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TGF-β1 PATHWAY AND AGE-RELATED MACULAR DEGENERATION

PhD thesis

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LIST OF ABBREVATIONS

RPE	Retinal pigment epithelium
VA	Visual acuity
BCVA	Best-corrected VA
PH	Pinhole VA
CNV	Choroideal neo-vascularization
RAPD	Relative afferent pupillary defect
FA	Fluorescein angiography
CCD	Charge-coupled device
FAZ	Foveal avascular zone
DR	Diabetic retinopathy
RVO	Retinal vein occlusion
ICGA	Indocyanine green angiography
PCV	Polypoidal choroidal vasculopathy
OCT	Optical coherence tomography
SS	Swept-source
CSR	Central serous retinopathy
FAF	Fundus autofluorescence
AMD	Age-related macular degeneration
GA	Geographic atrophy
PED	Retinal pigment epithelial detachment
RAP	Retinal angiomatous proliferation
CFH	Complement factor H
SNP	Single nucleotide polymorphism
ARMS2	Age-related mavulopathy susceptibility 2
AREDS	Age-related eye disease study
VEGF	Vascular endothelial growth factor
PDT	Photodynamic therapy
PIGF	Placental growth factor
rtPA	recombinant tissue plasminogen activator
AD	Alzheimer’s disease

Aβ	Amyloid β
APP	Amyloid precursor protein
BACE-1	beta-secretase 1
PEDF	Pigment epithelium-derived factor
SERPINF1	Serpin family F member 1
ARPE-19	Adult retinal pigment epithelial cell line-19
RAGEs	Receptor for advanced glycation end products
NF-kB	Nuclear factor- kappa B
EC	Esterified cholesterol
PC	Phosphatidylcholine
MCP-1	Monocyte chemoattractant protein-1
C3	Complement component 3
C5	Complement component 5
iC3b	inactivated C3b
IL-1β	Interleukin-1beta
TNF-α	Tumor necrosis factor alpha
IL-6	Interleukin-6
IL-8	Interleukin-8
MMP2	Matrix metalloproteinase 2
MMP9	Matrix metalloproteinase 9
ROS	Reactive oxygen species
$RSAD_2$	Radical S-adenosyl methionine domain containing 2
mRNA	messenger RNA
PET	Positron emission tomography
PiB	Pittsburgh compound-B
TGFβ-1	Trasforming growth factor beta-1
TGFβ-2	Trasforming growth factor beta-2
TGFβ-3	Trasforming growth factor beta-3
LAP	Latency-associated peptide
ALK5	Activin-like kinase 5
TGFβRI	TGFβ receptor I

TGFβRII TGFβ receptor II

SMAD Small mother against decapentaplegic

R-SMAD Small mother against decapentaplegic receptor

ERK Extracellular-regulated kinase

PI3K/AKT Phosphatidylinositol-3-kinase/protein kinase B

CSF Cerebrospinal fluid

MCI Mild cognitive impairment

hAPP human beta-amyloid precursor

NFT Neurofibrillary tangles

ALK5 Activin-like kinase 5

NGF Nerve growth factor

BDNF Brain-derived neurotrophic factor

GDNF Glial-derived neurotrophic factor

TRKB Tropomyosin receptor kinase B

Bcl-2 B cell lymphoma-2

Bcl-xl B cell lymphoma-extra large

INTRODUCTION

Anatomical landmarks

The macula is a round area at the posterior pole, lying inside the temporal vascular arcades. It measures between 5 and 6 mm in diameter, and subserves the central 15-20° of the visual field. Histologically, it shows more than one layer of ganglion cells, in contrast to the single ganglion cell layer of the peripheral retina. The inner layers of the macula contain the yellow xanthophyll carotenoid pigments lutein and zeaxanthin in far higher concentration than the peripheral retina (hence the full name 'macula lutea'- yellow plaque).

- **The fovea** is a depression in the retinal surface at the centre of the macula, with a diameter of 1.5 mm - about the same as the optic disc.
- **The foveola** forms the central floor of the fovea and has a diameter of 0.35 mm. It is the thinnest part of the retina and is devoid of ganglion cells, consisting only of a high density of cone photoreceptors and their nuclei (Fig. 1), together with Mŏller cells.
- **The umbo** is a depression in the very centre of the foveola which corresponds to the foveolar light reflect, loss of which may be an early sign of damage.
- **The foveal avascular zone**, a central area containing no blood vessels but surrounded by a continuous network of capillaries, is located within the fovea but extends beyond the foveola. The exact diameter varies with age and in disease, and its limits can be determined with accuracy only by fluorescein angiography (average .6 mm)

Retinal pigment epithelium

The retinal pigment epithelium (RPE) is composed of a single layer of cells that are hexagonal in cross-section. The cell consist of an outer non-pigmented basal element containing the nucleus, and an inner pigmented apical section containing abundant melanosomes.
The cell base is in contact with Bruch membrane, and at the cell apices multiple thread-like villous processes extend between the outer segments of the photoreceptors.
at the posterior pole, particularly at the fovea, RPE cells are taller and thinner, more regular in shape and contain more numerous and larger melanosomes than in the periphery.
RPE cells and intervening tight junctional complexes (zonula occludentes) constitute the outer blood-retinal barrier, preventing extracellular fluid leaking into the subretinal space from the choriocapillaris, and actively pumping ions and water out of the subretinal space. Its integrity is important for continued adhesion between the two, thanks to a combination of osmotic and hydrostatic forces, possibly with the aid of hemidesmosomal attachments. Facilitation of photoreceptor turnover by the phagocytosis and lysosomal degradation of outer segments following shedding.
Maintenance of the outer blood-retinal barrier is a key factor, as are the inward transport of metabolites (primarily small molecules such as amino acids and glucose) and the outward transport of metabolic waste products. The dense RPE pigment serves to absorb stray light.

Bruch membrane

The Bruch membrane separates the RPE from the choriocapillaris and on electron microscopy consists of five distinct elements:

1) The basal lamina of the RPE
2) An inner collagenous layer
3) A thicker band of elastic fibres
4) An outer collagenous layer
5) The basal lamina of the inner layer of the choriocapillaris

The RPE utilizes Bruch membrane as a route for the transport of metabolic waste products out of the retinal environment. Changes in its structure are thought to be important in the pathogenesis of many macular disorders .

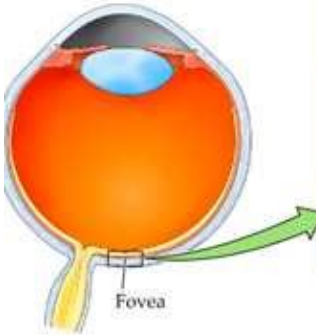

Fig.1 : Cross-section of the fovea

CLINICAL EVALUATION OF MACULAR DISEASE

The main characteristic symptoms:

- Blurried vision and difficulty with close work may be an early symptom.
- A positive scotoma , in which patients complain of something obstructing central vision, is a symptom of more severe disease. The optic neuropathy, however, leads to a missing area in the visual field(negative scotoma).
- Micropsia (decrease in image size) is caused by spreading apart of foveal cones, and is less common.
- Macropsia (increase in image size) is due to crowding together of foveal cones, and is uncommon.

- Metamorphopsia (distortion of perceived images) is a common symptom that is virtually never present in optic neuropathy.
- Colour discrimination may be disturbed, but is generally less evident than in even relatively mild optic neurophaty.
- Difficulties related to dark adaptation, such as poor vision in dim light and persistence of after-images, may occur.

VISUAL ACUITY

Distance visual acuity (VA) is directly related to the minimum angle of separation (subtended at the nodal point of the eye) between two objects that allow them to be perceived as distinct. In ptactice, it is most commonly carried out using a Snellen chart, which utilizes black letters or symbols (optotypes) of a range of sizes set on a white chart (Fig.2), with the subject reading the chart from a standard distance.

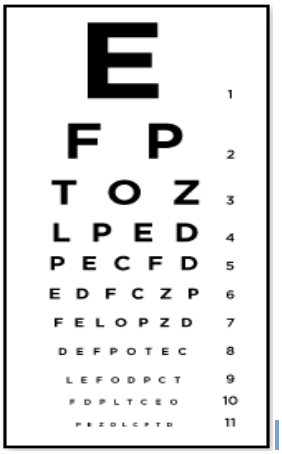

Fig.2 : Snellen visual acuity chart

- **Normal monocular VA** equates to 6/6 (metric notation) on Snellen testing. Normal corrected VA in young adults is often superior to 6/6.
- **Best-corrected VA** (BCVA) denotes the level achieved with optimal refractive correction.
- **Pinhole VA** a pinhole (PH) aperture compensates for the effect of refractive errors, and consists of an opaque occlude perforated by one or more holes of about 1 mm diameter. Howewer, PH acuity in patients with macular disease and posterior lens opacities may be worse than with spectacle correction.
- **Binocular VA** is usually superior to the better monocular VA of each eye, at least where both eyes have roughly equal vision.

CONTRAST SENSITIVITY

Is a measure of the ability of the visual system to distinguish an object against its background. A target must be sufficiently large to be seen, but must also be of high enough contrast with its background; a light grey letter will be less well seen against a white background than a black letter. Contrast sensitivity represents a different aspectof visual function to that tested by the spatial resolution tests described above, which all use high-contrast optotypes.
A lot of conditions reduce both contrast sensitivity and visual acuity (e.g. amblyopia, optic

neuropathy, some cataracts).
The Pelli-Robson contrast sensitivity letter chart is viewed and 1 metre and consists of rows of letters of equal size but with decreasing contrast of 0.15 log units for groups of three letters.

NEAR VISUAL ACUITY

Near vision testing can be a sensitive indicator of the presence of macular disease. The chart is held at a comfortable reading distanceand this is measured and noted. The patient wears any necessary distance correction together with a presbyopia correction if applicable

AMSLER GRID

Evaluates the 20° of the visual field centred on fixation (Fig.3). It is principally useful in screening for and monitoring macular disease, but will demonstrate central visual field defects originating elsewhere.

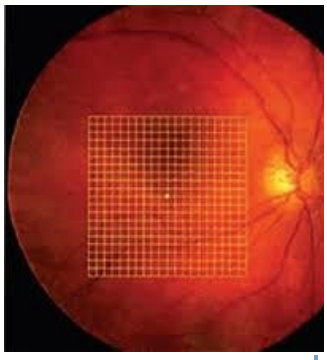

Fig.3 : Amsler grid superimposed on the macula

PUPILS

The pupillary reactions to light are usually normal in eyes with macular disease, although extensive pathology such as a large area of CNV (choroideal neo-vascularization) can give a relative afferent pupillary defect (RAPD). In contrast, an RAPD occurs in relatively mild cases of asymmetrical optic neuropathy.

COLOUR VISION

Colour vision is commonly affected only in proportion to the decrease in visual acuity in macular disease, again in contrast to optic neuropathy were subtle colour desaturation is an early sign.

INVESTIGATION OF MACULAR DISEASE

MICROPERIMETRY

This is a newer investigative technique that has hitherto been used principally in research but may increasingly be incorporated into clinical practice. It measures sensitivity at finely spaced central retinal loci, including in patients with poor fixation, and uses a tracking system based on image registration to facilitate serial monitoring, allowing detection of subtle change.

FUNDUS FLUORESCEIN ANGIOGRAPHY

Fluorescein angiography (FA) should be performed only if the findings are likely to influence management.

- **Fluorescence** is the property of certain molecules to emit light of a longer wavelength when stimulated by light of a shorter wavelength. The excitation peak for fluorescein is about 490 nm (in the blue part of the spectrum). Stimulated molecules will emit yellow-green light of about 530 nm (Fig.4)

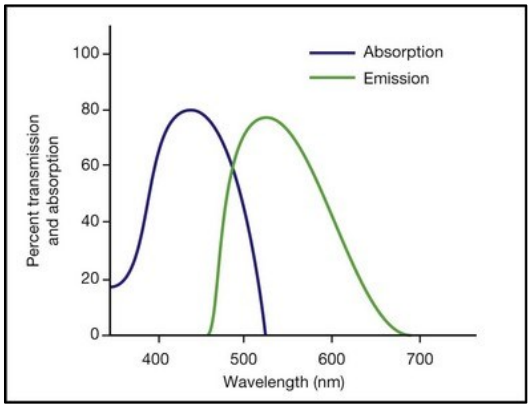

Fig.4: Absorption and emission curves of sodium fluorescein dye. The peak absorption (excitation) is at 465–490 nm (blue light). The peak emission occurs at 520–530 nm (yellow–green light).

- **Fluorescein** (sodium fluorescein) is an orange water-soluble dye that, when injected intravenously, remains largely intravascular. It is excreted in the urine over 24-36 hours.
- **Outer blood-retinal barrier.** The major choroidal vessels are impermeable to both bound and free fluorescein. However, the walls of the choriocapillaris contain fenestrations through which unbound molecules escape into the extravascular space, crossing Bruch membrane but on reaching the RPE are blocked by intracellular complexes termed tight junctions or zonula occludentes.
- **Inner blood-retinal barrier** is composed principally of the tight junctions between retinal capillary endothelial cells, across which neither bound nor free fluorescein can pass; the basement membrane and pericytes play only a minor role in this regard. Disruption of the

inner blood-retinal barrier permits leakage of both bound and free fluorescein into the extravascular space

- **Filters**
  - cobalt blue excitation: incident white light from the camera is filtered so that blue light enters the eye, exciting the fluorescein molecules in the retinal and choroidal circulations.
  - Yellow-green barrier filter blocks any blue light reflected from the eye, allowing only yellow-green emitted light to pass.
- **Image capture** in modern digital cameras uses a charge-coupled device (CCD). Modern device typically require a lower concentration of injected fluorescein to obtain high-quality images, with a lower incidence of adverse effects.

Technique

Adequate pharmacological mydriasis is important to obtain high-quality images; media opacity such as cataract may reduce picture quality. It is important to mention common and serious adverse effects, particularly the discoloration of skin and urine; the patient should be seated in front of the fundus camera, and colour photographs, red-free (green incident light, to enhance red detail)and autofluorescence images taken as indicated.
An intravenous cannula is inserted and fluorescein (usually 5 ml of 10% solution) is injected in 5-10 seconds; a 5 ml vial of 10% (100 mg/ml) sodium fluorescein contains 500 mg and pictures should be taken over 20-60 minutes following ingestion.

Angiografic phases

Fluorescein enters the eye through the ophthalmic artery, passing into the choroidal circulation through the short posterior ciliary arteries and into the retinal circulation through the central retinal artery. The angiogram consists of the following overlapping phases:
The choroidal (pre-arterial) phase typically occurs 9-15 seconds after dye injection and is characterized by patchy lobular filling of the choroid due to leakage of free fluorescein from the fenestrated choriocapillaris.
The arterial phase start about a second after the onset of choroidal fluorescence, and shows retinal arteriolar filling and the continuation of choroidal fillin.
The arteriovenous (capillary) phase shows complete filling of the arteries and capillaries with early laminar flow in the veins in which the dye appears to line the venous wall leaving an axial hypofluorescent strip. This phenomenon reflects initial drainage from posterior pole capillaries filling the venous margins, as well as the small-vessel velocity profile, with faster plasma flow adjacent to vessel walls where cellular concentration is lower.
The venous phase. Laminar venous flow pregresses to complete filling, with late venous phase featuring reducing arterial fluorescence.
The late (recirculation) phase demonstrates the effects of continuous recirculation, dilution and elimination of the dye. Fluorescein is absent from the retinal vasculature after about 10 minutes.
The dark appearance of the fovea is caused by three factors:

- absence of blood vessels in the FAZ;
- blockage of background choroidal fluorescence due to the high density of xanthophyll at the fovea
- blockage of background choroidal fluorescence by the RPE cells at the fovea, which are larger and contain more melanin and lipofuscin than elsewhere in the retina.

Causes of hyperfluorescence

- Autofluorescentcompounds absorb blue light and emit yellow-green light in a similar fashion to fluorescein, but much more weakly. Autofluorescent lesions classically include optic nerve head drusen and astrocytic hamartoma.
- Pseudofluorescence (false fluorescence) refers to non-fluorescent reflected light visible prior to fluorescein injection.
- A window defect is caused by atrophy or absence of the RPE as in atrophic age-related macular degeneration, a full-thickness macular hole, RPE tears and some drusen.
- Pooling in an anatomical space occurs due to breakdown of the outer blood-retinal barrier (RPE tight junction). As regards the subretinal space, it is characterized by early hyperfluorescence, which, as the responsible leak tends to be only small, slowly increases in intensity and area. Instead , in the sub-RPE space occurs an early hyperfluorescence that increases in intensity but not in size.
- Leakage of dye is characterized by fairly early hyperfluorescence, increasing with time in both area and intensity. It occurs as a result of breakdown of the inner blood-retinal barrier due to: dysfunction or loss of existing vascular endothelial tight junctions as in background diabetic retinopathy (DR), retinal vein occlusion (RVO) and papilloedema; primary absence of vascular endothelial tight junctions as in CNV, proliferative diabetic retinopathy and tumours.

