



Università Campus Bio-Medico di Roma

Corso di dottorato di ricerca in  
IMMUNOLOGIA OCULARE

XXIII ciclo anno 2008

**DALLA NEUROIMMUNOLOGIA ALLA NEUROPROTEZIONE:**

**DAL SEGMENTO ANTERIORE AL POLO POSTERIORE**

**Dr. Marco Coassin**

Coordinatore

Chiar.mo Prof. Stefano Bonini

Tutore

Prof. Alessandro Lambiase

24 marzo 2011

## INDICE

1. Introduzione	3
1.1 Una nuova scienza: la Neuroimmunologia	3
1.2 Neurodegenerazione e neuroprotezione	5
1.3 I fattori neurotrofici ed il sistema immunitario	7
1.4 La Neuroimmunologia ed il sistema visivo	9
2. Aspetti neuroimmunologici della cheratite da Herpes simplex virus	12
3. Correlazione tra encefalite e retinite da Herpes simplex virus	21
4. Aspetti di neuroprotezione nella patologia glaucomatosa	31
5. Aspetti di neuroprotezione nella retinopatia diabetica	41
6. Future terapie: l'ipotermia al di là della neuroprotezione	50
7. Conclusioni	62
8. Bibliografia	64
9. Pubblicazioni scientifiche nel corso del dottorato	73
10. Articoli scientifici allegati	74

- Lambiase A, Coassin M, et al. Topical treatment with NGF in an animal model of herpetic keratitis. Graefes Arch Clin Exp Ophthalmol 2008;246:121-7.

- Coassin M, et al. Retinal p75 and Bax overexpression is associated with RGCs apoptosis in a rat model of glaucoma. Graefes Arch 2008;246:1743-9.

- Coassin M, et al. Hypothermia reduces the release of VEGF by retinal pigment epithelium. Br J Ophthalmol 2010;94:1678-83.



## 1. Introduzione

### 1.1 Una nuova scienza: la Neuroimmunologia

La neuroimmunologia è una disciplina che applica le metodologie dell'immunologia alle scienze neurologiche studiando le interazioni tra il sistema nervoso ed il sistema immunitario (Murphy, Travers et al. 2008). Lo scopo di questa nuova scienza è quello di spiegare la fisiopatologia di alcune malattie neurologiche (sclerosi multipal e miastenia gravis, solo per citare gli esempi più noti) e di scoprire terapie innovative non attualmente disponibili per i pazienti.

Ecco come le caratteristiche della neuroimmunologia vengono descritte dall'Istituto Nazionale della Salute degli Stati Uniti (US National Institute of Health):

"Despite the brain's status as an immune privileged site, an extensive bi-directional communication takes place between the nervous and the immune system in both health and disease. Immune cells and neuroimmune molecules such as cytokines, chemokines, and growth factors modulate brain function through multiple signaling pathways throughout the lifespan. Immunological, physiological and psychological stressors engage cytokines and other immune molecules as mediators of interactions with neuroendocrine, neuropeptide, and neurotransmitter systems. For example, brain cytokine levels increase following stress exposure, while treatments designed to alleviate stress reverse this effect.

"Neuroinflammation and neuroimmune activation have been shown to play a role in the etiology of a variety of neurological disorders such as stroke, Parkinson's and Alzheimer's

*Marco Coassin*

disease, multiple sclerosis, pain, and AIDS-associated dementia. However, cytokines and chemokines also modulate CNS function in the absence of overt immunological, physiological, or psychological challenges. For example, cytokines and cytokine receptor inhibitors affect cognitive and emotional processes. Recent evidence suggests that immune molecules modulate brain systems differently across the lifespan. Cytokines and chemokines regulate neurotrophins and other molecules critical to neurodevelopmental processes, and exposure to certain neuroimmune challenges early in life affects brain development. In adults, cytokines and chemokines affect synaptic plasticity and other ongoing neural processes, which may change in aging brains. Finally, interactions of immune molecules with the hypothalamic-pituitary-gonadal system indicate that sex differences are a significant factor determining the impact of neuroimmune influences on brain function and behavior."

Per molti anni il cervello è stato considerato come un sito immunologicamente privilegiato, dove la sorveglianza immunitaria da parte delle cellule infiammatorie non poteva aver luogo (Murphy, Travers et al. 2008). Questa ipotesi aveva ottenuto favore a causa della mancanza del sistema linfatico a livello cerebrale e della scoperta dell'esistenza della barriera emato-encefalica che, a livello della vascolatura celebrale, impedisce la fuoriuscita di cellule immunitarie. Inoltre, fatta eccezione per gli astrociti, non vi è espressione costitutiva di molecole MHC nelle cellule del sistema nervoso centrale. Infine, il trapianto di tessuto allogenico a livello cerebrale sopravvive per lunghi periodi di tempo. Tuttavia, è probabile che la presentazione dell'antigene avvenga a livello del sistema nervoso centrale tramite l'espressione di molecole di MHC di classe I e II da parte di cellule che non le esprimono costitutivamente, tipo gli astrociti e le cellule della glia, in caso di infezione, trauma o infiammazione cronica (Antel and Prat 2000). Infine, è stato dimostrato che in alcune situazioni, grazie all'espressione di alcune molecole di adesione,

*Marco Coassin*

i linfociti T possono attraversare la barriera emato-encefalica. Una volta entrati nel sistema nervoso centrale, è stato dimostrato che le cellule immunitarie si muovono attraverso tratti di sostanza bianca e le zone perivacolari della sostanza grigia. Successivamente alla loro attivazione, le cellule infiammatorie potranno rilasciare chemochine, citochine e fattori di crescita (Kerschensteiner, Meinl et al. 2009).

## 1.2 Neurodegenerazione e neuroprotezione

Sorpassato lo schematico storico che faceva del cervello un sito di privilegio immunitario assoluto, si riconosce che il sistema nervoso è costantemente monitorato da parte del sistema immunitario innato ed adattativo. Quando il dialogo tra queste due entità viene compromesso, vengono poste le basi per lo sviluppo di patologie autoimmunitarie e la neurodegenerazione. In questo senso, il collegamento tra neuroimmunologia e patologie neurologiche è molto stretto (Murphy, Travers et al. 2008).

Prendiamo ad esempio la sclerosi multipla: anche se senza dubbio il sistema immunitario controlla il verificarsi delle riacutizzazioni della malattia, il progressivo accumularsi di disabilità è dovuto allo sviluppo di processi neurodegenerativi a livello della placca demielinizzata (Stuerzebecher and Martin 2000). Qualsiasi sia il meccanismo patogenetico - danno immunitario cellulo-mediato, complemento- o anticorpo- mediato oppure distrofia primaria dell'oligodendroglia - le attuali terapie (i.e., quella con interferone) sono rivolte alla correzione degli aspetti infiammatori e demielinizzanti della sclerosi multipla (Noseworthy, Lucchinetti et al. 2000). Ciononostante, è opinione comune che la vera frontiera del trattamento della sclerosi multiple sia il contrasto dei suoi aspetti neurodegenerativi precoci (Fox 2010).



L'ischemia cerebrale è un altro degli interessi della neuroimmunologia. Infatti, è dimostrato che l'insulto ischemico a livello del sistema nervoso centrale porta ad un'attivazione precoce del sistema immunitario, seguita da una ritardata immunosoppressione endogena (Kriz 2006). Anche se i meccanismi di questo processo sono ancora da chiarire, è certo che l'infiammazione post-ischemica contribuisce alla neurodegenerazione. In questo senso, è stato dimostrato che cellule staminali o precursori trapiantati sperimentalmente hanno un'attività immunomodulante molto forte in grado di ridurre l'infiammazione post-ischemica (Schwartz, Litwak et al. 2008). Di conseguenza, negli ultimi anni lo studio della neuroprotezione tramite immunoregolatori ha riscosso un grande entusiasmo.

Per neuroprotezione si intendono tutti quegli interventi mirati a ridurre il danno precoce e tardivo alle cellule neuronali successivamente ad un danno tissutale. Considerato l'aumento dell'aspettativa media di vita nel mondo occidentale, in futuro assisteremo ad un consistente aumento dell'incidenza delle malattie neurologiche e neurodegenerative. Una maggior conoscenza delle loro basi patogenetiche è imprescindibile per sviluppare delle opportune strategie terapeutiche. Varie alterazioni di proteine sinaptiche, apoptosi mediata da proteine dello stress, modificazioni delle proteine protagoniste della risposta infiammatoria e su tutto lo stress ossidativo sono state chiamate in causa per spiegare i fenomeni neurodegenerativi. Di conseguenza, ai fini neuroprotettivi, sono stati studiati il ruolo dei recettori nicotinici dell'acetilcolina e dell'acetilcolinesterasi, il sistema purinergico, le cascate di segnalazione dello stress segnalazione, le proteine patogene ( $\beta$ -amiloide e prioni) e la neuroinfiammazione (Schliebs 2004).

Moltissimi approcci terapeutici sono stati intentati a scopo di proteggere i neuroni e/o ritardarne la progressiva degenerazione e l'apoptosi: moltissime molecole che

*Marco Coassin*

interagiscono con i recettori presenti sui neuroni, l'eritropoietina, i corticosteroidi, l'ipotermia e svariati fattori di crescita proteici. Tra questi ultimi, i più interessanti sono quelli di stampo neurotrofico: il Nerve growth factor (NGF), il Brain derived neurotrophic factor (BDNF) e le neurotrofine 3, 4 e 5.

### 1.3 I fattori neurotrofici ed il sistema immunitario

Il Nerve growth factor (NGF), capostipite delle neurotrofine, esercita una funzione fondamentale nello sviluppo, il mantenimento e la rigenerazione del sistema nervoso centrale e periferico (Levi-Montalcini 1987). Il NGF è una proteina dal peso molecolare di 130 kD composta da 118 aminoacidi il cui gene è localizzato sul braccio corto prossimale del cromosoma 1 dell'uomo. Delle sue tre sub-unità, la Beta è responsabile dell'attività biologica della molecola, la Gamma è una proteasi che modifica il pre-NGF nella sua forma matura e l'Alfa sembra essere inattiva. NGF è prodotto da diverse celle nel corpo e si accumula in diversi tessuti periferici.

Per la trasduzione del segnale, NGF si lega a due distinti recettori: il recettore più specifico trkA (una tirosin-chinasi) ed il recettore per le neurotrofine p75 (Sofroniew, Howe et al. 2001). La maggior parte delle attività biologiche del NGF sono dovute all'autofosforilazione del trkA e alla successiva attivazione di diverse cascate di trasduzione del segnale, tra cui la via della proteina-kinasi Ras-ERK, della fosfolipasi CY1 e P31 chinasi. Il p75, un membro della superfamiglia del recettore del fattore di necrosi tumorale (TNF), è di per sé funzionale attraverso una via di trasduzione specifica che coinvolge il fattore nucleare kappa B, la chinasi c-jun (JNK) e la maggiore produzione di ceramide. Il salvataggio delle cellule mediata dal trkA comprende l'attivazione di segnali di sopravvivenza o la

*Marco Coassin*

soppressione di segnali apoptotici mediati dal p75. p75NTR morte [14]. La durata e la potenza dell'effetto del NGF dipende dal rapporto reciproco tra trkA e p75 .

Il NGF non solo ha un'azione sulle cellule nervose, ma è anche in grado di modulare le attività immuno-endocrine (Lambiase, Micera et al. 2004). Esiste una mole di evidenze in letteratura che il NGF è prodotto dalle cellule del sistema immunitario (monociti/macrofagi, neutrofili, granulociti e linfociti T e B) che a loro volta esprimono i suoi due recettori e che ha un ruolo chiave nella comunicazione bidirezionale tra il sistema nervoso e quello immunitario. In particolare, l'aumentata espressione di NGF è stata dimostrata nei linfociti T helper e nelle cellule B attivate; inoltre, il NGF modula la proliferazione dei linfociti T e B in modo autocrino. Il NGF è coinvolto nella differenziazione delle cellule del sistema immunitario e ematopoietico, in particolare controllando la sopravvivenza delle cellule B ed il mantenimento della memoria immunologica. Infine, il NGF è in grado di influenzare la sopravvivenza e l'attivazione dei mastociti ed eosinofili, cellule chiave nella risposta infiammatoria di tipo allergico.

Altri studi in vivo e in vitro hanno dimostrato che il NGF non solo ha un'azione di supporto trofico e differenziativo nei confronti delle cellule immunitarie, ma anche un ruolo diretto nell'infiammazione. Il NGF induce il rilascio di mediatori da parte dei basofili, eosinofili e neutrofili (Kanna Y. et al., 1991; Bishoff SC et al., 1992; Takafuji S. et al., 1992), agisce come fattore chemiotattico per i leucociti polimorfonucleati (Gee AP et al., 1983; Boyle MDP et al., 1985) e potenzia la fagocitosi neutrofila (Kanna Y. et al., 1991). Il Nerve Growth Factor è in grado di regolare l'espressione di numerose citochine (Stanisz A et al., 1987). In particolare, il NGF determina un incremento di IL-1 beta e di IL-10 e una diminuzione di IL-2.

*Marco Coassin*



D'altra parte, la somministrazione di elevate concentrazioni di NGF purificato nella cavità articolare di roditori non induce alcun fenomeno infiammatorio: questa evidenza ha supportato l'ipotesi che il NGF non abbia un ruolo pro-infiammatorio ma che sia, piuttosto, coinvolto nella modulazione della risposta immunitaria. Si è ipotizzato che il NGF sia una "molecola sentinella": in condizioni fisiologiche, la sintesi di NGF determina un'attivazione delle cellule immunitarie che si preparano a rispondere più tempestivamente alle aggressioni provenienti dall'esterno. I livelli di NGF sono alterati nelle malattie autoimmuni. Infine, è possibile che il NGF protegga anche alcuni citotipi che non dovrebbero risentire dei fenomeni distruttivi dell'infiammazione, quali i neuroni e gli oligodendrociti. In questo senso si potrebbero leggere le evidenze che il NGF favorisce il processo di cicatrizzazione e, quindi, i processi riparativi del danno provocato dall'infiammazione (Li et al., 1980; Otten, 1991).

#### **1.4 La Neuroimmunologia ed il sistema visivo**

Alla stregua del sistema nervoso centrale, anche l'occhio è stato considerato storicamente un sito di privilegio immunitario. Oggigiorno sappiamo che il privilegio immunitario è molto di più di una struttura anatomica che passivamente blocca il sistema immune. Il microambiente oculare coinvolge continuamente ed attivamente il sistema immunitario con meccanismi di tipo immunosoppressivo (Taylor and Kaplan 2010). Le caratteristiche uniche del privilegio immunitario oculare sembrano destinate a proteggere gli occhi e preservare la vista. Tuttavia, a volte la protezione non è sufficiente e si manifesta una forma di malattia del sistema immunitario a livello intraoculare: l'uveite. Essa può essere idiopatica o associata a malattie sistemiche o infezioni. Altre volte le patologie oculari si manifestano con caratteristiche più proprie delle malattie neurodegenerative.

*Marco Coassin*

Esempio principe di malattia dai connotati neurodegenerativi è la patologia glaucomatosa. Il glaucoma è un'otticopatia degenerativa che porta alla perdita progressiva di cellule ganglionari a livello retinico e di fibre nervose a livello del nervo ottico. Le cause del tipo di glaucoma più diffuso (quella cronico ad angolo aperto) non sono conosciute. L'aumento della pressione intraoculare appare più come un fattore di rischio che un elemento per spiegare l'origine delle forme di glaucoma cronico ad angolo aperto. Mentre la teoria vascolare appare poco convincente, si ipotizza che le ragioni della malattia glaucomatosa siano piuttosto di ordine immunologico (Grus and Sun 2008). Il carattere neurodegenerativo cronico del glaucoma ha spinto gli scienziati alla ricerca di un trattamento neuroprotettivo che rallentasse o inibisse la progressione di questa invalidante malattia. L'incrocio di aspetti neurologici ed immunologici unici, insieme alla facilità con cui l'organo della vista può essere raggiunto dai farmaci topici, ha fatto del glaucoma un argomento principe della neuroimmunologia e dei trattamenti sperimentali neuroprotettivi.

Numerose sono le evidenze che le neurotrofine ed in particolare il NGF agiscano su cellule dell'occhio sia durante lo sviluppo che nello stato adulto (Bonini, Aloe et al. 2002; Micera, Lambiase et al. 2004). La sintesi di NGF e la presenza dei suoi recettori è stata dimostrata sia nel segmento anteriore dell'occhio sia in quello posteriore, sia nell'uomo che negli animali. Viene spontaneo pensare che possa avere un ruolo nel restaurare l'integrità delle cellule dell'apparato visivo nelle diverse patologie oculari.

Il NGF svolge un ruolo cruciale per lo sviluppo e la differenziazione della retina ed è stata dimostrata la produzione di NGF e l'espressione dei suoi recettori da parte delle cellule gangliari, delle cellule di Muller e dell'epitelio pigmentato retinico (Chakrabarti et al., 1990).

In uno studio condotto su un ceppo mutante di topo, il C3H, utilizzato come modello sperimentale di retinite pigmentosa, la somministrazione di NGF esogeno sia per via intravitreale che retro-oculare era in grado di ritardare la degenerazione dei fotorecettori (Lambiase e Aloe, 1996). Numerosi studi su modelli animali hanno riguardato il ruolo del NGF nel proteggere le cellule gangliari da un danno di natura meccanica (in particolare, sezione del nervo ottico), ischemica o ipertensiva in diverse specie animali: le salamandre (Turner e Delaney, 1979), i pesci (Turner et al., 1980), i ratti (Carmignoto et al., 1989), i gatti (Siliprandi et al., 1993), i conigli (Lambiase et al., 1997). Tutti questi studi hanno messo in evidenza un'efficacia del neuropeptide nell'inibire la degenerazione di queste cellule retiniche.

Il segmento anteriore dell'occhio presenta uno straordinario intreccio tra aspetti neurologici (la cornea è il tessuto più innervato del corpo umano) ed immunologici (la congiuntiva è una mucosa). La prima evidenza della presenza di NGF a livello corneale la ottenne Ebendal (1988) nei polli. In seguito, è stata dimostrata la presenza di NGF e del suo recettore (TrkA) a livello dell'epitelio e dell'endotelio corneale (Lambiase et al, 1998) e dei cheratociti (Kruse et al., 2000). Queste osservazioni suggerivano un ruolo della neurotrofina nel trofismo e nel mantenimento dell'integrità dell'epitelio corneale in condizioni basali. Sulla base di queste evidenze sperimentali, sono stati condotti alcuni trials clinici utilizzando l'applicazione topica del NGF nel trattamento delle ulcere corneali neurotrofiche ed autoimmuni di grado severo. La somministrazione topica del NGF ha determinato una riepitelizzazione dell'ulcera con un recupero della sensibilità corneale di tutti i pazienti trattati senza effetti collaterali locali o sistemici (Lambiase, Rama et al. 1998).

## 2. Aspetti neuroimmunologici della cheratite da Herpes simplex virus

La cheratite da Herpes simplex virus (HSV), una delle principali cause di cecità nel mondo, è caratterizzata da episodi cronici di riattivazione virale che porta a cicatrici corneali e deficit visivo permanente. Durante l'infezione primaria, la latenza si stabilisce nei neuroni quando HSV raggiunge i gangli sensitivi attraverso il trasporto assonale retrogrado. La riattivazione di HSV latente sembra essere legato alla perdita del sostegno trofico fornito dal bersaglio periferico. Secondo questa ipotesi, diversi tipi di stress, tra cui la resezione dei nervi corneali, cheratectomia e l'esposizione ai raggi UV può causare la recidiva di cheratite erpetica. Gli stratagemmi terapeutici mirano a mantenere il virus latente ed alcuni vaccini sono stati proposti con risultati contrastanti.

