play a pivotal role in posttranscriptional mechanisms and are involved in the pathogenesis of several psychiatric and neurologic disorders. HuR, the ubiquitously expressed member of the family, is implicated in many cellular functions, including survival, inflammation and proper functioning of the immune system. We previously documented the potential entanglement of HuR in MS pathogenesis. In the present work, we explored HuR protein levels in peripheral blood mononuclear cells (PBMCs) from MS patients before and after DMF treatment compared to healthy controls (HC). Considering that HuR may act on various targets, playing a protective role against oxidative stress, our main goals were to evaluate whether manganese-dependent superoxide dismutase transcript (SOD2) could represent a new molecular target of HuR and to study the potential influence of DMF treatment on this interaction.

Methods: PBMCs from 20 patients with MS and 20 frequency-matched HC by sex and age were used to evaluate HuR, MnSOD (the protein coded by SOD2) and Nrf2 protein content by Western blot, before and after 12 months of DMF treatment.

Immunoprecipitation experiments coupled with RNA extraction in PBMCs were performed to explore whether SOD2 mRNA could be physically bound by HuR and whether the expression of MnSOD protein could be affected by 12 months of DMF treatment.

Results: In PBMCs, HuR protein binds SOD2 transcript in HC and in MS patients naïve to disease modifying treatment. The expression of MnSOD protein is positively affected by 12 months of DMF treatment. PBMCs from MS patients have a lower HuR and MnSOD protein content compared to matched HC (HuR: $p<0.01$, MnSOD: $p<0.01$). Of interest, 12 months of DMF treatment in MS patients restores the amount of both HuR protein and MnSOD enzyme to the levels observed in HC. We also confirmed that Nrf2 is an HuR target, and we report that its levels are significantly increased in MS patients naïve to disease modifying treatment and remain elevated following DMF administration.

Conclusion: SOD2 transcript is a new target of HuR protein. DMF induces an increased expression of HuR protein, which ultimately interacts more strongly with SOD2 transcript promoting the expression of this antioxidant protein. The activation of this molecular cascade can constitute an additional tool that the cells can exploit to counteract the oxidative stress associated with MS development, and can account for the multifaceted molecular mechanisms underlying DMF efficacy in MS.

Keywords: Multiple Sclerosis, HuR, SOD2/MnSOD, Nrf2, Dimethyl Fumarate

Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS), representing one of the most significant neurological diseases in terms of incidence, prevalence, age of onset and potential for neurological disability. Pathogenic processes underlying MS include CNS inflammation, demyelination and neurodegeneration; these phenomena occur simultaneously, but with different expression during the course of the disease (Mallucci et al., 2015).

Dimethyl fumarate (DMF) is an oral drug approved for the treatment of relapsing remitting MS (MS) (Fox et al., 2012; Gold et al., 2012). The mechanisms of action of DMF comprise immunomodulatory and cytoprotective effects (Pistono et al., 2017). In the presence of oxidative stress, DMF activates the nuclear factor erythroid 2-related factor (Nrf2) pathway (Suneetha and Raja Rajeswari, 2016), and the consequent down-stream transcription of genes that code for antioxidant proteins, including the manganese-dependent superoxide dismutase (MnSOD;

coded by SOD2 gene) (Mills et al., 2018). Of note, MnSOD has been demonstrated to be able to detoxify cells from oxidative stress especially in the context of neurodegenerative disorders (Kokoszka et al., 2001). Despite DMF being known to activate the Nrf2 pathway, Nrf2-independent mechanisms have been also described and novel insights on its mode of action are still emerging (Yadav et al., 2019).

ELAV proteins are a small family of evolutionarily conserved RNA-binding proteins (RBPs) able to impact on trafficking and metabolism of the targeted mRNAs. Indeed, following intra- and extracellular inputs, these proteins mainly induce a rise in the cytoplasmic stability and/or rate of translation of the target transcripts, by preferentially binding to ARE (adenine-uracil-rich elements) cis-acting elements located within their sequence, although other consensus elements may be implicated (Pascale and Govoni, 2012; Talman et al., 2016).

Among ELAV, HuR protein is of particular interest, given its involvement in the onset of inflammatory processes and the essential contribution in oxidative stress (Poganik et al., 2019; Srikantan and Gorospe, 2012). Recently, we demonstrated the potential entanglement of HuR in MS pathogenesis by documenting that peripheral blood mononuclear cells (PBMCs) from MS patients display a lower HuR protein content compared to healthy controls (HC) (Pistono et al., 2020).