Causes of hypofluorescence

- Masking of retinal fluorescence. Preretinal lesions such as blood will block all fluorescence.
- Masking of background choroidal fluorescence allows persistence of fluorescence from superficial retinal vessels: deeper retinal lesions, subretinal or sub-RPE lesions, increased density of the RPE, choroidal lesions
- Filling defects may results from: vascular occlusion, which may involve the retinal arteries, veins or capillaries; loss of the vascular bed as in myopic degeneration and choroideremia.

INDOCYANINE GREEN ANGIOGRAPHY

Whilst FA is an excellent method of studying the retinal circulation, it is of limited use in delineating the choroidal vasculature , due principally to masking by the RPE. In contrast, the near-infrared light utilized in indocyanine green angiography (ICGA) penetrates ocular pigments such as melanin and xanthophyll, as well as exudate and thin layers of subretinal blood, making this technique eminently suitable. An additional factor is that about 98% of ICG molecules bind to serum protein (mainly albumin), considerably higher than the binding of fluorescein.
ICG fluorescence is only $1/25^{th}$ that of fluorescein so modern digital ICGA uses high-sensitivity videoangiographic image capture by means of an appropriately adapted camera. The technique is similar to that of FA, but with an increased emphasis on the acquisition of later images than that FA.

Diagnosis

Examples of the pathological images are shown under the discussion of individual conditions where relevant.

Hyperfluorescence

- A window defect similar to those seen with FA.
- Leakage from retinal or choroidal vessels, the optic nerve head or the RPE; this gives rise to tissue staining or to pooling.
- Abnormal retinal or choroidal vessels with an anomalous morphology and/or exhibiting greater fluorescence than normal.

Hypofluorescence: blockage of fluorescence. Pigment and blood are self-evident causes, but fibrosis, infiltrate, exudate and serous fluid also block fluorescence.

Indications

- **Polypoidal choroidal vasculopathy (PCV)**: ICGA is far superior to FA for the imaging of PCV.
- **Exudative age-related macular degeneration**: conventional FA remains the primary method of assessment, but ICGA can be a useful adjunct, particularly if PCV is suspected.
- **Chronic central serous chorioretinopathy**: in which it is often difficult to interpret areas of leakage on FA.
- **Posterior uveitis**: ICGA can provide useful information beyond that available from FA in relation to diagnosis and the extent of disease involvement.
- **Choroidal tumours**.
- **Breaks in Bruch membrane**.
- **If FA is contraindicated**.

OPTICAL COHERENCE TOMOGRAPHY

Optical coherence tomography (OCT) is a non-invasive, non-contact imaging system providing high resolution cross-sectional images of the posterior segment. Imaging of the anterior segment has also been increasingly adopted. OCT is analogous to B-scan ultrasonography but uses near-infrared light interferometry rather than sounds waves, with images created by the analysis of interference between reflected reference waves and those reflected by tissue. As regards instruments promising newer modalities include swept-source (SS) OCT that can acquire images at a much higher rate and with extremely high retinal element resolution and better imaging depth; choroidal definition is improving rapidly.

Applications

- Macula: the diagnosis and monitoring of macular pathology has been revolutionized by the advent of OCT imaging, e.g. AMD, diabeti maculopathy, macular hole, epiretinal membrane and vitreomacular traction, CSR and retinal venous occlusion.
- Glaucoma: the widespread availability of OCT in ophthalmology suites for the assessment of medical retinal disease has contributed to its increased adoption as an adjunct to clinical and perimetric assessment in the management of glaucoma.
- Retinal detachment: distinction of retinal detachment from retinoschisis.

- Anterior segment OCT: has an expanding range of clinical applications such as suspected angle-closure glaucoma and corneal analysis.

FUNDUS AUTOFLUORESCENCE

Imaging of fundus autofluorescence (FAF) using an enhanced fundus camera or scanning laser ophthalmoscopy permits visualisation of accumulated lipofuscin in the retinal pigment epithelium.the scope of its place in the clinical management of macular degeneration and other conditions has not yet been clearly defined. It can be useful, for instance, to demonstrate more extensive macular disease than is visible clinically, in order either to determine the cause of unexplained poor visual acuity or to establish the reason for substantial visual symptoms despite good measured acuity. There is speculation that it may have greater utility in the future in the management of dry AMD once effective therapies become available.

AGE-RELATED MACULAR DEGENERATION

Age-related macular degeneration (AMD) is a degenerative disorder affecting the macula. It is characterized by the presence of specific clinical findings, including drusen and RPE changes, in the absence of another disorder. Later stages of the disease are associated with impairment of vision.

Classification

Conventionally AMD has been divided into two main types:

I. **Dry** (non-exudative, non-neovascular) AMD is the most common form, comprising around 90% of diagnosed disease. Geographic atrophy (GA) is the advanced stage of dry AMD; it has been authoritatively suggest that the term "dry AMD" be used only to describe GA rather than earlier stages of AMD.

II. **Wet** (exudative, neovascular) AMD is much less common than dry, but is associated with more rapid progression to advanced sight loss. The main manifestations are CNV and PED, though in recent years at least two additional conditions, retinal angiomatous proliferation (RAP) and polypoidal choroidal vasculopathy (PVC), have been included under the umbrella of neurovascular AMD.

A recent expert consensus committee has provided a clinical classification of AMD (table 1)

Epidemiology

AMD is the most common cause of irreversible visual loss in industrialized countries. In the USA, it is responsible for around 54% of severe sight loss (better eye worse than 6/60) in Caucasian, 14% Hispanic and 4% in black individuals. The prevalence increases with age and symptoms are rare in patients under 50 years of age. In the UK, significant visual impairment (binocularly 6/18 or worse) from AMD affects about 4% of the population aged over 75 years and 14% of those over 90, with 1.6% over 75 having binocular acuity of less than 6/60.
Patients with late AMD in one eye, or even moderate vision loss due to non-advanced AMD in one eye, have about a 50% chance of developing advanced AMD in the fellow eye within 5 years.

Risk factors

AMD is multifactorial in aetiology, and is thought to involve a complex interaction between polygenic, lifestyle and environmental factors:

- Age: is the major risk factor.
- Race: late AMD is more common in white individuals than those of other races.
- Heredity: family history is important; the risk of AMD is up to three times as high if a first-degree relative has the disease. Variants in many genes have been implicated in AMD risk and protection such as the complement factor H gene *CFH*, which helps to protect cells from complement-mediated damage, with several times the risk of AMD for homozygotes with a particular single nucleotide polymorphism (SNP), and the *ARMS2* gene on chromosome 10. Genes related to lipid metabolism are also thought to be important.
- Smoking: doubles the risk of AMD.
- Hypertension
- Dietary factors: high fat intake and obesity may promote AMD, with high antioxidant intake having a protective effect in some groups.

- Other factors: such as cataract surgery, blue iris colour, high sunlight exposure and female gender are suspected, but their influence remains less certain.

Table 1: clinical classification of age-related macular degeneration (AMD)

Category	Definition **(based on presence of lesions within two disc diameters of the fovea in either eye)**
No apparent ageing changes	No drusen No AMD pigmentary abnormalities
Normal ageing changes	Only drupelets No AMD pigmentary abnormalities
Early AMD	Medium drusen (>63 µm but <125 µm) No AMD pigmentary abnormalities
Intermediate AMD	Large drusen (>125µm). Any AMD pigmentary abnormalities
Late AMD	Neovascular AMD and/or any geographic atrophy

DRUSEN

Histopathology

Drusen are extracellular deposits located at the interface between the RPE and Bruch membrane. The material of which are composed has a broad range of constituents, and is thought to be derived from immune-mediated and metabolic processes in the RPE. Their precise role in the pathogenesis of AMD is unclear, but is positively associated with the size of lesions and the presence or absence of associated pigmentary abnormalities. Age-related drusen are rare prior to the age of 40, but are common by the sixth decade. The distributions is highly variable and they may be confined to the fovea, may encircle it or form a band around the macular periphery.

Clinical features

There is a strong association between the size of drusen and the risk of developing late AMD over 5-year period.

a) *Small drusen(drupelets)*, sometimes termed "hard" drusen, are typically well-defined white-yellow and by definition measure <63 µm in diameter.
b) *Intermediate drusen* are fairly well-defined yellow-white focal deposits at the level of the RPE measuring between 63 µm and 125 µm.
c) *Large drusen* are less well delineated yellow-white deep retinal lesions measuring over 125 µm in diameter. The presence of large drusen in both eyes is associated with a 13% risk of progression to late AMD over 5 years, but with accompanying bilateral pigmentary abnormalities this rises to about 50%.

OCT

Medium-sized and large drusen are seen as hyper-reflective irregular nodules beneath the RPE, located on or within the Bruch membrane.

Fluorescein angiography

FA findings depend on the state of the overlying RPE and on the affinity of the drusen for fluorescein. Hyperfluorescence can be caused by a window defect due to atrophy of the overlying RPE, or by late

staining.Hypofluorescencedrusen masking background fluorescence are hydrophobic, with a high lipid content, and tend not to stain.

ANTIOXIDANT SUPPLEMENTATION

There is substantial evidence, notably from the Age-Related Eye Disease Study (AREDS, now known as $AREDS_1$) and the follow-up $AREDS_2$, that taking high-dose antioxidant vitamins and minerals on a regular basis can decrease the risk of the development of advanced AMD in individuals with certain dry AMD features.
The regimen used in $AREDS_1$ consisted of vitamin C, vitamin E, the beta-carotene form of vitamin A, and 80 mg daily of zinc. Because high doses of zinc and beta carotene create problems to other districts, $AREDS_2$ looked at adjusting this components. $AREDS_2$ found that lutein and zeaxanthin are a safe alternative to beta-carotene, and are probably superior (18% reduction in risk of advanced AMD). Therefore recommended daily supplementation based on $AREDS_2$, are:

- Vitamin E (400 IU)
- Vitamin C (500 mg)
- Lutein (10 mg)
- Zeaxanthin (2 mg)
- Zinc (25-80 mg)
- Copper (2 mg)

NON-EXUDATIVE (dry, non-neovascular) AMD

Diagnosis

Symptomsconsist of gradual impairment of vision over month or years. Both eyes are usually affected, but often asymmetrically.
Sygnsin approximately chronological order:
- numerous intermediate-large soft drusen
- focal hyper-and/or hypopigmentation of the RPE
- sharply circumscribed areas of RPE atrophy associated with variable loss of the retina and choriocapillaris
- drusenoid RPE detachment.

OCT

Loss of RPE and morphological alterations of the overlying retina of increasing severity are seen in GA. Outer retina tubulations may be seen; outer retinal corrugations. This recently described phenomenon is an undulating hyper-reflective layer on OCT thought to correspond to the histological finding of basal *laminar* deposit, a layer that accumulates between the RPE and the RPE basement membrane in AMD. Basal *linear* deposit is a distinct finding consisting of membranous debris laid down between the RPE basement membrane and the inner collagenous layer of the Bruch membrane that may progress focally to form drusen.

FA

FA of atrophic areas shows a window defect due to unmasking of background choroidal fluorescence, if the choriocapillaris is still intact.

Potential new therapies

An extensive range of therapies shows promise for the treatment of dry AMD. Lampalizumab, for example, is a complement-inhibiting monoclonal antibody injected intravitreally on a monthly basis reduced progression of GA by 44%. More, preliminary evidence suggest a neuroprotective effect of saffron (20 mg/day). Others therapies include subretinal stem cell transplantation and intravitreal injection of a range of drugs including ciliary neurotrophic factor, steroid insert and neuroprotective drugs including brimonidine.

RETINAL PIGMENT EPITHELIAL DETACHMENT

Pigment epithelial detachment (PED) is the condition in which there is fluid beneath the retinal pigment epithelium (RPE). PED has many causes but the most common are AMD and central serous choroidopathy. It is caused by disruption of the physiological forces maintaining adhesion. There are different types of PED:

- *Seruos PED*: blurred central vision and metamorphosia; an orange dome-shaped elevation with sharply delineated edges, often with a paler margin of subretinal fluid. Are also associated blood and lipid exudation. As regards the therapies, intravitreal injections of vascular endothelial growth factor (VEGF) inhibitor may stabilize or improve vision; combining photodynamic therapy (PDT) with intravitreal anti-VEGF can also be effective.
- *Fibrovascular PED*: by definition fibrovascular PED represents a form of "occult" CNV. It is much more irregular in outline and elevation than serous PED.
- *Drusenoid PED*: develops from confluent large soft drusen, and is often bilateral. There are shallow elevated pale areas with irregular edges. The outlook is usually better than other forms of PED, with only gradual visual loss
- *Haemorrhagic PED*: virtually every haemorrhagic PED has underlying CNV or polypoidal choroidal vasculopathy (PCV). It is characterized by: sudden impairment of central vision, elevated dark red dome-shaped lesion with a well-defined outline and blood may break through into the subretinal space, assuming a more diffuse outline and a lighter red colour.

RETINAL PIGMENT EPITHELIAL TEAR

Tears may occur spontaneously , following laser or after intravitreal injection. Older patients and large irregular PEDs associated with CNV are at higher risk. Occurs a sudden fall in vision with foveal involvement. A crescent-shaped pale area of RPE dehiscence is seen, next to a darker area corresponding to the retracted and folded flap.

CHOROIDAL NEOVASCULARIZATION (CNV)

Choroidal neurovascularization (CNV) consists of a blood vessel complex that extends through Bruch membrane from the choriocapillaris into the sub-RPE (type 1) or subretinal (type 2) space. It occurs in many different disorders, usually when Bruch membrane and/or RPE function has been compromised by a degenerative, inflammatory, traumatic or neoplastic process. AMD is the most common causative association, followed by myopic degeneration. Often the CNV is the first lesion in neovascular AMD; understanding of aetiopathogenesis has improved over recent years. The promotion and inhibition of blood vessel growth by cytokines is important, particularly vascular endothelial growth factor (VEGF). It binds to endothelial cell receptors, promoting proliferation and vascular leakage.
There is an acute or subacute painless blurring of vision, usually with metamorphosia. The CNV itself may be identifiable as a grey-green or pinkish-yellow lesion and medium-large drusenare a typical finding in the same or fellow eye. Also haemorrhage is common with an intra- and subretinal lipid deposition, sometimes extensive.

Treatment with anti-VEGF agents

Inhibitor of VEGF block its interaction with receptors on the endothelial cell surface and so retard or reverse vessel growth. They have become the predominant means of treatment for CNV. Intravitreal injection is the standard method of administration, notable risks including retinal detachment, damage to lens, RPE tears and endophthalmitis. All available anti-VEGF agents seems to have potential for benefit in a range of vascular eye diseases. Every CNV subtypes respond to anti-VEGF therapy, but benefits is only likely in the presence of active disease.
Alfibercept (Eylea®) is a recombinant fusion protein that binds to VEGF-A, VEGF-B and placental growth factor (PIGF). It was adopted rapidly into clinical practice, principally because the recommended maintenance regimen consists of one injection every 2 months in contrast to the monthly injections recommended with ranibizumaband bevacizumab. The standard dose is 2 mg in 0.05 ml.
Ranibizumab (Lucentis®) is a humanized monoclonal antibody fragment developed specifically for use in the eye, though is derived from the same parent mouse antibody as bevacizumab. It non-selectively binds and inhibits all isoform of VEGF-A. The usual dose is 0.5 mg in 0.05 ml. Three main treatment strategies are adopted in AMD.
Bevacizumab (Avastin®) is a complete antibody originally developed to target blood vessel growth in metastatic cancer deposits. Treatment strategies in AMD are similar to those used for ranibizumab. The dose of bevacizumab is usually 1.25 mg/0.05 ml.
Pegaptanib (Macugen®) was the first anti-VEGF agent approved by regulatory authorities for ocular treatment.

Treatment with photodynamic therapy (PDT)

Verteporfin is a light-activated compound preferentially taken up by dividing cells including neovascular tissue. It is infused intravenously and then activated by diode laser to cause thrombosis. With the avvention of anti-VEGF treatment, PDT is now rarely used for CNV.

