Executive Summary:
Diabetic Retinopathy (DR), the leading cause of blindness among working-age individuals in developed countries, has been classically considered a microcirculatory disease of the retina. However, there is growing evidence suggesting that retinal neurodegeneration is an early event in the pathogenesis of DR. For this reason, it is reasonable to hypothesize that therapeutic strategies based on neuroprotection will be effective not only in preventing or arresting retinal neurodegeneration but also in preventing the development and progression of the early stages of DR (i.e. micro-aneurysms and/or retinal thickness).

EUROCONDOR (European Consortium for the Early Treatment of Diabetic Retinopathy) is a solid and well-balanced consortium (ophthalmologists, endocrinologists, basic researchers, a biopharmaceutical SME and a pan-European diabetes association) which has been created to implement the first clinical trial...
SME and a pan-European diabetes association) which has been created to implement the first clinical trial using eye drops for treatment of the early stages of DR.

The main objective was to assess whether the selected neuroprotective drugs (Brimonidine and Somatostatin) administered topically are able to prevent or arrest neurodegeneration, as well as the development and progression of the early stages of DR.

The main conclusions of the preliminary statistical analyses are the following:

- Neurodegeneration is not present in 1/3 of type 2 diabetic patients with early microvascular disease (ETDRS 20-35).
- From the mfERG parameters, P1 amplitude is more sensitive than implicit time (IT) for detecting neurodegeneration.
- Topical treatment with somatostatin and brimonidine seems not useful for preventing the development of neurodegeneration, at least in a period of 2 years of follow-up. However, these neuroprotective treatments are effective in arresting the progression in those patients in whom some degree of neurodegeneration is already present.
- Somatostatin has a positive effect in microvascular disease by arresting or maintaining the number of microaneurysms in comparison with placebo.

In conclusion, our results pave the way to new clinical trials based on topical administration of neuroprotective and/or vasculotropic agents. The identification of patients in whom neurodegeneration is present in early stages of DR will permit us to implement a more personalized and efficient medicine which could reduce the economic burden associated with DR.

Project Context and Objectives:
Epidemiology and current treatment of diabetic retinopathy

Diabetic retinopathy (DR) is the leading cause of visual impairment and preventable blindness, representing a significant socioeconomic cost for healthcare systems worldwide. DR prevalence in the diabetic population is approximately one-third, and 10% have vision-threatening states such as diabetic macular edema or proliferative diabetic retinopathy (PDR) (Yau et al. Diabetes Care 2012;35(3):556-64). In addition, given that DR is the most common complication of diabetes and that diabetes is expected to increase from 388 million in 2013 to 592 million by 2030, DR will become an even more serious problem in the future (Guariguata et al. International Diabetes Federation. Diabetes Atlas. International Diabetes Federation 2014, Brussels, Belgium). The potentially substantial worldwide public health burden of DR highlights the importance of searching for new approaches beyond current standards of diabetes care.

The tight control of blood glucose levels and blood pressure are essential in preventing DR development or arresting its progression. However, at present we are doing nothing specifically addressed to the diabetic eye until very advanced stages when laser photocoagulation, intravitreal injections of corticosteroids or anti-VEGF agents and vitreoretinal surgery are implemented. All these treatments are expensive, require a vitreoretinal specialist and have a significant number of secondary effects. Therefore, new treatments for the early stages of DR are needed (Simó and Hernández. Novel approaches for treating diabetic retinopathy based on recent pathogenic evidence. Prog Retin Eye Res. 2015;48:160-80).
Neurodegeneration: an early event of DR

DR has been classically considered a microcirculatory disease of the retina. However, there is emerging evidence to suggest that retinal neurodegeneration is an early event in the pathogenesis of DR which could participate in the development of microvascular abnormalities (Abcouwer and Gardner. Ann N Y Acad Sci. 2014 Apr;1311:174-90; Simó R, Hernández C. European Consortium for the Early Treatment of Diabetic Retinopathy (EUROCONDOR). Trends. Endocrinol. Metab 2014;25:23-33). Therefore, the study of the underlying mechanisms leading to neurodegeneration and the identification of the mediators in the cross-talk between neurodegeneration and microangiopathy is essential for the development of new therapeutic strategies.

Neuronal integrity is essential for vision. In the early stages of diabetes, a routine clinical evaluation of a patient’s sensory capacity will, in a high proportion of patients, reveal deficiencies that they are commonly unaware of in daily life. These deficits include decreased hue discrimination and contrast sensitivity, delayed dark adaptation and abnormal visual fields. Abnormalities in retinal function evaluated by means of electroretinography (ERG) or other electrophysiological as well as psychophysical methods have been found in diabetic patients without any evidence of microvascular abnormalities. In this regard, the presence of neuroretinal dysfunction assessed by mfERG (multifocal electroretinography) in type 1 diabetic patients even without any subtle blood-retinal barrier leakage measured by vitreous fluorometry has recently been reported. In addition, and more importantly, several authors have found that an increase of the implicit time in mfERG is a predictor for the development of visible vascular abnormalities over 1-year, and 3-year periods (reviewed by Simó and Hernandez on behalf of the EUROCONDOR consortium in Trends Endocrinol Metab 25:23-33, 2014).