The presence of predicted cis-acting elements that can be bound by RBPs were identified in the primary sequence of Nrf2 (Poganik et al., 2019) and SOD2 (Church, 1990) transcripts. Recently, it has been disclosed, *in vitro*, that the transcript of Nrf2 is a target of HuR (Poganik et al., 2019); however, as of today, no study has yet analysed weather the transcript of SOD2 could be a target of HuR as well.

The goals of this study were to assess the effect of DMF on the expression of the ubiquitous HuR protein in MS patients naïve to disease modifying treatments. Additionally, we aimed at disclosing whether SOD2 mRNA could be a novel target of HuR by exploring the potential interaction between HuR protein and SOD2 transcript and analysing its protein expression in PBMCs from HC versus MS patients naïve to disease modifying treatments before and after 12 months of DMF administration. Finally, we pursued to confirm, also in PBMCs from human subjects, Nrf2 as a HuR target and to evaluate possible protein expression changes following DMF administration.

Materials and methods

This study was approved by the local ethics committee (IRCCS OSM, Pavia, Italy: number P-20170028029) and was conducted in accordance with principles expressed in the Declaration of Helsinki.

Subjects

A total of 20 relapsing remitting MS patients were consecutively enrolled in the study from beginning of 2018 to end of 2019. MS patients were ≥18 years old, diagnosed with MS in accordance with McDonald Criteria 2010 (Polman et al., 2011), had to be naïve to disease modifying treatments and had to start DMF according to clinical practice. Additionally, a minimum of 30 days washout after the last steroid treatment was required. Exclusion criteria for MS patients were autoimmune comorbidities and active systemic infections. MS patients were prospectively followed-up for 12 months.

One healthy control (HC) for each MS case was frequency-matched by age and sex selected from the database of healthy subjects. HCs were provided by the Immunogenetics Laboratory, Immunohematology and Transfusion Centre, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy. All recruited subjects signed an informed consent form.

Clinical data

According to clinical practice, a neurological examination was performed at DMF start (T0) and after 12 months of DMF treatment (T12). At T0, medical history, MS history, demographic and clinical data were also recorded. Expanded Disability Status Scale (EDSS), presence and frequency of acute relapses, presence and timing of confirmed disability worsening, signs of MRI activity and adverse events (AE) were also recorded at both time-points (T0 and T12). Additionally, a “rebaseline MRI scan” with gadolinium was performed according to clinical practice within 6 months after DMF start and signs of MRI activity were analysed.

Annualized relapse rate (ARR) one year and two years before T0 were calculated and MS severity scale (MSSS) at T0 and T12 were assessed (Roxburgh et al., 2005).

A relapse was defined as an episode of neurological symptoms lasting at least 24 hours in the absence of fever and infection. Confirmed disability worsening was defined as i) ≥1.5-point increase if EDSS = 0 at baseline, or ii) ≥1.0-point increase if EDSS = 0.5–4.5 at baseline, or iii) ≥0.5-point increase if EDSS≥ 5.0 at baseline, confirmed at 3 months. Signs of MRI activity were defined as gadolinium-enhancing lesions (Gd+) or the appearance of new T2-hyperintense lesions, compared with the previous scan. All MRI findings underwent quality control check and incomplete reports were excluded. NEDA-3 (No Evidence of Disease Activity) status and EDA-3 (Evidence of Disease Activity) status were reported at 12 months. In detail, the three assessed components were: i) no confirmed disability worsening, ii) no relapse activity, and iii) no MRI activity (Giovannoni et al., 2017).

PBMCs isolation from blood and sample preparation

Whole blood was drawn from MS patients naïve to disease modifying treatments at T0 and T12; whole blood was drawn (20-27 mL to each) without any preparation required from the patients and collected by venipuncture in Vacutainer tubes containing EDTA.

PBMCs were isolated from the whole blood of HC and MS patients. Blood was diluted 1:1 with physiological solution (sodium chloride 0.9%). After that, blood was transferred in a 50 mL Falcon tube containing 15 mL of LymphoprepTM (Alere Technologies AS) and centrifuged at 800 x g for 30 min (without brake). PBMCs above the Ficoll ring were harvested and washed twice with phosphate-buffered saline. The cellular pellets, after resuspension in fetal bovine serum plus 10% DMSO, were stored at − 80°C until further analysis.