HAEMORRAGIC AMD

The visual prognosis for most eyes with extensive subretinal or sub-RPE haemorrhage is relatively poor. Some results superior to the untreated course have been reported for intravitreal anti-VEGF injection alone, and liquefaction of blood by intravitreal recombinant tissue plasminogen activator (rtPA) and pneumatic displacement may be appropriate for large or thick haemorrhage. If the patient takes a coumarin anticoagulant, liaison with the prescribing physician is worthwhile to assess if this could reasonably be stopped- there is an association with massive macular haemorrhage. Antiplatelet drugs do not usually require discontinuation, though aspirin may be associated with a greater risk of CNV than other agents.

AGE-RELATED MACULAR DEGENERATION AND ALZHEIMER'S DISEASE

Both diseases age-relatedmacular degeneration of the eye (AMD) and Alzheimer's disease of the brain (AD) share a common risk: the age factor. These are two diseases whichcause irreversible damage: AMD is the most common cause of blindness.[Gehrs et al., 2006], AD is the most common form of dementiain the world[Hirtz et al.,2007].
The brain and the retina are derived from the neural tube and both have blood tissue barriers, for these reasons they have many common features.

Alzheimer's disease is.mainly characterized by memory loss, with disoriented behaviour and impairments in language, comprehension, and spatial skills also characterizing this disorder. Neuropsychiatric symptoms, such as agitation and psychosis are also frequent in people with AD, and are a common precipitant of institutional care.

There are different clinical and pathological features that pool these two diseases, such as events of oxidative stress and inflammation or the molecular similaritiesof the deposits that are typically accumulated. Also, the condition of oxidative stress and inflammation leads to the activation of the protein aggregation especially in aged post-mitotic cells, such as neurons and RPE [Kaarniranta et al.,2011].

Molecular similarities and molecular links between age-related macular degeneration and AD

The most important feature that links AMD and AD is the presence and composition of extracellular deposits, that in AMD are called drusen and in AD are referred to as senile plaques. The presence of large and confluent drusen is a strong risk factor for developing choroidal neovascularisation (CNV), a complication of the wet type of AMD[Bressler et al.,1990]. The typical plaques of AD are deposited in the hippocampus and in the brain cortex and they can be diffuse, primitive, cored or compact amyloid plaques [Atwood et al.,2002].

Amyloid β (Aβ)

The Aβs are 36- to 43-amino acid peptides as the natural products of metabolism. There are two isoforms of secreted Aβ: the $A\beta_{1-40}$ and $A\beta_{1-42}$. The proteolysis of amyloid precursor protein (APP) generates Aβ peptides by some sequentially enzymatic hydrolysis at the β-sites by APP-cleaving

enzyme 1 (BACE-1), β- and γ-secretase, and protein complexes that contain presenilin1 at the catalytic core [Haass et al., 2007]. There are two physical forms of aggregates of Aβ: one of them is characterized by 2-6 peptide oligomers that create intermediate assemblies. Another form is represented by fibrils organised into β-pleated sheets forming insoluble fibres of advanced amyloid plaques. In normal retina and in normal brain there are small amounts of Aβ[Anderton et al.,1997] and these levels are subject to increase with age. In the retina these deposits take place primarily among the photoreceptor outer segments and on the interface between the RPE and Bruch's membrane, but also Aβ is deposited in the vascular network of the inner and the outer retina.The first scientists who identify Aβ drusen in the eye with AMD were Johnson et al.in 2002 [Johnson et al.,2002] and showed that the Aβ was part of a substructural vesicular component within the drusen. Also Dentchev et al. [Dentchev et al.,2003] showed these results demonstrating that Aβ accumulations were exclusive of drusen in eyes with AMD too.
Anderson et al. [Anderson et al., 2004]demonstrated, through the immunoelectron microscopy, the structural features of the Aβ-containing material in drusen. They showed the ultrastructure of the spherical Aβ-containing elements, which were composed of a central core, with one or more concentric inner rings. The most of the Aβ immunoreactive mature fibrils were associated with the outer layers, which consisted of densely-packed spherical subunits. Luibl et al [Luibl et al.,2006]through the use of an anti-oligomer antibody proved the presence of toxic nonfibrillar oligomers in the drusen, especially in the centre of drusen. They called this structure "amyloid oligomer cores"; usually the size of these cores is the same in both large and small drusen, sometimes in large drusenoccurs coalescence of smaller drusen.
It is still unclear which form of Aβ is more prevalent in normal human retina and in the drusen of AMD patients between $A\beta_{1-40}$ or $A\beta_{1-42}$, in fact Prakasam et al.[Prakasam et al.,2010], through the use of a Aβ ELISA kit measured the levels of this two forms in different eye tissues from bovine and mouse. Also Dutescu et al. [Dutescu et al.,2009]showed that Aβ was not detectable in the retina or the RPE, but significant amounts of $A\beta_{1-40}$ or $A\beta_{1-42}$were present in the aqueous and vitreous humors of all the eyes examined. Anyway the $A\beta_{1-40}$ might be the predominant form in the eye. As regards the brain, the Aβ levels are normally low because usually it is secreted in the cerebrospinal fluid. According to this scenario it would berelevant to understand the role of Aβ in development of AMD. Yoshida et al. [Yoshida et al.,2005]demonstrated that human RPE cells express constitutively all of the genes that are involved in Aβ production: APP, α-, β-, γ-secretase and neprilysin. Also,it is interesting the evidence that the exposure of cultured human RPE cells to Aβ induced a significant increase in the expression of VEGF and a significant decrease in the expression of pigment epithelium-derived factor (PEDF). PEDF is also known as serpin F1 (SERPINF1),it is a multifunctional secreted protein that has anti-angiogenic, anti-tumorigenic, and neurotrophic functions[Filleur et al., 2009]. It was originally identified in the retina[Dawson et al., 1999]and was secreted by RPE cells[Tombran-Tink et al.,1991] .Yoshida et al. have reported that there is an important equilibrium shift between VEGF and PEDF and this balance is crucial for the development of AMD. Neprilysin is a zinc-dependent metalloprotease enzyme that degrades a series of small secreted peptides, in particular the peptide Aβ [Pardossi-Piquard et al.,2006]; it has been demonstrated, through immunohistochemical analyses, that in senescent neprilysin gene-deficient mice (neprilysin -/- mice) there was an up-regulation of VEGF and a down-regulation of PEDF in comparison to wild type mice. Moreover, Ma et al. [Ma et al.,2007]showed that oligomeric forms of Aβ up-regulated VEGF secretion in ARPE-19 cellsthrough the binding to receptors for advanced glycation end products (RAGEs); this mechanism was widely dependent on NF-κBsignalling pathway.
Proteomic analyses revealed an high similarity between the molecular components of senile plaques and drusen, as regards proteoglycans, inflammatory mediators, metal ions (Fe,Cu,Zn), proteases and

clearance-related elements, α2-macroglobulin, cholinesterases, serum amyloid P component, apolipoprotein E, immunoglobulin and basement membrane matrices. Also a lot of complement activators are present in both elements (plaques and drusen)in association with complement components and complement regulatory proteins [Anderson et al.,2010; Hageman et al.,2005; Johnson et al.,2001; Johnson et al.,2006; Eikelenboom et al., 1982; Eikelenboom et al.,1996; Reichwald et al., 2009;Zanjani et al.,2005]. Obviously this suggests the existence of possible similar and common pathways involved in the aetiologiesof both diseases. Recently, Wang et al. [Wang et al.,2010] identified the amount of lipids and proteins present in drusen from 36 human retinas obtained >6h after death; the major components of drusen were esterified cholesterol (EC) and phosphatidylcholine (PC). This suggests that alterations of metabolism of cholesterol and other related molecules could contribute to the genesis of AMD by increasing the production of Aβ.Also in patients at early stage of AD an increased level of total cholesterol has been found [Wolozin et al., 2004].

The deposition of Aβ causes AMD and AD

- *Role of Chronic inflammation*

Chronic inflammation seems to be a common factor in both diseases[Donoso et al., 2006; Penfold et al., 2001; Cameron et al.,2010; Mandrekar-Colucci et al., 2010]. There are a lot of scientific evidence that demonstratean important role of complement activation in the pathogenesis of AMD [Bonifati et al., 2007; Anderson et al., 2010; Gehrs et al., 2006]and AD, in fact various kind of complement components exists in the brains of AD patients and in the drusen of AMD eyes. All the scientific evidences available, confirm that the complement activation at the level of Bruch's membrane is a core process in drusen formation, and demolition of the integrity of Bruch's membrane is linked to wet AMD.
Aβ is known to influence the alternative complement activation pathway; human RPE cells express viz., C3, C5, factor B, factor D, factor H and factor I. Some experiments showed that Aβ binds directly to complement factor I, which blocks its ability to cleave C3b and inactivate iC3b (factor I and factor H are soluble complement-activation inhibitors) [Wang et al., 2008]. This suggests that Aβ triggers the complement system by stopping the activity of factor I, bringing to chronic inflammation in the subretinal tissues. As regard the main activator of the alternative complement pathway (factor B) it has been shown that Aβ did not modulate the expression of factor B in RPE cells, but it directly enhanced the production of monocyte chemoattractant protein-1 (MCP-1)[Wang et al., 2009; Hageman et al., 2005].Aβ also increased the production of IL-1β, TNF-α in macrophages/microglia and the exposure of RPE cells to IL-1β and TNF-α significantly up-regulated factor B. ThereforeAβ stimulates RPE cells to produce MCP-1 , it recruits macrophages/microglia which produce cytokines that acts on factor B in RPE cells, up-regulating its expression. Scholl et al. [Scholl et al., 2008]measured the concentrations of complement activation products in plasma of AMD patients and found that Ba and C3d (markers of chronic complement activation) were significantly elevated, compared to controls.

- *The role of microglia and other inflammatory cells*

Microglial activation is one of the mechanisms involved in the pathologenesis of AD, in fact Aβs are excellent activators of microglial cells. These cells and reactive astrocytes migrate to fibrillary plaques and the inflammation marker linked to these cells are elevated in the brains of AD patients [Wyss-Coray et al., 2002]. As in all inflammatory processes the phagocytic microglia engulf and degrade Aβwith a subsequentand large production of chemokines (IL-1, IL-6 and TNFα) [Akiyama et al., 2000].Also, microglia express RAGEs which bind Aβ, stimulating in this way the production of

cytokines, glutamate and nitric oxide [Li et al., 2003; Yan et al., 1996].The RPE cells are important for the maintenance of immune balance in the subretinal space with the production of immunosuppressive factors; this normal condition is also maintained by the absence of microglial cells in this site [Chen et al., 2002]. Retinal microglial cells move from their normal position to the inner retina to get close to the RPE in the subretinal space, in AMD eyes and in animal models of AMD[Combadiere et al., 2007]. The activated condition of retinal microglial cells, observed in AMD eyes, have ameboid morphological structures that represent their activated status [Luibl et al., 2006] . Ma et al. [Ma et al., 2009]studied the effects of retinal microglia on RPE cells by co-culturing RPE cells and activated microglial cells. These onescaused changes in the structure and composition of RPE cells, increasing the expression and secretion of pro-inflammatory, chemotactic and proangiogenic molecules, with an increase of VEGF, MMP2, MMP9, too. All these variations of the normal immunological and molecular equilibrium would then enhance the progression of AMD.

- *Oxidative stress*

It's a common condition among AMD [Beatty et al., 2000; Brennan et al., 2009] and AD [Darvesh et al., 2010; Querfurth et al., 2010]and it's linked to age. Photoreceptors are easily exposed to oxidative stress because they contain lipofuscin (a photo-inducible generator of ROS)[Feeney-Burns et al., 1984; Feeney, 1978]and Aβ is a potent mitochondrial toxin that affects the neurosynaptic pool [Priller et al., 2006]in fact induces mitochondrial dysfunction and oxidative stress in RPE cells [Butterfield et al., 2004].

- *Alteration of RPE cells gene expression*

Microglia and neural cells are the main targets in the brain for the deposition of Aβ. Instead in the retina the major cell type affected by Aβ is RPE cells. Some scientists studied the genome-wide changes in gene expression of RPE cells stimulated with $A\beta_{1-40}$ by gene microarray and RT-PCR [Kurji et al., 2010]. Essentially the up-regulate genes were member of inflammatory and immune categories, mainly IL-1β, RSAD2 (Radical S-Adenosyl Methionine Domain Containing 2) and IL-8, which are involved also in the angiogenic responses in the RPE/choroidal layers. As regard the $A\beta_{1-42}$Bruban et al. [Bruban et al., 2009]showed that it induces a disorganisation of cytoskeletal actin filament with also a decrease expression of tight junction proteins (occludin and zonula occludens-1) in RPE cells. The loss of cell adhesion involves in the irreversible damage of the blood-retina barrier.

Genetic background

Several research groups have studied the genetic basis of AD and AMD diseases. AMD was the first in which the GWAS (genome-wide association) identified *CFH* gene as the responsible gene [Edwards et al., 2005;Haines et al., 2005; Henning et al., 2005]. Mainly, variations in regulatory region for complement activation (which contains multiple haplotype) alter the risk of AMD. In fact, for example, modifications in the C3 locus are significantly associated with the development of this disease [**Spencer** et al., 2008; **Yates et al.,** 2007].This observation further support for the involvement of the alternative pathway of complement activation in the pathogenesis of AMD. The uncontrolled activation of the alternative pathway of complement at the level of Bruch's membrane is thought to be a key element in the process of drusen formation and a major contributing factor to the pathogenesis of AMD [Gehrs et al., 2010].

Patients with AD and AMD

It was not yet perfectly defined the AMD frequency among patients with AD. However, it was reported a common condition of abnormalities and damages of visual pathways in patients with AD, like optic nerve degeneration, ganglion cell degeneration and decreased thickness of the retinal nerve fibre layer [Parisi et al., 2001]. In the retinas of AD patients Aβ is deposited around the major retinal vessels in the inner surface of the retina, as it was showed in amyloid angiophaty of the brain.Aβ depositsare known to damage the ganglion cell layer, cause the death of the ganglion cells with a thinning of nerve fibre layer, mimicking glaucoma.

The Aβ deposition in the brain and macular area of retina

It is clear that the accumulation of Aβ due to loss of balance between its production and clearance, may be the initiating factor bothin AD and AMD. There are two enzymes that regulate the steady-state levels of Aβ: proteases neprilysin and insulin-degrading enzyme. Neprilysin degrades Aβ monomers and oligomers [Shirotani et al., 2001] , in fact its reduction causes a cerebral accumulation of Aβ [Iwata et al., 2001]. Iwata et al. [Iwata et al., 2002]showed that the neprilysin levels were lower in 132-week-old mice compared to 10-week-old. The mRNA level of this enzyme was significantly lower in AD brains than in control human brains [Russo et al., 2005], so the down-regulation of neprilysin is linked to the deposition of Aβ in normal aging brain and in AD disease.

Imaging studies of AD and AMD

The detection of Aβ deposition is essential to diagnose AD and AMD at an early stage. The most successful non-invasive technique is probably positron emission tomography (PET) with ^{11}C-labelled Pittsburgh Compound-B (PiB) [Klunk et al., 2004]. Instead, Higuchi et al. [Higuchi et al.,2005]used^{19}F- and ^{1}H-MRI to resolve the negative aspects of PET. This technique has relatively high resolution and the elimination of radiation exposure.
The retina can be directly observed through the pupil of the eye. Today , improvements in ocular imaging, e.g., spectral domain optical coherence tomography make possible the identification of retinal Aβ. It would be extremely beneficial to detect the presence of Aβ, and in particular Aβ oligomers, within the drusen of AMD patients.

Common therapies to AMD and AD

The therapies, with anti-amyloid agents, developed for the treatment of AD may also be used for AMD, considering the similitudes between these two disease.Butovsky et al. [Butovsky et al., 2006]showed some interesting results as regards a treatment with Copaxone (glatiramer acetate). The T cell-based vaccination with Copaxone in AD mouse model led a reduction of the cognitive decline, elimination of plaque formation and induction of neurogenesis.
A lot of clinical trials are in progress: γ-secretase inhibitors (LY450139, BMS-708163, GSI-953, E2012, PF-3084014, NIC5-15), α-secretase activators (EHT0202, MK-0952, MEM1414/R1533) and β-secretase inhibitors (CTS-21166, HPP854). In particular, one therapeutic approach (phase 3 of clinical trial) is underway: two monoclonal antibodies against Aβ (AAB-001, LY2062430) and 10% intravenous immune globulin. These antibodies bind Aβ activating the complement system and Fc-receptor-mediated phagocytosis by microglia[Fu et al., 2010]. It would be interesting to consider a drug that enhances the activity of neprilysin,too.