Diabetes increases apoptosis in neurons, especially in the inner retina, where retinal ganglion cells (RGC) are located. This loss of neural cells results in a reduction in the thickness of the retinal nerve fibre layer, which has been detected in rats with STZ-induced diabetes and in clinical studies using scanning laser polarimetry or optical coherence tomography (OCT) (reviewed by Simó and Hernandez on behalf of the EUROCONDOR consortium in Trends Endocrinol Metab 25:23-33, 2014). It should be noted that this thinning of the ganglion cell layer has also been found in diabetic patients without any microcirculatory abnormalities appearing in the ophthalmoscopic examinations performed during the year before death. In several experimental models of diabetic retinopathy it has been shown that activation of death receptors and mitochondrial damage by oxidative and endoplasmic reticulum stressors are major triggers of apoptosis that ultimately lead to cellular damage. It is worth mentioning that we have been recently demonstrated that in the early stages of DR an imbalance between proapoptotic and survival signaling exists in the neuroretina of diabetic patients (Valverde et al., Mol Vis. 2013;19:47-53).

Somatostatin

Somatostatin (SST) is one of the most important neuroprotective factors synthesized by the retina, retinal pigment epithelium (RPE) being its main source in the human eye. The human retina produces significant amounts of SST, as deduced by the strikingly high levels reported within the vitreous fluid (Simó et al., Diabetes Care 25:2282–2286, 2002; Hernández et al., Diabetes Care 2005;28:1941–1947).
Besides SST, its receptors (SSTRs) are also expressed in the retina, with SSTR1 and SSTR2 being the most widely expressed (Cervia et al., Mol Cell Endocrinol 2008;286:112–122). The production of both SST and its receptors simultaneously suggests a relevant autocrine action in the human retina. SST acts as a neuromodulator in the retina through multiple pathways, including intracellular Ca2+ signaling, nitric oxide function, and glutamate release from the photoreceptors. Apart from neuroprotection, SST has potent antiangiogenic properties and regulates various ion/water transport systems. Therefore, SST seems to be essential in preventing both proliferative DR (PDR) and diabetic macular edema (DME).

In the early stages of DR, there is a downregulation of SST that is associated with retinal neurodegeneration. In fact, it has recently been reported that intravitreal administration of SST and SST analogs protects the retina from AMPA-induced neurotoxicity. In addition, the lower expression of SST in RPE and neuroretina is associated with a dramatic decrease of intavitreal SST levels in both DME and PDR. As a result, the physiological role of SST in preventing both fluid accumulation within the retina and neovascularization could be reduced, and consequently, the development of DME and PDR would be favored. For all these reasons, SST replacement treatment can be considered a new target not only for preventing the neurodegenerative process but also for more advanced stages of DR such as DME and PDR.

Brimonidine

Brimonidine is a highly selective alpha 2-adrenergic agonist that was introduced in 1996 for glaucoma treatment. Brimonidine reduces intraocular pressure decreasing aqueous humor production and increasing uveoscleral outflow. A lot of clinical studies have documented its safety and efficacy in lowering intraocular pressure. Interestingly, there are experimental evidences suggesting a neuroprotective effect of brimonidine beyond intraocular pressure lowering (Saylor et al Arch Ophthalmol 2009;127:402-406). Brimonidine increases in a dose-dependent manner rat retinal ganglion cell (RGC) survival in the presence of glutamate, oxidative stress, and hypoxia (Lee et al. Mol Vision 2010;16:246-51). Proteomic analyses of retinal explants cultured with or without brimonidine revealed that it downregulates GFAP (glial fibrillar acidic protein), a sensitive marker of glial activation (Prokosh et al. Invest Ophthalmol Vis Sci 2010;51:6688-99). Studies in animals have found that treatment of chronic ocular hypertension in rats with brimonidine drops resulted in a reduction in RGC loss, a decrease in the level of GFAP immunoreactivity, and an increase in BDNF mRNA and p-Akt levels (Kim et al. Vis Neurosci 2007;24:127-39).

Based on these and other preclinical and clinical studies on the efficacy of SST and brimonidine in the retina, the EUROCONDOR project aimed at addressing new insights in the mechanisms of neuroprotection of brimonidine/SST. For this goal we have used a plethora of cellular systems of the retina as well as diabetic mice with DR.

OBJECTIVES

The main objectives of the project are the following:

Primary objective:
To assess whether the selected neuroprotective drugs (Brimonidine and Somatostatin) administered topically are able to prevent or arrest neurodegeneration, as well as the development and progression of the early stages of DR.

Secondary objectives:

- To determine the prevalence of functional abnormalities related to neurodegeneration in those patients without or with minimal microvascular damage under ophthalmoscopic examination.
- To compare the effectiveness of the selected drugs.
- To evaluate the local and systemic adverse effects of the selected drugs.
- To identify those patients most prone to progressive worsening (characterization of phenotypes and circulating biomarkers).
- To determine the molecular mechanisms by which the selected drugs exert their beneficial effects.

Project Results:
1. CLINICAL TRIAL

1.1 Study population

European Consortium for the Early Treatment of Diabetic Retinopathy (EUROCONDOR, NCT01726075 (278040); EudraCT Number: 2012-001200-38) is a multicentre, 2-year prospective, interventional, phase II-III, randomized controlled clinical trial.