Western blotting

PBMCs samples were allowed to thaw and then they were centrifuged at 800 x g to obtain PBMC pellets. PBMCs were then treated with a homogenization buffer containing 20 mM Tris, 2 mM EDTA, 0.5 mM EGTA, 5 mM 2-β-mercaptoethanol, 0.32 M sucrose (pH 7.4) and a protease inhibitor cocktail (Sigma-Aldrich). Successively, they were homogenized with a glass-teflon homogenizer (Potter-Elvehjem) or directly into an Eppendorf with a Pellet Pestle (Kimble Chase) when the pellets were more visible. The samples subsequently underwent sonication 3 times for 20 seconds each time. The protein content of all the samples was determined by the Bradford protein assay method, employing

bovine serum albumin as internal standard. Proteins were diluted in 2X SDS protein gel loading solution, boiled for 5 min and separated onto 12% SDS-PAGE at room temperature. As a molecular weight marker, a standard mixture with coloured proteins having a known molecular weight (Amersham Pharmacia Biotech Rainbow marker, Amersham) was used. The anti-HuR (Santa Cruz Biotech) and the anti-MnSOD (= anti-SOD2; Santa Cruz Biotech) mouse monoclonal antibodies were diluted 1:1000 and 1:200 respectively, based on each datasheet. The anti-Nrf2 rabbit (Cell Signaling Technology) was diluted 1:1000. The horseradish peroxidase-conjugated secondary antibodies were diluted in TBS-T buffer (10mM Tris-HCl, 100mM NaCl, 0,1% Tween, pH 7,5) containing 6% of milk. The proteins were then transferred onto a nitrocellulose membrane (porosity: 0.2 µm) at 4°C for 2 hours and at a constant electrical current of 250 mA. The nitrocellulose membrane signals were detected by chemiluminescence (by using WesternBright® ECL HRP substrate, Advansta) by means of an Imager Amersham 680 detection system. The same membranes were re-probed with α-tubulin monoclonal antibody (Thermo Fisher Scientific) and used to normalize the data. Statistical analysis of Western blot data was performed on the densitometric values obtained using ImageJ, an NIH software, after image acquisition.

Immunoprecipitation

Immunoprecipitation on PBMCs pellets was performed according to a previously published protocol with minor modifications (Amadio et al., 2008). For each group (HC, T0 and T12) a pool of few pellets was obtained. Immunoprecipitation was repeated 4 times using different pools of samples for each experiment. Briefly, immunoprecipitation was performed at room temperature for 2 hours using 1 µg of anti-HuR antibody per 300 µg of proteins diluted with an immunoprecipitation buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM MgCl2, 0.05% Igepal, 20 mM EDTA, 100 mM DTT, protease inhibitor cocktail and an RNAase inhibitor) in the presence of 50 µl of protein A/G plus agarose (Santa Cruz Biotech). The samples were finally subjected to RNA extraction. One hundred microliters of the immunoprecipitation mix were immediately collected from each sample and used as ‘‘input signals’’ to normalize the RT-PCR data.

RNA extraction and quantitative real-time RT-PCR

RNA was extracted from immunoprecipitated pellets and relative ‘‘input signals’’ by using RNeasy Micro Plus Kit (Qiagen). The reverse transcription was performed following standard procedures. PCR amplifications were carried out using the Rotor-Gene Q (Qiagen) in the presence of QuantiTect SYBR Green PCR mix (Qiagen) with primers designed by Sigma-Aldrich. Primer human sequences were as follows: SOD2, 5’- ATCATACCCTAATGATCCCAG -3’ (forward), 5’-AGGACCTTATAGGGTTTTCAG -3’ (reverse); Nrf2: Hs NFLE2L2 (Qiagen); GAPDH, 5’- CAGCAAGAGCAAGAGGAAG-3’ (forward), 5’-CAACTGTGAGGAGGGGAGATT-3’ (reverse). The GAPDH mRNA was chosen as the reference on which the SOD2 and Nrf2 values were normalized because it remained relatively stable during all the treatments, and it does not bear ARE sequences (not shown).

Statistical analysis

Demographic and clinical characteristics of MS patients were reported using mean and standard deviation (SD) or median and interquartile (IQ) range for the quantitative variables and absolute/relative frequency values for the qualitative ones.

Data of the protein expression between HC, MS patients at T0 and MS patients at T12 were subjected to analysis of variance (ANOVA) followed, when significant, by a *post hoc* (Dunnett's Multiple Comparisons test) analysis.

Comparison of quantitative expression of HuR, MnSOD and Nrf2 at T0 between presence versus absence of gadolinium enhancing lesions was tested using non-parametric Mann-Whitney U test.

Comparison of quantitative expression of HuR, MnSOD and Nrf2 at T12 between NEDA-3 status group and EDA-3 status group was tested using non-parametric Mann-Whitney U test.