Since it was shown that Alzheimer's Aβ is also an important factor in AMD[Yoshida et al., 2005] many more studies have focused on the similarities in the pathogenesis and characteristics of AD and AMD, starting from accumulation of the Aβ up to the consequences caused by this deposits.

Growing evidence suggests that a deficit of neurotrophic factors such as Transforming-Growth-Factor-β1 (TGF-β1) can significantly contribute to the pathogenesis of amyloid-related neurodegenerative disorders such as Alzheimeir's disease. The deficiency of TGF-β1 signaling has been shown to increase both Aβ accumulation and Aβ-induced neurodegeneration in AD models (Caraci et al. 2011).

Presently no studies have been conducted to examine TGF-β1 levels in AMD. Nevertheless TGF-β1signaling is required to maintain retinal vascular stability and retinal function in adult mice [Walshe et al., 2009], and TGF-β1 signaling has been implicated in several retinal diseases [Carmeliet and Jain, 2011]. It is therefore important to understand how TGF-β1signaling in the CNS and in particular in the retina is regulated, because rescue of TGF-β1 signaling might represents a new strategy to promote neuroprotection in amyloid-related neurodegenerative disorders such as AD and AMD.

TGF-β1 signaling pathway: smad and non-smad dependent pathways

It has been hypothesized that neurotoxicity of Aß in vivo is limited by the presence of endogenous protective factors that may be lacking in the AD brain such as TGF-β1 [Caraci et al. 2011]. Transgenic mice lacking TGF-β1 show enhanced neuronal susceptibility to different neurotoxic insults [Brionne et al.,2003].

TGF-β1 is a member of TGF-beta superfamily, which consists of several groups of highly conserved multifunctional cell-cell signaling proteins of key importance in the control of tissue homeostasis [Ten Dijke et al., 2004].

The TGF-β subfamily includes three isoforms in mammals, TGF-β1, 2 and 3, which are important modulators of cell survival, inflammation, and apoptosis [Taipale et al, 1998], and also exert a central role in immune suppression, and repair after injury [Li et al., 2006]. The three TGFβs are all synthesized as homodimeric proproteins (proTGFβ) that are around 400 amino acids in size and products of separate genes. The proTGFβs are cleaved intracellularly by furin into a larger C-terminal pro-region also known as latency-associated peptide (LAP), and a shorter N-terminal active peptide, which forms the mature homodimers (25-kDa). LAP remains non-covalently associated with the mature TGFβ 25-kDa dimer before the complex is secreted [Dubois et al., 1995]. The association between the TGF-β1, 2, and 3 prodomains (LAPs) and the corresponding mature growth factors prevents signaling through the TGF-β high affinity receptors [Lawrence et al.,1984]. Thus, TGF-bioactivity requires dissociation from LAP, a process termed latent TGF-β activation. '

Extracellular activation of TGF-β is a critical but incompletely understood process in vivo. In particular, an important and unresolved issue in TGF-β biology regards the connection between matrix incorporation and activation of the latent TGF-β. A variety of molecules, from protons to different proteases, such as plasmin and trombospondin, have been described as latent TGFβ activators [Annes et al.,2003]. It seems that inactive TGF-β stored in tissues can be activated in response to iniury and subsequent extracellular matrix perturbations. After TGF-β is released from its latency-associated peptide, it becomes able to initiate its diverse cellular responses by binding to, and activating specific cell surface receptors that have intrinsic serine/threonine kinase activity.

All three TGF-β isoforms interact with a high-affinity transmembrane receptor complex consisting of the activin-like kinase 5 (ALK5)/TGF-β type I receptor and the TGF-β type II receptor (TβRII) subunits [Caraci et al. 2011]. Several studies have demonstrated that ligand binding to TβRII induces the assembly of type I and type II receptors into complexes with the subsequent phosphorylation and activation of ALK5, which then propagates the signal inside the cell through the phosphorylation of receptor-regulated Smads (R-Smads: Smad2, Smad3, Smad5 and Smad8). The interaction between R-Smads and (ALK5)/TGF-β type I receptor is facilitated by the Smad anchor for receptor activation (SARA) [Shi et al., 2003]. Phosphorylated R-Smads form heteromeric complexes with Smad4. These complexes accumulate in the nucleus, where they regulate gene expression in a cell-type-specific and ligand dose-dependent manner through interactions with transcription factors and specific promoter elements of target genes.

Smad6 and Smad7 are inhibitory Smads, which are known to counteract the signalling of R-Smads through different mechanisms [Ten et al., 2004]. Inhibitory Smads bind to activated type I receptors, thus inhibiting the phosphorylation and the following nuclear translocation of R-Smads. Furthermore, they can recruit E3-ubiquitin ligases targeting the receptor complex to the ubiquitin degradation pathway with the following inhibition of TGF-β/Smad signaling cascade.

Recent evidence suggest that TGF-□1 can also exert its biological effects through the activation of smad-independent pathways such as the extracellular-regulated kinase (ERK) pathways [Caraci et al., 2008], the nuclear factor κB (NF-κB) pathway [König et al., 2005], and the phosphatidylinositol-3-kinase (PI3K)/Akt pathway [Caraci et al., 2008].

Role of TGF-β1 in the brain and in amyloid-related neurodegenerative disorders: the example of AD

In the CNS TGF-β2 and 3 isoforms account for almost all the TGF-β immunoreactivity, while TGF-β1 expression has been found to be constitutive only in the meninges and choroid plexus and, most importantly, in some specific brain regions such as the hippocampus and the cortex [Vivien et al., 2006]. Interestingly, TGF-β1 expression and release increase significantly in response to CNS lesions. Astrocytes and microglia seem to be the major sources of TGF-β1 in the injured brain [Finch et al., 1993], and several studies have shown that TGF-β1 induction during iniury exerts a central role in preventing neurodegeneration [Caraci et al.2011].

An increased expression of TGF-β1 has been observed with age [Finch et al. 1993], and a protective role has been suggested for this neurotrophic factor in longevity [Salvioli et al., 2009]. Aging is characterized by an increased level of pro-inflammatory markers such as IL-6, TNF-α or IL-1β [Franceschi et al., 2000]. This state of sub-clinical, chronic inflammation has been called "inflamm-ageing", and seems to be involved in the pathogenesis of several age-related disorders such as cancer, diabetes, cardiovascular pathologies and AD [Franceschi et al., 2000]. The protective role of TGF-β in aging and longevity has been suggested by in vitro and in vivo studies [Carrieri et al.,2004]. Increased plasma levels of bio-active TGF-β1 have been found in both male and female centenarians as compared to younger control subjects [Carrieri et al. 2004]. Similar results have been obtained by Forsey et al. [Forsey et al., 2003] in octogenarian and nonagenarian subjects. Salvioli et al. [Salvioli et al., 2009] have also proposed that this age-related increase of TGF-β1 might counteract the pro-inflammatory status observed during aging, thus preventing the development of age-related disorders such as cancer and AD.

Changes in TGF-β1 serum and cerebrospinal fluid (CSF) levels have also been analyzed in AD. In particular, increased TGF-β1 levels have been found in CSF of AD patients [Tarkowski et al., 2002; Chao et al., 1994], whereas a reduction of both its active (25 kDa) and inactive (50 kDa) forms has been reported in AD plasma [Mocali et al., 2004].

Recently a single nucleotide polymorphisms (SNPs) at codon +10 (T(C) and +25 (G/C) that affects the levels of expression of TGF-β1 has been associated with an increased conversion of Mild Cognitive Impairment (MCI) in AD [Arosio et al., 2007]. Other studies have demonstrated that that both the +10 C allele and the CC genotype are over-represented in AD when compared to HC, and, that CC genotype might act as a risk factor for the development of Late-Onset AD (LOAD), independently of apolipoprotein status [Caraci et al. 2012].

Many reports also describe a significant impairment of TGF-β1 signaling in AD brain [Wyss-Coray et al., 2006; Caraci et al. 2011; Lee et al. 2006; Ueberham et al. 2006; Chalmers et al. 2007; Tesseur et al. 2006]. The study by Tesseur et al.[2006] strongly points to a causal role for of TGF-β signalling dysfunction in age-dependent neurodegeneration and AD pathogenesis . The authors found that the expression of TGF-β type II receptor (TβRII) by neurons is reduced very early in the course of AD, and this alteration seemed to be specific for AD and was not observed in other neurodegenerative conditions such as Parkinson's disease, frontotemporal dementia, or Lewy body dementia. The authors also found that a deficiency of TGF-β signalling, in a mouse model of AD, promoted both Aβ deposition and neuronal loss [Tesseur et al. 2006]. Moreover, Tesseur et al. [2006] have shown that the impairment of TGF-β signaling in neuroblastoma cells resulted in neuritic dystrophy and increased levels of secreted Aβ and β-secretase-cleaved soluble amyloid precursor protein. These data suggest that a deficiency of TGF-β/TβRII signaling axis might exert a pathogenetic role in AD, depriving cortical neurons of trophic support, and finally promoting Aβ-induced neurodegeneration.

However, the role of TGF-ß1 in AD pathophysiology is not unequivocal, and conflicting results have been reported recently. TGF-ß1 is known to induce the expression of the APP gene in several different cell culture systems [Lesne et al. 2003] and might thus increase Aß production. The co-expression of TGF-ß1 in transgenic AD mice accelerates the deposition of Aß in cerebral blood vessels [Wyss-Coray et al. 1997], and transgenic mice overexpressing TGF-ß1 develop AD-like vascular alterations [Gaertner et al. 2004]. In addition, vessel-derived TGF-ß1 has been suggested to contribute to inflammatory processes in the AD brain [Harris-white et al. 1998; Grammas et al. 2002]. Town et al. [2008] have found that blocking TGF-β-Smad 2/3 signaling reduces cerebrovascular β-amyloid deposits and Aβ abundance in Tg2576 mice, and these events result in promotion of Smad1/5/8 signaling with increased infiltration of Aβ-containing peripheral macrophages around cerebral vessels and β-amyloid plaques.

Overall data from the literature seem to suggest that TGF-ß1 can promote Aβ deposition in cerebral blood vessels, but reduces Aβ accumulation in the brain parenchyma [Wyss-Coray et al., 2006]. In particular, it has been demonstrated that a modest increase in astroglial TGF-β1 production in aged transgenic mice expressing the human beta-amyloid precursor protein (hAPP) results in a 50% reduction of Aβ load in the hippocampus, and a decrease in the number of dystrophic neurites [Wyss-Coray et al., 2001].

Deficiency of TGF-ß1 signaling is also involved in tau pathology and NFT formation. Luterman et al. [2000] found that low levels of TGF-ß1 mRNA negatively correlated with NFT in the AD brain, thus suggesting that a deficiency of TGF-ß1 might also contribute to the cascade of events that result in the development of NFT-bearing neurons. The relationship between tau hyperphosphorylation and TGF-

ß1 signaling has been studied in the temporal lobe in AD [50]. Interestingly NFT can sequester phosphorylated Smad3 in AD brain, thus preventing its translocation into the nucleus and the induction of gene transcription [Chalmers et al.2007].

Other groups report an impairment of Smad-dependent TGF-ß1 signaling in AD brain [Lee et al. 2006; Ueberham et al. 2006;], with an aberrant localization of phosphorylatedSmad2 to the cytoplasm rather than the nucleus of hippocampal neurons and a specific colocalizationwith amyloid plaques and NFT. These data suggest a dysfunction of Smad signalling in AD brain, and, interestingly, a recent *in vitro* study has demonstrated that Aβ can inhibit TGF-ß1 signaling by inducing the expression of Smad 7 [Lee et al. 2005].

Taken together, these data might explain the paradox observed in the AD brain, where TGF-ß1 levels in CSF are found to be high; however this neurotrophic factor might not exert its neuroprotective action for an impairment of Smad signalling.

We believe that a deficiency in TGF-ß1 signaling might exert a central role not only in AD pathogenesis, but also in age-related macular degneration via different mechanisms that finally lead to Aβ accumulation and/or neuroinflammation with an ensuing neurodegeneration.

Neuroprotective effects of TGF-β1 against Aβ-induced neurodegeneration

TGF-ß1 is known to protect neurons against a diverse number of insults, including excitotoxicity, hypoxia, ischemia, and deprivation of trophic factors [Caraci et al. 2011; Vivien et al. 2006; Dhandapani et al. 2003; Flanders et al. 1998]. Several studies have suggested that TGF-ß1 also exerts a neuroprotective role against Aβ toxicity by selectively interfering with different steps of the Aß-induced death cascade. In cultured neurons, estrogen-stimulated release of TGF-ß1 from glial cells [Sortino et al. 2004] or application of recombinant TGF-ß1 [Prehn et al. 1996; Ren et al. 1996; Ren et al. 1997; Kim et al. 1998; Caraci et al. 2011] reduce Aß-induced neurodegeneration.

The neuroprotective effects of endogenous TGF-β1 signaling in the rat brain have been demonstrated after intracerebral injection of synthetic Aß [Caraci et al. 2008]. Aß injection into the dorsal hippocampus produced only a small extent of neuronal loss in the pyramical layer of the CA1 region. However, Aß neurotoxicity was amplified by i.c.v. injection of SB431542, which behaves as a selective inhibitor of the activin-like kinase 5 (ALK5) TGF-β type I receptor [Inman et al. 2002].

Different molecular mechanisms have been implicated in the neuroprotective effects of TGF-β1 against Aß toxicity. TGF-β1 receptors are expressed both in glial cells and neurons [Flanders et al. 1998], and, therefore TGF-β1 might exert its protective effects by acting on both cell types.

TGF-β1 has a constitutive role in the suppression of inflammation, and appears to control the degree of microglial activation in the CNS Caraci et al. 2011]. Inhibition of TGF-β1 in different models of neurodegenerative disorders is associated with local inflammation mediated by macrophage/microglia and T cells [Caraci et al. 2011; Boche et al. 2006]. Inflammatory responses elicited by elevated Aβ peptides play an important role in the progression of AD, and microglia activation is an early event in AD pathogenesis and can be already detected in patients with MCI [Okello et al. 2009]. Aß can activate microglia to release pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α [Maccioni et al. 2009], which can contribute to neuronal death in the AD brain. Interestingly, several studies have

demonstrated that TGF-β1 reduces microglia activation and promotes the degradation of Aβ by the microglia [Wyss-Coray et al. 2001, Magnus et al. 2002].

TGF-β1 might also affect neuronal survival through other mechanisms because it acts synergistically with other neurotrophins and is required for a full neuroprotective activity of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial-derived neurotrophic factor (GDNF) [Unsicker K, Krieglstein 2002; Svhobe et al. 2007]. The levels of BDNF and its receptor, tropomyosin receptor kinase B (TRKB), are reduced in the AD brain, and deficiency of BDNF signalling has been related to neurodegeneration and cognitive dysfunction in AD [Cotman, 2005]. Interestingly, TGF-β1 enhances the expression of BDNF and TrkB in rat neuronal cultures [Sometani et al. 2001].

It might be possible that the contemporary failure of both BDNF and TGF-β1 signaling in the AD brain enhances neuronal vulnerability to Aß, thus accelerating the progression of AD.

Finally, a component of the neuroprotective action of TGF-β1 is mediated by the activation of neuronal TGF-β receptors. TGF-β1 is known to prevent apoptotic cell death in neurons through the inhibition of caspase-3 activation [Zhu et al. 2001]. In addition, TGF-β1 maintains mitochondrial membrane potential and increases the expression of anti-apoptotic proteins, such as Bcl-2 and Bcl-xl [Prehn et al. 1996]. TGF-β1 can also activate the extracellular-regulated kinase (ERK) pathway in hippocampal neurons, thus promoting the phosphorylation and subsequent inhibition of the pro-apoptotic protein, Bad [Zhu et al. 2002]. Furthermore, TGF-β1 can increase the transcriptional activity of the anti-apoptotic transcriptional factor, NF-kappaB, through the PI3K/Akt and ERK signaling pathways [Zhu et al. 2004; Caraci et al. 2011].

Design of the present research

Based on the reviewed data, the present thesis has focused on the role of Aβ in the pathogenesis of AMD with the aim to develop an*in vivo* model of AMD and to assess the retinal damage induced by Aβ oligomers.In addition, we have investigated the neuroprotective effects of TGF-β1 against Aβ-induced neurodegeneration in retina.

The following aspects were addressed:

1. To develop an in vivo model in rat of amyloid-related retinal neurodegeneration induced by Aβ oligomers that mimics AMD
2. To identify gene pathways possibly involved in AMD or AD.
3. To assess the neuroprotective effects of TGF-β1. In an vivo model of AMD
4. To develop a new topical nano-technological formulation able to deliver TGF-β1 into the posterior segment of the eye.