This outpatient study population will consist of a representative group of male and female type 2 diabetic patients (n= 450) with diabetic retinal disease with ETDRS level < 20 (50% of enrolled patients) or ETDRS levels 20 or 35 with presence of at least one microaneurysm in Field 2 between the superior and inferior arcades (50% of enrolled patients) in the Study Eye as determined by the Reading Centre.

Inclusion Criteria:

1. Patients with type 2 diabetes mellitus
2. Diabetes duration ≥ 5 years
3. Aged between 45-75 years-old
4. ETDRS level < 20 (microaneurysms absent) (50% of enrolled patients)
   Or
   ETDRS levels 20 or 35 with presence of at least one microaneurysm in Field 2 between the superior and inferior arcades (50% of enrolled patients) in the Study Eye as determined by the Reading Centre.
5. Informed Consent

Exclusion Criteria:

1. Previous laser photocoagulation
2. Other diseases which may induce retinal degeneration (e.g. glaucoma)
3. Subject with a refractive error ≥ ± 5 diopter
3. Subject with a refractive error ≥ ± 5 diopter
4. Inadequate ocular media and/or pupil dilatation that do not permit good quality fundus photography.
5. Renal failure (creatinine > 1.4 mg/dl)
6. HbA1C > 10% in the previous 6 months and at Screening
7. Subjects taking somatostatin or brimonidine, for any indication, in the previous 3 months
8. Subject has a condition or is in a situation which may put the subject at significant risk, may confound the study results or may interfere significantly with the patient’s participation in the study.
9. Pregnancy or nursing
10. Hypersensitivity to the active substances to be tested or to any of the excipients
11. Subject receiving systemic monoamine oxidase (MAO) inhibitor therapy or antidepressants which affect noradrenergic transmission (e.g. tricyclic antidepressants and mianserin)

Eligible patients were included in the Clinical Trial and randomly allocated in a 1:1:1 ratio to one of the 3 arms:

- Group A: placebo (1 drop BID in each eye)
- Group B: somatostatin 0.1% (1 drop BID in each eye)
- Group C: brimonidine tartrate 0.2% (1 drop BID in each eye)

Patients will be followed and treated during a 96-weeks period (24 months).

The Clinical Sites has been conducted the Clinical Trial in accordance with the Protocol, the ICH-GCP Guideline and regulatory requirements according to the Monitoring Reports.

The Clinical Trial has an Adverse Effects Sub-Committee (AESC) who evaluates on a continuous basis the adverse effects and looks for the potential relationship with the treatment. Moreover, the Independent Data Monitoring Committee (IDMC) evaluates safety and efficacy (treatment compliance) outcomes on a 6-month basis.

1.2 The main objectives and the corresponding outcomes are the following:

Primary Objectives:

- To assess whether somatostatin, administered topically, is able to prevent or arrest the development and progression of neurodegenerative changes.
- To assess whether, brimonidine, administered topically, is able to prevent or arrest the development and progression of neurodegenerative changes.

Primary Outcomes:

- Changes in the Implicit Time assessed by mfERG (IT-mfERG)

Secondary Objectives:

To determine the prevalence and to characterise functional and structural abnormalities related to
- To determine the prevalence and to characterise functional and structural abnormalities related to neurodegeneration in those patients with or without detectable microvascular damage.
- To identify those patients most prone to progressive worsening of the retinopathy by identifying progression of DR using the ETDRS severity scale, BCVA, microvascular disease activity (MA turnover and retinal thickness) and neurodegenerative changes.
- To assess the correlation between the presence and progression of neuronal and glial alterations (mfERG abnormalities and ganglion cell layer thickness) and the appearance and progression of the microvascular lesions (MA turnover and overall retinal thickness).
- To assess whether there is an effect on the visual-related quality of life in the early stages of nonproliferative DR as measured by the VFQ-25.
- To evaluate the local and systemic adverse events of the selected drugs.

Secondary Outcomes:

- Neurodegenerative variables:
  - Retinal Nerve Fiber Layer (RNFL) assessed by SD-OCT
  - Ganglion Cell Layer (GCL) assessed by SD-OCT

- Microvascular variables:
  - Microaneurysm turnover assessed by Colour Fundus Photography
  - Retinal thickness assessed by SD-OCT
  - Central retinal thickness assessed by SD-OCT
  - DR severity assessed by ETDRS scale

Other analyzed variables:

- BCVA assessed by ETDRS scale
- Visual Fields defects assessed by Visual Fields Test
- Visual-related Quality of Life assessed by Visual Function Questionnaire (VFQ-25)
- Adverse Events assessed by inquiry and ophthalmological examination
- Need for rescue treatment

1.3 Normative database

As essential preliminary task of the clinical trial we generated a multicentric and multinational mfERG normative database in an adult Caucasian population. For this purpose, healthy volunteers were recruited from 11 centers (7 countries) included in the EUROCONOR consortium and belonging to the European Vision Institute Clinical Research Network (EVICR.net). To the best of our knowledge, this is largest multicenter study to date using mfERG in a normal population including and analyzing data from 103 hexagons. These results have been submitted for publication to PloS ONE (under review).