Il NGF è un fattore pleiotropico in grado di esercitare una funzione trofica sul sistema nervoso periferico, un'azione di modulazione della risposta immunitaria e indurre la risoluzione delle ulcere corneali. Inoltre, esperimenti in vivo e in vitro hanno dimostrato che il NGF esercita un'attività antivirale e si ipotizza che NGF potrebbe essere il fattore trofico responsabile del mantenimento della latenza nei neuroni. Per valutare il ruolo del NGF endogeno nella infezione da HSV corneale e gli effetti del trattamento topico con NGF sulla cheratite erpetica, l'infezione corneale è stata indotta in 40 conigli con inoculazione di HSV-1 (ceppo McKrae). Gli animali sono stati divisi in 4 gruppi sperimentali: trattamento con anticorpi anti-NGF, con NGF, con acyclovir in pomata o con soluzione fisiologica (controllo). Gli anticorpi anti-NGF hanno indotto un quadro peggiore di cheratite e due degli animali svilupparono un'encefalite letale. Il trattamento con NGF, invece, ha indotto un miglioramento di tutti i parametri clinici e di laboratorio quando confrontato col gruppo controllo. Non sono state notate differenze significative tra i gruppi NGF e acyclovir.

Questo studio ha dimostrato il ruolo cruciale del NGF come modulatore della latenza virale, della risposta immunitaria e della riparazione tissutale nella cheratite erpetica ed è un punto di partenza per future nuove terapie antivirali con le neurotrofine..

## 2.1 Introduction

Herpes Simplex Virus (HSV) keratitis, a primary cause of blindness worldwide, results from chronic episodes of viral reactivation leading to permanent corneal scarring, vision impairment and blindness [16,44]. During primary infection, latency is established in neurons when HSV reaches sensitive ganglia by retrograde axonal transport [12,44]. Reactivation of latent HSV seems to be related to the loss of trophic support provided by the peripheral target [40]. According to this hypothesis, several types of stress, including corneal nerve resection, keratectomy and UV exposure may cause herpetic keratitis recurrence that ultimately leads to corneal scarring [12,24]. Corneal opacity is a difficult challenge for the ophthalmologist, since corneal grafts are often unsuccessful due to the reactivation of latent HSV and rejection reactions [9,15]. Prophylactic treatment with oral acyclovir significantly improves the prognosis and rate of recurrence of herpetic keratitis, as well as the survival of corneal graft, but there is no general agreement regarding the duration of treatment and the risk of side effects in case of prolonged treatment [2,7,23,35,48,49]. Novel and long acting treatment for HSV keratitis aims to keep the virus latent, and some vaccines have been proposed with contrasting results [18,39,53]. In recent years, nerve growth factor (NGF) has been described as a pleiotropic factor exerting a trophic function on the peripheral nervous system, a modulating action on immune response and wound healing activity on corneal ulcers [11,27,31,32]. In vivo and in vitro studies have shown that NGF also exerts antiviral activity. In vitro, NGF has a cyto-protective effect, as demonstrated on HSV infected cell cultures [52]. Indeed NGF treatment preserves HSV latency in rat sympathetic neuronal cell cultures, while



neutralizing anti-NGF antibodies induce virus reactivation [57]. This antiviral action of NGF has been confirmed by in vivo studies showing that NGF treatment decreases neuronal damage induced in young rat cervical ganglia in an animal model of HSV encephalitis [23]. Moreover, systemic treatment with neutralizing anti-NGF antibodies induces reactivation of HSV keratitis in a rabbit model of HSV infection [24]. Price and Schmitz hypothesized that NGF may be the trophic factor responsible for maintaining HSV latency in neurons [40,55,56]. Several studies support this hypothesis, since (1) NGF-dependent neurons [36,50] are documented sites of HSV latency [17,34] and (2) since treatments that produce a decrease of NGF retrograde transport, such as axotomy and central rhizotomy, result in reactivation of latent HSV.[41,45,54]

The aims of this study were to evaluate the role of endogenous NGF in HSV corneal infection and the effects of topical NGF treatment on herpetic keratitis.

## **2.2 Methods**

This study was performed according to the guidelines of the European Community Council Directive of 24 of November 1986 (86/609/EEC), the Declaration of Helsinki for research involving animal models and the Guide for the Care and Use of laboratory animals (USPHS). All analytical grade reagents and plasticware were from SIAL (Milan, Italy), SERVA (Weidelberg, Germany), Celbio (Milan, Italy) and NUNC (Roskilde, Denmark), when not specified differently in the text.

### In vitro HSV-1 Virus propagation

HSV-1 MacKrae strain ( $10^6$  PFU/mL) was expanded on VERO cells cultured in monolayer. After 40 hrs, total virus was collected, frozen-thawed three times and spun down to eliminate cellular debris. Virus was titred by plaque assay on these same cell monolayers and stored at  $-80^{\circ}\text{C}$  in  $10^8$  PFU/mL aliquots until inoculation.

### Animals and experimental model

*Manuela Coassin*

Forty New Zealand female rabbits (~2.5Kg, purchased from Charles-River, Como, Italy) were used for the study. Following corneal scarification, one eye was infected with 25µL of a suspension containing HSV-1 McKrae strain ( $1-2 \times 10^5$  PFU/mL) [24]. Three days after infection, animals were randomly divided into 4 groups (10 animals per group) that were treated in one eye five times daily as follows: group 1 (referred to as NGF) received NGF ointment (100 µg/mL) [13]; group 2 (anti-NGF) received a neutralizing anti-NGF antibody ointment (1000 µg/mL) [28]; group 3 (Acyclovir) received 3% Acyclovir ointment; group 4 received the ointment vehicle (Control). All groups were treated for 12 days.

Murine NGF was purified according to the method devised by Angeletti and Bocchini, dissolved in paraffin oil at 37 °C and mixed 1:2 with Vaseline ointment [10].

Animals were evaluated every day by bio-microscopic examination and eye photographs were taken. After fluorescein staining, corneal lesions were scored by a masked observer as follows: 0.0-0.5: normal to non-specific, random superficial lesions; 0.6-0.9: punctate ulcerations; 1.0-1.9: one or more dendritic ulcerations; 2.0-2.9: geographic ulcerations or trophic erosions with less than 50% of cornea involved; 3.0-3.9: geographic ulcerations or trophic erosions with more than 50% of the cornea involved [4].

Fourteen days post-infection, animals were sacrificed (overdose of pentobarbital) and corneas collected for biochemical and molecular evaluations. HSV infection was assessed in corneas by immunohistochemical and PCR techniques, in order to evaluate the grade of infection/replication in both untreated and treated eyes.

#### Biochemical evaluation of herpetic infection

For immunohistochemical analysis, corneas were pre-fixed in 4% buffered paraformaldehyde, cut into 0.3 µm sections (Microm HM325, Bioptica, Milan, Italy), placed onto electrostatically charged slides (BDH, Bioptica), de-waxed and quenched with 0.3% H<sub>2</sub>O<sub>2</sub> in 10 mM phosphate buffered saline (PBS) for 15 min. The sections were then blocked/permeabilized (0.8% bovine serum albumin/0.5% Triton-X100 in PBS) and probed

*Marco Coassin*

with a mouse anti-HSV-1 glycoprotein D antibody (MCA220, Serotec, Milan, Italy) according to the avidin-biotin-complex peroxidase technique.

### Molecular evaluation of herpetic infection

Molecular analysis was performed on corneas using a nested PCR system. Half of the corneal button was used for total DNA extraction (Gentra Systems, Minnesota, USA). DNA was dissolved in depc-water, quantified at 260nm and electrophoresed on a 1% agarose gel. Normalized samples were assayed at a concentration of 3µg per reaction mixture. Amplification was performed in a programmable PTC-100 thermocycler (MJ Research, Watertown, MA, USA) on a volume of 20µL containing 3µL DNA (for GpD) or 1µL DNA (for α-actin) and 17µL master mix containing 10µL of 10XSYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 0.5µL of each primer (10pM) and 6 µL depc-treated water. PCR amplification conditions for α-actin were 15min/95°C (hot start activation), followed by 30 cycles of 30sec/95°C, 25sec/55°C, 30sec/72°C, and further elongation for 10min/72°C. PCR amplification conditions for GpD were 15min/95°C (hot start activation), followed by 29 cycles of 30sec/94°C, 30sec/55°C, 60sec/72°C, and further elongation for 2min/72°C. A 100bp sequence of the rabbit α-actin gene (forward 5'-CCG GAC GCC ATC CTG CGT CT-3' and reverse 5'-CGC TCG GCC GTG GTG GTG AA-3': designed by Primer3 software, available on line at the [http://www-genome.wi.mit.edu/cgi-bin/primer/primer3\\_www.cgi](http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and prepared by MWG Biotech, Ebersberg, Germany) was amplified from each DNA sample. HSV-1 glycoprotein D (GpD) gene amplification was performed according to the nested procedure with primers described by Aurelius and coworkers, and prepared by MWG [6].. The first amplification was carried out at 30sec/55°C with the forward primer 5'- ATCACGGTAGCCCGGCCGTGTGACA-3' and the reverse primer 5'- CATACCGGAACGCACCACACAA-3'. Ten µL of the first amplification were used as template for the second offset of primers (forward: 5'- CCATACCGACCACACCGACGA -3' and reverse: 5'-GGTAGTTGGTCGTTTCGCGCTGAA-

*Marco Coassin*



3') for a final 145 bps of PCR product. PCR products were visualized on a 2.5% agarose gel and photographed by a Kodak imager station (Kodak 550, Eastman Kodak Company, Sci. Imaging Systems, Rochester, NY). Negative controls were composed of depc water, HSV-free corneas from the eye bank, as well as a cell line of human fibroblasts (MRC-5, ATCC, Rockville, MD).

### Evaluation of herpetic encephalomyelitis

The number of animals that died during treatment was used as a parameter in determining treatment efficacy. Histological (hematoxylin and eosin staining; Biotica) and immunofluorescent evaluations (HSV-1 specific staining) were routinely performed on the brain of animals that died during the study to confirm the diagnosis of herpetic encephalitis.

### Statistical analysis

ANOVA for repeated measures was performed for analysing differences among treatment groups. Post-hoc comparisons within logical sets of means were performed using the Tukey-Kramer test. Analyses were performed using the statistical package SPSS 13.0 for Windows (SPSS Inc., USA). A probability  $<0.05$  was considered statistically significant.

## **2.3 Results**

In animals treated with inactivation of endogenous NGF, the herpetic keratitis was significantly exacerbated compared to the other three groups ( $p < 0.0001$ , figure 1). In particular, 8 out of 9 animals treated with neutralizing anti-NGF antibodies had ulcers at day 14 (figure 1 and 2). Two rabbits of the anti-NGF treated group died before the end of the experiment. Histological and immunohistochemical studies carried out on brain sections demonstrated that these animals died from HSV encephalitis (figure 3). Conversely, NGF treatment induced a significant improvement in corneal signs of herpetic keratitis. As shown in figures 1 and 2, NGF treatment accelerated corneal wound healing

*Marco Coassin*

when compared to placebo (ANOVA, repeated measures x treatment:  $F = 21.137$ ;  $p < 0.01$ ): in the NGF treated group all the corneas were healed at day 12, while the control corneas were healed at day 14.

No significant difference was observed between NGF and acyclovir treatment ( $p > 0.05$ ) in terms of time required to achieve complete corneal healing. However, the NGF treated group showed significantly more improvement in keratitis when compared to acyclovir treated animals at days 6, 7 and 8 ( $p < 0.05$ ; fig. 1).

These clinical data were confirmed by biochemical and molecular evaluation of HSV expression in the cornea. In fact, HSV glycoprotein D (GpD), produced early in viral replication and required for HSV-1 cell entry and fusion [14,43], and GpD mRNA were markedly expressed in the corneas of control and anti-NGF treated groups and only slightly expressed in the NGF and acyclovir treated groups, as demonstrated by immunohistochemistry and PCR (figure 4).

## 2.4 Discussion

This masked, negatively and positively controlled animal study demonstrated the role of endogenous NGF and the efficacy of NGF topical treatment in herpetic keratitis. Despite the increasing number of effective antiviral drugs, HSV keratitis is still one of the main causes of blindness in developed countries, as a result of chronic episodes of viral reactivation leading to permanent scarring and visual impairment [16,44]. Corneal transplantation has a poor prognosis in patients with herpetic keratitis due to the high rate of viral recurrence that causes progressive opacity of the implanted corneal graft [9,15]. Systemic therapy is effective in preventing herpetic infection recurrence but may present adverse side-effects [25]. New antiviral drugs with different mechanisms of action are required to guarantee an effective treatment and prevention of recurrence with minor side effects. With this perspective, we sought in this study to investigate the role of endogenous

*Marco Coassin*

NGF in an animal model of herpetic keratitis, and to compare NGF treatment to conventional antiviral therapy.

We observed a worsening of the clinical outcome of herpetic keratitis and the spreading of HSV infection to the brain in 20% of animals following neutralization of endogenous NGF by the topical administration of specific anti-NGF antibodies. This data is consistent with the observation of an increased corneal production of NGF in an experimental model of epithelial injury [28], similar to the corneal scarification used in this model. The protective role of endogenous NGF in limiting HSV damage is well known. In fact, in vitro interruption of the NGF supply for even 1 hr by neutralizing anti-NGF antibodies is followed by a rapid reactivation of HSV infection in sympathetic and sensory neurons of rat, rabbit and human [5,24,42], and anti-NGF treatment in vivo stimulates reactivation of ocular HSV in latently infected rabbit eye, suggesting a correlation between deficits in NGF synthesis and ocular virus reactivation and infection [24]. Interestingly, Dicou and co-workers reported increased titers of anti-NGF in the sera of HSV-infected patients with active herpetic disease, compared to latently infected patients who had no sign of disease [19].

Since inhibition of endogenous NGF exacerbates herpetic keratitis, we investigated the effects of topical NGF treatment in this disease. Clinically, NGF administration induced an improvement in outcome of the viral infection when compared to placebo, with an efficacy comparable to acyclovir. In addition, biochemical and molecular data derived from corneas demonstrated that NGF inhibited the productive phase of the virus. These results are in line with the antiviral effects of NGF previously demonstrated in vitro and in vivo. In vitro NGF administration to cell line cultures inhibited cell death and HSV replication in a dose dependent fashion [52], and in vivo NGF administration to developing rodents significantly delayed virus expression and neuronal damage to the superior cervical ganglia [3].

Apparently our data are in contrast with the findings of Laycock and co-workers, which demonstrated that sub-conjunctival NGF administration failed to suppress HSV



reactivation in a mice model of herpetic keratitis [30]. This discrepancy may be related to differences between species, as well as to the different formulation and route of NGF administration. In fact, we used an NGF ointment instead of a sub-conjunctival injection, the latter of which was found to be a potent stimulator of HSV reactivation (Laycock data). The mechanisms underlying NGF's inhibition of HSV replication are still unclear. Some studies indicate that this NGF effect is specific and second messenger pathway mediated [47]. NGF may also influence HSV keratitis by modulating corneal wound healing and the immune reaction. In fact, experimental and clinical studies have demonstrated that topical NGF treatment induces corneal healing and sensitivity restoration in neurotrophic, autoimmune and traumatic corneal injury [27,28,29]. In addition to its known neurotrophic activity, NGF exerts various effects on immune-competent cells that have a crucial role in herpetic infection [32]. For example, NGF enhances proliferation of T and B lymphocytes, regulates antibody production from B cells [37,51] and induces differentiation of monocytes into macrophages [20,33]. Recently, it has been reported that NGF affects the production of several cytokines (such as TNF $\alpha$  and INFs) and chemokines that exert a pivotal role in activating and recruiting leukocytes to the sites of viral infection [1,8,21,22,26,38,46].

In conclusion, this study demonstrated the crucial role of endogenous NGF and the efficacy of topical NGF in an animal model of HSV keratitis. As well as modulating the outcome of this disease, endogenous NGF released by corneal injury seemed to protect animals from the spreading of viral infection, since two animals treated with anti-NGF died of herpetic encephalomyelitis.

The demonstrated role of NGF in maintaining HSV latency in neurons [40,55,56] lead us to evaluate in a future study the efficacy of NGF as a long term treatment for HSV keratitis, in order to prevent infection recurrences.

At the light of the evidences that NGF maintains HSV latency in neurons [40, 55, 56], further investigation on the efficacy of NGF in herpetic keratitis recurrences is warrant.

*Marco Coassin*

### 3. Correlazione tra encefalite e retinite da Herpes simplex virus

L'encefalite da Herpes simplex (HSE) è la causa più comune di encefalite acuta sporadica negli adulti, con una mortalità del 70% se non trattata e una prognosi infausta anche se trattata. HSV-1 è l'agente eziologico della HSE nel 90% degli adulti immunocompetenti, con il 70% di questi casi che sembrano dovuti alla riattivazione del virus latente endogeno. L'encefalite da HSV-2, invece, sembra essere correlata ad una infezione primaria ed è più comune negli adulti immunocompromessi.

La coesistenza di retinite ed encefalite da herpes simplex negli adulti è stata inizialmente riportata da Minckler nel 1976. L'infezione della retina da HSV porta rapidamente alla necrosi della retina periferica, con vasculopatia occlusiva e severa infiammazione, un quadro clinico noto come necrosi retinica acuta (acute retinal necrosis, ARN). In un recente studio, HSV è stata rilevata come agente causale in circa il 50% dei pazienti con ARN.

Tuttavia, ARN è una malattia rara e l'associazione con l'encefalite non è ben conosciuta. Di recente, è stata ipotizzato che l'encefalite sia un vero e proprio fattore di rischio per la retinite da HSV (HSR). Lo scopo di questo studio era di chiarire alcuni aspetti della patogenesi dell'ARN e sottolineare i dettagli della relazione tra le due malattie. La presente revisione della letteratura comprende tutti i pazienti che hanno avuto encefalite e retinite da HSV nel corso della loro vita (con esclusione di coloro che hanno manifestato entrambe le patologie nei primi tre mesi di vita).

Dai 44 articoli selezionati (61 pazienti, 81 occhi), emerge che i pazienti possono essere suddivisi in 4 gruppi:

1) gruppo 1: HSE e HSR contemporaneamente. Gruppo composto da 7 adulti, in 5 l'agente causale era HSV-2;



2) gruppo 2: HSE neonatale e HSR ritardata. Gruppo composto da 6 pazienti. Intervallo medio tra le due patologie: 6.2 anni. In 3 casi l'agente causale era HSV-1, in uno HSV-1 e in 2 non riportato;

3) gruppo 3: HSE in adulti/ragazzi e HSR ritardata. Gruppo composto da 39 pazienti. Intervallo medio tra le due patologie: 28.1 mesi. Nel 80% dei casi l'agente causale era HSV-1;

4) gruppo 4: HSR precede HSE. Gruppo composto da 4 pazienti. Intervallo medio tra le due patologie: 53 giorni. In tutti i casi l'agente causale era HSV-1, tutti i pazienti erano immunocompromessi e tutti sono morti entro tre mesi.

Se invece l'analisi dei sottogruppi veniva fatta considerando solo i casi in cui HSE precedeva HSR (gruppi 1 e 2 riuniti), si deduce che il 48.9% dei pazienti ha avuto la retinite 3 mesi dopo l'encefalite (early-onset group) mentre il 51.1% dopo un tempo variabile che va da un minimo di due anni ad un massimo di 20 anni (late-onset group). Questa revisione della letteratura permette di dedurre straordinarie considerazioni sulla correlazione tra encefalite e retinite erpetica, gettando nuova luce sulla patogenesi delle due malattie.