CHAPTER I

TGF-β1 prevents rat retinalinsult induced by amyloid-β (1-42) oligomers

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Key words: macular degeneration; retina; Alzheimer's disease; TGF-β1

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Abstract

To set up a retinal degenerative model in rat that mimics pathologic conditions such as age-related macular degeneration (AMD) using amyloid-β(Aβ)oligomers, and assess the effect of TGF-β1. Sprague-Dawley male rats were used. Human $A\beta_{1-42}$ oligomers were intravitreally (ITV) injected (10 μM) in the presence or in the absence of recombinant human TGF-β1 (1ng/μl ITV injected). After 48h, the animals were sacrificed and the eyes removed and dissected. The apoptotic markers Bax and Bcl-2 were assessed by western blot analysis in retina lysates. Gene-pathway network analysis was carried out in order to identify pathways involved in AMD. Treatment with Aβ oligomers induced a strong increase in Bax protein level (about 4-fold; $p<0.01$) and a significant reduction in Bcl-2 protein level (about 2-fold; $p<0.05$). Co-injection of TGF-β1 triggered a significant reduction of Bax protein induced by Aβ oligomers. Bioinformatic analysis revealed that Bcl-2 and PI3K-Akt are the most connected nodes, for genes and pathways respectively, in the enriched gene-pathway network common to AMD and Alzheimer disease (AD). Overall, these data indicate that ITV injection of $A\beta_{1-42}$ oligomers in rat induces molecular changes associated with apoptosis in rat retina,highlighting a potential pathogenetic role of Aβ oligomers in AMD. Bioinformatics analysis confirmsthat apoptosis pathways can take part in AMD.Furthermore, these findings suggest that human recombinant TGF-β1 can prevent retinal damage elicited by Aβ oligomers.

1. **Introduction**

Age-related macular degeneration (AMD) is the leading cause of irreversible central vision loss in elderly populations in developed countries. Two main forms of AMD exist, the dry and the wet one. Dry AMD is characterized by drusen bodies (cellular debris) that accumulate between choroid and retina; wet AMD includes abnormal growth of choroidal blood vessels leading to detachment of retina along with edema due to vascular leakage.

Drusen are extracellular deposits that accumulate underthe basement membrane of the retinal pigmented epithelium (RPE) and the inner collagenous layer of the Bruch membrane (Fig. 1). An age-related accumulation of amyloid-β (Aβ) in the normal mouse retina and human retina has been recently demonstrated (Hoh Kam et al., 2010).Many protein and lipid constituents of drusen are similar to those found in deposits characteristic of other age-related degenerative disorders such as Alzheimer disease (AD).Several studies have led to the comprehension that prefibrillar soluble oligomers, rather than amyloid fibrils, might be the primary toxic agents in AD brain (Kayed et al., 2003; Kayed et al., 2004; Lambert et al., 1998). The presence of prefibrillar oligomers in drusen has been demonstrated (Luibl et al., 2006), suggesting that amyloid oligomers may be involved in drusen biogenesis and/or participate directly in local RPE toxicity(Isas et al., 2010).

Transforming-growth-factor-β1 (TGFβ1) is an anti-inflammatory cytokine with neurotrophic and neuroprotective properties (Caraci et al., 2011; ten Dijke and Hill, 2004). It has been proposed that TGF-β1-TGFβ receptor I (TβRI) axis plays a key role in the function of retinal vascular barrier, by promoting endothelial cell survival and homeostasis (Walshe et al., 2009).

Based on these grounds, we set up an *in vivo* model of AMD using Aβ oligomers to induce retinal damage and investigate the potential protective role of TGF-β1. Furthermore, we carried out a bioinformatics analysis in order to sort out gene pathways commonly related to AMD and AD.

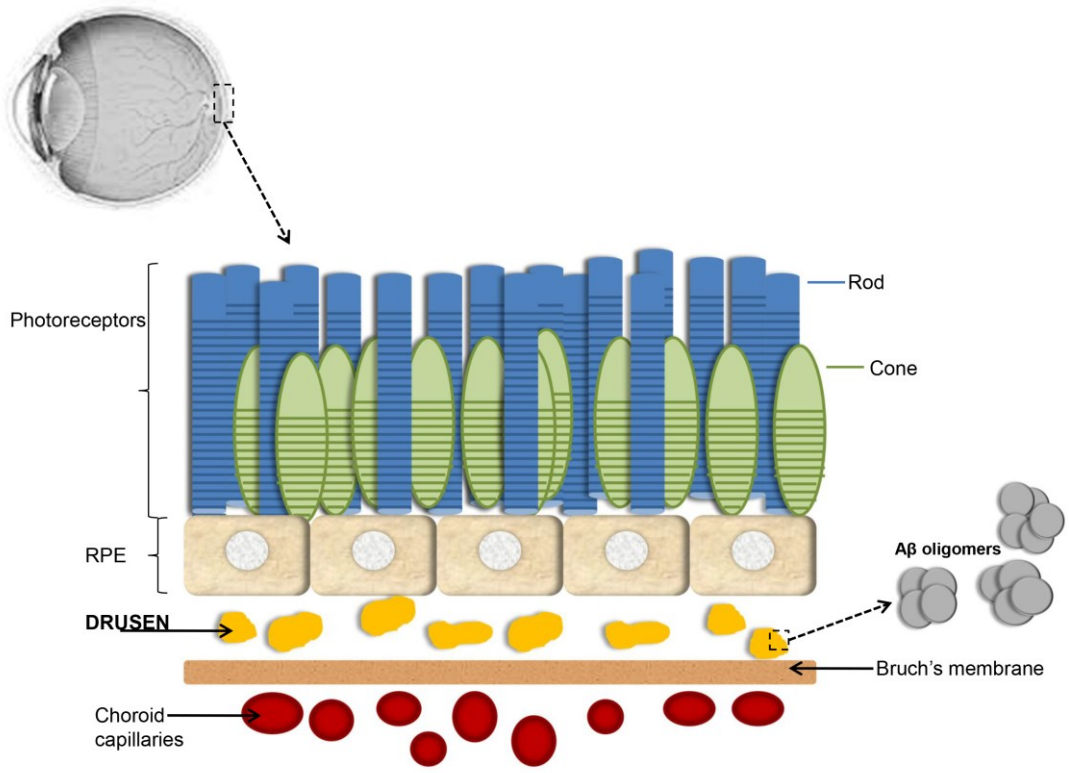

Figure 1. Schematic diagram of the age-related macular degeneration.

2. Material and methods

2.1. Reagents

SB431542, a selective inhibitor of TGFβ-1 receptor, and protease inhibitors cocktail were purchased from Sigma-Aldrich (St Louis, MO). Rabbit polyclonal antibody against Bax, and rabbit monoclonal antibodies against Bcl-2, and GAPDH were purchased from Cell Signaling Technology (Danvers, MA); secondary goat anti-rabbit IRDye 680conjugated antibody was purchased from LI-COR (Lincoln, US).

2.2. Amyloid-β oligomers

Human $A\beta_{1\text{-}42}$ oligomers were prepared according to the original protocol of Klein's group (Caraci et al., 2015b; Gong et al., 2003). Briefly, the $A\beta_{1\text{–}42}$ lyophilized peptide, purchased from Novus Biologicals (Littleton, USA), was dissolved in trifluoroacetic acid (TFA, 1 mg/ml) and sonicated in a water bath sonicator for 10 min. Then TFA was evaporated under a

gentle stream of argon and 1 ml1,1,1,3,3,3-hexafluoro-2-propanol(HFIP) was added to the peptide. After 1 h incubation at 37°C, the peptide solution was dried under a stream of argon, and then solubilized again by adding 2 ml of HFIP. Finally, HFIP was removed by argon streaming followed by further drying in a lyophilizer for 1 h and $A\beta_{1-42}$re-suspended in 5 mM anhydrous dimethyl sulfoxide (DMSO), before dilution to 100 µM in ice-cold PBS. Aliquots of 100 µM $A\beta_{1-42}$ were incubated for 72 h at 4°C and then stored at -20° until use. For *in vivo* experiments human $A\beta_{1-42}$ oligomers were diluted in sterile PBS and 1 µl intravitreally (ITV) injected at the final concentration of 10µM.

2.3. Animal treatment

Male Sprague-Dawley rats (250-300 g) were purchased from Harlan (Udine, Italy). The animals were fed on standard laboratory food and were allowed free access to water in an air conditioned room with a 12-h light/12-h dark cycle. The animals were randomly divided in four experimental groups: 1) control; 2) ITV injected with 1µl Aβ at a concentration of 10µM in PBS; 3) ITV injected with 1µl of Aβ and TGFβ1 (1ng/µl; the dose was selected based on previous work, (Caraci et al., 2015a); 4) ITV injected with 1µl of Aβ, TGFβ1 and the inhibitor of TβRI(SB431542; 20µM; the dose was selected based on previous work)(Caraci et al., 2015a). Before ITV injection, animals were anesthetized by intravenous injection of 5 mg/kg Zoletil (tiletamine HCl and zolazepamHCl, Virbac, Milano, Italy); and 1 drop in the eye of the local anesthetic Novesina (Novartis, Origgio, Italy) .Animals were sacrificed 48 hours after treatment, and retinas were dissected. Housing and treatments were in accordance to Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Visual Research.

2.4. Western blotting

Retinas from control and treated rats were homogenized and sonicated in RIPA buffer (Life Technologies, Monza, Italy) in the presence of protease inhibitor cocktail (Sigma P2714), serine/threonine phosphatase inhibitors (Sigma P0044) and tyrosine protein phosphatase inhibitors (Sigma P5726). Protein concentrations were determined by Bradford's method using bovine serum albumin as standard. Retina lysates(40 µg protein) were loaded into SDS-PAGE, blotted and probed for different target proteins. Membranes were incubated with primary antibodies against total Bcl-2 (Rabbit monoclonal, 1:1,000 dilution), Bax (rabbit polyclonal, 1:1,000 dilution), GAPDH (Rabbit, monoclonal 1:1,000 dilution). The

membranes were then incubated with secondary fluorescent antibodies (1:20,000 dilution) for 1 h at room temperature, and the immunocomplexes were detected by Odyssey imaging system (LI-COR, Lincoln, NE). All blots were controlled for equal loading by probing with GAPDH. Densitometric analysis was performed using Image J software.

2.5. Statistical analysis

Statistical significance between two groups was analyzed by Student's *t-test*. One-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test, was used for multiple comparisons. P values < 0.05 were considered as statistically significant.

2.6.Bioinformatics analysis

Gene(s) to pathway(s) information was derived from the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway database. AMD is a complex disease and gene association studies highlighted a series of genes involved in development of AMD: *HGS*, *TNF*, *RAD51B*, *CFH*, *CFB*, *C3*, *ARMS2*, *COL8A1*, *CX3CR1*, *FBLN5*. Analysis of pathways, and pathways interconnection, regulated by these genes was carried out with the web application KENeV-KEGG Enriched Network Visualizer (KENev)(Pilalis et al., 2015). However, output associated to this list of genes was not informative enough. Therefore, an enrichment information strategy was carried out. In a previous work (Romano et al., 2015) we have looked at pathways that are common to neurodegenerative diseases (glaucoma, AMD and AD), with particular focus on glaucoma. Results were obtained through bioinformatic prediction of microRNAs (miRNA) involved in such diseases.The enrichment analysis was carried out with the following steps, because one miRNA can regulate more than one gene:

- searching of miRNAs known to be deregulated in AMD (Romano et al., 2015);
- searching of miRNAs that putatively target genes, retrieved from gene association studies (Romano et al., 2015). This search was carried out throughthe web server microRNA.org (Betel et al., 2008). Only miRNAs commonly deregulated both in AMD and AD were analyzed;
- searchingfor biochemical pathway commonly regulated by miRNAs, through DIANA-miRPath, by using the miRTarBase algorithm(Vlachos et al., 2012);
- retrieving of genes that are targets of selected miRNA, which regulate pathways known to have a known role in AMD and AD;
- building of enriched gene-pathway network interaction with KENev.

Output from KENev, a genes-pathways network, was analyzed and visualized with Cytoscape 3.2.1(Shannon et al., 2003). Genes and pathways are represented as nodes in the network, if a gene regulates a pathway then an edge between two nodes is created.

2. Results

Bcl-2 and Bax are cytoplasmic proteins involved in the regulation of apoptosis. This family of apoptosis regulatory genes affects mitochondrial membrane dysfunction during programmed cell death. In the early stage, pro-apoptotic Bcl-2 family member such as Bax are translocated to mitochondrial membranes where they increase mitochondrial permeability (Mattson, 2000). Bcl-2 proteins prevent apoptotic death in cells, while Bax proteins induce the opening of mitochondrial permeability transition pores and the subsequent release of cytochrome C, which results in cell apoptosis (Garcia et al., 1992; Vander Heiden et al., 1999). We observed a significant ($p<0.01$) reduced level of Bcl2 and a significant ($p<0.01$) increase in the level of Bax in retina of $A\beta_{1-42}$ oligomers treated rats (Fig. 2).

As a result, intraocularinjectionof $A\beta_{1-42}$ oligomers significantly ($p<0.01$) increased retinal Bax/Bcl2 ratio in Aβ-treated rats in comparison with control group. Interestingly, TGFβ1 significantly prevented ($p<0.01$) both Bax induction and the Aβ-induced decrease ofBax/Bcl2 ratio, suggesting an anti-apoptotic effect of this neurotrophic factor in our experimental model of AMD.Furthermore, co-treatment withTGFβ1 and SB431542, a selective inhibitor of ALK5/TβRI, blunted the effect of TGFβ1 on Bax/Bcl-2 ratio confirming that the protective effect of exogenous TGFβ1 was exerted through the activation of TβRI(Fig. 2).

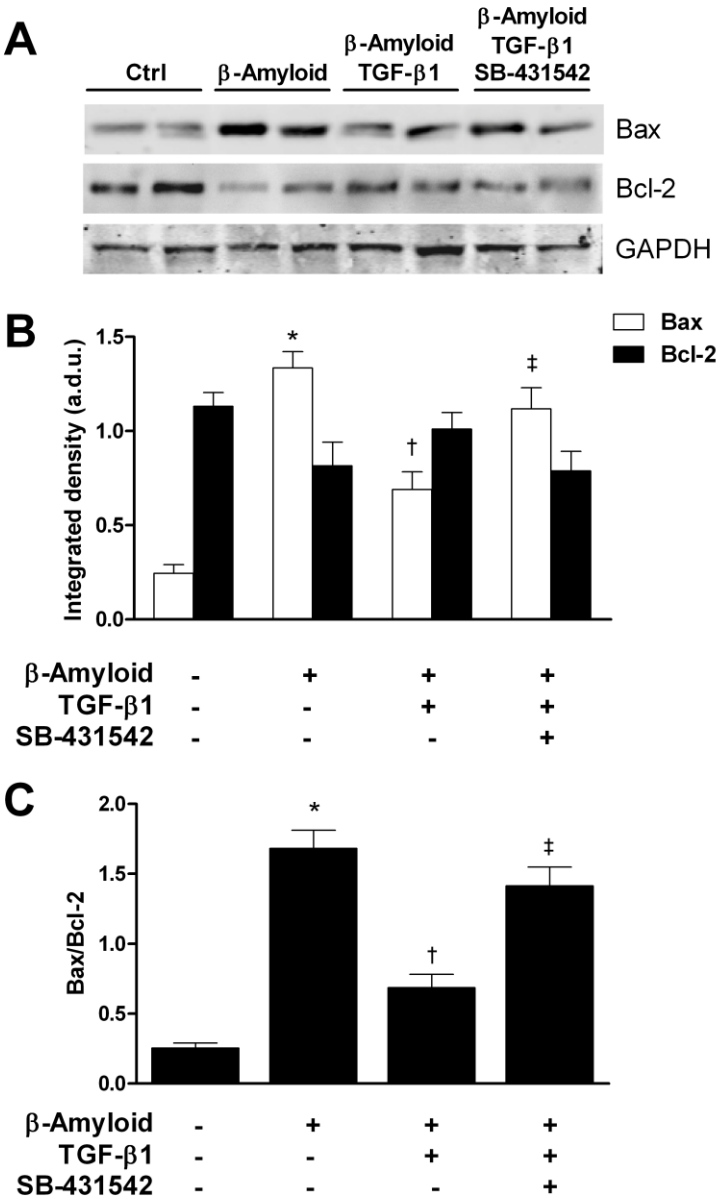

Figure 2. Western blot analysis of Bax and Bcl-2 in Aβ-treated rats with or without TGF-β1 in the presence or absence of selective inhibitor of TβRI (SB431542).Representative western blotting of Bax, Bcl-2 and GAPDH are shown (panel A). The values, expressed as arbitrary densitometric units (a.d.u.), were obtained by the reading of blots with the Image J program and are means ± SD of at least three independent experiments, each performed in triplicate (panel B). Bax/Bcl-2 ratio (panel C). *$p<0.01$ *vs.* control;†$p<0.01$ *vs.* Aβ-treatedrats, ‡$p<0.01$ *vs*. Aβ +TGF-β1treated rats.