1.4 Preliminary Results

1.4.1 Evaluation of the prevalence of functional abnormalities related to neurodegeneration in those patients without or with minimal microvascular damage
The data collected from baseline EUROCONDOR study shown alterations of the mfERG in almost 60% of type 2 diabetic patients with no apparent fundus abnormalities (ETDRS level 10). These mfERG changes were found in a higher percentage (65%) when mild DR (ETDRS levels 20 and 35) was present.

From the mfERG parameters, P1 amplitude is more sensitive than implicit time (IT) for detecting neurodegeneration.

Correlations between the diameters of the retinal arterioles (CRAE) and venules (CRVE) with early neurodegeneration were found. In specific, CRAE was associated with macular ganglion cell layer thickness, and CRVE was correlated with macular retinal thickness and retinal nerve fiber layer thickness. This indicates a close relation between vascular and neurogenic parameters at a very early stage of DR.

All these findings support the concept that functional impairment related to neurodegeneration is an early event in the diabetic retina which antedates microvascular structural abnormalities.

1.4.2 Vision-related quality of life (VRQoL)

Evaluation of Quality of Life was included in EUROCONDOR to verify if subtle changes in some at least of the subscales explored by the NEI VFQ-25 questionnaire could be detected at baseline and possibly modified as a result of either disease progression or changes in visual function, intervention-induced or not, that the trial aimed at exploring. Indeed, evidence for impaired Vision Specific Mental Health and Role Difficulties in patients with mild non-symptomatic DR may highlight some aspects of discomfort in everyday life, despite preserved BCVA. More in line with expectations, reduced retinal thickness at the ganglion cell layer, as assessed by OCT, was associated with a minor decrease in visual acuity, though not with retinopathy, and worse scores for such VRQoL dimensions as Ocular Pain, General Vision, Difficulties with Colour Vision and Difficulties with Peripheral Vision.

The NEI VFQ-25 was able to detect subtle changes in patients’ perception of visual function, despite absent/minimal DR. We suggest that it is important to be aware of even minor changes in the perception of disease among patients, especially because psychological dimensions are often coupled with socio-economic difficulties and inequalities in access to health as the involvement of people with diabetes is crucial to delivering screening and preventing eye complications.

In conclusion, we found that the NEI VFQ-25 could detect subtle changes in patients’ perception of visual function, despite absent/minimal DR.

1.4.3 Assessment of the effectiveness of the selected drugs (Brimonidine and Somatostatin) in preventing or arresting DR development.

The main conclusions of the preliminary statistical analyses of the EUROCONDOR clinical trial are the following:

Topical treatment with somatostatin and brimonidine seems not useful for preventing the development of neurodegeneration, at least in a period of 2 years of follow-up.
Topical treatment with somatostatin and brimonidine is effective in arresting the progression of neurodegeneration after excluding patients in the low quartile of IT values (best retinal function). That is, in those patients in whom some degree of neurodegeneration is already present.

Somatostatin has a positive effect in microvascular disease by arresting or maintaining the number of microaneurysms in comparison with placebo.

1.4.4 Identification of serum biomarkers

The serum biomarkers measured in the clinical trial were Laminin-P1 (LamP1), N-carboxymethyl-lysine (CML) and asymmetric dimethylarginine (ADMA).

Laminin is a non-collagenous glycoprotein of basement membranes which is up-regulated by high glucose concentrations. CML is the most abundant of circulating advanced glycation products (AGEs). ADMA is involved in the nitric oxide pathway and serum levels of ADMA have been found elevated in diabetic patients with DR.

The samples to measure these biomarkers have been collected at visit 1, visit 4 (6 month) and visit 6 (12 months). All the biomarkers were assessed by ELISA.

At baseline, carboxy methyl lisine (CML) levels were independently associated with implicit time and retinal thickness, and the increase in CML concentration throughout the study was related to an impairment of mfERG-Implicit Time.

Baseline levels of laminin were independently associated with baseline IT value, and were directly related to the increase of retinal thickness during the follow-up (2 years).

Laminin levels decreased in patients treated with somatostatin, thus suggesting a direct effect of somatostatin on this essential component of basal membrane.

In summary, CML and LamP1 levels could help us to identifying patients with early retinal abnormalities (functional and microvascular). Furthermore, the assessment of LamP1 serum level could be an additional tool for monitoring the effectiveness of somatostatin treatment. Therefore, the increase of these biomarkers could be a complementary tool for monitoring the impairment of neurodegeneration and retinal thickness.

In conclusion, our results pave the way to new clinical trials based on topical administration of neuroprotective and/or vasculotropic agents. The identification of patients in whom neurodegeneration is present in early stages of DR will permit us to implement a more personalized and efficient medicine which could reduce the economic burden associated with DR.

2. BASIC RESEARCH. MECHANISMS OF ACTION OF THE SELECTED NEUROPROTECTIVE DRUGS
To get new mechanistic evidences on the effects of somatostatin (SST) in the retina, we have used different experimental models:

1) In vitro cellular models: cell cultures of retinal ganglion cells (RGC-5), retinal pigment epithelium cells (ARPE-19), photoreceptors (661W), pericytes, endothelial cells and microglia (Bv.2 cells).
2) Ex vivo models: mouse and porcine retinal explants.
3) In vivo animal models: diabetic rats, db/db mice and IRS2-deficient mice.
Also, some determinations have been performed in retinas from diabetic donors.