### 3.1 Introduction

Herpes simplex encephalitis (HSE) is the most common cause of acute sporadic encephalitis in adults, with a mortality of 70% if not treated and a poor prognosis even with treatment. HSV-1 is the causative agent of HSE in 90% of immunocompetent adults, with 70% of these cases reflecting reactivation of endogenous latent virus. HSV-2 encephalitis appears to be related to a primary infection and is most common in immunocompromised adults (Tyler 2004).

The coexistence of herpes simplex retinitis (HSR) and encephalitis in adults was initially reported by Minckler in 1976 (Minckler, McLean et al. 1976). Infection of the retina by HSV



rapidly leads to peripheral retinal necrosis, with occlusive vasculopathy and prominent inflammation in the vitreous and anterior chamber, a clinical picture known as acute retinal necrosis (ARN) (Margolis and Atherton 1996). Although ARN is a clinical picture that could have also other causative agents (i.e., the Varicella-zoster virus), most of the cases occurred in immunocompetent adults were caused by HSV (Walters and James 2001). HSV retinitis is a rare disease that has been often reported in association with HSV encephalitis. The relationship between them is not well documented. This review intends to clear some aspects of the pathogenesis of the HSV retinitis and to investigate possible mechanisms that link herpetic encephalitis and retinitis in adults. We included all the cases present in the literature where someone had both HSE and HSR in his/her lifespan. We decided to exclude from the review all the cases of HSR and HSE where both the diseases occurred in newborns during their first 90 days of life (see Discussion). A review of the literature on concomitant HSE and HSR in newborns has been published recently and this topic is not included in this review 51.

### **3.2 Methods**

Our review includes all the published articles of herpes simplex encephalitis and retinitis in adults and children present in Medline through March 2009. The keywords were herpes simplex/herpetic/viral encephalitis/encephalopathy, herpes simplex/herpetic/viral retinitis/retinopathy and acute retinal necrosis. Publications were examined for references until no further studies were found. Clinical case reports and case series, patient notes from articles describing diagnostic procedures and one extract from a book chapter were included. The major inclusion criterion was the presence of HSE and HSR diagnosed at the same time (defined as “concomitant”) or at different time in the lifespan of the patients. Only the congenital (diagnosed within 10 days from the birth) or neonatal (diagnosed between 10 and 90 days of age) cases of concomitant HSE and HSR were excluded.

*Marco Coassin*

Articles with generic diagnosis of “herpetic virus” (with no specification of the genus or specie) were also excluded.

When available, patient age and gender, HSV type, eye involved, interval of time between encephalitis and retinitis (or vice versa), final visual acuity (converted in feet), therapy, patient survival, eventual immunosuppression and optic nerve involvement were recorded and reported in Table 1.

The laboratory technique used for the diagnosis of the disease in each article is reported in Table 1 as well. Of note, Polymerase Chain Reaction (PCR) of cerebrospinal fluid (CSF) is considered the gold standard for diagnosing HSE. PCR is even more sensitive and specific than appropriate histological and cultural analysis of a brain biopsy (Boivin 2004; Tyler 2004). Also a fourfold increase of CSF anti-HSV IgG or a serum-to-CSF ratio <20 can help retrospectively to diagnose HSE (these two parameters are positive in 80% of cases of HSE) (Nahmias, Whitley et al. 1982; Boivin 2004). Quantitative anti-HSV IgM and IgG levels in serum have less diagnostic significance in HSE (Nahmias, Whitley et al. 1982; Tyler 2004). Specific changes at the magnetic resonance imaging (MRI) have been reported in 90% of PCR-positive patients. Computerized tomography (CT) and electroencephalography (EEG) provide limited diagnostic information (Tyler 2004). CSF cultures are rarely positive in adult cases of HSE (Boivin 2004) and demonstration of peripheral excretion of HSV is of no diagnostic value in HSE (Nahmias, Whitley et al. 1982).

Tissue culture isolation is considered the best way to diagnose HSR and equally useful are virus cultures of the aqueous, vitreous, subretinal fluid, serum and CSF, but their relative sensitivity is unknown (Margolis and Atherton 1996). Currently, PCR techniques for detecting HSV DNA in ocular samples are considered the most reliable, sensitive and specific tool for HSR diagnosis (Chan, Shen et al. 2005). Although the sensitivity of serum anti-HSV antibody level alone is questionable, the presence of intraocular anti-HSV



antibody (Sarkies, Gregor et al. 1986) and the calculation of the Goldmann-Witmer coefficient (intraocular-serum ratio) yield a higher diagnostic value (Davis, Feuer et al. 1995; de Boer, Verhagen et al. 1996; Margolis and Atherton 1996; Van Gelder, Willig et al. 2001).

Histological and ultrastructural analysis, if not supported by immunohistochemistry (IHC) and immunofluorescence (IF), cannot distinguish among different viruses of the herpetic family (Margolis and Atherton 1996).

No critical exclusions of articles were made because of their limits in demonstrating HSV infection. Diagnosis of HSE was classified "Clinical" when based on CT, MRI, EEG, clinical signs or symptoms. In the Table 1, "History of HSE" states that no detailed clinical information was provided. Diagnosis of HSR was classified "Clinical" when supported only by ophthalmoscopic examination.

### 3.3 Results

We found 44 papers (Minckler, McLean et al. 1976; Bloom, Katz et al. 1977; Johnson and Wisotzky 1977; Cibis, Flynn et al. 1978; Savir, Grosswasser et al. 1980; Colin, Bodereau et al. 1981; Partamian, Morse et al. 1981; Uninsky, Jampol et al. 1983; Pepose, Hilborne et al. 1984; Pepose, Kreiger et al. 1985; Freeman, Thomas et al. 1986; Sarkies, Gregor et al. 1986; Wessel, Wietholter et al. 1987; Watanabe, Ashida et al. 1989; Fabricius, Lipp et al. 1990; Manderieux, Boukhobza et al. 1990; Ahmadieh, Sajjadi et al. 1991; Gartry, Spalton et al. 1991; Sekizawa, Hara et al. 1991; Feinman, Friedberg et al. 1993; Thompson, Culbertson et al. 1994; Davis, Feuer et al. 1995; Cunningham, Short et al. 1996; Margolis and Atherton 1996; Schlingemann, Bruinenberg et al. 1996; McDonnell, Silvestri et al. 1997; Pavesio, Conrad et al. 1997; Knox, Chandler et al. 1998; Perry, Girkin et al. 1998; Levinson, Reidy et al. 1999; Verma, Venkatesh et al. 1999; Ganatra, Chandler et al. 2000; Kamel, Galloway et al. 2000; Bergua, Schmidt et al. 2001; Gaynor, Wade et al.

*Marco Coassin*

2001; Maertzdorf, Van der Lelij et al. 2001; Van Gelder, Willig et al. 2001; Gain, Chiquet et al. 2002; Hadden and Barry 2002; Kim and Yoon 2002; Cardine, Chaze et al. 2004; Diaz De Durana Santa Coloma, Vazquez Cruchaga et al. 2004; Preiser, Doerr et al. 2004; Landry, Mullangi et al. 2005) describing 61 patients with HSV retinitis and previous, concomitant or subsequent diagnosis of HSV encephalitis. Thirty-seven were male, 21 female and in 3 the gender was not reported. The age of the patients ranged from 18 months to 80 years (mean 40.8 years; 3 cases with no provided age). Twenty patients had both eyes involved (34.4%) and 41 patients had only one eye affected (65.6%), for a total of 81 eyes described. HSV type 1 infection was the found in 30 cases, HSV type 2 in 12 cases and in 19 patients the subtype of the involved HSV was not determined.

The diagnosis of HSV retinitis was made for 28 cases with PCR analysis, nine by detecting intraocular antibodies, two with immunohistochemistry of the ocular tissue, one culturing the sub-retinal fluid, four with TEM, and one with light microscopy. The diagnosis of HSR was clinical in 15 instances.

Diagnosis of HSV encephalitis was made by PCR analysis in nine cases, by detecting CSF antibodies in four, detecting blood antibodies in seven and by detecting both CSF and serum antibodies in eight; three with immunohistochemistry and one with light microscopy of the brain tissue; culturing brain tissue biopsies in four, and CSF in two. In 10 instances the diagnosis of HSE was clinical; 12 times only "history of HSE" was described.

Among all these 61 reported cases, eight had both HSR and HSE confirmed with PCR analysis.

In five cases, the time-course between the HSE and HSR was not provided. In seven patients the two infections occurred at the same time and five of these where due to HSV type 2. In four cases, the retinitis preceded the encephalitis (mean interval 53 days, ranging from 10 to 90 days). In 45 patients, the retinitis followed the encephalitis. Among them, the time interval between HSE and HSR was as follow: within 3 days, one patient;

*Marco Coassin*

within 3 days and 1 month, 10 patients; within 1 and 3 months, 11 patients; within 3 and 12 months, two patients; within 2 and 10 years, 17 patients; after more than 10 years, four patients.

The final visual acuity in 50 of the 81 eyes was reported as follows: 18 eyes with no light perception, four light perception, five hand motion, one counting fingers (for a total of 27 eyes (44.2%) with none or very poor visual acuity); seven eyes had a final visual acuity between 4/60 and 20/40; 15 eyes had a final visual acuity better than 20/40.

Systemic therapy with intravenous acyclovir was given in 34 cases (starting from 1984 (Pepose, Hilborne et al. 1984)), and in one case each with vidarabine (Bloom, Katz et al. 1977), adenine arabinoside (Colin, Bodereau et al. 1981) or foscarnet (Cunningham, Short et al. 1996). In 24 cases, anti-viral therapy was not given or was un-reported.

Thirteen patients were immunodepressed: six had AIDS, five were on immunosuppressive therapy for other medical conditions and one had intrathecal steroid injection for narrowing of the vertebral canal.

The time interval between the infection and the death of the patient was reported in seven cases, with a mean survival from the starting of symptoms of 7.5 weeks.

The ocular and brain histopathologic findings were reported in seven cases post-mortem (Minckler, McLean et al. 1976; Bloom, Katz et al. 1977; Cibis, Flynn et al. 1978; Partamian, Morse et al. 1981; Pepose, Hilborne et al. 1984; Pepose, Kreiger et al. 1985; Freeman, Thomas et al. 1986). In five of these, involvement of the intraorbital portion of optic nerve was demonstrated.

### **3.4 Discussion**

Herpes simplex encephalitis is the most common cause of sporadic viral encephalitis, with a range of clinical presentations from aseptic meningitis and fever to a severe, rapidly progressive, fatal form. It can occur either through hematogenous spread or neuronal

transmission. In adults infected with HSV-1, the neuronal spread of the latent virus occurs from the peripheral neuron in retrograde fashion to the brain, usually through the trigeminal nerve. Reactivation of latent virus within other parts of the brain has also been postulated. In neonates, the initial infection occurs in the birth canal and spreads hematogenously, with the virus diffusing through the blood-brain barrier (Tyler 2004).

Herpes simplex retinitis is characterized by marked retinal edema, exudates, hemorrhage and vascular occlusion, associated with optic disk swelling and late rhegmatogenous retinal detachment. The disease may start in the posterior pole, equator or periphery and can involve both eyes, although not always simultaneously (Margolis and Atherton 1996). HSV retinal infection may also present with the clinical features of ARN; i.e., peripheral necrosis, vitritis and retinal vasculitis. In such case. the ophthalmoscopic appearance of confluent, well-demarcated areas of retinal whitening in the pre-equatorial zone and occasional exudative retinal detachment are characteristic (Margolis and Atherton 1996; Walters and James 2001).

The evidences present in the literature, as reviewed by Margolis and Atherton (Margolis and Atherton 1996), delineate the pathogenesis of the HSR as follows: (1) HSV, either from a primary infection or reactivation of a latent source (trigeminal, superior cervical or ciliary ganglion) causes an anterior uveitis; (2) the immune system cannot suppress the spreading to CNS by retrograde axonal transport via the parasympathetic component of the oculomotor nerve; (3) spreading along axonal pathways to the suprachiasmatic nuclei and returns to one or both eyes by retrograde axonal transport along the optic nerve; (4) virus replication in the ganglion cell layer and retina destruction.

In this review of the literature, we could not find well-defined risk factors in immunocompetent adults. Carefully looking for any stressor in the medical history, we could find a history of recent head trauma in two cases (Johnson and Wisotzkey 1977;

Thompson, Culbertson et al. 1994) and one patient who had undergone a craniotomy (Perry, Girkin et al. 1998).

Based on analysis of the data from the literature, it appears that subjects who had HSE and HSR could be subdivided in four sub-populations (Tab. 2). The first group is composed of seven adults who had HSE and HSR at the same time: five of these had HSV-2 infection. The second group is composed of six patients who had neonatal HSV encephalitis and delayed retinitis (mean interval between the two diseases: 6.2 years): three of them had HSV-2, one HSV-1 and in two cases the type was not reported.

Consistent with these data, it has been recently reported that ARN caused by HSV-2 can be correlated with neonatal herpes (Landry, Mullangi et al. 2005). The third group is composed of 39 cases of HSE in childhood or adulthood followed by HSR after an average time of 28.1 months: among these cases, eight (20.5%) were caused by HSV-2. The fourth group is composed by four patients in whom the HSV retinitis preceded the encephalitis (mean interval: 53 days). In all four cases, HSV-1 was the etiologic agent. Two of these patients had AIDS, one was being treated with immunosuppressors after a renal transplant and one was immunocompetent. All four patients died within 3 months. This suggests that intense medical treatment and close scrutiny for encephalitis should be considered in immunosuppressed patients with retinitis.

Considering all the patients who had HSR after HSE (the second and third groups together), 48.9% of the patients had HSR within 3 months from the HSE; 4.4% had HSR within 3 and 12 months from the HSE, none between 12 and 24 months; 46.7% had HSR after minimum 2 years from the HSE. In other words, of all the patients who developed HSV retinitis after HSV encephalitis, almost half developed HSR within 3 months (early-onset group), whereas the remaining patients developed HSR between 2 and 20 years later (late-onset group).

Considering the publication date and the delay between HSE and HSR, the patients belonging to the late-onset retinitis group had encephalitis before 1980 (Pavesio, Conrad et al. 1997; Kamel, Galloway et al. 2000). It is notable that systemic acyclovir started to be available for clinical use in the late 1970s, greatly increasing the survival of patients with herpetic encephalitis. Only in four cases (Thompson, Culbertson et al. 1994; Levinson, Reidy et al. 1999; Verma, Venkatesh et al. 1999; Kim and Yoon 2002) of the late-onset retinitis group was the use of acyclovir specifically reported as a treatment for the previous encephalitis. Only one patient of the late-onset group received corticosteroids during HSE (Verma, Venkatesh et al. 1999). In the remaining 16 cases, the treatment for the previous HSE is not mentioned.

The late-onset retinitis after HSE may have parallels in cases of subacute sclerosing panencephalitis, where an aberrant immune response fails to control measles infection, leading to viral persistence; however, the lack of bilateral eye involvement in any of the late-onset cases does not support this possible pathogenesis.

One limitation of this study is the possibility that the encephalitis and the retinitis were not caused by the same HSV strain or that the second infection may have been due to a successive primary infection. The lack of any cerebral involvement during the retinitis following HSE is also of interest, even if some authors have reported MRI enhancement or CSF anti-HSV antibody but no symptoms of encephalitis during acute retinal necrosis caused by HSV (Walters and James 2001).

Further clinical and experimental studies to explain the relation between HSR and HSE and to delineate possible risk factors may provide a better understanding of herpetic infections in humans. The recent epidemic of genital HSV-2 infection may result in an increase in neonatal herpes infection and in delayed cases of ARN 53.

#### 4. Aspetti di neuroprotezione nella patologia glaucomatosa

È ampiamente documentato che l'amministrazione di neurotrofine induce la sopravvivenza delle cellule della retina nel corso di una serie di insulti. Al tempo stesso, un maggiore livello di neurotrofine, come il fattore di crescita nervosa (NGF), è stato trovato in modelli sperimentali di glaucoma. Tuttavia, è indubitabile che la perdita di cellule della retina si verifica nel corso di ipertensione oculare. Pertanto, nel presente studio abbiamo cercato di chiarire se i cambiamenti nei livelli di NGF ed espressione dei suoi recettori possa spiegare la progressione del danno retinico durante il glaucoma.

Per fare questo, abbiamo indotto un'elevata pressione intraoculare nei ratti, iniettando soluzione salina ipertoniche nella vena episclerale. I ratti sono stati sacrificati dopo giorni 10, 20 e 35 dall'iniezione. Successivamente abbiamo preso in esame il tasso di apoptosi delle cellule ganglionari retiniche (RGCs), la sintesi del NGF a livello retinico così come l'espressione di Bcl-2, Bax, trkA e p75. Particolare attenzione è stata posta sull'equilibrio tra trkA e p75, in considerazione del loro ruolo anti e pro-apoptotico, rispettivamente.

Abbiamo dimostrato che nel nostro modello sperimentale di glaucoma, la perdita di cellule ganglionari della retina è accompagnata da un aumento iniziale di NGF endogeno a livello retinico. Inoltre, abbiamo riscontrato che a livello di RNA messaggero il rapporto tra trkA e p75 e tra Bcl-2 e Bax era diminuito in entrambi i casi, indicando una sovra espressione di p75 e Bax. Abbiamo concluso che il NGF è inizialmente sovra-espresso in condizioni di glaucoma sperimentale, ma che questo aumento NGF non è sufficiente a sostenere la sopravvivenza delle cellule ganglionari. Una possibile spiegazione del mancato sostegno trofico da parte del NGF potrebbe essere la progressiva up-regulation del p75 in relazione al trkA.

Ad ogni modo, questo è stato il primo di una serie di studi che hanno definito il ruolo del



NGF in collirio come agente neuroprotettivo nel glaucoma sperimentale nel ratto ed anche nel glaucoma umano in fase terminale (Lambiase, Aloe et al. 2009; Colafrancesco, Parisi et al. 2011).

#### 4.1 Introduction

Glaucoma is a sight-threatening disease characterized by progressive degeneration of the Retinal Ganglion Cells (RGCs), the neurons that constitute the optic nerve (Quigley, Dunkelberger et al. 1989). RGCs loss may be explained as a consequence of the ocular hypertension that leads to functional axotomy with impairment of the neurotrophic support (Pease, McKinnon et al. 2000).

Nerve growth factor (NGF) is a neurotrophin that regulates development, growth, survival, differentiation, functional maintenance and even apoptosis of neuronal cells and has raised potential on clinical application to neurodegenerative diseases (Levi-Montalcini 1987; Hefti 1994). NGF activities are mediated by binding to two structurally unrelated receptors: the tropomyosin receptor ( $trkA^{NGFR}$ ) and the pan-neurotrophin p75 receptor ( $p75^{NTR}$ ). NGF binding to  $trkA^{NGFR}$  stimulates the tyrosine kinase domain, leading to proliferation, differentiation and survival of neurons (Saragovi, Zheng et al. 1998). NGF interaction with  $p75^{NTR}$ , a member of the Tumor Necrosis Factor family, leads mainly to activation of caspases and therefore mediates cell apoptosis, even if cell differentiation is also reported (Chao, Casaccia-Bonofil et al. 1998). On the same cell-type, the choice between survival or death depends on the balance between surface  $trkA^{NGFR}/p75^{NTR}$  expression, as well as on their hetero or homo -dimeric cell surface sharing (Casaccia-Bonofil, Gu et al. 1999). The role of NGF and its receptors in glaucoma but also in other degenerative diseases of ganglion cells are controversial. NGF intraocular administration increases the survival of RGCs after optic nerve axotomy in the rat (Carmignoto, Maffei et al. 1989) and after retinal ischemia in the cat (Siliprandi, Canella et al. 1993), as well as in a model of acute





glaucoma in the rabbit (Lambiase, Centofanti et al. 1997). For other authors, NGF might be not such effective in protecting RGCs (Cui, Lu et al. 1999; Shi, Birman et al. 2007).