Spearman's rank correlation was used to identify a correlation between protein expression at T0 and ARR one year or ARR two years before starting DMF and to identify a correlation between protein expression at T0 or T12 and MS severity (i.e. MSSS).

A p-value below 0.05 was considered statistically significant. The statistical analysis was performed using GraphPad Prism statistical package (version 8 GraphPad software, San Diego).

Results

Demographic and Clinical Characteristics of MS Patients and HC

A total of 20 MS patients naïve to disease modifying treatments were included in the study, 66% (n=15) were female. At T0, mean age was 28.70 ± 8.70 years, mean MS duration was 5.65 ± 6.38 years and median EDSS was 1.5 (min, max: 0, 5.0). The median MSSS was 2.438 (min, max: 0.2330, 6.326). Eighteen out of the 20 enrolled MS patients concluded the 12 months study. Reasons for DMF withdrawal were pregnancy planning in one case and therapy failure due severe disease activity in another case. No serious adverse effects (AEs) were reported/observed in this group of patients. None of the participants was excluded from the analysis. After 12 months of DMF treatment, 66.6 % (n=12) of MS patients had a NEDA-3 status.

HuR protein expression in PBMCs after DMF treatment

At baseline, before starting DMF, western blot analysis showed that HuR expression was different between HC (median 931.0; IQ 810.5-1253) and MS patients naïve to disease modifying treatments (median 381.6; IQ 0-727.0). In detail, MS patients naïve to disease modifying treatments expressed lower levels of HuR with respect to HC group ($p<0.001$). After 12 months of DMF treatment, the expression of HuR was restored to the levels observed in HC subjects (median 954.6; IQ 755.6-1276); **Figure 1**.

At baseline, we observed no difference in HuR expression according to Gd+ lesions at MRI scan (i.e presence versus absence of contrast enhancing lesions) (Mann Whitney p =0.12); at T0 there was also no correlation between HuR expression and ARR 1 year before DMF start or HuR expression and ARR 2 years before DMF start (Spearman r -0.07; p=0.76, Spearman r -0.12; p=0.59, respectively). We also do not report any correlation between HuR expression at T0 and MSSS at T0 (Spearman r -0.12; p=0.60).

After 12 months of treatment with DMF, HuR expression was 988.6 (IQ 855.1-1262) in the MS group with the status of NEDA-3 and 860 (IQ 691.3-1286) in the group of MS with the status of EDA-3 (Mann Whitney p =0.42). We did not

observe any correlation between HuR expression at T12 and MSSS at T12 (Spearman r 0.35; p=0.14).

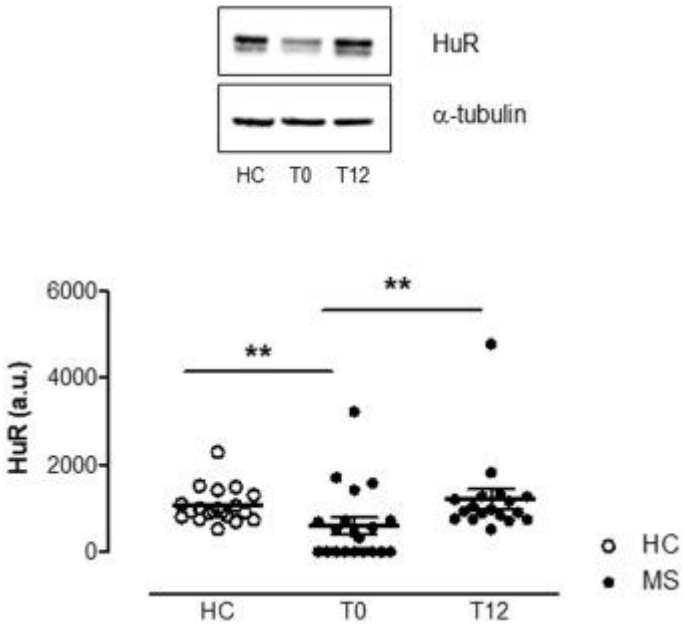


Figure 1. HuR protein levels in PBMCs from HC and MS patients. Upper: Representative Western blot images of HuR and α-tubulin protein content in PBMCs from HC and MS patients (T0 and T12). Lower: Scatter dot plot of the HuR protein expression levels in PBMCs from HC (white, n = 20) and MS patients (black, T0, n=20; T12, n=18). The results are expressed as mean grey levels ratios x 103 (mean ± S.E.M.) of HuR/α-tubulin immunoreactivities measured by Western blotting. a.u. = arbitrary units. Data were analysed by two-way ANOVA followed by post hoc analysis; ** p< 0.01. HC= healthy controls; T0= MS patients naïve to disease modifying treatments; T12: MS patients after 12 months of DMF treatment.