2.1 Effect of SST on SST receptors expression

We have explored the expression of SST receptors in cell cultures (RGC-5, ARPE-19 and pericytes), diabetic mice and human retinas. It must be noted that we found the expression of SSTRs in pericytes by the first time, and that SSTRs expression in the retina is downregulated by diabetes.

2.2 Effects of SST in the permeability of outer blood retinal barrier

We have explored the effects of SST in outer blood retinal barrier (BRB) function by means of the assessment of transelectrical resistance and dextran permeability in ARPE-19 cells treated with a medium that mimics the diabetic milieu. In addition, the effect of SST on the expression (mRNA and protein) of tight junctions-related proteins (occludin, ZO and claudin-1) has been evaluated. We found that SST abrogates the increase of permeability induced by the diabetic milieu in ARPE-19 cells. The optimal concentration for detecting this beneficial effect was 1x10^{-7}M.

We have assessed whether SST alter the expression of proteins of the tight junctions in ARPE-19 cells. We did not observe changes in the expression of occludin and ZO-1 under different conditions by Western blot or RT-PCR.

Treatment with IL-1beta (two doses of 10 ng/mL each 24 h) increased claudin-1 expression as we previously reported (Villarroel et al., Exp Eye Res. 2009, 89(6):913-20). However, under SST treatment, claudin-1 expression was reduced. The same effect was obtained by RT-PCR.

2.3 Effects of SST and BRM on the microvascular part of the retina: cross talk between pericytes, endothelial and ganglion cells

Human retinal pericytes (HRP) were first used as a cellular model. Dose-response experiments were performed, and proliferation and apoptosis used as readouts of drug activity. The effects of both drugs were also tested in conditions of hyperglycemia/hypoxia to mimic the diabetic microenvironment. Hypoxia, alone and combined with hyperglycaemia, was able to increase HRP apoptosis and decreased their proliferation. Addition of somatostatin did not determine any variation. Neither SST nor BRM altered the proliferation or survival of HRP.

Co-culture systems of HRP with endothelial cells (EC) or ganglion cells (RGC-5), as a model of retinal-blood barrier, were subsequently established. HRP were exposed intermittently at 48 hour intervals to
blood barrier, were subsequently established. HRP were exposed intermittently at 48-hour intervals to high/physiological glucose concentration (intHG) and/or hypoxia, with/without SST or BRM. Conditioned media (CM) from the final two days were collected and used to culture either EC or RGC-5. Vice versa, CM from EC and RGC-5 cultured in the same diabetic-like conditions were used to culture HRP. Control cells were cultured directly in physiological (NG), high glucose (HG), intHG, hypoxia, with/without SST/BRM. Cell proliferation and apoptosis were assessed. SST and BRM, when added to EC, did not show any relevant effect on pericyte proliferation or apoptosis. SST seems to enhance the proliferative effect on EC of CM from pericyte cultures. BRM under physiological conditions may increase pericyte control on endothelial cells proliferation. No adverse effects of either SST or BRM were found on EC or RGC-5 cells, as mediated by pericytes, nor on HRP, as mediated by EC or RGC-5 cells.

IntHG conditions, both direct and mediated by HRP, decrease the proliferation of RGC-5 cells and increase their apoptosis, in comparison with control, behavior very similar to that shown by HRP. As it is known that HRP are sensitive to intermittent (but not stable) high glucose, it can be hypothesized that HRP in stress conditions may release soluble factor(s) affecting RGC-5 viability.

We next addressed the effects of hypoxia combined with high glucose on HRP and the expression of molecules involved in the pro-apoptotic and survival pathways in order to clarify the mechanisms of action of these diabetic-like stress stimuli. The expression of pro-apoptotic molecules, such as FasL, caspase-8, t-Bid, Bax and p53, was increased in HRP cultured in intermittent, but not stable HG. No differences were observed in the expression of survival markers in intHG, while there was a slight increase in stable HG. Diabetic–like conditions (intHG and hypoxia) were able to stimulate pericyte apoptosis through activation of pro-apoptotic molecules, thus leading to an imbalance between pro-apoptotic and survival signaling pathways.

2.4 Effects of SST on cellular viability of the retinal ganglion cell, photoreceptors and retinal explants: new insights in the molecular mechanisms involved

We addressed the direct effects of SST in the balance between apoptosis and cell death in retinal ganglion cells (RCG-5 cell line) cultured under HG. In these experimental conditions, cellular viability of RCG-5 cells was significantly decreased at both 24 and 48 hours. Likewise, the percentage of apoptotic cells was significantly increased, reflecting the deleterious effect of hyperglycemia in retinal ganglion cells. SST at 10-6 M concentration was able to ameliorate apoptosis and increase survival.

To get mechanistic insights on the effect of SST on cellular viability, we analyzed the levels of the 35 kDa fragment of calpain-2 and the expression pattern of its substrate, PTP1B. PTP1B is a tyrosine phosphatase that dephosphorylates the IGF-IR and switches off the IGF-IR/Akt survival pathway (Buckler et al., Mol Cell Biol. 2002; 22:1998–2010). We found that PTP1B inhibition in photoreceptors protects against the deleterious effects of pro-inflammatory cytokines on the IGF-IR/Akt signaling (Arroba and Valverde. IOVS 2015; 56(13):8031-44). RGC-5 cells cultured under HG, the decrease in the 35 kDa calpain-2 fragment detected after 24 h of culture under HG was coincident with the cleavage of PTP1B which was detected at 48 h. Interestingly, treatment with SST prevented both effects.