These controversial findings may be explained by the observation that RGCs express both  $\text{trkA}^{\text{NGFR}}$  and  $\text{p75}^{\text{NTR}}$  receptors (Carmignoto, Comelli et al. 1991). NGF might have different actions on RGCs because of different relative expression of  $\text{trkA}^{\text{NGFR}}$  and  $\text{p75}^{\text{NTR}}$  by RGCs upon different circumstances (Rudzinski, Wong et al. 2004; Shi, Birman et al. 2007).

To better understand the relation between NGF and RGCs degeneration, we used a well-characterized experimental model of glaucoma (Morrison, Moore et al. 1997). Morrison and coworkers previously demonstrated that the injection of hypertonic saline into the episcleral vein of rats induces sclerosis of the aqueous outflow pathways, chronic elevation of Intraocular Pressure (IOP), inner retinal atrophy and optic nerve degeneration, resembling human glaucoma (Morrison, Moore et al. 1997; Morrison, Johnson et al. 2005). We measured the retinal expression of NGF,  $\text{trkA}^{\text{NGFR}}$  and  $\text{p75}^{\text{NTR}}$  after 10, 20 and 35 days of elevated IOP, and correlated to the expression of two genes involved in apoptotic pathway, Bcl-2 and Bax.

## 4.2 Methods

All experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were obtained from Charles River (Calco, Como, Italy). All sterile tissue culture plastic-ware and reagents were from Iwaki (Tokyo, Japan), NUNC (Roskilde, Denmark), SIAL (Rome, Italy), Celbio (Milano, Italy) and GIBCO-Invitrogen (Carlsbad, CA, USA), otherwise specified in the text.

### Experimental glaucoma and tissue sampling

Unilateral elevation of IOP was produced in male Brown Norway rats by saline injections into the episcleral veins, as previously described (Morrison, Moore et al. 1997; Morrison, Johnson et al. 2005). In brief, while under general anesthesia (1 mL/kg rat cocktail: 5:2.5:1



ketamine [100 mg/mL], xylazine [20 mg/ mL], and acepromazine [10 mg/mL]), the right eye of each animal was injected with 50  $\mu$ L of a 1.75 M hypertonic saline solution through the superior episcleral vein. Rats were housed in a constant low-light environment (40-90 lux) to minimize IOP circadian oscillations (Morrison, Johnson et al. 2005). The calibrated TonoPen XL tonometer (Mentor, Norwell, MA) was used for daily monitoring of IOP under topical anesthesia (Moore, Epley et al. 1995; Jia, Cepurna et al. 2000). Each daily IOP value was determined as the mean of 10 valid readings (Morrison, Moore et al. 1997). The animals that did not achieve a sustained elevated IOP (an increase of IOP equal or greater than 20% of their baseline IOP) after the first 7 days were excluded.

For the experiments, 27 animals were used and sacrificed after 10, 20 and 35 days from the saline injection (3 groups of n=9 rats/group). The fellow eye was used as control. At the time of sampling, anesthetized rats were trans-cardially perfused with 4% p-formaldehyde, for morphological studies. Eye globes including the optic nerve attached portion (n=3rats/group), were removed, post-fixed (18-hrs), embedded in paraffin and sliced in 3 $\mu$ m sagittal sections (HM325 Microtome; Microm Bioptica, Milan, Italy). Sections were mounted twice per slide, yielding approximately 20 slides. Parallel slides were prepared with the adjacent retina (control eye). For ELISA and real time RT-PCR analysis, retinas (n=3rats/group per analysis) were detached from the eye, under a dissecting microscope (Nikon, Tokyo, Japan), and placed into specific microtubes for quickly processing.

#### Immunohistochemistry and TUNEL analysis.

De-waxed sections were processed for immunostaining, according to the Avidin-Biotin-Peroxidase complex procedure (Lambiase, Bonini et al. 1998).

Apoptosis was assessed by in situ Terminal deoxynucleotidyl-transferase-mediated (TdT) dUTP-biotin Nick-End Labeling (TUNEL), which detect the DNA fragmentation, a characteristic feature of apoptotic cells (Johnson, Deppmeier et al. 2000). TUNEL assay was performed on four retina sections, according to the manufacturer's instruction with minor

*Manco*

modification. Briefly, de-waxed sections were 3.7% p-formaldehyde fixed and 0.1% TritonX100 permeabilized. The slides were then incubated with exogenous rTdT and biotin 14-dATP (both from GIBCO-Invitrogen), 50µL/section for 3hrs, for repair of 3'-hydroxyl DNA ends. For positive controls, sections were treated with DNase I (2U/mL DNase solution) for 30min at 37°C. For negative controls, sections were incubated with buffer lacking the rTdT enzyme. The apoptotic nuclei were labeled with peroxidase-DAB, according to the ABC technique. To determine the percentage of cell undergoing apoptosis five fields per section were evaluated by a masked observer who identified and counted the intensely dark brown, condensed, TUNEL<sup>+</sup> nuclei (chromatin condensation and clumping). The percentage of apoptosis was calculated as follows: [(number of apoptotic cells/number of total cells) x 100]. All RGC layer nuclei, with the exception of spindle-shaped endothelial nuclei, were counted. Data are expressed as the ratio of TUNEL<sup>+</sup> labeled RGCs to total number of RGCs examined (Johnson, Deppmeier et al. 2000).

#### NGF-ELISA assay

Retinas were ultra-sonicated (Ika, NC, USA) in extraction buffer (20mM tris-acetate, pH7.5, 150mM NaCl, 1mM Ethylenediamine-Tetraacetic acid (EDTA), 1mM Ethyleneglycol-Tetraacetic acid (EGTA), 2.5mM Sodium-Pyrophosphate, 1mM Ortovanadate, 1mM β-Glycerolphosphate, 100mM NaF, 1mM Phenylmethylsulfonyl Fluoride (PMSF), 1µg/mL Leupeptin) and supernatant was clarified at 4°C/13000rpm for 10 min. Tissue concentration of NGF was measured by a highly sensitive two-site enzyme immunoassay ELISA kit ("NGF Emax<sup>tm</sup> ImmunoAssay System, G7631; Promega, Madison, WI), following the instructions provided by the manufacturer.

#### Relative real time RT-PCR.

Bcl2, Bax, NGF, trkA<sup>NGFR</sup> and p75<sup>NTR</sup> mRNA were measured in not-pooled retinas (average 0.010mg wet weight for each sample). Tissues were pre-treated with Proteinase K (20mg/mL; Finnzyme, Milan, Italy) in HIRT buffer at 56°C/3hrs and total RNA was extracted

*Manuela*

from each samples using the Puregene RNA purification kit (Gentra Systems, Minnesota, USA). The resulting total RNA was re-suspended in 25 $\mu$ L diethyl pyrocarbonate-treated water (ICN, Germany) and treated with RNase-Free DNase I according to the supplier's protocol (2 U/ $\mu$ L Turbo DNA free kit AM-1907; Ambion Ltd., Cambridgeshire, UK), to eliminate any genomic DNA contamination. Total RNA samples were checked for both RNA quantity (Nanodrop; Celbio, Milan, Italy), purity (>1.6) and absence of any RNA degradation (RIN~8). Equivalent amounts of RNA (3 $\mu$ g) per sample were used as a template in normalized cDNA synthesis. Reverse transcription was performed according to the standardized Mu-MLV protocol (final volume reaction of 20  $\mu$ L, using 50 pM oligo dT<sub>21</sub>-primer, 1 mM dNTP mix and 200 U reverse transcriptase; Mu-MLV, F-605L; Finnzyme), in a PTC-100 programmable thermocycler (MJ Research, Watertown, MA). The resulting cDNA was amplified using the SYBR Green PCR core reagent kit (Applied Biosystems, Foster City, CA) using an Opticon2 MJ Research system (MJ Research). The reaction contained 10  $\mu$ L of SYBR reagent, 3  $\mu$ L of cDNA (for the target) or 1  $\mu$ L of cDNA (for the referring gene), and 20 nM primers in a 20- $\mu$ L final volume. The temperature profile included initial denaturation at 95°C/15min, followed by 35-47 cycles of denaturation at 95°C/30sec, annealing at 55-60°C/25sec (the annealing time depended on the primer's T<sub>m</sub>), elongation at 72°C/30sec, fluorescence monitoring at 60-90°C, 0.01°C for 0.3sec, and further incubation at 72°C/5m. Specific primers for Bcl2 (for: CTC CCA ATA CTG GCT CTG TC; rev: CCT GAT GCT CTG GGT AAC TC; 111bps; 55°C Ta; U34964), Bax (for: TGC CAG CAA ACT GGT GCT; rev: ACC CAA CCA CCC TGG TCT T; 129bps; 55°C Ta; U34963); NGF (for: CTG GCC ACA CTG AGG TGC AT; rev: TCC TGC AGG GAC ATT GCT CTC; 120bps; 53°C Ta; BC011123), trkA<sup>NGFR</sup> (for: AAC AAC GGC AAC TAC AC; rev: CCT GTT TCT CCG TCC AC; 137bps; 58°C Ta; M23102), p75<sup>NTR</sup> (for: GAG GCA CCA CCG ACA ACC TC; rev: TGC TTG CAG CTG TTC CAC CT; 131bps; 55°C Ta; AF187064) and the referring gene GAPDH (for: TGA CTC TAC CCA CGG CAA G; rev: TAC TCA GCA CCA

*Marco Coassin*

GCA TCA CC; 135bps; 53°C Ta; BC059110) were designed using Prime3 software (<http://www.prime3.com>). GenBank software was used to select the complete mRNA sequence of each gene investigated (<http://www.ncbi.nlm.nih.gov/Genbank>; provided by the National Center for Biotechnology Information, Bethesda, MD). Primer specificities were set in pilot experiments and further confirmed by the single melting curves obtained during each amplification. Negative controls (without template) were produced for each run. Experiments were performed in duplicate for each data point. Quantitative values were obtained from the threshold cycle value (Ct), which is the point where a significant increase of fluorescence is first detected. According to the REST© software (Pfaffl, Horgan et al. 2002), results are expressed as N-fold difference (increase or decrease) in target gene expression, GAPDH normalized and compared to controls (fellow eye).

Indeed, ratios between  $trkA^{NGFR}/p75^{NTR}$  and Bcl2/Bax were calculated according to the single Ct values, taking into consideration that Cts are inversely proportional to mRNA expression.

### Statistical Analysis

Data are represented in the graphics as mean  $\pm$  standard deviation (SD). Each evaluation was performed three times, in triplicate or quadruplicate. The statistical package used was StatView II for PC (Abacus Concepts. Inc., USA). Parametric ANOVA followed by a Tukey-Kramer post hoc analysis was used. The Kendall's rank correlation coefficient (Tau) was also calculated to identify relationships between ratios. A confidence of 95% ( $p \leq 0.05$ ) was presumed to reflect significant difference between group mean values.

## **4.3 Results**

During the entire experiment, the mean IOP detected in un-injected fellow eyes (controls) was  $26.9 \pm 0.6$  mmHg. The saline injection resulted in a rapid increase of IOP at all the time-point evaluated, with a mean value of  $35.7 \pm 0.5$  mmHg. Parallel, TUNEL<sup>+</sup> rate of 1.62:1000



was also observed at day 35, in comparison to 0.094:1000 rate in control eyes ( $p < .01$ ; Fig. 1A). At day 10 and 20, the RGCs apoptotic rate did not show a statistically significant difference when compared to control sections.

Molecular analysis carried out on retinas showed that at 35 days from saline injection, the expression of the "anti-apoptotic" marker Bcl-2mRNA was markedly down-regulated (59 folds) while the expression of the "pro-apoptotic" marker BaxmRNA was up-regulated (12 folds), when compared to controls ( $p < .05$ ; Fig. 1B).

Biochemical analysis of retinas at 10, 20 and 35 days from saline injection showed a significant increase of NGF protein only after 35 days from injection ( $259.9 \pm 44.4$  pg/g,  $77.5 \pm 23.8$  pg/g, as compared to controls,  $p < .05$ ; Fig. 2A). Interestingly, real-time PCR analysis showed a progressive time-dependent NGFmRNA increase in injected retinas, as compared to un-injected ones (Fig. 2B). According to the biochemical data, the greater NGFmRNA expression was detected at 35 days from injection.

To understand the meaning of these increased NGF levels, we evaluated the levels  $trkA^{NGFR}$  and  $p75^{NTR}$  receptors. Immunohistochemistry demonstrated both  $trkA^{NGFR}$  and  $p75^{NTR}$  expression in RGCs in control animals (Fig. 3). Molecular analysis showed that  $trkA$ mRNA - the promoting cell survival receptor- was slightly decreased at 10 days, mildly increased at 20 days and over-expressed at 35 days from injection, when compared to controls (Fig. 4A). On the other hand, the level of  $p75$ mRNA- the pro-apoptotic receptor - progressively increased during the course of experimental glaucoma and particularly at 35 days from injection,  $p75$ mRNA expression was 42-times greater than the one in the controls (Fig. 4A).

In order to compare NGF receptor expression with apoptotic markers at all the time-points, independent  $trkA^{NGFR}/p75^{NTR}$  and Bcl2/Bax ratios were calculated according to the Ct values. Interestingly,  $trkA^{NGFR}/p75^{NTR}$  mRNAs ratio progressively decreased during experimental glaucoma (Fig. 4B) and it was significantly correlated to Bcl2/Bax mRNAs

*Marco Coassin*

ratio (Tau=0,833;  $p < .05$ ; Fig. 4C). Since Ct values are inversely proportional to mRNA expression, this result is inverted in the graphics where Ct values were used.

#### 4.4 Discussion

In the present study we show that in an experimental model of glaucoma: i. RGCs apoptosis and increased BaxmRNA occurred after hypertonic saline injection; ii. NGF protein increases at later stages (35 days); iii. p75<sup>NTR</sup> is significantly up-regulated compared with trkA<sup>NGFR</sup>.

Glaucoma is an optic neuropathy characterized by apoptosis of RGCs and optic nerve degeneration (Quigley, Dunkelberger et al. 1989). In our study, the progressive increase of NGFmRNA in the retina might be explained as a tissue adaptive/reparative consequence of the retinal insult as well as an attempt to support RGCs survival. This hypothesis is based on the demonstrated block of the axonal transport along optic nerve (Pease, McKinnon et al. 2000) and on the well known effects of neurotrophins, including NGF, in supporting RGCs during different pathological condition (Aguayo, Clarke et al. 1996). In fact, the role of NGF in the survival of RGCs has been previously reported and the protective function of NGF intraocular administration has been clearly demonstrated in experimental models of ischemic, traumatic and hypertensive injury (Carmignoto, Maffei et al. 1989; Siliprandi, Canella et al. 1993; Lambiase, Centofanti et al. 1997).

This observation arise the question on the mechanism(s) for which the retinal increase of NGF is unable to prevent RGCs apoptosis. A possible explanation of the failure of NGF trophic support may be related to the progressive decrease of trkA<sup>NGFR</sup>/p75<sup>NTR</sup> ratio. In fact, the magnitude of NGF signaling depends on the ratio of trkA<sup>NGFR</sup> and p75<sup>NTR</sup> activity (Yoon, Casaccia-Bonnet et al. 1998; Casaccia-Bonnet, Gu et al. 1999), and, in particular, trkA<sup>NGFR</sup> mediates survival of some neurons by turning-off an ongoing p75<sup>NTR</sup>-mediated apoptotic signal (Majdan, Walsh et al. 2001). In our animal model the decrease



of retinal  $\text{trkA}^{\text{NGFR}}$  expression was associated with a progressive increase of  $\text{p75}^{\text{NTR}}$ , that paralleled RGCs apoptosis, as detected by TUNEL. The progression of RGCs loss was also associated to a decrease of the anti-apoptotic Bcl2 transcript and an increase in Bax transcript (that stimulates cell susceptibility to death stimuli, including increased IOP (Nickells 1999)). As previously reported, specific NGF binding to  $\text{trkA}^{\text{NGFR}}$  up-regulates Bcl-2 protein, that protects cells from apoptosis by preventing caspases activation (la Sala, Corinti et al. 2000). On the other hand, neurotrophins' binding to  $\text{p75}^{\text{NTR}}$  may induce caspase-mediated cell apoptosis (Tabassum, Khwaja et al. 2003), also in RGCs (Troy, Friedman et al. 2002).

One limit of this paper is that is difficult to prove that the increase in RGC apoptosis is directly related to the changes in the level of the retinal NGF receptors. Indirectly, we may assume that in our model the damage to the RGCs is selective, as demonstrated by Morrison and coworkers [29]. Moreover, no other changes were seen in the retina sections although in some sections the inner nuclear layer appeared thinner.

In this experimental model of glaucoma, retinal NGF is over-expressed in experimental glaucoma but this NGF increase is not sufficient to support RGCs survival. A possible explanation for failure of NGF trophic support might be associated with the progressive up-regulation of  $\text{p75}^{\text{NTR}}$  in relation to  $\text{trkA}^{\text{NGFR}}$ . Anyway, the role of NGF as neuroprotective tool for RGCs survival during glaucoma warrants further investigations.



## 5. Aspetti di neuroprotezione nella retinopatia diabetica

Un'altra patologia oculare molto diffusa in cui gli aspetti neurodegenerativi sono strettamente legati agli aspetti vascolari è la retinopatia diabetica. Infatti, è caratterizzata dalla formazione di nuovi vasi sanguigni, dall'aumento della permeabilità vascolare, dall'occlusione vascolare dei capillari e dalla degenerazione delle cellule retiniche. I livelli di un certo numero di fattori di crescita, tra cui NGF e VEGF, sono stati trovati alterati nella retinopatia diabetica. Il NGF è una proteina solubile che è prodotta da e agisce su molte cellule nervose, cellule vascolari e cellule del sistema visivo, comprese le cellule della retina. Il VEGF è una citochina multifunzionale prodotta dalle cellule endoteliali dei vasi retinici che agisce come un induttore della permeabilità vascolare, un mitogeno delle cellule endoteliali e come un mediatore pro-infiammatorio. È stato dimostrato che il livello di VEGF e NGF aumenta con l'avanzare della retinopatia diabetica e che il NGF protegge le cellule endoteliali da apoptosi indotta dal diabete. Vi sono inoltre prove che il NGF previene l'apoptosi cellulare retinica e la patologia capillare retinica nel diabete sperimentale. Il NGF e il VEGF possono avere quindi effetti sovrapponibili e distinti nei sistemi vascolare e nervoso.: tuttavia, il loro preciso ruolo e la loro interazione nello sviluppo della retinopatia diabetica non sono noti.

Nel presente studio, abbiamo valutato se le alterazioni dei livelli endogeni di VEGF e di NGF sono associati con il danno alle cellule retiniche in corso di diabete in un modello animale. I nostri risultati hanno mostrato che il diabete provoca un aumento immediato e persistente di VEGF, e un aumento ritardato e temporaneo di NGF. Abbiamo trovato che gli anticorpi anti-NGF peggiorano gli effetti dannosi della retinopatia mentre la somministrazione di NGF protegge le cellule gangliari della retina. Questi dati suggeriscono che la disponibilità prolungata di NGF potrebbe attenuare gli effetti del DR.