In order to test the hypothesis that genes related to PIK3-Akt signaling or cell cycle activation are dysregulated in AMD we built a gene-pathway network. In a first step we tried to build a gene-pathway network starting from genes retrieved from gene association studies related to AMD; however, retrieved pathways did not give any information about AMD. Therefore, considering that miRNAs can target more than one gene, and deregulated miRNAs can regulate biochemical pathways with combinatorial effects, we proceeded to identify miRNAs known to be deregulated in AMD. Additional miRNAs, commonly deregulated in AMD and AD, where predicted to regulate genes associated to AMD (Romano et al., 2015, Table 1).

Table 1. Human microRNAs associated to AMD and AD.

AMD*	**AMD*∩ AD**	**GAS§∩ AD**
hsa-miR-30b	hsa-miR-9	hsa-miR-107
hsa-miR-23a	hsa-miR-146a	hsa-miR-137
hsa-miR-9	hsa-miR-21	hsa-miR-146a
hsa-miR-146a	hsa-miR-34	hsa-miR-181c
hsa-miR-146b		hsa-miR-197
hsa-miR-31		hsa-miR-21
hsa-miR-21		hsa-miR-22
hsa-miR-184		hsa-miR-328
hsa-miR-34a		hsa-miR-590

Age related macular degeneration (AMD), Alzheimer's disease (AD), gene association studies (GAS). * miRNA retrieved from literature (Romano et al., 2015).hsa-miR is the tag for human miRNA
§ These miRNAs are common to AMDand AD, furthermore these miRNAs are predicted to target genes retrieved from GAS for AMD. In GAS∩ AD column, miRNA were underlined in order to highlight those predicted miRNA that have been also experimentally validated(hsa-miR-146a and hsa-miR-21 are also in AMD∩ AD column)

These miRNAs (table 1) can impact the following pathways: cell cycle, Toll-like, RIG-1 like, NF-kB, Apoptosis, p53 signaling, Notch signaling, PI3K-Akt, Focal Adhesion, Neurotrophins (NT), HIF-1, Adherence junctions, Wnt signaling.

In particular hsa-miR-107 targets VEGFA, hsa-miR-146a targets NF-kB. Interestingly hsa-miR-181c, upregulated in AD, is predicted to down-regulate Bcl-2, then leading to apoptosis.

An enriched list of 104 genes(supplemental material), that are predicted to be targeted by miRNAs listed in table 1, was created from miRNA-pathway analysis carried out with DIANA-miRPath. These genes were used for building an enriched gene-pathway network. Submission of these genes to the KENeV web application gave the result illustrated in Figure 3.Genes-pathway interaction network was visualized with a circular layout, increasing closeness centrality was used as hierarchical parameter. High closeness centrality nodes have the shortest distance from other nodes of the network; thus, high closeness centrality nodes are connected through few links to any other node of the network. MYC and Bcl-2 are the genes with the higher closeness centrality.The most high closeness centrality pathway is PI3K-Akt, along with "miRNA in cancer" and "pathways in cancer". This network, built from genes targeted by miRNAs listed in table 1, supports the hypothesis that dysregulation of Bcl-2 and PI3K-Akt pathways would lead to apoptotic events occurring in both AMD and AD.

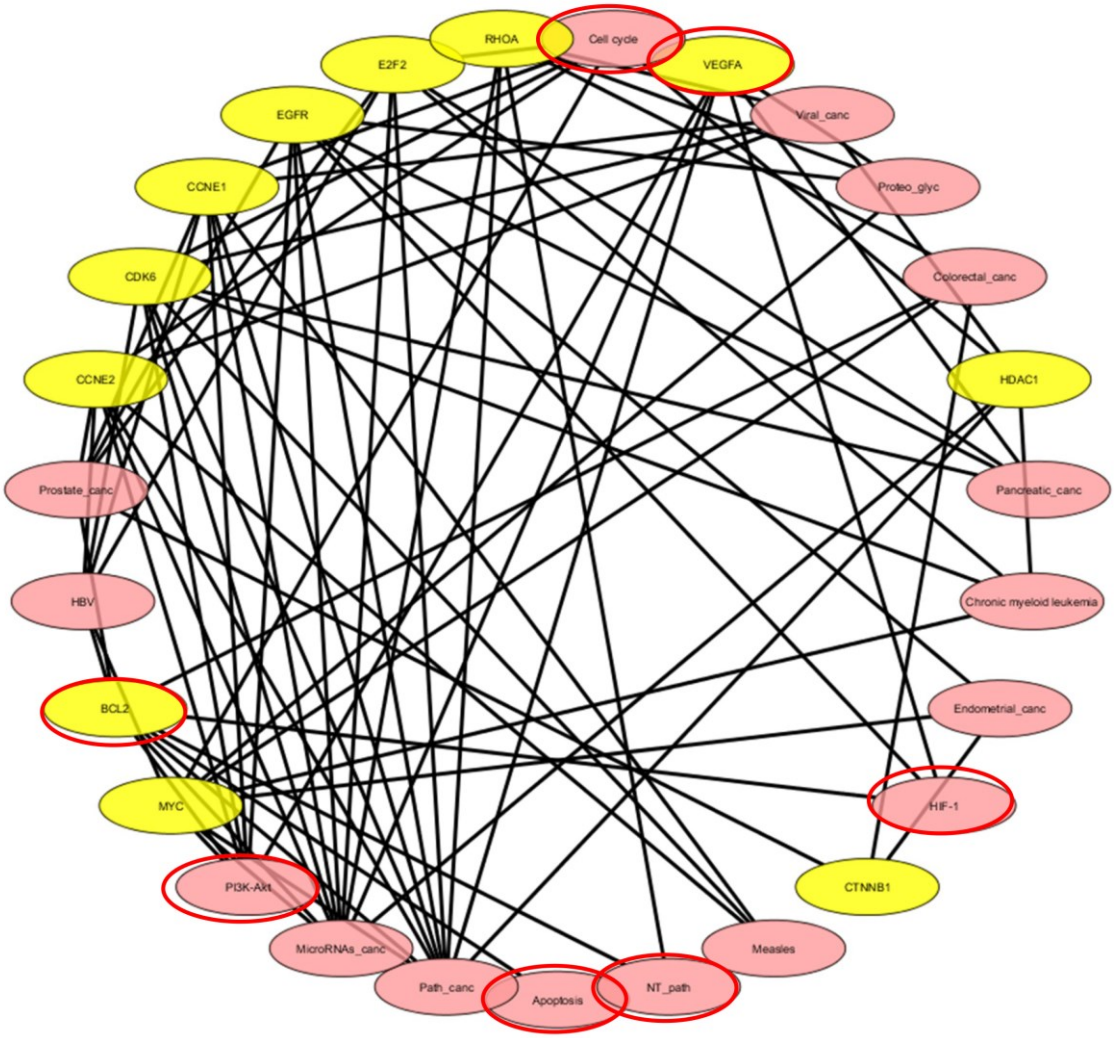

Figure 3. Gene-pathway network. Genes are the yellow nodes. Pathways are the pink nodes. Genes are named with their "HUGO gene nomenclature committee" symbols.Other abbreviations: viral carcinogenesis (viral_canc), proteoglycan in cancer (proteo_glyc), colorectal cancer (colorectal_canc), pancreatic cancer (pancreatic_canc), endometrial cancer (endometrial_canc), hypoxia inducible factor HIF signaling pathway (HIF-1), neurotrophins signaling pathway (NT_pathway), pathways in cancer (path_canc), microRNA in cancer (microRNA_canc), prostate cancer (prostate_canc), hepatitis B virus (HBV).

4. Discussion

Complex and multi-factorial diseases, such AMD, need appropriate experimental models for identification of new pharmacological targets and development of new neuroprotective drugs. Besides the validity of genetic animal models for AMD, animal models of choroidal neovascularization are among the most simple and diffused models of wet AMD (Pennesi et al., 2012). Consistent with the neurodegenerative hypothesis of AMD, Liu and co-workers (2013) developed a model of early stages of AMD (Liu et al., 2013). This model was based on intravitreal injection of toxic fragments of $A\beta_{1-40}$ in Long Evans rats; Aβ treatment led to upregulation of proinflammatory cytokines (e.g. IL-6, TNF-α, IL-1β, and IL-18)in the RPE and neuroretina (Liu et al., 2013).

Different groups have demonstrated the neurotoxic effects of amyloid-β peptides ($A\beta_{1-40}$ and $A\beta_{1-42}$)in cortical neurons(Jen et al., 1998; Paradis et al., 1996). In particular, in human neuron primary cultures, $A\beta_{1-40}$ and $A\beta_{1-42}$ can trigger the execution phase of apoptotic death via induction of Bax protein and downregulation of Bcl-2 (Paradis et al., 1996). Furthermore, Jen and co-workers (1998) showed that treatment with $A\beta_{1-40}$ and $A\beta_{1-42}$ severely decrease Bcl-2 immunoreactivity in Müller glial cells(Jen et al., 1998).In the present study wedeveloped an animal model of AMD characterized by apoptotic events elicited by intravitreal injection of $A\beta_{1-42}$ oligomers. Recently, the presence of a wide spectrum of amyloid structures in drusen, from patients with AMD,has been demonstrated; in particular non-fibrillar oligomers were detected (Isas et al., 2010). In an early phase of AD, soluble Aβ monomers aggregate into soluble Aβ oligomers and insoluble Aβ plaques, both considered as toxic to neurons (Klein, 2013). Furthermore, it has been suggested that Aβ oligomers might represent the primary neurotoxic species in amyloid-related neurodegeneration in AMD. Therefore, we challengedrat retina with prefibrillar small soluble Aβoligomers,because

theyare known to induce apoptotic death in neuronal cultures (Giuffrida et al., 2009), to set up a model of AMD.

Here we show, for the first time, that Aβ oligomers increase the ratio of Bax/Bcl2 in this new experimental model of AMD. We hypothesize that Aβ oligomers convert into fibrils during the 48 h of treatment, which could finally contribute to apoptotic retinal cell death; in this context, the ratio of pro-apoptotic Bax to anti-apoptotic Bcl2 can be taken as an indexof the extent of apoptosis. Furthermore, we showed that retinal damage is reversed by exogenous TGF-β1 treatment.Treatment with human recombinant TGF-β1 reversed both the induction of Baxand the reduction of retinal Bcl-2 in rats treated with$A\beta_{1-42}$oligomers, leading to a reduction of the ratio of Bax/Bcl2, which reduces execution phase of apoptotic cell death.

An impairment of TGF-β1 signaling pathway plays a central role in the pathogenesis of AD and it contributes to Aβ accumulation and microglia activation in animal models of AD(Chen et al., 2015; Tichauer and von Bernhardi, 2012). Accordingly, exogenous TGF-β1 is neuroprotective against Aβ toxicity both in *in vitro* and *in vivo* models of AD (Caraci et al., 2011). TGF-β1 has been shown to protect cortical neurons from Aβ-induced neurodegeneration, through activation of the neuroprotective pathway PI3K-Akt (Caraci et al., 2008). However, the role of TGF-β1 in promotion of either cell survival or apoptosis in retina is not completely understood. The TGF-β1 signaling pathway could induce either apoptosis or cell survival, most likely depending on other cell signaling pathways, such as PI3K-Akt pathway that can be activated independently of Smad- signaling (Wilkes et al., 2005).

Members of the TGF superfamily act through a receptor complex constituted by the activin-like kinase 5 (ALK5)/TGF-β type I receptor (TβRI) and TGF-β type II receptor (TβRII), strongly expressed in the CNS, particularly in hippocampus(Derynck and Zhang, 2003). TGF-β1 binding to TβRII induces the assembly of TβRI and TβRII receptors into a complex, with the subsequent transphosphorylation of TβRI by the type II receptor kinase. The subsequent activation of TβRI receptor leads to phosphorylation of selected SMAD proteins that, in turn, translocate into the nucleus and regulate the expression of different target genes involved in cell survival and proliferation (ten Dijke and Hill, 2004, Fig. 4). Besides Smad-mediated gene transcription, TGFβ activates Smad-independent pathways, including NF-kB (Konig et al., 2005), and PI3K/Akt (Bakin et al., 2000; Caraci et al., 2008). TGF-β/Smad-independent pathways play a key role in mediating different biological effects of TGF-β such as cell cycle inhibition, epithelial-to-mesenchymal trans-differentiation, immune suppression and neuroprotective effects (Caraci et al., 2008; Derynck and Zhang, 2003).

Yin and co-workers (2011) have reported that upregulation of Akt prevents alteration of Bcl-2 family members (including Bcl-xL, Bcl-w, Bad, and Bax) elicited by Aβ and that overexpression of Akt significantly attenuates Aβ-induced apoptosis, while simultaneous inhibition of PI3K, the immediate upstream regulator of Akt, abolishes the protective effect of Akt activation(Yin et al., 2011). Therefore, because TGFβ1 activates PIK3-Akt signaling(Zhu et al., 2004)which is anti-apoptotic, its protective effect could be attributed to the activation of this pathway; however, other mechanism/s of cell protection by TGFβ1 through SMAD2 cannot be ruled out (Zhu et al., 2004, Fig. 4).

We carried out a bioinformatics analysis, characterized by several enrichment information steps, in order to verify the importance of PI3K-Akt pathway in retinal cell survival.The genes-pathways network, built with the web-application KENeV, revealed that Bcl-2 and PI3K-Akt are respectively the gene and the pathway that can be dysregulated in AMD (Fig. 3). This bioinformatics analysis included also the assumption that AMD and AD share common pathogenic mechanisms. The KEGG pathway related to TGFβ signaling suggests that p-SMAD2 a TGF-β1 downstream pathway effector, would regulate apoptosis (Fig. 4). Recently,we demonstrated that p-SMAD2 is involved in the effects of TGF-β1 on long term potentiation (LTP) formation (Caraci et al., 2015a). Aβ oligomers are known to induce a severe impairment of Smad-dependent TGF-β1 signaling in AD brain (Caraci et al., 2012). Thus, we speculate that activation of the PI3K/Akt pathway by exogenous TGF-β1 in AMD might counteract the deficit of Smad-dependent TGF-β1induced by Aβ oligomers. Further studies are needed to elucidate whether or not the PI3K/Akt pathwaycontributes to the neuroprotective effects of TGF-β1 in AMD, under conditions of defective activation of Smad-dependent TGF-β1 signaling similar to that occuring in AD brain.