The direct effect of SST on the IGF-IR/Akt survival pathway was also evaluated. We found that SST directly induced the phosphorylation of the IGF-IR and Akt in both RCG-5 cells and retinal explants. These
Directly induced the phosphorylation of the IGF-IR and Akt in both RCG-5 cells and retinal explants. These results suggest that SST directly or indirectly (probably through the induction of IGF-I secretion) is able to induce Akt phosphorylation that is a key transducer of survival signaling (Taniguchi et al. Nat Rev Mol Cell Biol. 2006; 7: 85–96).

2.5 Effects of SST and BRM in protection against neuroinflammation in the retina: role of microglia

The role of SST in neuroprotection in the retina was evaluated since many neurodegenerative conditions (i.e.: Alzheimer Disease and Parkinson) begin with early neuroinflammation. It has been proposed that in neuroinflammation microglia becomes activated and produce inflammatory mediators. Microglia, serving as resident macrophages of the retina, has multiple functional states and carries out diverse functions. Capable of rapid dynamism and motility, microglial cells synthesize and release cytokines, chemokines, neurotrophic factors, and neurotransmitters that interact with multiple cell types in the CNS and exert cytotoxic, cytoprotective and scavenger effects depending on the tissue context (Lynch MA Molecular neurobiology 2009; 40: 139-15).

In the retina, microglia cells have the ability to orient an inflammatory response towards M1 (proinflammatory) or M2 (anti-inflammatory/ tissue repair) polarization stages depending on the tissue context. As an in vitro experimental model that mimics the proinflammatory environment of the retina, the Bv.2 microglia cell line has been used and these cells were stimulated with LPS as a stimulus that induces a M1 proinflammatory response. SST was able to reduce the expression and secretion of M1 (pro-inflammatory) cytokines.

In contrast, SST did not induce a M2 response (anti-inflammatory). Also, SST was able to reduce the expression of the M1 response induced by a diabetic milieu (glucose (25 mM) plus H2O2 (300 mM) plus IL1 beta (20 ng/mL)). We confirmed these results by the analysis of the M1 marker iNOS. SST and also BRM decreased LPS-induced iNOS protein levels in BV.2 microglial cells.

2.6 Metabolomic approach to identify changes in metabolites during DR and their modulation by SST: relationship with oxidative stress-mediated mitochondrial dysfunction

During the last reporting period, we have focused on the Irs2-deficient mouse as a model for defining the effects of diabetic metabolism on the retina. Lack of IRS2 signals causes insulin resistance and hyperglycemia, similar to type 2 diabetic mechanisms in humans. We have performed experiments in which female WT controls and pre-diabetic (fasting glucose <150) KO were treated with SST administered directly to the eye in drops (10 mg/ml).

Whole eyes were collected at end of treatment protocol and processed for metabolomics analysis. This technique provides high sensitivity and offers the possibility to determine 1) how insulin resistance and pre-diabetes alters metabolites (ATP, lactate, creatine, cholesterol, phospholipids, etc) and neurotransmitters (glumate, asparatate, GABA) in the retina; 2) whether SST administration alters the metabolic profile in the retina. Moreover, this approach replicates the procedure employed with patients in clinical trial of EUROCONDOR. Changes in these metabolites have been associated with neurodegeneration in various experimental models and patients.
Mitochondrial dysfunction contributes to a range of neurodegenerative diseases, making mitochondria a potential target for pharmacological-based therapies for pathologies such as diabetic retinopathy. Using the same experimental approach as described above, retinas were collected from control and mutant mice treated with SST and processed for mtDNA and protein extraction. PGC-1 alpha stimulates mitochondrial biogenesis and thus, was assessed as a biological readout of mitochondrial status in our models.

Based on preliminary experiments, direct administration of SST to the eye in drops did not alter the expression levels of PGC-1 alpha. We are currently analyzing other genes and markers of mitochondrial biogenesis in these samples.

2.7 Topical administration of somatostatin prevents diabetes-induced retinal neurodegeneration in diabetic murine models

Rats with streptozotocin-induced diabetes mellitus (STZ-DM) were treated with either SST eye drops or vehicle for 15 days. Nondiabetic rats treated with vehicle served as a control group. Functional abnormalities were assessed by electroretinography (ERG), and neurodegeneration was assessed by measuring glial activation and the apoptotic rate. In addition, proapoptotic (FasL, Bid, and activation of caspase-8 and caspase-3) and survival signaling pathways (BclxL) were examined. Intraretinal concentrations of glutamate and its main transporter glutamate/aspartate transporter (GLAST) were also determined. Treatment with SST eye drops prevented ERG abnormalities, glial activation, apoptosis, and the misbalance between proapoptotic and survival signaling detected in STZ-DM rats. In addition, SST eye drops inhibited glutamate accumulation in the retina and GLAST downregulation induced by diabetes mellitus (Hernández et al. Diabetes. 2013, 62(7):2569-78).