Un altro studio preliminare ha dimostrato che il NGF esogeno somministrato come collirio potrebbe ridurre direttamente i livelli di VEGF, inducendo un miglioramento della retinopatia diabetica e dell'edema maculare diabetico (ARVO abstract 2011).

## 5.1 Introduction

Diabetic retinopathy (DR) is a common complication of diabetes, characterized by the formation of new blood vessels, increased vascular permeability, vascular occlusion and retinal cells degeneration.[1] The levels of a number of growth factors, including NGF and VEGF, have been found altered in DR.[2] NGF is a soluble protein that is produced by and acts upon a number of nerve cells, vascular cells and cells of the visual system, including retinal cells [3-8]. VEGF is a multifunctional cytokine produced by endothelial and retinal cells that acts as an inducer of vascular permeability, an endothelial cell-specific mitogen, and as a pro-inflammatory mediator.[9-11] It has been shown that the level of VEGF and NGF increases as DR advances, and that NGF protects endothelial cells from apoptosis induced by diabetes.[12] There is also evidence that NGF prevents neuroretinal cell death and capillary pathology in experimental diabetes[3] and that VEGF promotes the survival of retinal neurons during ischemic injury.[9] These observations indicate that NGF and VEGF can have overlapping and distinct effects within the vascular and nervous systems.[13] However, what role they play in the development of DR is not known.

In the present study, we investigated whether alterations of endogenous levels of VEGF and NGF are associated with diabetic-related impairment in the retina. Our results showed that diabetes causes an early and persistent increase of VEGF, and a delayed and temporary increase of NGF. We found that anti-NGF antibodies worsen DR and that NGF administration protects retinal ganglion cells (RGCs). These data suggest that prolonged availability of NGF may mitigate the effects of DR.

## 5.2 Methods

Streptozotocin (STZ) (Sigma), 3,3'-diaminobenzidine (DAB) (Sigma), Rat  $\beta$ -NGF DuoSet ELISA kit (R&D), Quantikine Rat VEGF Immunoassay (R&D), rabbit polyclonal IgG anti-TrkA receptor (Upstate Biotechnology), biotinylated anti-rabbit IgG (Vector) and Avidin-conjugated Horseradish Peroxidase Complex (Vector) were obtained from commercial distributors. NGF was purified from adult male mouse submandibular glands following the method of Bocchini and Angeletti[14] and anti-NGF antibody was produced in rabbit and purified by affinity chromatography.[15]

### Animals and treatments

All procedures with animals were performed in accordance with the 'Principles of laboratory animal care' (NIH publication no. 85-23, revised 1985;

<http://grants1.nih.gov/grants/olaw/references/phspol.htm>). Three month old male Sprague-Dawley rats (n=88), raised in our animal facility and weighting 180-200 g, were fastened for 12 hours. Diabetes was induced by intraperitoneal injection of freshly prepared STZ (70 mg/kg body weight) dissolved in citrate-phosphate buffer, pH 4.5.[16] Detection of blood glucose levels > 350 mg/dl were used as markers of diabetes.

Eighty rats received equivalent volumes of buffer solution and served as the control group.

Rats were maintained on a 12-hour light-dark cycle, provided with food and water ad libitum and sacrificed with an overdose of Nembutal.

### Anti-NGF antibody administration

Diabetic (n = 24) and non-diabetic (n = 20) rats received intravitreal injections of anti-NGF antibody, at a concentration of 100  $\mu$ g/eye in 5  $\mu$ l of physiological solution, 7 weeks after the induction of diabetes. An equal number of diabetic and non-diabetic rats received equivalent amounts of goat IgG and served as controls. Animals were killed with an overdose of Nembutal 3 days after anti-NGF antibody administration.

### NGF administration

Eight weeks after induction of diabetes, diabetic (n = 14) and non-diabetic (n = 14) rats were treated twice a day with eye drops of 20  $\mu$ l of purified NGF solution (200  $\mu$ g of NGF dissolved in 1 ml of 0.9% NaCl). As control, diabetic and non-diabetic rats received equivalent amounts cytochrome C (a molecule with physiochemical properties similar to NGF, but lacking its biological activities [5]). Animals were killed with an overdose of Nembutal 3 week after NGF treatment (11 weeks after STZ administration).

### NGF and VEGF determination

Retinas were ultra-sonicated (Ika, NC, USA) in extraction buffer and the supernatant was clarified by centrifugation at 12000 rpm for 20 min. The tissue concentration of NGF was measured using the DuoSet ELISA Development kit, following the instructions provided by the manufacturer. Data are represented as pg/g tissue and all assays were performed in duplicate. The sensitivity of the assay was 15 pg/ml of NGF. The tissue concentration of VEGF protein was determined using an ELISA assay (Quantikine Rat VEGF Immunoassay) following the instructions provided by the manufacturer.

### Histological analysis

Eye globi (n = 8) with the optic nerve attached were removed from each experimental group and fixed in Bouin fluid. Ten-micrometer thick sections of the eyes were then cut with a cryostat (Leica CM 1850 UV, Germany) at -20° C, taking care that the cross section of each retina was performed in the same orientation. Sections were stained with hematoxylin-eosin and the number of retinal ganglion cells (RGCs) located approximately 2 mm from the optic disc was determined under a Zeiss microscope equipped with a 40X objective. RGCs with a well-defined nucleus were counted in a masked manner in 15 distinct areas of each retina/rat and the results were averaged, converted into cells per mm<sup>2</sup>, and expressed as a percentage of the control.

For electron microscopy studies, retinal tissue was processed as previously described.[22]

*Marco Coassin*

### Immunohistochemical analyses

Eye globi (n=6) were fixed in 4% paraformaldehyde and cut in coded serial 10- $\mu$ m thick sections, all containing the retina and optic nerve in the same orientation. The sections were then immunostained against the NGF-receptor TrkA to visualize NGF-responsive retinal cells, as previously described.[5] Immunostained RGCs, located about 2 mm from the optic disc, were determined under a Zeiss microscope equipped with a 40X objective. All immunostained RGCs present in 15 distinct areas of each rat retina were counted in a masked manner and the results were averaged, converted to cells per  $\text{mm}^2$ , and expressed as a percentage of the control.

### Statistical analysis

All statistical evaluations were performed using the StatView package for Windows. The data are expressed as a mean  $\pm$  SEM. A post-hoc comparison within logical sets of means was performed using Tukey's test. A p-value of less than 0.05 was considered significant.

## **5.3 Results**

A single administration of STZ causes a significant decrease in body weight (Fig. 1A) and up-regulation of glucose blood levels (Fig. 1B) both 7 (\*p < 0.05) and 11 weeks (\*\*p < 0.01) later.

STZ-induced diabetes causes retinal cell degeneration. Histological analysis reported in Fig.1 showed a significant decrease in the number of RGCs of STZ-treated rats, both after 7 (Fig. 1F) and 11 (Fig. 1G) weeks, as compared to control (Fig. 1E). Quantitative evaluation revealed that the reduction of RGCs is statistically significant (\*p < 0.05; \*\*p < 0.01).

Electron microscopic examination revealed marked signs of retinal degeneration, particularly of the photoreceptor cell and RGC layers (data not shown).

STZ-induced diabetes alters the expression of VEGF and NGF in the retina. As reported in Fig. 1, the retina of diabetic rats expressed elevated levels of VEGF both at 7 (276 $\pm$ 34 vs.

*Manuela*

104±21 pg/g;  $p < 0.01$ ) and 11 weeks (257±28 vs. 104±21 pg/g;  $p < 0.01$ ) after administration of STZ, compared to the level in the retina of untreated rats (Fig. 1C). The level of NGF is elevated after 7 weeks (187±9 vs. 86±12 pg/g;  $p < 0.01$ ), but is lower than that of control retina after 11 weeks (43±14 vs. 86±12 pg/g;  $p < 0.01$ ) (Fig. 1D). Table 1 summarizes the effect of STZ administration on retinal NGF and VEGF at different time points.

The increase of VEGF precedes the increase of NGF. Since VEGF and NGF can have both distinct and overlapping effects,[13] the next question we addressed was whether VEGF precedes the increase of NGF. A time course analysis showed that 10 days after administration of STZ the level of VEGF in the diabetic retina is significantly elevated as compared to control (181±19 vs 104±21 pg/g;  $p < 0.01$ ), while the levels of NGF were comparable to the control levels (90±15 vs. 86±12 pg/g;  $p > 0.05$ ). This observation indicates that the increase of VEGF precedes the increase of NGF and that their local increases are most likely not functionally correlated.

Effect of anti-NGF administration in diabetic retina. To further explore the interrelationship between VEGF and NGF, diabetic rats were treated with intraocular administration of anti-NGF antibody and killed 3 days later. The results reported in Fig.2 show that anti-NGF antibody has no significant effects on the levels of VEGF in diabetic rats (Fig. 2A). The anti-NGF antibody administration significantly reduces the level of NGF as compared to STZ treatment alone (16±11 vs. 187±9 pg/g;  $p < 0.01$ ) and to the control levels (16±11 vs. 86±12 pg/g;  $p < 0.01$ ) (Fig. 2B). These results indicate that the expression of NGF in the retina does not stimulate expression of VEGF and that the two growth factors may have distinct roles in the diabetic retina.

NGF administration by eye drops enhances the presence of NGF in the retina of diabetic rats. Since NGF has been shown to have protective action on RGCs[4] and to reach the retina when administrated to the conjunctiva as eye drops [18,23], we investigated the effect

of topical NGF ocular administration to our diabetic rats. Fig.2 shows the results of daily NGF eye drops application for 3 weeks (from week 9 to 11). This treatment significantly enhances the presence of retinal NGF as compared to controls and to diabetic non-treated rats ( $178 \pm 10$  vs.  $86 \pm 12$  vs.  $43 \pm 14$  pg/g;  $p < 0.01$ ) (Fig. 2C).

Effect of NGF eye drops on expression of TrkA by Retinal ganglion cell. Fig. 2 shows representative sections of retina of control rats (Fig. 2D), STZ rats (Fig. 2E) and NGF-treated STZ rats (Fig. 2F) immunostained for TrkA, the high-affinity NGF receptor. Eleven weeks after STZ administration, the retina showed marked reduction of TrkA-receptor expression in the retinal ganglion cell (RGC) layer (arrows in Fig. 2E). NGF topical administration to diabetic rats from week 9 to 11 prevented the loss of TrkA-positive cells in the RGC layer (arrows in Fig. 2F). These differences are statistically significant as shown in Fig. 2G ( $p < 0.01$ ).

#### 5.4 Discussion

Hyperglycemia is considered the major determinant of DR, causing pericyte death and blood vessels wall damage.[1] Studies have shown that DR is characterized by altered expression of NGF and VEGF, two biological mediators which, in addition to their distinct action on endothelial and nerve cells, can have both neurotrophic and vascular effects.[11-13] What is less understood is whether they have similar, distinct, or overlapping mechanisms of action in DR. This is a critical issue, as both VEGF[1,9] and NGF[17-18] have recently gained increased clinical interest. Using an animal model of diabetes, the present study showed that NGF and VEGF are expressed in the retina and that the level of retinal VEGF increases during the early development of the disease. The VEGF protein is over-expressed 10 days after induction of diabetes and remains elevated for over 11 weeks. Instead, the level of NGF after 10 days is comparable to the controls, it is over-expressed at 7 weeks and is down-regulated 11 weeks after induction of diabetes.

*Marco Coassin*

If NGF is elevated in diabetic retinas, it is not clear if this growth factor induces apoptosis of RGCs throughout its p75 neurotrophin receptor [19] or reduces cell death [20]. This ambivalent effect of NGF is well known.[3,17] In our study, we found that NGF first increases and lately decreases in diabetic retina and that, at that time, the number of RGCs is reduced. We demonstrated that topical eye NGF administration not only can reach the retina (as previously demonstrated [17,18,21]), but also enhances the presence of NGF protein in the retina, the expression of its receptor TrkA and prevents the damage to RGCs. Our diabetic rats were also treated with anti-NGF antibody. We thought that by inhibiting the endogenous expression of NGF in the diabetic retina we could obtain clearer information regarding the distinct, similar or overlapping role of VEGF and NGF. The concentration of VEGF after anti-NGF antibody administration did not change, supporting the hypothesis that the mechanism leading to over-expression of VEGF in the diabetic retina is not modulated by local up-regulation of NGF, but is most likely controlled by different mechanisms. The present results reinforce the hypothesis that NGF is able to prevent retinal cell death and provide further evidence that topical NGF administration is able to protect RGCs not only in glaucoma[17,18] but also in diabetic retinopathy. The protective effect of NGF on RGCs is suggested by evidences that NGF is able to promote functional recovery of damaged RGC and that RGCs express NGF-receptors.[3,4] Because DR is characterized by altered local expression of a number of growth factors,[1] it is possible that other growth factors were involved in RGC protection. Why does NGF first increase and then decrease in the retina of diabetic rats? It has been shown that NGF over-expression occurs in degenerative events involving NGF-responsive cells and it has been hypothesized that this mechanism represents a short-lived protective response against early cell damages that is not effective in the long run.[3] Recent studies have shown that endogenous NGF may be not sufficient in preserving retinal cells from apoptosis because of the downregulation of his high-affinity receptor TrkA [17]. It seems

*Marco Coassin*



that administration of exogenous NGF might preserve retinal cells from death by enhancing the expression of TrkA and support cells trophism in the long run. It has been found that in rabbit ocular hypertension, characterized by initial up-regulation of NGF and retinal cells degeneration, administration of NGF protects RGCs.[5] Likewise, skin wounds,[12,22] cardiac ischemia,[23] and corneal ulcers [7] (all characterized by increased local levels of NGF and cell degeneration) have been shown to benefit from exogenous administration of NGF. Consistently with these observations, our findings indicate that the up-regulation of endogenous NGF does not last and is not not sufficient to protect degenerating RGCs. They also show that exogenous administration of NGF might useful to protect these cells from degeneration.

Our working hypothesis is that NGF either alone or cooperatively with other growth factors can exert a protective action on diabetic retina. Our data suggest that the increase of NGF in diabetic retina could be an endogenous response to maintain retinal cell integrity and that exogenous administration of NGF might help to prevent and/or reduce RGC damage induced by diabetic retinopathy.



## 6. Future terapie: l'ipotermia al di là della neuroprotezione

La perdita visiva dovuta alla degenerazione maculare senile (AMD) spesso si sviluppa secondariamente alla neovascolarizzazione coroideale. Un regolatore critico dell'angiogenesi retinica e della permeabilità vascolare è il Fattore di crescita dell'endotelio vascolare (VEGF), specialmente in condizioni di ipossia. Abbiamo ipotizzato che l'ipotermia possa ridurre il metabolismo dell'epitelio pigmentato retinico (RPE) e, di conseguenza, i livelli di secrezione di VEGF da parte di cellule RPE in coltura in condizioni di ipossia. Gli effetti di ipotermia sono stati confrontati con gli effetti di inibizione del metabolismo causati da alcuni farmaci, quali il tiopental e la nicotinamide.

L'ipotermia è stata usata diffusamente in molti campi della medicina, in quanto capace di ridurre l'attività metabolica cellulare e proteggere i tessuti neurali. L'ipotermia ritarda la deplezione di energia, riduce l'acidosi intracellulare, riduce l'accumulo di neurotrasmettitori eccitotossici indotto dall'ischemia ed attenua l'afflusso di calcio intracellulare. Inoltre l'ipotermia sopprime la produzione di radicali liberi dell'ossigeno coinvolti nel danno secondario associato a ri-perfusione. L'arresto circolatorio ipotermico viene utilizzato in chirurgia toracica e neurochirurgia per proteggere il cervello durante il bypass cardiopolmonare. L'ipotermia è in corso di valutazione per il trattamento delle lesioni cerebrali traumatiche, l'ictus ischemico e l'asfissia neonatale. In oftalmologia, Machemer e altri hanno dimostrato che l'ipotermia legata ai fluidi di infusione proteggeva la retina durante la vitrectomia. Gli effetti neuroprotettivi di ipotermia sulle cellule della retina sono state dimostrate in vitro e confermate in modelli animali da altri studiosi. Se l'ipotermia riduce al minimo il danno retinico di ipossia con una riduzione del metabolismo cellulare, l'ipotermia potrebbe anche indurre una inibizione del metabolismo del RPE e ridurre la secrezione di VEGF, anche in condizioni di ipossia.

*Marco Coassin*

Le cellule di tipo ARPE-19 sono state tenute in coltura per un massimo di 5 giorni in condizioni normossiche (20% O<sub>2</sub>) e di ipossia (1% O<sub>2</sub>), con temperature che variavano da 27 a 40 gradi centigradi.

Abbiamo trovato che l'ipotermia riduceva il metabolismo delle cellule e la loro secrezione di VEGF in un modo dipendente dalla temperatura. La secrezione del VEGF è stata ridotta del 38% a 34 gradi C rispetto alle cellule coltivate a 37°C. Al contrario, la secrezione di VEGF delle cellule è aumentata del 32% a 40°C rispetto alle cellule coltivate a 37°C. In condizioni di ipossia, come atteso, abbiamo rilevato un aumento di secrezione di VEGF dell'84% a 37°C. Tuttavia, l'ipotermia è stata in grado di diminuire l'aumento di secrezione di VEGF indotta dall'ipossia del 30% (confrontata al controllo). Inoltre, l'effetto dell'ipotermia sulla secrezione di VEGF da parte delle cellule di RPE è reversibile dopo 4 giorni. A differenza dell'ipotermia, il tiopental e la nicotinammide sono stati in grado di rallentare il metabolismo delle cellule dell'epitelio pigmentato retinico, ma non la secrezione di VEGF dimostrando che questo era un effetto specifico dell'ipotermia. In conclusione, l'ipotermia potrebbe avere un ruolo fondamentale nel controllare le maculopatie degenerative attraverso una regolazione del VEGF. Questa nuova proprietà si aggiunge alle sue già ben note caratteristiche neuroprotettive sulle cellule retiniche. La coesistenza di queste due vie di protezione del tessuto retinico potrebbe essere particolarmente efficace nel trattamento dell'ostruzione dell'arteria centrale della retina e di altre patologie vascolari della macula che attualmente mancano di una cura efficace.