SOD2 as a new HuR target

We first analysed the SOD2 primary sequence, and we identified the presence of multiple cis-acting elements that could be potentially bound by HuR protein. Through a bioinformatic analysis that returns prediction of the interaction between RNA and proteins (http://pridb.gdcb.iastate.edu/RPISeq/results.php; made available to the scientific community by Iowa State University), we calculated the probability of interaction between SOD2 transcript and HuR protein. The table in **panel A** of **Figure 2** shows the relative position within the transcript of the ARE sequences (corresponding to potential binding sites) and the percentage of mRNA-HuR (Score) binding affinity obtained using an alternative software (http://rbpdb.ccbr.utoront.ca/).

Subsequently, we performed immunoprecipitation experiments coupled with RNA extraction and RT-PCR in PBMCs to explore whether SOD2 mRNA could

be physically bound by HuR and whether this binding could be affected by DMF treatment. As depicted in panel B of **Figure 2**, we found that HuR protein can interact with SOD2 transcript. Furthermore, our analysis showed that the HuR protein/ SOD2 mRNA binding does not differ between HC and MS patients naïve to disease modifying treatment. However, the aforementioned interaction increased after 12 months of DMF treatment ($p<0.01$). This finding indicates that DMF treatment for 12 months increases the amount of complex between HuR protein and SOD2 transcript in PBMCs.

A

Start	End	Matching sequence	Score
464	467	GUUU	100%
862	865	GUUU	100%
120	123	GUUU	100%
907	910	GUUU	100%
526	529	GUUU	100%
684	687	AUUU	86%
889	892	AUUU	86%
856	859	AUUU	86%
372	375	AUUU	86%
885	888	AUUU	86%

B

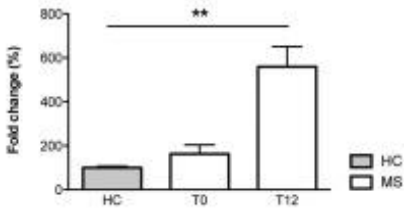


Figure 2. SOD2 transcript as a new target of the RNA-binding HuR protein. **A)** Table reporting the position of the ARE sequences within the SOD2 transcript and the percentage of mRNA-HuR binding affinity (Score) obtained from the RBPDB online database (http://rbpdb.ccbr.utoronto.ca). **B)** Fold enrichment detected by quantitative RT-PCR of SOD2 mRNA in PBMCs from HC (grey) and MS (white), following immunoprecipitation with anti-HuR antibody. The values have been normalized to the level of GAPDH mRNA and expressed as mean percentages ± S.E.M. with respect to HC (100%); **p<0.01. HC= healthy controls; T0= MS patients naïve to disease modifying treatments; T12: MS patients after 12 months of DMF treatment.

We then investigated the protein content of MnSOD. At baseline, the western blot analysis shows that MnSOD expression in MS patients naïve to disease modifying treatments is lower (median 401.0; IQ 138.8-600.3) compared to the HC group (median 1086; IQ 809.0-1859; $p<0.01$). However, after 12 months of DMF treatment, the expression of MnSOD increases up to the levels observed in HC (median 1047; IQ 483.8-1960); **Figure 3**.

At DMF starts, we observed no difference in MnSOD expression according to Gd+ lesions at MRI scan (i.e presence versus absence of contrast enhancing lesions) (Mann Whitney $p =0.49$); at T0 there was also no correlation between MnSOD expression and ARR 1 year before DMF start or MnSOD expression and ARR 2 years before DMF start (Spearman r -0.08; $p=0.73$, Spearman r -0.18; $p=0.43$). At baseline, no correlation was observed between MnSOD expression at T0 and MSSS at T0 (Spearman r -0.02; $p=0.90$).

After 12 months of treatment with DMF, MnSOD expression was 899.0 (IQ 502.1-1700) in the MS group with the status of NEDA-3 and 1674 (IQ 385.8-3391) in the MS group with the status of EDA-3 (Mann Whitney p =0.60). However, we observed a negative correlation between MnSOD expression at T12 and MSSS at T12 (Spearman r -0.51; p=0.03).