The experimental model currently used to study retinal neurodegeneration in DR is the rat with STZ-DM. However, since STZ is neurotoxic itself (Phipps et al. Invest Ophthalmol Vis Sci 2004;45:4592–4600), a debate has arisen regarding the appropriateness of this model for examining retinal neurodegeneration shortly after STZ administration. A second rodent model, the Ins2Akita (Akita) mouse, which contains a dominant point mutation in the gene encoding for insulin-2 that induces spontaneous type 1 diabetes in the B6 mouse strain, reproduces some findings of the neurodegenerative process that occurs in the human diabetic retina. However, both STZ-DM and Akita mouse are models of type 1 diabetes and further characterization of the neurodegenerative process in type 2 models is needed. In the setting of this project, we have characterized the neurodegenerative process that occurs in the retinas of db/db mice (Bogdanov et al. PlosOne 2014). We found that the db/db mouse reproduces the features of the neurodegenerative process that occurs in the human diabetic eye. Thus, our results suggest that the db/db mouse is an appropriate experimental model for testing neuroprotective agents in DR.

We found that topical administration (eye drops) of SST prevents retinal neurodegeneration (glial activation, neural apoptosis and electroretinographical abnormalities) in db/db mice (a murine model that develops spontaneous type 2 diabetes). These findings confirmed results commented above obtained in rats with STZ-DM.

A transcriptomic analysis comparing retinas between db/db mice treated with SST eye drops and db/db mice treated with placebo has been performed and the biostatistical analysis is ongoing.
mice treated with placebo has been performed and the biostatistical analysis is ongoing. In summary, we have demonstrated that topical administration (eye drops) of SST prevents retinal neurodegeneration (glial activation, neural apoptosis and electoretinographical abnormalities). This effect can be attributed to a significant reduction of extracellular glutamate and an increase of prosurvival signaling pathways.

We also analyzed ex vivo retinal explants from 8 weeks old db/db mice treated with SST that showed a marked reduction in GFAP immunostaining, strongly suggesting that SST reduces reactive gliosis.

Potential Impact:

SCIENTIFIC COMMUNITY

At present it is unknown whether retinal neurodegeneration antedates and promotes early microvascular abnormalities in DR. We found that the neurodegeneration is not present in 1/3 of type 2 diabetic patients with early microvascular disease, but when neurodegeneration is present there is a link with early microvascular impairment. Notably, topical treatment with somatostatin and brimonidine was effective in arresting the progression of neurodegeneration in those patients in whom some degree of neurodegeneration was already present. Therefore, the identification of patients in whom neurodegeneration is present in early stages of DR will permit us to implement a more personalized and efficient medicine which could reduce the economic burden associated with DR.

Innovative methods (mfERG, FD-OCT) have been used for the first time in a large cohort of diabetic patients to monitor neurodegeneration. The standardization process has delivered validated results and encourage their widespread use in clinical practice. Used together with the study of serum biomarkers, they could be of great diagnostic and screening value for DR. Finally, these biomarkers could also be used to evaluate new drug candidates for DR.

Moreover, the insights gained on neurodegeneration processes in this project may be helpful for the understanding of other ocular diseases (i.e. glaucoma, age-related macular degeneration), and certain neurodegenerative disorders such as Alzheimer’s disease.

The EUROCONDOR project has been the trigger and backbone of other proposal based on neuroprotection for the treatment of DR (EuroTarget-DR: European Consortium for Treatment of Early Stages of Diabetic Retinopathy by Targeting GLP-1R) which was presented in the Call H2020-SC1-2016-RTD. This project has been very well evaluated and clearly surpassed the thresholds of all the items, but finally has not been funded. However all the consortium agrees that this is an excellent project and we are prepared for sending it again in the coming Calls.

HEALTH CARE SYSTEMS

EUROCONDOR will help reduce the incidence, cost and devastating effects of DR. The opening of a new non-invasive route to administer neuroprotective and/or anti-angiogenic treatments in ocular disease will eventually improve the patient-oriented treatment.
In addition, by establishing the prevalence of functional (mfERG) and morphologic (FD-OCT) changes associated with retinal neurodegeneration would allow us to propose a new flowchart for the screening of DR. The message of considering neurodegeneration as a new target to screen DR at earlier stages could be one of the most relevant impacts of our project which fits very well with the emerging concept of personalized medicine.

The cost of such a screening must be compared to the health benefits and good use of health care resources achieved with recommended diabetes interventions. Moreover, targeting prevention is usually more cost-effective than assuming the considerable medical and social costs related to treatment (laser photocoagulation, vitrectomy or intravitreous anti-VEGF therapy) and legal blindness. Nevertheless, specific pharmaco-economic studies will be necessary to obtain accurate information on this issue.

INDUSTRY

The results obtained in EUROCONDOR will enhance European competitiveness through the transformation of research into commercially successful products in the field of DR. The beneficial effects of the topical administration of Somatostatin and/or Brimonidine open a new scenario in the prevention and treatment of DR. In fact, EUROCONDOR could trigger the development of novel therapeutic strategies based on neuroprotection for treating early stages of DR, thus engaging with the pharmaceutical industry on two levels: 1) the development of new neuroprotective topical formulations based on other neuroprotective agents. This would result in the emerging of new biomedical companies with positive consequences for economic growth and employment. 2) the validation of both mfERG and FD-OCT for performing the screening of DR. These would favour economic growth and an increase in investment in innovation in those industries specialising in the manufacture of these devices. In addition, it is expected that new companies interested in this field will appear.