## 6.1 Introduction

Age related macular degeneration (AMD) is the leading cause of visual loss in the Western world.(de Jong 2006) Most visual loss in AMD develops secondary to neovascularization beneath the retina that leads to hemorrhage, accumulation of subretinal fluid and, inevitably, replacement of macular tissue with a scar.(Bressler, Bressler et al. 1988) A



critical regulator of retinal angiogenesis and vascular permeability is vascular endothelial growth factor (VEGF).(Aiello, Pierce et al. 1995) VEGF is a potent and specific endothelial-cell-specific mitogen that plays a unique role in the regulation of physiological and pathological angiogenesis.(Leung, Cachianes et al. 1989; Ferrara 2000) Although many factors increase VEGF *in vitro*, a major pathophysiological stimulus that leads to high levels of its expression in the retina is hypoxia.(Shweiki, Itin et al. 1992; Pournaras, Miller et al. 1997; Mousa, Lorelli et al. 1999) *In vivo*, experiments selectively correcting hypoxia in ischemic retinas with 100% inhaled oxygen have shown a reduction in VEGF expression in the monkey retina.(Pournaras, Miller et al. 1997)

The retina is a neural tissue with high metabolism and the highest oxygen consumption per unit weight of all human tissues.(Whikehart 2003) Retinal pigment epithelium (RPE) is the cellular layer lying between the retina and the choroid and acts as a primary regulator of the balance between pro- and anti- angiogenic factors at this level.(Sakamoto, Sakamoto et al. 1995; Das and McGuire 2003) In particular, it is well-known that RPE cells express and secrete VEGF in response to hypoxia.(Ghiso, Rohan et al. 1999; Young, Wang et al. 2005) In retinal hypoxia, reduction in the supply of oxygen to the tissue leads to adenosine triphosphate (ATP) depletion. Subsequently, a myriad of mechanism may mediate tissue damage: membrane depolarization, release of neurotransmitters, calcium overload, acidosis, free radical generation, nitric oxide overproduction and activation of caspases.(Osborne, Casson et al. 2004) Retinal hypoxia is implicated in visual loss in a broad array of retinovascluar diseases, including diabetic retinopathy, branch and central vein occlusion, retinopathy of prematurity, sickle retinopathy and others. Whether hypoxia plays a role in causing increased levels of VEGF in AMD remains unclear; however, it is postulated that oxygen reduction in the aging retina may stimulate the RPE to release VEGF and induce retina neovascularization from the choroid.(Mousa, Lorelli et al. 1999; Schlingemann 2004; Zhang and Ma 2007)



Therapeutic interventions to treat retinal hypoxic tissue injury have generally not addressed its underlying cause. Unlike treatment of cardiac ischemia where revascularization surgery can be performed to increase tissue oxygenation, the small chorioretinal vasculature and Bruch's membrane are not currently accessible to surgical intervention to improve retinal oxygenation. Given the imbalance between oxygen supply and demand in retinovascular disease and possibly, AMD, it is appealing to consider reducing retinal demand as a means to mitigate ischemia and its consequences. Were demand to be reduced and made commensurate with reduced oxygen supply, then the imbalance that promotes VEGF secretion might be prevented and VEGF mediated vascular leakage and neovascularization could be potentially reversed. One potential means to reduce retinal demand for oxygen is hypothermia.

Hypothermia is used safely in other fields of medicine because metabolic inhibition is protective for neural tissues.(Singer and Bretschneider 1990; Singer and Bretschneider 1990; Geiser 2004; Liu and Yenari 2007) It retards energy depletion, reduces intracellular acidosis, lessens the ischemia induced accumulation of excitotoxic neurotransmitters, and attenuates the influx of intracellular calcium.(Globus, Alonso et al. 1995; Pang and Geddes 1997) Additionally, hypothermia suppresses the generation of oxygen free radicals involved in secondary damage associated with reperfusion.(Maier, Sun et al. 2002; Horiguchi, Shimizu et al. 2003) It also suppresses the mechanisms related to blood-brain barrier degeneration (Chi, Liu et al. 2001) and postischemic remodeling.(Liu and Yenari 2007) Deep hypothermic circulatory arrest is used in thoracic and neuro- surgery to protect the brain during cardiopulmonary bypass.(Dorotta, Kimball-Jones et al. 2007)

Hypothermia is under evaluation for the treatment of the traumatic brain injuries,(Wang, Larner et al. 2006; Jiang and Yang 2007) acute ischemic stroke,(Hemmen and Lyden 2007; Sacco, Chong et al. 2007) brain injury during cardiac arrest(Froehler and Geocadin

2007) and neonatal hypoxic ischemic encephalopathy (birth asphyxia).(Schulzke, Rao et al. 2007; Shah, Ohlsson et al. 2007)

In ophthalmology, Machemer and others have demonstrated the safety of hypothermic infusion fluid and its ability to reduce the risk of light damage to the retina during intraocular surgery.(Rinkoff, Machemer et al. 1986; Zilis, Chandler et al. 1990) Local hypothermia during intraocular surgery seems to inhibit the breakdown of the blood-ocular barrier and the postoperative inflammation.(Tamai, Majima et al. 1995; Tamai, Toumoto et al. 1997) The neuroprotective effects of hypothermia on retinal cells have been demonstrated *in vitro*(Maynard, Quinones-Hinojosa et al. 1999; Quinones-Hinojosa, Malek et al. 2003; Kunikata, Abe et al. 2006; Kunikata, Tomita et al. 2007) and confirmed in animal models.(Faberowski, Stefansson et al. 1989; Tamai, Toumoto et al. 1997; Tamai, Toumoto et al. 1997; Wang, Niwa et al. 2002) In particular, in a model of retinal ischemia, lowering mice body temperature to 35°C during the insult period significantly inhibited retinal cell apoptosis.(Wang, Niwa et al. 2002) Clinically, this may be useful in blinding diseases with no current available treatment, such as the central retinal artery occlusion.(Chen and Lee 2008)

If hypothermia minimizes the retinal damage of hypoxia by a reduction of cellular metabolism, hypothermia might also induce a metabolic inhibition of the RPE and reduce VEGF secretion, even under hypoxic condition. In the present study, we hypothesized that hypothermia might reduce RPE metabolism and consequently the release of VEGF in hypoxic *in vitro* conditions. To demonstrate this correlation, as a first approach we studied the effect of hypothermia on the RPE VEGF secretion under normoxic conditions. Then, we investigated the effect of temperature on VEGF expression by RPE cells in normoxic versus hypoxic conditions.

To demonstrate the specificity of hypothermia in reducing RPE cell metabolism and VEGF production, we compared the effects of hypothermia to those caused by other inhibitors of



cellular metabolic activity (i.e., thiopental and nicotinamide) (Pulido, Fukuda et al. 1989; Shen, Huang et al. 2004).

## 6.2 Methods

ARPE-19 cells (American Type Culture Center) were revived in a mixture of 90% 1:1 of Dulbecco's modified Eagle's medium (DMEM; Invitrogen, New York, NY) and F-12 medium with 3 mM L-Glutamine, and 10% fetal bovine serum (FBS) and incubated at 37 °C in 5% CO<sub>2</sub>. Medium was changed within 24 hours of seeding and then every three days thereafter. The RPE cells were seeded on 48-well plates at the concentration of 5000 cells/well and used for the experiments described below once they reached confluence (2-3 weeks). In the experiments described below media was changed every 24 hours. Cells were cultured in hypoxic conditions using the Modular Incubator Chamber (MIC-101) (Billups-Rothenberg, Inc., Del Mar, CA). Hypoxia was induced by gassing the chamber with 1% O<sub>2</sub>, 5% CO<sub>2</sub>, 94% N<sub>2</sub> as recommended by the manufacturer. Briefly, inlet and outlet ports were opened and the gas was input to the chamber for 4 minutes. After the chamber was purged with gas at a rate of 20 liters/min for 4 minutes, the chamber was sealed by closing the clamps. The chamber was repurged with gas every 12 hours for the duration of the experiment. Standard incubator conditions were used for normoxic treatment of cells, 5% CO<sub>2</sub>, 95% ambient air. For experiments with pharmacological agents, doses of thiopental (1 mM; Sigma) and nicotinamide (1 mM; Sigma) known to be elicit responses in other tissues were added to the media [48, 49].

### Cell Metabolism Assay

ARPE-19 cells were cultured under various oxygen and temperature conditions. ARPE-19 cell metabolic activity was assessed with the Vybrant Cell Metabolic Assay Kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Cells were incubated

with C<sub>12</sub>-reazurin for 45 minutes and fluorescence was measured with the SpectraMax Gemini XS ROM v.4.55 spectrofluorometric microplate reader (Molecular Devices, Sunnyvale, CA) at the absorption and emission of 563 and 587 nm wavelength, respectively.

### VEGF ELISA Assay

ARPE-19 cells were cultured under various oxygen and temperature conditions. At each timepoint, all media were collected from wells and stored frozen until assayed. VEGF levels were measured using a Quantikine VEGF ELISA Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions with some minor modifications. Briefly, standards and cell culture medium (50 µL) were pipetted into an antibody-coated, 96-well plate containing 50 µL assay diluent and incubated for 2 hours at RT on a shaker. The wells were then washed, 100 µL of VEGF conjugate was added, and the samples were again incubated for 2 hours at RT. Samples were washed again, 100 µL substrate buffer was added, the samples were incubated for 30 minutes at RT, the reaction was stopped, and the absorption was measured with an ELISA reader (Emax; Molecular Devices, Sunnyvale, CA) at 450 nm with lambda correction at 570 nm. All measurements were performed in duplicate. Standards included 0.5 log dilutions of VEGF from 5 pg to 100 ng/well. The tissue sample concentration was calculated from the standard curve and corrected for total protein. VEGF concentrations ranged from about 500 to 5000 pg/ml, depending on the experimental conditions.

### **6.3 Results**

We first determined the effect of hypothermia on ARPE-19 cell metabolism. Cellular metabolism was assessed in ARPE-19 cells cultured for 96 hours at 27°C, 34°C, and 37°C under normoxic conditions. The results are shown in Figure 1. ARPE-19 cell metabolism



was significantly reduced by 31% ( $212 \pm 19$ ) and 17% ( $256 \pm 23$ ) respectively when grown at 27°C and 34°C compared to cells grown at 37°C ( $309 \pm 24$ ) (Figure 1; N= 24,  $p < 0.0001$ ). Next, we determined the effect of hypothermia on ARPE-19 cell VEGF secretion. VEGF secretion was assessed in ARPE-19 cells cultured for 96 hours at 34°C, 37°C, and 40°C under normoxic conditions. The results of 3 experiments were combined and are shown in Figure 2. ARPE-19 cell VEGF secretion was significantly reduced by 38% ( $1420 \pm 73$ ) (Figure 2; N=12,  $p < 0.0001$ ) at 34°C compared to cells grown at 37 °C ( $2293 \pm 60$ ) (N=15). Conversely, ARPE-19 cell VEGF secretion was significantly increased by 32% ( $3026 \pm 14$ ) (Figure 2; N=9,  $p < 0.0001$ ) when grown at 40 °C compared to cells grown at 37 °C ( $2293 \pm 60$ ) (N=15).

Next we assessed ARPE-19 cell VEGF secretion under hypoxic and normoxic conditions. VEGF secretion was compared in ARPE-19 cells cultured for 96 hours at 37°C under normoxic (20%O<sub>2</sub>) and hypoxic (1%O<sub>2</sub>) conditions. ARPE-19 cell VEGF secretion increased by 84% under hypoxic conditions ( $3775 \pm 111$ ) (Figure 3; N= 24,  $p < 0.0001$ ) compared to cells cultured under normoxic conditions ( $2052 \pm 67$ ). The effect of hypothermia on ARPE-19 cell VEGF secretion under hypoxic and normoxic conditions was also investigated. VEGF secretion was assessed in ARPE-19 cells cultured for 96 hours at 34°C under normoxic (20%O<sub>2</sub>) and hypoxic (1%O<sub>2</sub>) conditions. The results are also shown in Figure 3. Under normoxic conditions ARPE-19 cell VEGF secretion was significantly decreased by 50% at 34°C (Figure 3; N=22,  $p < 0.0001$ ) compared to cells grown at 37°C. Importantly, under hypoxic conditions ARPE-19 cell VEGF secretion was decreased by 30% at 34°C (Figure 3; N=22,  $p < 0.0001$ ) compared to cells grown under hypoxic conditions at 37°C.

We also determined whether the effects of hypothermia on ARPE-19 cell VEGF secretion are reversible. Identical plates of ARPE-19 cells were grown for 4 days at 34°C and 37°C under normoxic conditions. After 96 hours, both plates were grown at 37°C for 1 day after

which VEGF secretion was assessed as described above. The results are shown in Figure 4. ARPE-19 cell VEGF secretion was significantly reduced by 55% by day 1 at 34°C compared to cells grown at 37°C (N=6,  $p<0.001$ ). This inhibition persisted through day 4. When the ARPE-19 cells grown at 34°C were transferred to 37°C, VEGF secretion returned to normal (Figure 4; N=4,  $p=0.17$ ).

To demonstrate the specificity of hypothermia in reducing both RPE cell metabolism and VEGF production, we compared the effects of hypothermia on VEGF secretion to other inhibitors of cell metabolism. In a separate experiment, the effects of thiopental and nicotinamide on ARPE-19 cell metabolism and VEGF secretion were investigated. Both thiopental and nicotinamide reduced cell metabolism by about 24% in ARPE-19 cells cultured for 96 hours at 37°C under normoxic conditions (Figure 5; N=8,  $p<0.0001$ ). Unlike hypothermia, these drugs did not reduce VEGF secretion by ARPE-19 cells cultured under normoxic conditions (Figure 6; N=8,  $p>0.89$ ).

#### 6.4 Discussion

In the present study we demonstrate that hypothermia decreases both VEGF secretion and cellular metabolism of cultured human RPE cells compared to physiologic temperature. Furthermore, the effect of hypoxia on increasing VEGF secretion by these cells is mitigated by hypothermia. Indeed, a 3°C reduction in temperature reduced RPE VEGF secretion under hypoxic conditions to similar levels observed under normoxic conditions at 37° C. While prolonged effects of hypothermia were not studied in these cells, 4 days of hypothermia did not cause irreversible changes in VEGF secretion when the cells were returned to 37° C.

To demonstrate the specificity of hypothermia in reducing both RPE cell metabolism and VEGF production, we compared the effects of hypothermia to those induced by other inhibitors of cellular metabolic activity (i.e., thiopental and nicotinamide). It might be



expected that all agents, hypothermia included, that reduce cell metabolism would lead to a global reduction in protein synthesis. We demonstrate that at least some agents that inhibit metabolic activity in RPE cells do not reduce VEGF secretion.

Barbiturates and nicotinamide have been used to reduce tissue energy requirements and shown to protect against hypoxia.(Pulido, Fukuda et al. 1989; Shen, Huang et al. 2004) However, it is generally accepted that pharmacologic attempts at reducing cellular energy requirements are less successful than hypothermia.(Maynard, Quinones-Hinojosa et al. 1999) In the in vitro retina, for example, although thiopental reduces retinal function, it only marginally reduced oxygen and glucose consumption compared with hypothermia.(Zager and Ames 1988) Hypothermia may depress cellular function by causing a reduction in energy demands, in a way that only a combination of drugs might achieve.(Maynard, Quinones-Hinojosa et al. 1999) Moreover, it is well known that moderate hypothermia may induce mammalian cells to respond to stresses (i.e., hypoxia) with different strategies from those at 37° C, promoting expression of proteins involved in cell survival, while inhibiting induction of apoptosis.(Fujita 1999; Ning, Chi et al. 2007)

One limit of our study is that we do not know if hypothermia inhibits or promotes the secretory pathways of other growth factors. It has been previously demonstrated that hypothermia tends to downregulate proinflammatory and proapoptotic genes and to upregulate cell survival and trophic genes.(Han and Yenari 2007; Ohta, Terao et al. 2007) Mediators of the inflammatory cascade such as the cytokine IL-6 are reduced by hypothermia.(Gundersen, Vaagenes et al. 2001) Among growth factors, hypothermia increases the expression of Brain-derived neurotrophic factor.(Vosler, Logue et al. 2005) Hypoxia-inducible transcription factor-1 (HIF-1) plays a major role in regulating hypoxia-induced gene responses, such as those involved in angiogenesis, and it is modulated by hypothermia.(Rissanen, Tranberg et al. 2006) When cells were incubated at 32 °C for 24 h under hypoxia, HIF-1 protein accumulation and the expression of VEGF mRNA were

*Marco Coassin*

significantly reduced when compared to normal temperature.(Tanaka, Wakamatsu et al. 2009) As we demonstrated, a generalized reduction of cellular metabolism and protein synthesis alone can not explain the reduction of VEGF secretion by RPE. It is possible that the effect of hypothermia on RPE cells is mediated by the modulation of HIF-1 and we postulate that other proteins were influenced as previously demonstrated in other cell types.(Gundersen, Vaagenes et al. 2001; Vosler, Logue et al. 2005; Rissanen, Tranberg et al. 2006; Han and Yenari 2007; Ohta, Terao et al. 2007; Tanaka, Wakamatsu et al. 2009) The current focus on anti-angiogenic AMD therapy is to inhibit the effects of VEGF, a growth factor secreted in response to ischemia and inflammation. Anti-angiogenic therapy is also used in such conditions as diabetic macular edema(Scott, Edwards et al. 2007) and macular edema resulting from retinal vein occlusions(Hsu, Kaiser et al. 2007; Rabena, Pieramici et al. 2007) where ischemia also probably induces VEGF secretion. An alternative approach to treating these conditions would be to reduce retinal energy requirements such that VEGF secretion would be diminished. This has been indirectly demonstrated by the fact that hypothermia reduce the VEGF expression by brain derived microvascular endothelial cells during hypoxia.(Fischer, Renz et al. 1999) Hyperoxia may be a way to furnish more energy to suffering cells in some retinal pathologies, such as vascular occlusive diseases, where tissue damage is widespread.(Wangsa-Wirawan and Linsenmeier 2003) This has already been proven in an animal model of central retinal vein occlusion in monkeys(Pournaras, Miller et al. 1997) and in patients affected by diabetic macular edema.(Nguyen, Shah et al. 2004) Unfortunately, in AMD high concentration of oxygen may be deleterious, because it may induce oxidative damage to RPE cells(Lu, Hackett et al. 2006) and photoreceptor cells.(Beatty, Koh et al. 2000) On the contrary, hypothermia might have therapeutic effects in AMD by reducing: i) the energy consumption of the suffering photoreceptors; ii) the metabolic activity of RPE; iii) the secretion of VEGF by the RPE cells and the consequent neovascularization.

*Marco Coassin*

While appealing and anatomically accessible, creating a local hypothermic environment in the retina is challenging. A major physiologic function for the high-flow choroidal circulation is the stabilization of the temperature environment of the retina.(Parver, Auker et al. 1982) The choroid is so effective in keeping the retina temperature constant that changing external temperatures from -20 to +40°C affects the retinal temperature by less than 2°C.(Geiser, Bonvin et al. 2004) If the choroidal circulation itself could be cooled, then perhaps, RPE metabolism could be reduced and VEGF levels diminished. Although the protective effects of hypothermia have been established during vitrectomy in animal models,(Faberowski, Stefansson et al. 1989; Tamai, Toumoto et al. 1997; Tamai, Toumoto et al. 1997) there are no data on the safety/effects of hypothermia when applied for longer periods of time. Chronic reduction in VEGF levels induced by hypothermia might be problematic because VEGF is essential for the development of choriocapillaris(Hasegawa, McLeod et al. 2007) and that choriocapillaris is frequently atrophic in elderly patients with AMD.(Lutty, Grunwald et al. 1999; Zarbin 2004) Furthermore, although we demonstrate that the effects of hypothermia are reversible in the short term, long term effects of hypothermia on visual function remain unknown. Nevertheless, our results are a first step in the recognition of importance of the RPE metabolism in the control of VEGF secretion. In summary, the present study demonstrates that hypothermia reduces metabolism and VEGF secretion in cultured human RPE cells. Moderate hypothermia may induce an almost physiologic release of VEGF by RPE, even under hypoxic conditions. Taken together, these data imply that hypothermia might protect the outer retina and reduce the neovascular stimulus in patients with AMD. Although hypothermia could not be easily used to cure retina diseases, this paper represents a first step towards the demonstration that the reduction of RPE metabolism can induce a decrease of VEGF secretion, even under hypoxic conditions. The targeting the RPE metabolism may represent a future strategy of pharmacological research in AMD.