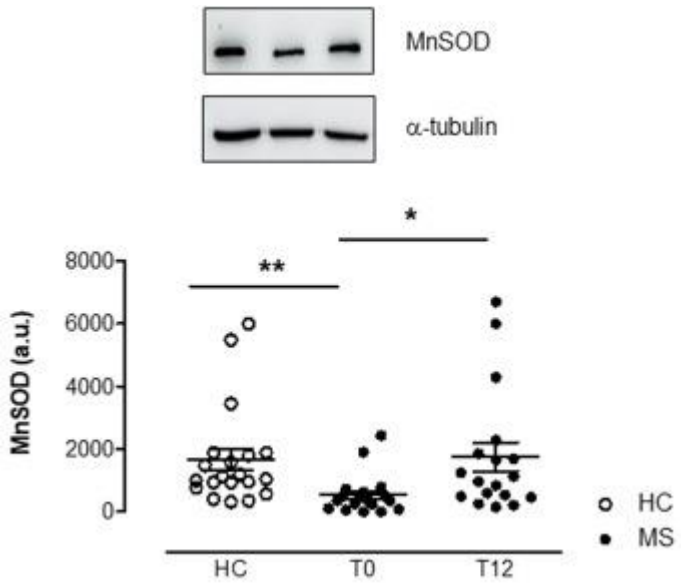


Figure 3. MnSOD protein levels in PBMCs from HC and MS patients. Upper: Representative Western blot images of MnSOD and α-tubulin protein content in PBMCs from HC and MS patients (T0 and T12). Lower: Scatter dot plot of the MnSOD protein expression levels in PBMCs from HC (white, n=20) and MS patients (black, T0, n=20; T12, n=18). The results are expressed as mean grey levels ratios x 103 (mean ± S.E.M.) of MnSOD/α-tubulin immunoreactivities measured by Western blotting. a.u. = arbitrary units. Data were analysed by two-way ANOVA followed by post hoc analysis, *p<0.05, ** p<0.01. HC= healthy controls; T0= MS patients naïve to disease modifying treatments; T12: MS patients after 12 months of DMF treatment.

Nrf2 expression in PBMCs after DMF treatment

As mentioned, a recent in vitro study documented that Nrf2 transcript is a HuR target (Poganik et al., 2019). First, through immunoprecipitation experiments coupled with RNA extraction and RT-PCR, we confirmed that HuR protein interacts with Nrf2 mRNA also in PBMCs (data not shown). Subsequently, we explored whether Nrf2 protein expression was affected in MS patients naïve to disease modifying treatments and the influence of DMF treatment on this parameter.

At DMF start, western blot analysis shows that Nrf2 expression is different between HC subjects (median 413.0; IQ 158.8-978.8) and MS naïve to disease

modifying treatments (median 1437; IQ 987.5-2114). In detail, MS naïve to disease modifying treatments expressed higher levels of Nrf2 with respect to HC group ($p<0.001$). Nrf2 protein content tends to remain elevated with respect to HC subjects (median 1038; IQ 501.3-1705) after 12 months of DMF treatment; **Figure 4**.

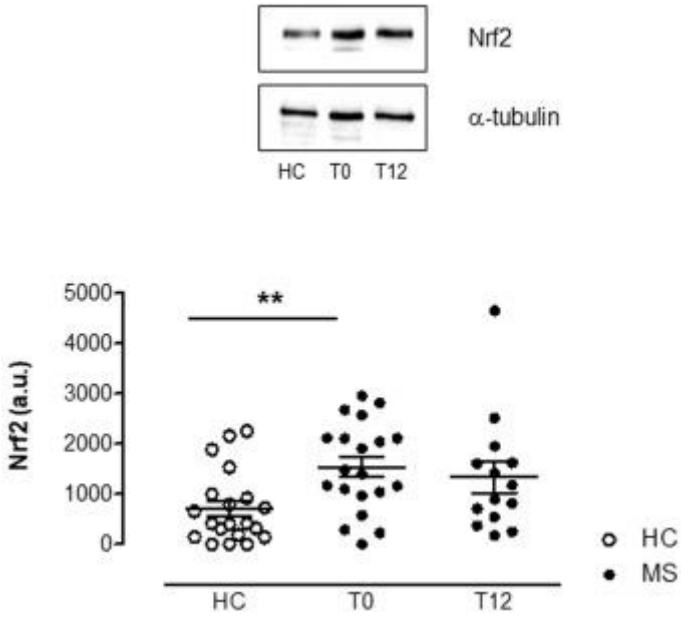


Figure 4. Nrf2 protein levels in PBMCs from healthy controls and MS patients. Upper: Representative Western blot images of Nrf2 and α-tubulin protein content in PBMCs from HC and MS patients (T0 and T12). Lower: Scatter dot plot of the Nrf2 protein expression levels in PBMCs from HC (white, n=18) and MS patients (black, T0, n=20; T12, n=14). The results are expressed as mean grey levels ratios x 103 (mean ± S.E.M.) of Nrf2/α-tubulin immunoreactivities measured by Western blotting. a.u. = arbitrary units. Data were analysed by two-way ANOVA followed by post hoc analysis; **p<0.01. HC= healthy controls; T0= MS patients naïve to disease modifying treatments; T12: MS patients after 12 months of DMF treatment.