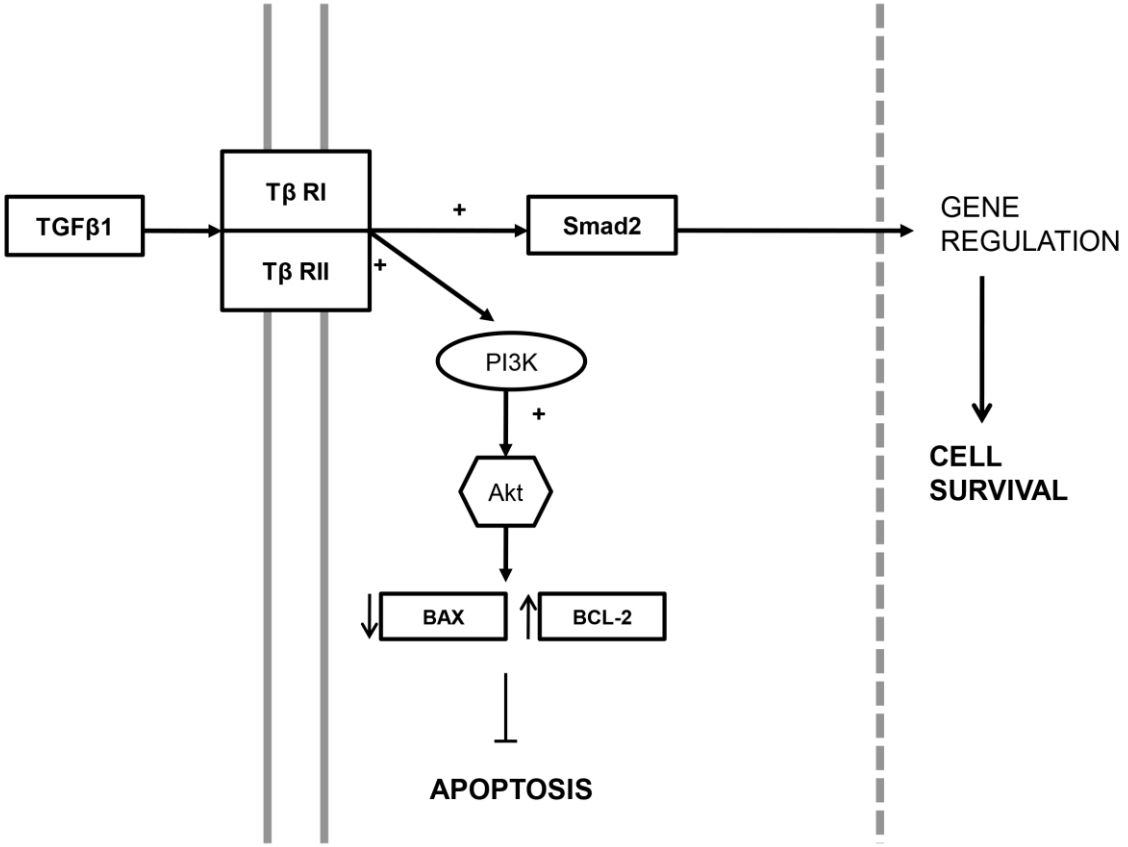

Figure 4. Smad-dependent and smad-independent TGF-β1 signaling pathways.

5. Conclusions

In conclusion, the novel experimental model hereby proposed can recapitulate the apoptotic events induced by Aβ oligomers occurring during AMD, and represents a tool to assess the pharmacological activity of new potential candidates for AMD management such as TGF-β1.

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CHAPTER II

Topical ocular delivery of TGF-β1 to the back of the eye: implications in age-related neurodegenerative diseases

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CNS Drug Target: **Submitted**

ABSTRACT

To develop a topical formulation of transforming growth factor β 1 (TGF-β1) and to assess the ocular pharmacokinetics profile. TGF-β1 has been formulated as encapsulated insmall unilamellar liposomes(SUV) in the presence of annexin V.

MATERIAL AND METHODS

SUV loaded with TGFβ1 were complemented by annexin V and Ca^{2+} prior topical administration to albino rabbits, that were used to evaluate the bioavailability of TGF-β1, in the vitreous at different time points (30, 60, 120, 180 and 240 min) after a single administration of eye drops (30µl, 450 ng TGF-β1) by a commercial ELISA kit. Ocular tolerability was also assessed by a modified Draize's test.

RESULTS

The pharmacokinetics profile demonstrated remarkable levels of TGF-β1 in the posterior segment of the eye ($AUC_{0-240}$was 4834 pg min /ml). In particular, we detected high levels of TGF-β1 (C_{max} 51.06 pg/ml) in the vitreous after 240 minutes (T_{max}) from the topical application of the eye drops. The tested formulation was well tolerated and the score for each parameter was zero at all time of observations.

CONCLUSION

In conclusion, we demonstrate that the novelformulation was able to deliver remarkable levels of TGF-β1 into the back of the eye. Therefore, this TGF-β1 delivery system may be useful in clinical practice to manage ophthalmic conditions such as age-related macular degeneration avoiding invasive intraocular injections.

Keywords: TGF-β1; age-related neurodegenerative diseases; retina; liposomes

1. INTRODUCTION

Transforming growth factor-β1 (TGF-β1) is a member of TGF-β superfamily, which includes several groups of highly conserved multifunctional cell-cell signalling proteins of key importance in the control of cell growth and differentiation, as well as immune suppression and repair after injury [1]. Within the mammalian TGF-β superfamily, TGF-β1, 2 and 3 are important modulators of cell survival and apoptosis [2]. All three TGFβs are synthesized as homodimeric proproteins (pro-TGF-β), which are then cleaved at intracellular level by furin into a larger C-terminal pro-region, also known as latency-associated peptide (LAP), and a shorter N-terminal active peptide, that forms the mature homodimers (25-kDa) of TGF-β1. The mature 25-kDa TGF-βdimer remains non-covalently associated with LAP before the complex is secreted [3, 4].In the central nervous system (CNS) TGF-β2 and 3 isoforms account for almost all the TGF-βimmunoreactivity, while TGF-β1 expression has been found to be constitutive only in the meninges and choroid plexus. Interestingly, TGF-β1 expression and release increase significantly in response to CNS lesions [5].Recently, a specific impairment of TGFβ1 signaling pathway has been demonstrated in Alzheimer's disease (AD) an amyloid-related neurodegenerative disorder, that share similar features with age-related macular degeneration (AMD) [6-8]. The deficiency of TGF-β1 signaling has been shown to increase both amyloid-β (Aβ)accumulation and Aβ-induced neurodegeneration in AD models [9]. Levels of TGF-β1 and of small latent TGF-β1 were found to be decreased in serum of AD patients [10, 11]. The potential use of growth factors for treatment of AD was extensively reviewed by Lauzon et al. in 2015 [12]. Growing evidence suggests a neuroprotective role for TGF-β1 against Aβtoxicity both in*in vitro* and *in vivo* models of AD [9, 13, 14]. Altogether, these studies, supported the hypothesis that either drugs capable of induce TGF-β1 secretion or TGF-β1 itself can be beneficial for neuroprotection. Based on these premises, rescue of TGF-β1 signaling might represents a new strategy to promote neuroprotection in AD as well as in other amyloid-related neurodegenerative disorders such as AMD.

More recently we demonstrated [8] that human recombinant TGF-β1 was able to prevent retinal damage elicited by Aβ oligomers. We injected human $A\beta_{1-42}$ oligomers into the rat's eye with or without TGFβ1 treatment; co-injection of TGFβ1significantly protect rat retina from Aβ-induceddamage. This was the first evidence that TGFβ1 could be useful in clinical practice to preserve retinal damage. However the intravitreal injection represents an invasive route, although it is currently used by ophthalmologists to delivery anti-VEGF drugs and corticosteroids. Intraocular administration has several drawbacks such as patient discomfort,

especially for multiple injections, and it could be risky because the potential endophthalmitis [15]. Therefore, our study was aimed at developing a topical nano-technological formulation able to deliver TGF-β1 into the posterior segment of the eye.

2. MATERIAL AND METHODS

2.1. Unilamellar vesicle preparation

The phospholipids used for the preparation of the vesicular systems were 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (Genzyme Pharmaceuticals, Liestal, Switzerland) and 1,2-dioleoyl-sn-glycero-3-phospho-L-serine sodium salt (DOPS) (Avanti Polar Lipids, Alabaster, AL, USA). Cholesterol (Chol) and phosphate buffered saline (PBS tablets, pH 7.4) were purchased from Sigma-Aldrich (Milan, Italy); methanol was a product from Riedel-DeHaën (Seelze, Germany), and chloroform was purchased from VWR PBI International (Milan, Italy); solvents were used as received. The multilamellar vescicle (MLV) liposomal suspensions were obtained by hydration of a phospholipid film using the thin layer evaporation (TLE) method [16]. Negatively charged vesicles, consisting of DPPC-Chol-DOPSin a 60:25:15 molar ratio, were produced. The lipids (1 mg total) were placed in a test tube and dissolved in 1 ml of a 1:1 (v/v) chloroform-methanol mixture. The solution was evaporated to dryness under a nitrogen stream and slow rotation, forming a thin lipid film at the bottom of the tube. To remove all the residual solvents, the tubes were placed in a Büchi T-50 oven at 30°C under high vacuum for 6-8 hours. The hydration process was accomplished by adding to the lipid film 2 ml of PBS (pH 7.4) (total volume) containing 250 ng/ml of TGF-β1 (human recombinant TGF-β1 cod. 240-B-010, R&D Systems Inc. USA). The tube was heated in a water bath at 50°C for 2 min under mild heating; the entire procedure was repeated three times. After hydration, samples were left to equilibrate at room temperature for 2 h. The MLV suspension was turned into a small univescicular(SUV) preparation by membrane extrusion, using a LiposofastTM system (Avestin, Ottawa, Canada). Each MLV sample was sequentially passed through two stacked polycarbonate membranes, with a nominal pore diameter of 400 nm and then 100 nm, pushing the suspension back and forth between two gastight glass syringes for 19 times, at room temperature. Soon before ocular topical administration we added to the TGF-β1 stock formulation recombinant human annexin V (#1005 purchased by BioVision, Milpitas, USA)

and $CaCl_2$ (purchased by Sigma-Aldrich, Milan, Italy). TGF-β1 final concentration was 125 ng/ml, annexin V was added to a final concentration of 15 μg/ml in order to obtain an average of 30 molecules per vesicle and $CaCl_2$ was added in order to obtain 2 mM concentration [17]. Each liposome batch was characterized within 24 h from preparation. The electrophoretic mobility and Zeta potential were determined by a particle electrophoresis analyzer (Zetasizer Nano ZS90, Malvern, UK). The apparatus consisted of a He-Ne laser with a maximal power of 4 mW, at a wavelength of 633 nm. Each sample was diluted 1:100 with HPLC-grade water for the test. Up to 100 measurements on each sample were recorded at room temperature to calculate the electrophoretic mobility andthe corresponding Zeta potential values, by using a Smoluchowski constant (Ka) value of 1.5. The mean size (Z-ave) and polydispersity index (PDI) were determined by dynamic light scattering using the same instrument. Samples were 10-fold diluted with HPLC-grade water before the analysis. The collected values are the mean ± SD of 90 measurements (three sets of 10 measurements in triplicate).

2.2. *In vivo* study

New Zealand albino rabbits have been obtained by Envigo (Udine, Italy). Rabbits, weighted approximately 2-2.2 Kg, have been housed for 1 week prior the study, while they were fed on standard laboratory food and allowed free access to water in a room with standard temperature and humidity conditions accordingly to the 12-h light/12-h dark cycle. Thirty microliters of the TGF-β1 liposomal formulation were topically administered in the rabbit eye. After 30, 60, 120, 180 and 240 min from TGF-β1 administration the rabbit was killed by intravenous injection of Tanax (0.3 mL/kg; Intervet Italia, Milano, Italy), and the vitreous collected and stored at -80 C°. Housing and treatments were in accordance to Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Visual Research.

2.3. TGF-β1 measurements

Vitreous samples were sonicated for 5 minutes in a ice-water bath and TGFβ1 levels were measured by ELISA kit (ADI-900-155, Enzo Life Bioscience, Farmingdale, NY, USA) as previously reported [18]. In order to measure only the exogenous TGFβ1 we avoided the acidification protocol that leads to dissociation of the LAP/TGFβ1 endogenous complex. TGFβ1 levels related to rabbit that received the TGFβ1 formulation have been compared to

the TGFβ1 levels detected in vitreous of control rabbits, in order to carry out the evaluation of TGFβ1 by means of the above described ELISA kit.

2.4. Ocular tolerability assessment

The potential ocular irritancy and/or damaging effects of the formulation were evaluated according to a modified Draize's test [19]. A slit lamp (mod. 4179T Sbisà, Florence, Italy) was used. Congestion, swelling, and discharge of the conjunctiva were graded on a scale from 0 to 3 (0=normal; 1, 2 and 3 = discrete, moderate and intense dilatation of conjunctival vessels, respectively), 0 to 4 (0 = normal; 1, 2, 3 and 4 = discrete, moderate, intense, intense + lid closure conjunctival swelling, respectively), and 0 to 3 (0 = normal; 1, 2 and 3 = discrete, moderate and intense discharge, respectively). Iris hyperemia was graded on a scale from 0 to 4 (0 = normal, 1 = discrete dilatation of iris vessels; 2 = moderate dilatation of iris vessels; 3 = intense iridal hyperemia with flare in the anterior chamber; 4 = intense iridal hyperemiawith flare in the anterior chamber and presence of fibrinous exudates). Corneal opacity was graded on a scale from 0 to 4 (0 = normal, 1 = some spots of opacity; 2 = diffuse cortical opacity; 3 = cortical and nuclear opacity; 4 = intense opacity plus posterior subcapsular opacity). Eye drops (30 μl) were topically administered in the right eye every 30 min for 6 h (12 treatments). At the end of the treatment, two observations at 10 min and 6 h were carried out to evaluate the ocular tissues. Observations were made by two independent observers in a masked way. Methylene blue staining was used to evaluate the corneal integrity, which allows an accurate determination of the extent of epithelial damage because of its poor diffusion through the stroma layer of the cornea.

2.5 Statistical analysis

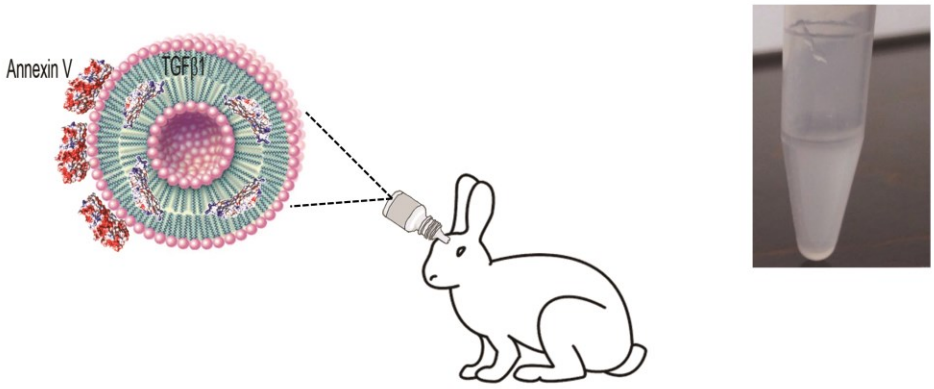
All data were expressed as means ± standard deviation. Statistical analysis was done using t-test followed by ANOVA. In all cases statistical significance was set at $p < 0.05$.

3. RESULTS AND DISCUSSION

The pharmacokinetics profile generated from this study showed a remarkable ocular bioavailability of TGF-β1 following single administration. In particular, TGF-β1 reached the

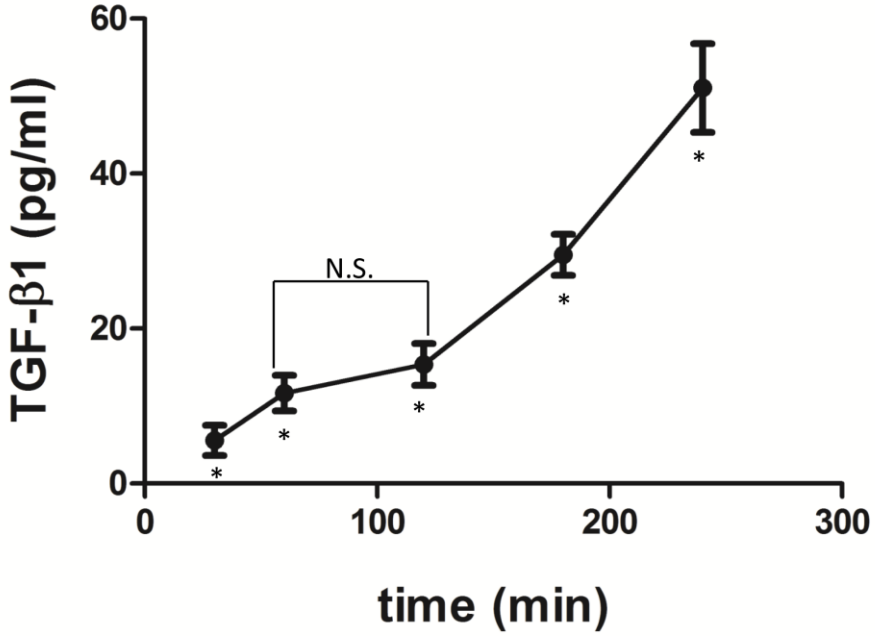
back of the eye after topical application of the drug delivery system into the conjunctival sac. These findings may have relevant clinical implications when considering that intravitreal injections are invasive and risky for patients. Previously studies from our lab demonstrated that intravitreal injection of TGFβ1was able to protect retinal tissue in an rat model of age-related macular degeneration[8]. We showed that intravitreal injection with Aβ oligomers induced a strong increase in Bax protein level and a significant reduction in Bcl-2 protein. Furthermore, we found that Bax/Bcl-2 ratio decreased when rats were treated with TGF-β1, while the beneficial effect of TGF-β1 was counteracted by inhibition of kinase ALK/TGF-β type I receptor. From these important studies we moved forward to explore the pharmacokinetics profile of a topical ocular formulation delivering TGF-β1. Using a classical TLE method, a heterogeneous population of MLV was obtained, with a mean size above 2 µm. Extrusion of these vesicles through polycarbonate membranes led to the formation of SUV with an average size of 154 nm and a very high size homogeneity (PDI= 0.067). The Zeta potential value was found to be markedly negative for the SUV containing the negatively charged DOPS (-16.6 ± 1.2 mV). Measurement of the Zeta potential before and after the extrusion of MLV to form the SUVs did not cause changes, confirming that this physical process did not affect the mean composition of the vesicles. After addition of annexin V, the SUV samples did not show relevant changes in the mean size (156 nm; PDI= 0.144), while the surface charge became more negative (-28.83 ± 0.9 mV), suggesting that the protein remained located on the external surface of the phospholipid vesicles (Fig.1).