*Marco Coassin*

## 7. Conclusioni

Nel corso dei tre anni di Dottorato in Immunologia Oculare, mi è stato possibile approfondire le interazioni del sistema nervoso e del sistema immunitario a livello dell'apparato visivo. Una maggiore comprensione delle interconnessioni tra i due sistemi, così come auspicata dalla neuroimmunologia, permette di spiegare meglio i meccanismi patogenetici alla base delle malattie e, di conseguenza, sviluppare nuove strategie terapeutiche. Il Dottorato mi ha dato modo di studiare alcuni aspetti neuroimmunologici del segmento anteriore (la cornea) e posteriore dell'occhio (la retina).

Nel caso della cheratite da Herpes simplex virus, abbiamo dimostrato nel modello animale come il Nerve growth factor abbia un ruolo chiave da un punto di vista neuro immunologico. Abbiamo evidenziato come il NGF abbia funzioni di modulazione della latenza virale, inducendo una rapida risoluzione della cheratite con un buon controllo della riparazione tissutale corneale.

Le interazioni tra sistema immunitario e nervoso sono ancora più evidenti nell'infezione da Herpes simplex virus che conduce all'encefalite e alla retinite. Abbiamo dimostrato in che modo le due malattie siano legate. La retinite erpetica può anticipare, essere concomitante o seguire l'encefalite. Il primo sembra essere il caso nei pazienti immunocompromessi e il virus di tipo 1 sembra essere l'agente causale. Nel secondo caso, l'agente causale sembra essere invece il HSV-2. Nel caso in cui la retinite segua l'encefalite (stragrande maggioranza dei casi), è possibile distinguere due gruppi: uno in cui l'insorgenza della retinite è precoce (entro tre mesi, 48.9%) ed uno in cui l'insorgenza è ritardata (dai 2 ai 20 anni, 51.1%). Questi dati e gli altri dettagli presenti nello studio caratterizzano in modo innovativo molti aspetti dell'encefalite e retinite erpetica e permetteranno di individuare i pazienti più a rischio.



Il glaucoma è un'altra malattia i cui aspetti neurodegenerativi sono di particolare interesse da un punto di vista neuroimmunologico. Nel nostro modello sperimentale di glaucoma, la perdita di cellule ganglionari della retina è accompagnata da un aumento iniziale di NGF endogeno a livello retinico. Tuttavia, la neurotrofina non era in grado di garantire la sopravvivenza delle cellule ganglionari nel lungo periodo, probabilmente a causa di una sovra espressione di p75 e Bax (proapoptotici) rispetto a trkA e Bcl-2 (anti-apoptotici). Questo è stato il primo di una serie di studi che hanno definito il ruolo del NGF in collirio come agente neuroprotettivo nel glaucoma sperimentale e nell'uomo.

Anche la retinopatia diabetica presenta aspetti neurodegenerativi di interesse per la neuroimmunologia. In un modello animale di diabete, la somministrazione di NGF in collirio è stata in grado di proteggere le cellule ganglionari retiniche in corso di retinopatia diabetica. Altri studi preliminari hanno dimostrato come il NGF esogeno possa ridurre i livelli di VEGF nella retinopatia diabetica. Perciò nuovi studi sono in corso per verificare se questa neurotrofina potrebbe essere in grado di curare l'edema maculare diabetico.

Infine, anche la degenerazione maculare senile presenta aspetti neurodegenerativi e nella sua patogenesi è stato chiamato in causa il sistema immunitario. Abbiamo dimostrato come l'ipotermia non sia solo in grado di avere effetti neuroprotettivi, come classicamente sostenuto, ma anche un diretto effetto sulla produzione di VEGF da parte dell'epitelio pigmentato retinico. Un calo della temperatura di pochi gradi centigradi (tre) è in grado di ridurre la produzione di VEGF da parte dell'EPR in maniera drastica, anche in condizioni ipossiche. Anche se difficilmente attuabile in pratica, l'ipotermia a livello del polo posteriore potrebbe rappresentare in futuro un trattamento di alcune patologie retiniche grazie alle sue proprietà neuroprotettive e di controllo del VEGF.

In conclusione, lo studio delle malattie oculari con una prospettiva neuroimmunologica ha permesso in modo esemplare di sviluppare nuove conoscenze fisiopatologiche e terapeutiche.



## 8. Bibliografia

- Aguayo, A. J., D. B. Clarke, et al. (1996). "Effects of neurotrophins on the survival and regrowth of injured retinal neurons." Ciba Found Symp 196: 135-44; discussion 144-8.
- Ahmadiéh, H., S. H. Sajjadi, et al. (1991). "Association of herpetic encephalitis with acute retinal necrosis syndrome." Ann Ophthalmol 23(6): 215-9.
- Aiello, L. P., E. A. Pierce, et al. (1995). "Suppression of retinal neovascularization in vivo by inhibition of vascular endothelial growth factor (VEGF) using soluble VEGF-receptor chimeric proteins." Proc Natl Acad Sci U S A 92(23): 10457-61.
- Antel, J. and A. Prat (2000). "Antigen and superantigen presentation in the human CNS." J Neuroimmunol 107(2): 118-23.
- Beatty, S., H. Koh, et al. (2000). "The role of oxidative stress in the pathogenesis of age-related macular degeneration." Surv Ophthalmol 45(2): 115-34.
- Bergua, A., B. Schmidt, et al. (2001). "[Fulminant acute retinal necrosis 10 months after ipsilateral herpes simplex virus encephalitis - a case report]." Klin Monatsbl Augenheilkd 218(11): 737-40.
- Bloom, J. N., J. I. Katz, et al. (1977). "Herpes simplex retinitis and encephalitis in an adult." Arch Ophthalmol 95(10): 1798-9.
- Boivin, G. (2004). "Diagnosis of herpesvirus infections of the central nervous system." Herpes 11 Suppl 2: 48A-56A.
- Bonini, S., L. Aloe, et al. (2002). "Nerve growth factor (NGF): an important molecule for trophism and healing of the ocular surface." Adv Exp Med Biol 506(Pt A): 531-7.
- Bressler, N. M., S. B. Bressler, et al. (1988). "Age-related macular degeneration." Surv Ophthalmol 32(6): 375-413.
- Cardine, S., P. A. Chaze, et al. (2004). "[Bilateral acute retinal necrosis syndrome associated with meningoencephalitis caused by herpes simplex virus 2. A case report]." J Fr Ophtalmol 27(7): 795-800.
- Carmignoto, G., M. C. Comelli, et al. (1991). "Expression of NGF receptor and NGF receptor mRNA in the developing and adult rat retina." Exp Neurol 111(3): 302-11.
- Carmignoto, G., L. Maffei, et al. (1989). "Effect of NGF on the survival of rat retinal ganglion cells following optic nerve section." J Neurosci 9(4): 1263-72.
- Casaccia-Bonnet, P., C. Gu, et al. (1999). "Neurotrophins in cell survival/death decisions." Adv Exp Med Biol 468: 275-82.
- Chan, C. C., D. Shen, et al. (2005). "Polymerase chain reaction in the diagnosis of uveitis." Int Ophthalmol Clin 45(2): 41-55.
- Chao, M., P. Casaccia-Bonnet, et al. (1998). "Neurotrophin receptors: mediators of life and death." Brain Res Brain Res Rev 26(2-3): 295-301.
- Chen, C. S. and A. W. Lee (2008). "Management of acute central retinal artery occlusion." Nat Clin Pract Neurol 4(7): 376-83.
- Chi, O. Z., X. Liu, et al. (2001). "Effects of mild hypothermia on blood-brain barrier disruption during isoflurane or pentobarbital anesthesia." Anesthesiology 95(4): 933-8.
- Cibis, G. W., J. T. Flynn, et al. (1978). "Herpes simplex retinitis." Arch Ophthalmol 96(2): 299-302.



- Colafrancesco, V., V. Parisi, et al. (2011). "Ocular application of nerve growth factor protects degenerating retinal ganglion cells in a rat model of glaucoma." J Glaucoma 20(2): 100-8.
- Colin, J., X. Bodereau, et al. (1981). "[Herpetic encephalitis and retinitis in an adult: apropos of a case]." Bull Soc Ophthalmol Fr 81(6-7): 701-3.
- Cui, Q., Q. Lu, et al. (1999). "CNTF, not other trophic factors, promotes axonal regeneration of axotomized retinal ganglion cells in adult hamsters." Invest Ophthalmol Vis Sci 40(3): 760-6.
- Cunningham, E. T., Jr., G. A. Short, et al. (1996). "Acquired immunodeficiency syndrome--associated herpes simplex virus retinitis. Clinical description and use of a polymerase chain reaction--based assay as a diagnostic tool." Arch Ophthalmol 114(7): 834-40.
- Das, A. and P. G. McGuire (2003). "Retinal and choroidal angiogenesis: pathophysiology and strategies for inhibition." Prog Retin Eye Res 22(6): 721-48.
- Davis, J. L., W. Feuer, et al. (1995). "Interpretation of intraocular and serum antibody levels in necrotizing retinitis." Retina 15(3): 233-40.
- de Boer, J. H., C. Verhagen, et al. (1996). "Serologic and polymerase chain reaction analysis of intraocular fluids in the diagnosis of infectious uveitis." Am J Ophthalmol 121(6): 650-8.
- de Jong, P. T. (2006). "Age-related macular degeneration." N Engl J Med 355(14): 1474-85.
- Diaz De Durana Santa Coloma, E., E. Vazquez Cruchaga, et al. (2004). "[Herpes simplex virus type 1 acute retinal necrosis two years after presumably herpetic meningoencephalitis]." Arch Soc Esp Oftalmol 79(1): 33-5.
- Dorotta, I., P. Kimball-Jones, et al. (2007). "Deep hypothermia and circulatory arrest in adults." Semin Cardiothorac Vasc Anesth 11(1): 66-76.
- Faberowski, N., E. Stefansson, et al. (1989). "Local hypothermia protects the retina from ischemia. A quantitative study in the rat." Invest Ophthalmol Vis Sci 30(11): 2309-13.
- Fabricius, E. M., T. Lipp, et al. (1990). "[Acute retinal necrosis and herpes encephalitis. The key role of the ophthalmologist in diagnosing opportunistic infections in AIDS, successful therapy with acyclovir (Zovirax)]." Klin Monatsbl Augenheilkd 196(3): 160-5.
- Feinman, M. C., M. A. Friedberg, et al. (1993). "Central nervous system herpesvirus infection in systemic lupus erythematosus: diagnosis by endoretinal biopsy." J Rheumatol 20(6): 1058-61.
- Ferrara, N. (2000). "Vascular endothelial growth factor and the regulation of angiogenesis." Recent Prog Horm Res 55: 15-35; discussion 35-6.
- Fischer, S., D. Renz, et al. (1999). "Hypothermia abolishes hypoxia-induced hyperpermeability in brain microvessel endothelial cells." Brain Res Mol Brain Res 74(1-2): 135-44.
- Fox, R. J. (2010). "Primary neuroprotection: the Holy Grail of multiple sclerosis therapy." Neurology 74(13): 1018-9.
- Freeman, W. R., E. L. Thomas, et al. (1986). "Demonstration of herpes group virus in acute retinal necrosis syndrome." Am J Ophthalmol 102(6): 701-9.
- Froehler, M. T. and R. G. Geocadin (2007). "Hypothermia for neuroprotection after cardiac arrest: mechanisms, clinical trials and patient care." J Neurol Sci 261(1-2): 118-26.
- Fujita, J. (1999). "Cold shock response in mammalian cells." J Mol Microbiol Biotechnol 1(2): 243-55.

- Gain, P., C. Chiquet, et al. (2002). "Herpes simplex virus type 1 encephalitis associated with acute retinal necrosis syndrome in an immunocompetent patient." Acta Ophthalmol Scand 80(5): 546-9.
- Ganatra, J. B., D. Chandler, et al. (2000). "Viral causes of the acute retinal necrosis syndrome." Am J Ophthalmol 129(2): 166-72.
- Gartry, D. S., D. J. Spalton, et al. (1991). "Acute retinal necrosis syndrome." Br J Ophthalmol 75(5): 292-7.
- Gaynor, B. D., N. K. Wade, et al. (2001). "Herpes simplex virus type 1 associated acute retinal necrosis following encephalitis." Retina 21(6): 688-90.
- Geiser, F. (2004). "Metabolic rate and body temperature reduction during hibernation and daily torpor." Annu Rev Physiol 66: 239-74.
- Geiser, M. H., M. Bonvin, et al. (2004). "Corneal and retinal temperatures under various ambient conditions: a model and experimental approach." Klin Monatsbl Augenheilkd 221(5): 311-4.
- Ghisso, N., R. M. Rohan, et al. (1999). "Suppression of hypoxia-associated vascular endothelial growth factor gene expression by nitric oxide via cGMP." Invest Ophthalmol Vis Sci 40(6): 1033-9.
- Globus, M. Y., O. Alonso, et al. (1995). "Glutamate release and free radical production following brain injury: effects of posttraumatic hypothermia." J Neurochem 65(4): 1704-11.
- Grus, F. and D. Sun (2008). "Immunological mechanisms in glaucoma." Semin Immunopathol 30(2): 121-6.
- Gundersen, Y., P. Vaagenes, et al. (2001). "Moderate hypothermia blunts the inflammatory response and reduces organ injury after acute haemorrhage." Acta Anaesthesiol Scand 45(8): 994-1001.
- Hadden, P. W. and C. J. Barry (2002). "Images in clinical medicine. Herpetic encephalitis and acute retinal necrosis." N Engl J Med 347(24): 1932.
- Han, H. S. and M. A. Yenari (2007). "Effect on gene expression of therapeutic hypothermia in cerebral ischemia." Future Neurology 2(4): 435-40.
- Hasegawa, T., D. S. McLeod, et al. (2007). "The embryonic human choriocapillaris develops by hemo-vasculogenesis." Dev Dyn 236(8): 2089-100.
- Hefti, F. (1994). "Neurotrophic factor therapy for nervous system degenerative diseases." J Neurobiol 25(11): 1418-35.
- Hemmen, T. M. and P. D. Lyden (2007). "Induced hypothermia for acute stroke." Stroke 38(2 Suppl): 794-9.
- Horiguchi, T., K. Shimizu, et al. (2003). "Postischemic hypothermia inhibits the generation of hydroxyl radical following transient forebrain ischemia in rats." J Neurotrauma 20(5): 511-20.
- Hsu, J., R. S. Kaiser, et al. (2007). "Intravitreal bevacizumab (avastin) in central retinal vein occlusion." Retina 27(8): 1013-9.
- Jia, L., W. O. Cepurna, et al. (2000). "Effect of general anesthetics on IOP in rats with experimental aqueous outflow obstruction." Invest Ophthalmol Vis Sci 41(11): 3415-9.
- Jiang, J. Y. and X. F. Yang (2007). "Current status of cerebral protection with mild-to-moderate hypothermia after traumatic brain injury." Curr Opin Crit Care 13(2): 153-5.
- Johnson, B. L. and H. M. Wisotzkey (1977). "Neuroretinitis associated with herpes simplex encephalitis in an adult." Am J Ophthalmol 83(4): 481-9.
- Johnson, E. C., L. M. Deppmeier, et al. (2000). "Chronology of optic nerve head and retinal responses to elevated intraocular pressure." Invest Ophthalmol Vis Sci 41(2): 431-42.

- Kamel, O. R., G. D. Galloway, et al. (2000). "Delayed onset acute retinal necrosis 20 years following herpetic encephalitis." Eye 14 Pt 5: 788-9.
- Kerschensteiner, M., E. Meinl, et al. (2009). "Neuro-immune crosstalk in CNS diseases." Neuroscience 158(3): 1122-32.
- Kim, C. and Y. H. Yoon (2002). "Unilateral acute retinal necrosis occurring 2 years after herpes simplex type 1 encephalitis." Ophthalmic Surg Lasers 33(3): 250-2.
- Knox, C. M., D. Chandler, et al. (1998). "Polymerase chain reaction-based assays of vitreous samples for the diagnosis of viral retinitis. Use in diagnostic dilemmas." Ophthalmology 105(1): 37-44; discussion 44-5.
- Kriz, J. (2006). "Inflammation in ischemic brain injury: timing is important." Crit Rev Neurobiol 18(1-2): 145-57.
- Kunikata, H., T. Abe, et al. (2006). "Hypothermia of 8 degrees C protects cultured retinal pigment epithelial cells and retinal ganglion cells against trypan blue toxicity." Am J Ophthalmol 141(4): 754-6.
- Kunikata, H., H. Tomita, et al. (2007). "Hypothermia protects cultured human retinal pigment epithelial cells against indocyanine green toxicity." J Ocul Pharmacol Ther 23(1): 35-9.
- la Sala, A., S. Corinti, et al. (2000). "Ligand activation of nerve growth factor receptor TrkA protects monocytes from apoptosis." J Leukoc Biol 68(1): 104-10.
- Lambiase, A., L. Aloe, et al. (2009). "Experimental and clinical evidence of neuroprotection by nerve growth factor eye drops: Implications for glaucoma." Proc Natl Acad Sci U S A.
- Lambiase, A., S. Bonini, et al. (1998). "Expression of nerve growth factor receptors on the ocular surface in healthy subjects and during manifestation of inflammatory diseases." Invest Ophthalmol Vis Sci 39(7): 1272-5.
- Lambiase, A., M. Centofanti, et al. (1997). "Nerve growth factor (NGF) reduces and NGF antibody exacerbates retinal damage induced in rabbit by experimental ocular hypertension." Graefes Arch Clin Exp Ophthalmol 235(12): 780-5.
- Lambiase, A., A. Micera, et al. (2004). "Nerve growth factor and the immune system: old and new concepts in the cross-talk between immune and resident cells during pathophysiological conditions." Curr Opin Allergy Clin Immunol 4(5): 425-30.
- Lambiase, A., P. Rama, et al. (1998). "Topical treatment with nerve growth factor for corneal neurotrophic ulcers." N Engl J Med 338(17): 1174-80.
- Landry, M. L., P. Mullangi, et al. (2005). "Herpes simplex virus type 2 acute retinal necrosis 9 years after neonatal herpes." J Pediatr 146(6): 836-8.
- Leung, D. W., G. Cachianes, et al. (1989). "Vascular endothelial growth factor is a secreted angiogenic mitogen." Science 246(4935): 1306-9.
- Levi-Montalcini, R. (1987). "The nerve growth factor 35 years later." Science 237(4819): 1154-62.
- Levinson, R. D., R. Reidy, et al. (1999). "Acute retinal necrosis after neonatal herpes encephalitis." Br J Ophthalmol 83(1): 123-4.
- Liu, L. and M. A. Yenari (2007). "Therapeutic hypothermia: neuroprotective mechanisms." Front Biosci 12: 816-25.
- Lu, L., S. F. Hackett, et al. (2006). "Effects of different types of oxidative stress in RPE cells." J Cell Physiol 206(1): 119-25.
- Lutty, G., J. Grunwald, et al. (1999). "Changes in choriocapillaris and retinal pigment epithelium in age-related macular degeneration." Mol Vis 5: 35.
- Maertzdorf, J., A. Van der Lelij, et al. (2001). "Herpes simplex virus type 1 (HSV-1)--induced retinitis following herpes simplex encephalitis: indications for brain-to-eye transmission of HSV-1." Ann Neurol 49(1): 104-6.