At baseline, there was no difference in Nrf2 expression according to Gd+ lesions at MRI scan (i.e presence versus absence of contrast enhancing lesions) (Mann Whitney p =0.12); at T0 there was also no correlation between Nrf2 expression and ARR 1 year before DMF start or Nrf2 expression and ARR 2 years before DMF start (Spearman r 0.12; p=0.59, Spearman r 0.11; p=0.62). No correlation was observed between Nrf2 expression at T0 and MSSS at T0 (Spearman r -0.36; p=0.11).

After 12 months of treatment with DMF, Nrf2 expression was 899.0 (IQ 478.5-2059) in the MS group with the status of NEDA-3 and 1419 (IQ 457.5-1787) in the MS group with the status of EDA-3 (Mann Whitney p =0.89). We did not

observe any correlation between Nrf2 expression at 12 months and MSSS at 12 months (Spearman r -0.18; p=0.52).

Discussion

DMF is a disease modifying treatment for MS, with pleiotropic mechanisms of action, that has long been known to have cytoprotective effects in immune cells, glia and neurons (Faissner and Gold, 2019). The neuroprotective effects and the reduction of the pro-inflammatory response in MS patients have been mainly ascribed to the activation of Nrf2 (Mills et al., 2018), which induces down-stream responses helping the cells to face oxidative stress. For instance, growing evidence implicates that the DMF neuroprotective effects arise from the alteration of the redox state of microglial cells through a Nrf2-dependent antioxidant action (Rosito et al., 2020). In addition, DMF has been shown to exert its immunomodulatory effect also via Nrf2-independent mechanisms, such as the one involving the inhibition of NF-kB (Gillard et al., 2015), the molecular driver of pro-inflammatory microglia. These effects, by downregulating pro-inflammatory genes and upregulating the anti-inflammatory ones, are compatible with a shift towards the anti-inflammatory and neuroprotective microglia phenotype. Our present results confirm a potential role of Nrf2 in MS pathogenesis, as MS patients naïve to disease modifying treatments show higher levels of Nrf2 protein, suggesting the need for these subjects of activating Nrf2-mediated down-stream defence mechanisms in PBMCs to counteract inflammation and oxidative stress events (Pegoretti et al., 2020). Nrf2 content was persistently high during DMF treatment, perhaps at least partially thanks to DMF itself, as suggested by literature data (Mills et al., 2018).

Interestingly, Nrf2 expression is controlled by HuR, an RNA binding protein (Poganik et al., 2019) which acts as a regulator of many cellular functions, including survival, inflammation and proper functioning of the immune system (Srikantan and Gorospe, 2012). Recently, we demonstrated the potential entanglement of HuR in MS pathogenesis (Pistono et al., 2020) by documenting a lower HuR protein content in PBMCs of MS patients as compared to healthy controls. This last finding was also confirmed by this work performed in a new population of MS patients, thus underscoring the robustness of the result. Further, in the present study, we analysed whether there was an interaction between HuR and SOD2, based on previous results demonstrating the presence of multiple cis-

acting elements in SOD2 primary sequence. Notably, MnSOD protein belongs to the superoxide dismutase family, a class of enzymes involved in the catalysing process of antioxidant reactions, which are responsible for degrading oxygen free radicals inside the cells where there is a sustained oxidative stress (Fridovich, 1995). Via this work, we were able to document in humans that SOD2 mRNA is indeed a novel target of HuR protein. Of note, we also found that, in PBMCs, this interaction is positively affected by DMF treatment, as demonstrated by immunoprecipitation experiments coupled with RNA extraction and RT-PCR showing that the formation of the HuR/ SOD2 complex is promoted by DMF administration.