Fig. 1 Description of topical nanoparticle formulation of TGF-β1



The pharmacokinetics profile generated in the present study (Fig. 2) demonstrated remarkable levels of TGF-β1 in the posterior segment of the eye ($AUC_{0\text{-}240}$was 4834 pg · min /ml). In particular, we detected high levels of TGFβ1 (C_{max} 51.06 pg/ml) in the vitreous after 240 minutes (T_{max}) from the topical application of the eye drops. It is worthy to note that these concentrations are biologically relevant and higher than serum levels detectable both in AD patients and healthy controls [10]. Interestingly TGF-β1 is known to exert in this range of concentration relevant neuroprotective effects in experimental models of neurodegenerative disorders [9]. Recently, Davis and co-authors (2014) developed a topical formulation of the monoclonal antibody bevacizumab (149 KDa) encapsulated in small unilamellar vesicles (SUVs) containing phosphatidylserine (PS), with adjunct of annexin V and Ca^{2+} extemporaneously to topical administration [17]. Bevacizumab was administered off-label, through intravitreal injection, to patients with age related macular degeneration, due to its ability to bind VEGFA thereby inhibiting VEGF pro-angiogenic activity.

Fig. 2 Rabbit vitreous bioavailability of TGFβ1.p-value of each time point is significantly different if compared to each other ($p<0.01$), with exception to concentration at 60' and 120'. Error bars indicate the standard deviation of six measurements. N.S. = not significant; n=6



Davis and co-authors (2014) exploited the ability of annexin V to cross the cell membrane in presence of calcium [20-23] and assessed the delivery of bevacizumab to the rabbit retina through topical administration. Following a similar protocol, we have developed a formulation of TGF-β1 encapsulated in SUVs[17]; the formulation has been supplemented withannexin V and calcium prior topical formulation (30 μl) to rabbit's eye. SUVs have been formulated with DPPC, DOPS and Chol at fixed concentrations (i.e. 60:15:25 molar ratio, respectively). TGF-β1 acts through a receptor complex constituted by the serine/threonine receptors ALK/TGF-β type I receptor and TGF-β type II receptor (TβRII), strongly expressed in the CNS. TGF-β1 binding to TGF-β type II receptorinduces the assembly of type I and type II receptors into a complex, with the subsequent transphosphorylationof type I receptor by the type II receptor kinase. The subsequent activation of type I receptor leads to

phosphorylation of selected SMAD proteins that, in turn, translocate into the nucleus in order to regulate the expression of different target genes involved in cell proliferation and neuronal survival [1, 9]. Besides SMAD-mediated gene transcription, TGF-β1 activates SMAD-independent pathways, including the extracellular-regulated kinase (ERK) pathway [24] and the PI-3-K/Akt pathway [8, 25]. Neurotrophic factor therapy represents a tough challenge for CNS drug discovery, because protein growth factors do not cross the blood–brain barrier and require intracerebral administration to be effective. Eye is considered an extension of brain and retina is part of CNS, therefore the ocular drug delivery shows challenges somehow similar to CNS delivery. Intravitreal injections are commonly used in clinical practice to delivery drugs to the posterior segment of the eye despite the risks of this invasive administration. The topical formulation of TGF-β1 was well tolerated and the score for each parameter was zero at all time of observations (Tab. 1). Therefore, the potential clinical interest of the present formulation is also related to its non-toxic effects other than ocular bioavailability.

Table 1. Ocular tolerability

Formulation	Conjunctiva			Iris	Corneal
	Congestion	Swelling	Discharge	hyperemia	opacity
SUV unloaded (10 min)	0	0	0	0	0
SUV unloaded (6 h)	0	0	0	0	0
SUV-TGF-β1 (10 min)	0	0	0	0	0
SUV-TGF-β1 (6 h)	0	0	0	0	0

4. CONCLUSION

In conclusion, we demonstrate that the novel liposomalformulation was able to deliver remarkable levels of TGF-β1 into the back of the eye after topical instillation. Therefore, this carrier may be useful in clinical practice to manage ophthalmic conditions such as age-related macular degeneration avoiding invasive intraocular injections.

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GENERAL DISCUSSION AND CONCLUSIONS

Complex and multi-factorial diseases, such AMD, need appropriate experimental models for identification of new pharmacological targets and development of new neuroprotective drugs. Besides the validity of genetic animal models for AMD, animal models of choroidal neovascularization are among the most simple and diffused models of wet AMD (Pennesi et al., 2012). Consistent with the neurodegenerative hypothesis of AMD, Liu and co-workers (2013) developed a model of early stages of AMD (Liu et al., 2013). This model was based on intravitreal injection of toxic fragments of $A\beta_{1-40}$ in Long Evans rats; Aβ treatment led to upregulation of proinflammatory cytokines (e.g. IL-6, TNF-α, IL-1β, and IL-18)in the RPE and neuroretina (Liu et al., 2013).

Different groups have demonstrated the neurotoxic effects of amyloid-β peptides ($A\beta_{1-40}$ and $A\beta_{1-42}$)in cortical neurons(Jen et al., 1998; Paradis et al., 1996). In particular, in human neuron primary cultures, $A\beta_{1-40}$ and $A\beta_{1-42}$ can trigger the execution phase of apoptotic death via induction of Bax protein and downregulation of Bcl-2 (Paradis et al., 1996). Furthermore, Jen and co-workers (1998) showed that treatment with $A\beta_{1-40}$ and $A\beta_{1-42}$ severely decrease Bcl-2 immunoreactivity in Müller glial cells(Jen et al., 1998).In the present study wedeveloped an animal model of AMD characterized by apoptotic events elicited by intravitreal injection of $A\beta_{1-42}$ oligomers. Recently, the presence of a wide spectrum of amyloid structures in drusen, from patients with AMD,has been demonstrated; in particular non-fibrillar oligomers were detected (Isas et al., 2010). In an early phase of AD, soluble Aβ monomers aggregate into soluble Aβ oligomers and insoluble Aβ plaques, both considered as toxic to neurons (Klein, 2013). Furthermore, it has been suggested that Aβ oligomers might represent the primary neurotoxic species in amyloid-related neurodegeneration in AMD. Therefore, Ichallengedrat retina with prefibrillar small soluble Aβoligomers,because they are known to induce apoptotic death in neuronal cultures (Giuffrida et al., 2009), to set up a model of AMD.

Here I show, for the first time, that Aβ oligomers increase the ratio of Bax/Bcl2 in this new experimental model of AMD. I hypothesize that Aβ oligomers convert into fibrils during the 48 h of treatment, which could finally contribute to apoptotic retinal cell death; in this context, the ratio of pro-apoptotic Bax to anti-apoptotic Bcl2 can be taken as an indexof the extent of apoptosis. Furthermore, I showed that retinal damage is reversed by exogenous

TGF-β1 treatment.Treatment with human recombinant TGF-β1 reversed both the induction of Baxand the reduction of retinal Bcl-2 in rats treated with$A\beta_{1-42}$oligomers, leading to a reduction of the ratio of Bax/Bcl2, which reduces execution phase of apoptotic cell death.

An impairment of TGF-β1 signaling pathway plays a central role in the pathogenesis of AD and it contributes to Aβ accumulation and microglia activation in animal models of AD(Chen et al., 2015; Tichauer and von Bernhardi, 2012). Accordingly, exogenous TGF-β1 is neuroprotective against Aβ toxicity both in *in vitro* and *in vivo* models of AD (Caraci et al., 2011). TGF-β1 has been shown to protect cortical neurons from Aβ-induced neurodegeneration, through activation of the neuroprotective pathway PI3K-Akt (Caraci et al., 2008). However, the role of TGF-β1 in promotion of either cell survival or apoptosis in retina is not completely understood. The TGF-β1 signaling pathway could induce either apoptosis or cell survival, most likely depending on other cell signaling pathways, such as PI3K-Akt pathway that can be activated independently of Smad- signaling (Wilkes et al., 2005).

Members of the TGF superfamily act through a receptor complex constituted by the activin-like kinase 5 (ALK5)/TGF-β type I receptor (TβRI) and TGF-β type II receptor (TβRII), strongly expressed in the CNS, particularly in hippocampus(Derynck and Zhang, 2003). TGF-β1 binding to TβRII induces the assembly of TβRI and TβRII receptors into a complex, with the subsequent transphosphorylation of TβRI by the type II receptor kinase. The subsequent activation of TβRI receptor leads to phosphorylation of selected SMAD proteins that, in turn, translocate into the nucleus and regulate the expression of different target genes involved in cell survival and proliferation (ten Dijke and Hill, 2004, Fig. 4). Besides Smad-mediated gene transcription, TGFβ activates Smad-independent pathways, including NF-kB (Konig et al., 2005), and PI3K/Akt (Bakin et al., 2000; Caraci et al., 2008). TGF-β/Smad-independent pathways play a key role in mediating different biological effects of TGF-β such as cell cycle inhibition, epithelial-to-mesenchymal trans-differentiation, immune suppression and neuroprotective effects (Caraci et al., 2008; Derynck and Zhang, 2003).

Yin and co-workers (2011) have reported that upregulation of Akt prevents alteration of Bcl-2 family members (including Bcl-xL, Bcl-w, Bad, and Bax) elicited by Aβ and that overexpression of Akt significantly attenuates Aβ-induced apoptosis, while simultaneous inhibition of PI3K, the immediate upstream regulator of Akt, abolishes the protective effect of Akt activation(Yin et al., 2011). Therefore, because TGFβ1 activates PIK3-Akt signaling(Zhu et al., 2004)which is anti-apoptotic, its protective effect could be attributed to

the activation of this pathway; however, other mechanism/s of cell protection by TGFβ1 through SMAD2 cannot be ruled out (Zhu et al., 2004, Fig. 4).

I carried out a bioinformatics analysis, characterized by several enrichment information steps, in order to verify the importance of PI3K-Akt pathway in retinal cell survival.The genes-pathways network, built with the web-application KENeV, revealed that Bcl-2 and PI3K-Akt are respectively the gene and the pathway that can be dysregulated in AMD (Fig. 3). This bioinformatics analysis included also the assumption that AMD and AD share common pathogenic mechanisms. The KEGG pathway related to TGFβ signaling suggests that p-SMAD2 a TGF-β1 downstream pathway effector, would regulate apoptosis (Fig. 4). Recently,it has beendemonstrated that p-SMAD2 is involved in the effects of TGF-β1 on long term potentiation (LTP) formation (Caraci et al., 2015a). Aβ oligomers are known to induce a severe impairment of Smad-dependent TGF-β1 signaling in AD brain (Caraci et al., 2012). Thus, I speculate that activation of the PI3K/Akt pathway by exogenous TGF-β1 in AMD might counteract the deficit of Smad-dependent TGF-β1induced by Aβ oligomers. Further studies are needed to elucidate whether or not the PI3K/Akt pathwaycontributes to the neuroprotective effects of TGF-β1 in AMD, under conditions of defective activation of Smad-dependent TGF-β1 signaling similar to that occurring in AD brain.

Along this line, small-molecule drugs that selectively activate specific elements of the TGF-β1 signaling pathway have been studied for the identification of drugs that can be beneficial in amyloid-related neurodegeneration (Zhang et al.2005). An alternative approach would be the use of centrally available drugs which are able to increase the local production of TGF-ß1.

Different drugs are able to increase TGF-β1 levels in the CNS and might be considered as new neuroprotective tools against Aβ-induced neurodegeneration (Caraci et al. 2011).

Estrogen treatment treatment has been shown to reduce the risk of AD when administered at the time of the menopause and continued over several years (Chen et al. 2006). Estrogens exert strong neuroprotective effects in hippocampal neurons when administered before Aβ treatment (Chen et al. 2006). Interestingly, estrogens act as neuroprotectants via an increased secretion of TGF-ß1 from astrocytes (Sortino et al.2004). TGF-ß1 released from astrocytes exposed to 17β-Estradiol prevents Aß toxicity in pure neuronal cultures by preventing the unscheduled activation of the cell cycle (Sortino et al.2004). These data suggest a possible role for TGF-ß1 in the neuroprotective activity of estrogen therapy in AD, but further evidence is needed to confirm this hypothesis.

Some cardiovascular drugs, such as aspirin and statins, can promote TGF-ß1 synthesis and release (Redondo et al.2007) and, interestingly, both of drugs are known to reduce the risk of developing AD [Nilsson et al. 2003; Sparks et al.2008]. Aspirin inhibits vascular smooth muscle cell proliferation via the TGF-ß1 pathway (Redondo et al.2003), and higher levels of active TGF-ß1 have been found in patients with coronary artery disease treated with aspirin (Grainger et al. 1995). Pravastatin has been found to increase TGF-ß1 synthesis and secretion in plaque monocytes from atherosclerotic patients (Porreca et al.2002). In addition, the combination of atorvastatin with aspirin in patients undergoing coronary artery bypass grafting (CABG) has been shown to decrease the risk of major adverse cardiac events via the suppression of inflammatory responses and an increased production of TGF-ß1 (Nakamura et al. 2006). Unfortunately, no studies have been carried out on the effects of these drugs on TGF-ß1 synthesis in the CNS.

Interestingly, some drugs used for the treatment of CNS disorders are known to promote TGF-ß1 synthesis in the brain. Glatiramer (GA) is a synthetic amino acid copolymer currently approved for the treatment of multiple sclerosis (MS) that reduces both relapse rate and progression of disability (Simpson et al.2002). Different mechanisms of action have been postulated for this drug in humans. Arnon and Aharoni (2004) have demonstrated that glatiramer in mice induces specific suppressor cells of the T helper (Th2) type that migrate to the brain where they express antinflammatory cytokines such as IL-10 and TGF-ß1 in addition to BDNF (Aharoni et al.2005). Furthermore GA-specific cells increase the expression of TGF-ß1 from glial cells in the cerebral cortex and hippocampus, two brain regions that are strongly implicated in the pathophysiology of AD. It could be interesting to examine the effects of glatiramer treatment on amyloid and tau pathology in AD models. Different antidepressants, including tianeptine (Breivik et al. 2006), sertraline (Sutcigil et al.2007), and venlafaxine (Vollmar et al. 2008), can increase TGF-ß1 production (Caraci et al.2011).

It is relevant to consider that TGF-ß1 is known to induce the transition of human lung fibroblasts to myofibroblasts, a primary event in the pathogenesis of idiopathic pulmonary fibrosis, a chronic interstitial lung disease of unknown etiology characterized by increased fibroblastic proliferation and extracellular matrix remodeling resulting into a loss of lung function and, eventually, respiratory failure (Phan, 2002). TGF-ß1 induces the phenotypic transformation of human lung fibroblasts into myofibroblasts via a sequence of molecular events that involves ERK1/2 activation, GSK-3 ß inhibition, and ß-catenin translocation into the nucleus, which may contribute to the pathophysiology of pulmonary fibrosis (Caraci et al.

2008b). This pro-fibrotic action of TGF-β1 should be considered when examining the potential neuroprotective efficacy of systemically administered drugs able to increase TGF-β1 levels in the CNS in age-related macular degeneration. Therefore a more feasible approach would be the use of topical formulation of TGF-β1 that are well tolerated and can be used to prevent retinal damage in age-related macular degeneration.

In the present thesis I have demonstrated that TGF-β1 can prevent the neurotoxic effects of Aβ oligomers in a new animal model of age-related macular degeneration. I have also examined a novel liposomal formulation of TGF-β1 able to deliver remarkable levels of human TGF-β1 into the back of the eye after topical instillation. I believe that this carrier might be further studied in animal models of age-related macular degeneration as well as in humans to be hopefully proposed as a new pharmacological tool in clinical practice for the management of age-related macular degeneration avoiding invasive intraocular injections.

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