SME industrial/ commercial involvement to ensure exploitation of the results

Somatostatin eye-drops

BCN Peptides holds the granted patent which protects the somatostatin eye drops formulation “Topical ophthalmic peptide formulation” in USA [US9216208(B2)], Europe (EP2515870 (B1) and Japan (JP5920928 (B2)] among other countries worldwide.

BCN Peptides S.A. is an API peptide manufacturer, EDQM/AEMPS and FDA approved. The company began manufacturing Somatostatin (holding a Certificate of Suitability from the EDQM) more than 20 years ago. In this regard, it should be noted that commercial manufacture (sometimes a pitfall in the industrial exploitation of new pharmaceutical products) will be not a problem because BCN Peptides will supply the API and has wide experience in peptide formulations. Something also to highlight is that the company is authorized to work as a Pharmaceutical/Control Laboratory for the analyses of final product formulations.

BCN Peptides invests more than 15% of its sales in R&D. The company has invested its own resources in the exploration of new potential therapeutic applications of somatostatin and analogues eye drops in the prevention of DR. BCN Peptides considers the EUROCONDOR project to be of outstanding importance.
prevention of DR, BCN Peptides considers the EUROCONDOR project to be of outstanding importance and impact on its pipeline. Thanks to EUROCONDOR and to the 7FP grant, the company has arrived till phase II/phase III with somatostatin eye drops, one of the products results from its proprietary research.

After confirmation of the positive results in preventing or delaying DR, the principal aim of BCN Peptides will be to go ahead for its registration in Europe and commercialise the SST eye drops. After consultation with the European and Spanish agencies, they suggest this possibility to BCN Peptides without further trials. For this reason BCN Peptides has acted as regulatory sponsor of the clinical trial, with the objective that the results and data of the clinical trial follow Good Clinical Practices and can be presented to EMEA and National Agencies in order to obtain a Register. Nevertheless for other countries like Russia, China and Japan specific trials with local patient population will be needed in order to get market authorization on those countries.

Regarding industrial exploitation, BCN Peptides will evaluate the results from EUROCONDOR trial to see if they would be enough to support registration to the EMEA and apply for market authorisation. This would determinate the exploitation approach that the company will follow for SST eye drops.

- If we get positive enough results on the efficacy of SST eye drops the company will register the product and apply for market authorization to the EMEA. On this scenario the company will try to keep commercialization rights in Spain and try to find a partner for commercialization in other EU countries and for the development in the rest of the world. This could be achieved by a deal with a single company for global right or finding multiple companies for local deals based on selected territories. In some countries with the market authorization granted by EMEA it would be enough in other it will be necessary to perform new clinical trials on local patients to get the authorization. In these cases the partner would be in charge for SST eye drops clinical development.

- If EUROCONDOR results are not conclusive enough to get authorization by the EMEA it will be necessary to perform a Phase III trial. In this scenario the approach will be different and the company will try to find another company interested to get global rights for SST eye drops and, consequently, this new player will be in charge for SST eye drops clinical development.

In all licensing scenarios the Company would keep an exclusivity agreement for SST API production and a deal structure based on upfront and milestones after regulatory achievements.

Brimonidine eye drops

Brimonidine is a generic product. It is a current treatment for glaucoma (an ocular disease characterized by an increase of intraocular pressure), which is common in diabetic patients.

Brimonidine eye drops might also be effective in preventing or arresting DR development. However due to the high proportion of ocular adverse effects found along the clinical trial in patients treated with brimonidine, and also reported in the information for the user leaflet, its exploitation as preventive treatment would be most probably discarded.

List of Websites:
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An internet/intranet portal was generated using a Web-based secured platform (restricted access for partners only). The intranet system was considered a portal to facilitate information flow among EUROCONDOR participants, communication, training, dissemination and management of knowledge and results. It allowed the members to browse the project’s progress, update and retrieve documents.

The project’s website (www.eurocondor.eu) includes notes and news on the progress, partners’ participation in events aimed at the scientific community (congresses, meetings, symposia...) or the general public (world sight day, world diabetes day...) as well as the list of publications related to, or written on behalf of, the project.

With the perspective of the availability of the final results, the website update was planned and the sections to be added were defined by VHIR and later validated by the Dissemination Board.

The material to be added to the website will be prepared once the final results of the Clinical Trial are available and have been properly analyzed. The expected timeline for this activity is aligned with the submission of the final results for presentation at the main scientific congresses of 2017, which means by September-October 2016. The update of the website is expected to be completed by October 2016.

Maintenance and server hosting of the website have also been planned and confirmed until 2018, so as to guarantee the visibility of the project through its dedicated website.

In parallel, partner IDF-Europe relayed the most prominent news and notes on their website (http://www.idf.org/regions/europe) and through their social media platforms (Facebook and Twitter), so as to give optimal visibility to the project among most of the European diabetes associations, advocacy groups and healthcare professional associations.

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