Furthermore, to better define HuR contribution to MS occurrence, we also investigated the expression of MnSOD in MS patients naïve to disease modifying treatments and explored the effect of DMF administration on both proteins. We observed a pronounced reduction in the levels of MnSOD enzyme in MS patients compared to HC, highlighting that the defence mechanisms capable of counteracting oxidative damage are impaired in MS. This finding is consistent with literature data reporting a significant decrease in MnSOD activity in PBMCs from MS patients not on disease modifying treatment (Emamgholipour et al., 2016). Interestingly, DMF treatment not only promotes the interaction between HuR protein and SOD2 transcript, but also exerts a positive effect on the expression of both HuR protein and MnSOD enzyme. We indeed demonstrated that 12 months of DMF administration in MS patients is able to restore the amount of both HuR protein and MnSOD enzyme to the levels observed in HC. Further, we also observed a negative correlation between MnSOD expression at T12 and MSSS at T12. This last finding might suggest that the presence of more elevated MnSOD levels, due to DMF treatment, allows to slow down the progression of the disease.

Taken together, these results suggest the existence of a novel molecular mechanism underlying the cytoprotective action of DMF. Specifically, DMF induces an increased expression of HuR protein in PBMCs, which ultimately gives rise to a greater amount of the HuR protein / SOD2 transcript complex finally promoting the expression of this antioxidant protein, which possibly contributes to slow down disease progression. The activation of this molecular cascade can thus constitute an additional tool that can be exploited at a cellular level to counteract the oxidative damage mediated by reactive oxygen species. The broad-spectrum profile of HuR-mediated effects makes this RNA-binding

protein a suitable target that might explain the multifaceted molecular mechanisms underlying the pharmacologic effectiveness of DMF in MS.

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List of Publications

1. Use of Dual-Flow Bioreactor to Develop a Simplified Model of Nervous-Cardiovascular Systems Crosstalk: A Preliminary Assessment. Marchesi N, Barbieri A, **Fahmideh F**, Govoni S, Ghidoni A, Parati G, Vanoli E, Pascale A, Calvillo L. *PLoS One*. 15(11): e0242627, 2020.

2. Non-Drug Interventions in Glaucoma: Putative Roles for Lifestyle, Diet and Nutritional Supplements. **Fahmideh F**, Marchesi N, Barbieri A, Govoni S, Pascale A. *Surv Ophthalmol*. S0039-6257(21)00185-5, 2021.

3. Ocular Neurodegenerative Diseases: Interconnection between Retina and Cortical Areas. Marchesi N, **Fahmideh F**, Boschi F, Pascale A, Barbieri A. *Cells*. 10(9):2394, 2021.

4. Pharmacological Versus Non-Pharmacological and Ancillary Mechanisms in Eye Drops Used in the Treatment of Glaucoma. Marchesi N, **Fahmideh F***, Barbieri A, Racchi M, Pascale A, Govoni S. *Drug Saf Regul*. 933471, 2022. *** (co-First Author)**

5. Effect of Troxerutin in Counteracting Hyperglycemia-Induced VEGF Upregulation in Endothelial Cells: A New Option to Target Early Stages of Diabetic Retinopathy?". **Fahmideh F**, Marchesi N, Campagnoli LIM, Landini L, Caramella C, Barbieri A, Govoni S, Pascale A. *Front in Pharmacol*. 951833, 2022.

6. Evidence for Novel Cell Defence Mechanisms Sustained by Dimethyl Fumarate in Multiple Sclerosis Patients: The HUR/SOD2 Cascade. Mallucci G, Marchesi N, Campagnoli LIM, Boschi F, **Fahmideh F**, Fusco S, Eleonora T, Govoni S, Bergamaschi R, Pascale A. *Mult Scler Relat Disord*. 104197, 2022.

7. The Interplay Between Gut Microbiota and Parkinson's Disease: Implications on Diagnosis and Treatment. Varesi A, Campagnoli LIM, **Fahmideh F**, Pierella E, Romeo M, Ricevuti G, Marchesi N, Chirumbolo S, Pascale A. *Int J Mol Sci*. 12289, 2022.

8. An Improved and Novel Stroke Model in Rats Based on Thromboembolism and Compliant with the Latest Standards in Preclinical

Trials. Pawletko K, Jędrzejowska–Szypułka H, Bogus K, Pascale A, **Fahmideh F**, Marchesi N, Grajoszek A, Olakowska E, Barski JJ. *Brainsci*, 2022. (Submitted)

9. Neuropathic Pain in Aged People; An Unresolved Issue Open to Novel Drug Approaches. Marchesi N, **Fahmideh F***, Pascale A, Allegri M, Govoni S. *Curr Neuropharmacol*, 2022. ***(co-First Author)** (Submitted)

- It should be mentioned that two more original articles are under preparation.