- Maier, C. M., G. H. Sun, et al. (2002). "Effects of mild hypothermia on superoxide anion production, superoxide dismutase expression, and activity following transient focal cerebral ischemia." Neurobiol Dis 11(1): 28-42.
- Majdan, M., G. S. Walsh, et al. (2001). "TrkA mediates developmental sympathetic neuron survival in vivo by silencing an ongoing p75NTR-mediated death signal." J Cell Biol 155(7): 1275-85.
- Manderieux, N., R. Boukhobza, et al. (1990). "[Retinal vascularity following herpetic encephalitis]." Ann Med Interne (Paris) 141(7): 631-3.
- Margolis, T. P. and S. S. Atherton (1996). Herpes Simplex Virus Diseases: posterior segment of the eye. Ocular infection and immunity. J. S. Pepose, G. N. Holland and K. R. Wilhenies. St. Louis, MO, Mosby: 1155-1166.
- Maynard, K. I., A. Quinones-Hinojosa, et al. (1999). "Neuroprotection against ischemia by metabolic inhibition revisited. A comparison of hypothermia, a pharmacologic cocktail and magnesium plus mexiletine." Ann N Y Acad Sci 890: 240-54.
- McDonnell, G. V., G. Silvestri, et al. (1997). "Acute visual disturbance in a young adult." Postgrad Med J 73(859): 307-9.
- Micera, A., A. Lambiase, et al. (2004). "Nerve growth factor involvement in the visual system: implications in allergic and neurodegenerative diseases." Cytokine Growth Factor Rev 15(6): 411-7.
- Minckler, D. S., E. B. McLean, et al. (1976). "Herpesvirus hominis encephalitis and retinitis." Arch Ophthalmol 94(1): 89-95.
- Moore, C. G., D. Epley, et al. (1995). "Long-term non-invasive measurement of intraocular pressure in the rat eye." Curr Eye Res 14(8): 711-7.
- Morrison, J. C., E. C. Johnson, et al. (2005). "Understanding mechanisms of pressure-induced optic nerve damage." Prog Retin Eye Res 24(2): 217-40.
- Morrison, J. C., C. G. Moore, et al. (1997). "A rat model of chronic pressure-induced optic nerve damage." Exp Eye Res 64(1): 85-96.
- Mousa, S. A., W. Lorelli, et al. (1999). "Role of hypoxia and extracellular matrix-integrin binding in the modulation of angiogenic growth factors secretion by retinal pigmented epithelial cells." J Cell Biochem 74(1): 135-43.
- Murphy, K., P. Travers, et al. (2008). Janeway's immunobiology. New York, Garland Science.
- Nahmias, A. J., R. J. Whitley, et al. (1982). "Herpes simplex virus encephalitis: laboratory evaluations and their diagnostic significance." J Infect Dis 145(6): 829-36.
- Nguyen, Q. D., S. M. Shah, et al. (2004). "Supplemental oxygen improves diabetic macular edema: a pilot study." Invest Ophthalmol Vis Sci 45(2): 617-24.
- Nickells, R. W. (1999). "Apoptosis of retinal ganglion cells in glaucoma: an update of the molecular pathways involved in cell death." Surv Ophthalmol 43 Suppl 1: S151-61.
- Ning, X. H., E. Y. Chi, et al. (2007). "Moderate hypothermia (30 degrees C) maintains myocardial integrity and modifies response of cell survival proteins after reperfusion." Am J Physiol Heart Circ Physiol 293(4): H2119-28.
- Noseworthy, J. H., C. Lucchinetti, et al. (2000). "Multiple sclerosis." N Engl J Med 343(13): 938-52.
- Ohta, H., Y. Terao, et al. (2007). "Therapeutic time window of post-ischemic mild hypothermia and the gene expression associated with the neuroprotection in rat focal cerebral ischemia." Neurosci Res 57(3): 424-33.
- Osborne, N. N., R. J. Casson, et al. (2004). "Retinal ischemia: mechanisms of damage and potential therapeutic strategies." Prog Retin Eye Res 23(1): 91-147.
- Pang, Z. and J. W. Geddes (1997). "Mechanisms of cell death induced by the mitochondrial toxin 3-nitropropionic acid: acute excitotoxic necrosis and delayed apoptosis." J Neurosci 17(9): 3064-73.

- Partamian, L. G., P. H. Morse, et al. (1981). "Herpes simplex type 1 retinitis in an adult with systemic herpes zoster." Am J Ophthalmol 92(2): 215-20.
- Parver, L. M., C. R. Auker, et al. (1982). "The stabilizing effect of the choroidal circulation on the temperature environment of the macula." Retina 2(2): 117-20.
- Pavesio, C. E., D. K. Conrad, et al. (1997). "Delayed acute retinal necrosis after herpetic encephalitis." Br J Ophthalmol 81(5): 415-6.
- Pease, M. E., S. J. McKinnon, et al. (2000). "Obstructed axonal transport of BDNF and its receptor TrkB in experimental glaucoma." Invest Ophthalmol Vis Sci 41(3): 764-74.
- Pepose, J. S., L. H. Hilborne, et al. (1984). "Concurrent herpes simplex and cytomegalovirus retinitis and encephalitis in the acquired immune deficiency syndrome (AIDS)." Ophthalmology 91(12): 1669-77.
- Pepose, J. S., A. E. Kreiger, et al. (1985). "Immunocytologic localization of herpes simplex type 1 viral antigens in herpetic retinitis and encephalitis in an adult." Ophthalmology 92(1): 160-6.
- Perry, J. D., C. A. Girkin, et al. (1998). "Herpes simplex encephalitis and bilateral acute retinal necrosis syndrome after craniotomy." Am J Ophthalmol 126(3): 456-60.
- Pfaffl, M. W., G. W. Horgan, et al. (2002). "Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR." Nucleic Acids Res 30(9): e36.
- Pournaras, C. J., J. W. Miller, et al. (1997). "Systemic hyperoxia decreases vascular endothelial growth factor gene expression in ischemic primate retina." Arch Ophthalmol 115(12): 1553-8.
- Preiser, W., H. W. Doerr, et al. (2004). "Acute retinal necrosis six years after herpes simplex encephalitis: an elusive immune deficit suggested by insufficient test sensitivity." J Med Virol 73(2): 250-5.
- Pulido, J. S., M. Fukuda, et al. (1989). "Barbiturates protect retinal cells from hypoxia in cell culture." Arch Ophthalmol 107(12): 1809-12.
- Quigley, H. A., G. R. Dunkelberger, et al. (1989). "Retinal ganglion cell atrophy correlated with automated perimetry in human eyes with glaucoma." Am J Ophthalmol 107(5): 453-64.
- Quinones-Hinojosa, A., J. Y. Malek, et al. (2003). "Metabolic effects of hypothermia and its neuroprotective effects on the recovery of metabolic and electrophysiological function in the ischemic retina in vitro." Neurosurgery 52(5): 1178-86; discussion 1186-7.
- Rabena, M. D., D. J. Pieramici, et al. (2007). "Intravitreal bevacizumab (Avastin) in the treatment of macular edema secondary to branch retinal vein occlusion." Retina 27(4): 419-25.
- Rinkoff, J., R. Machemer, et al. (1986). "Temperature-dependent light damage to the retina." Am J Ophthalmol 102(4): 452-62.
- Rissanen, E., H. K. Tranberg, et al. (2006). "Temperature regulates hypoxia-inducible factor-1 (HIF-1) in a poikilothermic vertebrate, crucian carp (*Carassius carassius*)." J Exp Biol 209(Pt 6): 994-1003.
- Rudzinski, M., T. P. Wong, et al. (2004). "Changes in retinal expression of neurotrophins and neurotrophin receptors induced by ocular hypertension." J Neurobiol 58(3): 341-54.
- Sacco, R. L., J. Y. Chong, et al. (2007). "Experimental treatments for acute ischaemic stroke." Lancet 369(9558): 331-41.
- Sakamoto, T., H. Sakamoto, et al. (1995). "Vessel formation by choroidal endothelial cells in vitro is modulated by retinal pigment epithelial cells." Arch Ophthalmol 113(4): 512-20.

- Saragovi, H. U., W. Zheng, et al. (1998). "A TrkA-selective, fast internalizing nerve growth factor-antibody complex induces trophic but not neuritogenic signals." J Biol Chem 273(52): 34933-40.
- Sarkies, N., Z. Gregor, et al. (1986). "Antibodies to herpes simplex virus type I in intraocular fluids of patients with acute retinal necrosis." Br J Ophthalmol 70(2): 81-4.
- Savir, H., Z. Grosswasser, et al. (1980). "Herpes virus hominis encephalomyelitis and retinal vasculitis in adults." Ann Ophthalmol 12(12): 1369-71.
- Schliebs, R. (2004). "Molecular mechanisms of neurodegeneration and neuroprotection--experimental approaches and the diseased brain." Int J Dev Neurosci 22(7): 441-2.
- Schlingemann, R. O. (2004). "Role of growth factors and the wound healing response in age-related macular degeneration." Graefes Arch Clin Exp Ophthalmol 242(1): 91-101.
- Schlingemann, R. O., M. Bruinenberg, et al. (1996). "Twenty years' delay of fellow eye involvement in herpes simplex virus type 2-associated bilateral acute retinal necrosis syndrome." Am J Ophthalmol 122(6): 891-2.
- Schulzke, S. M., S. Rao, et al. (2007). "A systematic review of cooling for neuroprotection in neonates with hypoxic ischemic encephalopathy - are we there yet?" BMC Pediatr 7: 30.
- Schwartz, S., S. Litwak, et al. (2008). "Hematopoietic stem cells reduce postischemic inflammation and ameliorate ischemic brain injury." Stroke 39(10): 2867-75.
- Scott, I. U., A. R. Edwards, et al. (2007). "A phase II randomized clinical trial of intravitreal bevacizumab for diabetic macular edema." Ophthalmology 114(10): 1860-7.
- Sekizawa, T., S. Hara, et al. (1991). "Acute retinitis 2 years after recovery from herpes simplex encephalitis." Neurology 41(3): 456.
- Shah, P. S., A. Ohlsson, et al. (2007). "Hypothermia to treat neonatal hypoxic ischemic encephalopathy: systematic review." Arch Pediatr Adolesc Med 161(10): 951-8.
- Shen, C. C., H. M. Huang, et al. (2004). "Protective effect of nicotinamide on neuronal cells under oxygen and glucose deprivation and hypoxia/reoxygenation." J Biomed Sci 11(4): 472-81.
- Shi, Z., E. Birman, et al. (2007). "Neurotrophic rationale in glaucoma: A TrkA agonist, but not NGF or a p75 antagonist, protects retinal ganglion cells in vivo." Dev Neurobiol 67(7): 884-94.
- Shweiki, D., A. Itin, et al. (1992). "Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis." Nature 359(6398): 843-5.
- Siliprandi, R., R. Canella, et al. (1993). "Nerve growth factor promotes functional recovery of retinal ganglion cells after ischemia." Invest Ophthalmol Vis Sci 34(12): 3232-45.
- Singer, D. and H. J. Bretschneider (1990). "Metabolic reduction in hypothermia: pathophysiological problems and natural examples--Part 1." Thorac Cardiovasc Surg 38(4): 205-11.
- Singer, D. and H. J. Bretschneider (1990). "Metabolic reduction in hypothermia: pathophysiological problems and natural examples--Part 2." Thorac Cardiovasc Surg 38(4): 212-9.
- Sofroniew, M. V., C. L. Howe, et al. (2001). "Nerve growth factor signaling, neuroprotection, and neural repair." Annu Rev Neurosci 24: 1217-81.
- Stuerzebecher, S. and R. Martin (2000). "Neuroimmunology of multiple sclerosis and experimental allergic encephalomyelitis." Neuroimaging Clin N Am 10(4): 649-68, vii-viii.
- Tabassum, A., F. Khwaja, et al. (2003). "The p75(NTR) tumor suppressor induces caspase-mediated apoptosis in bladder tumor cells." Int J Cancer 105(1): 47-52.

- Tamai, K., A. Majima, et al. (1995). "Effects of local hypothermia on uveal blood flow and postoperative inflammation in vitrectomy." Jpn J Ophthalmol 39(1): 43-8.
- Tamai, K., E. Toumoto, et al. (1997). "Local hypothermia protects the retina from ischaemic injury in vitrectomy." Br J Ophthalmol 81(9): 789-94.
- Tamai, K., E. Toumoto, et al. (1997). "Protective effects of local hypothermia in vitrectomy under fluctuating intraocular pressure." Exp Eye Res 65(6): 733-8.
- Tanaka, T., T. Wakamatsu, et al. (2009). "Persisting mild hypothermia suppresses hypoxia-inducible factor 1{alpha} protein synthesis and HIF-1-mediated gene expression." Am J Physiol Regul Integr Comp Physiol.
- Taylor, A. W. and H. J. Kaplan (2010). "Ocular immune privilege in the year 2010: ocular immune privilege and uveitis." Ocul Immunol Inflamm 18(6): 488-92.
- Thompson, W. S., W. W. Culbertson, et al. (1994). "Acute retinal necrosis caused by reactivation of herpes simplex virus type 2." Am J Ophthalmol 118(2): 205-11.
- Troy, C. M., J. E. Friedman, et al. (2002). "Mechanisms of p75-mediated death of hippocampal neurons. Role of caspases." J Biol Chem 277(37): 34295-302.
- Tyler, K. L. (2004). "Herpes simplex virus infections of the central nervous system: encephalitis and meningitis, including Mollaret's." Herpes 11 Suppl 2: 57A-64A.
- Uninsky, E., L. M. Jampol, et al. (1983). "Disseminated herpes simplex infection with retinitis in a renal allograft recipient." Ophthalmology 90(2): 175-8.
- Van Gelder, R. N., J. L. Willig, et al. (2001). "Herpes simplex virus type 2 as a cause of acute retinal necrosis syndrome in young patients." Ophthalmology 108(5): 869-76.
- Verma, L., P. Venkatesh, et al. (1999). "Bilateral necrotizing herpetic retinopathy three years after herpes simplex encephalitis following pulse corticosteroid treatment." Retina 19(5): 464-7.
- Vosler, P. S., E. S. Logue, et al. (2005). "Delayed hypothermia preferentially increases expression of brain-derived neurotrophic factor exon III in rat hippocampus after asphyxial cardiac arrest." Brain Res Mol Brain Res 135(1-2): 21-9.
- Walters, G. and T. E. James (2001). "Viral causes of the acute retinal necrosis syndrome." Curr Opin Ophthalmol 12(3): 191-5.
- Wang, K. K., S. F. Larner, et al. (2006). "Neuroprotection targets after traumatic brain injury." Curr Opin Neurol 19(6): 514-9.
- Wang, X., M. Niwa, et al. (2002). "Neuronal degradation in mouse retina after a transient ischemia and protective effect of hypothermia." Neurol Res 24(7): 730-5.
- Wangsa-Wirawan, N. D. and R. A. Linsenmeier (2003). "Retinal oxygen: fundamental and clinical aspects." Arch Ophthalmol 121(4): 547-57.
- Watanabe, J., M. Ashida, et al. (1989). "[A case of acute retinal necrosis syndrome caused by herpes simplex virus type 1]." Nippon Ganka Gakkai Zasshi 93(1): 65-71.
- Wessel, K., H. Wietholter, et al. (1987). "[Herpes simplex encephalitis with herpes simplex retinitis]." Nervenarzt 58(5): 317-21.
- Whikehart, D. R. (2003). Biochemistry of the eye. Philadelphia, Pa., Butterworth-Heinemann.
- Yoon, S. O., P. Casaccia-Bonnel, et al. (1998). "Competitive signaling between TrkA and p75 nerve growth factor receptors determines cell survival." J Neurosci 18(9): 3273-81.
- Young, T. A., H. Wang, et al. (2005). "Vascular endothelial growth factor expression and secretion by retinal pigment epithelial cells in high glucose and hypoxia is protein kinase C-dependent." Exp Eye Res 80(5): 651-62.
- Zager, E. L. and A. Ames, 3rd (1988). "Reduction of cellular energy requirements. Screening for agents that may protect against CNS ischemia." J Neurosurg 69(4): 568-79.

- Zarbin, M. A. (2004). "Current concepts in the pathogenesis of age-related macular degeneration." Arch Ophthalmol 122(4): 598-614.
- Zhang, S. X. and J. X. Ma (2007). "Ocular neovascularization: Implication of endogenous angiogenic inhibitors and potential therapy." Prog Retin Eye Res 26(1): 1-37.
- Zilis, J. D., D. Chandler, et al. (1990). "Clinical and histologic effects of extreme intraocular hypothermia." Am J Ophthalmol 109(4): 469-73.



## 9. Pubblicazioni scientifiche nel corso del dottorato

1. Lambiase A, **Coassin M**, Costa N, Lauretti P, Micera A, Ghinelli E, Aloe L, Rama P, Bonini S. *Topical treatment with nerve growth factor in an animal model of herpetic keratitis*. Graefes Arch Clin Exp Ophthalmol. 2008 Jan; 246(1):121-7.
2. **Coassin M**, Lambiase A, Sposato V, Micera A, Bonini S, Aloe L. *Retinal p75 and Bax overexpression is associated with retinal ganglion cells apoptosis in a rat model of glaucoma*. Graefes Arch Clin Exp Ophthalmol. 2008 Dec; 246(12):1743-9.
3. Sposato V, Bucci MG, **Coassin M**, Russo MA, Lambiase A, Aloe L. *Reduced NGF level and TrkA protein and TrkA gene expression in the optic nerve of rats with experimentally induced glaucoma*. Neurosci Lett. 2008 Nov; 446(1):20-4.
4. Sacchetti M, Lambiase A, **Coassin M**, Bonini S, Bonini S. *Toxic corneal ulcer: a frequent and sight-threatening disease*. Eur J Ophthalmol. 2009 Nov-Dec;19(6): 916-22.
5. Lambiase A, **Coassin M**, Tirassa P, Mantelli F, Aloe L. *Nerve growth factor eye drops improve visual acuity and electrofunctional activity in age-related macular degeneration: a case report*. Ann Ist Super Sanita. 2009;45(4):439-42.
6. Mattson MS, Huynh J, Wiseman ME, **Coassin M**, Kornfield JA, Schwartz D. *An in vitro intact globe expansion method for evaluation of cross-linking treatments*. Invest Ophthalmol Vis Sci. 2010 Jun;51(6):3120-8.
7. **Coassin M**, Duncan K, Bailey K, Singh A, Schwartz DM. *Hypothermia reduces the release of vascular endothelial growth factor by retinal pigment epithelium*. Br J Ophthalmol. 2010 Dec;94(12):1678-83.
8. Colafrancesco V, Parisi V, Sposato V, Rossi S, Russo MA, **Coassin M**, Lambiase A, Aloe L. *Ocular application of Nerve growth factor protects degenerating retinal ganglion cells in a rat model of glaucoma*. J Glaucoma. 2011 Feb;20:100-8.
9. Hu J, **Coassin M**, Stewart JM. *Fluocinolone Acetonide Implant (Retisert) for Chronic Cystoid Macular Edema in Two Patients with AIDS and Cytomegalovirus Retinitis*. Ocular Immunology & Inflammation 2011. In Press.
10. McFadden S, **Coassin M**, Mattson M, Howlett M, Kornfield J, Schwartz DM. *Scleral Cross-Linking Inhibits Ocular Elongation and Induces an Alternative Response to Form-Deprivation Myopia in the Guinea Pig Eye*. Invest Ophthalmol Vis Sci 2011. Submitted.
11. Colafrancesco F, **Coassin M**, Parisi V, Rossi S, Aloe L. *Nerve growth factor reduces retinal ganglion cells degeneration in diabetic rats*. Diabetologica 2011. Submitted.
12. **Coassin M**, Altamirano JC, Santos A. *The effect of intravitreal bevacizumab on the fellow untreated eye in exudative Age-related macular degeneration*. Arch Ophthalmol 2011. Submitted.
13. **Coassin M**, Ebrahimi KB, O'Brien JM, Stewart JM. *OCT imaging for retinal metastasis with unknown primary tumor*. Retina 2011. Submitted.

