# Bilateral inflammation after unilateral UVR-B irradiation

## Ocular cross-talk and immunomodulation between exposed and unexposed, contralateral eye

Doctoral thesis

to obtain a doctorate (PhD)

from the Faculty of Medicine

of the University of Bonn

**Janine Marx, born Groß**

Lahnstein, Germany

2021

Written with authorization of the Faculty of Medicine at the Rheinische Friedrich-Wilhelms-University of Bonn

First reviewer: PD Dr. rer. nat. Alfred R. Wegener

Second reviewer: Prof. Dr. med. Eicke Latz, MD PhD

Day of oral examination: 18, December 2020

For the clinic and policlinic for Ophthalmology

Director: Univ.-Prof. Dr. med. Frank G. Holz

# Table of Contents

## List of Abbreviations

| | |
|---|---|
| Ac | Anterior chamber |
| AEC | 3-amino-9-ethylcarbazole |
| AMD | Age-related macular degeneration |
| AqH | Aqueous humor |
| CNV | Corneal neovascularization |
| Cp | Ciliary processes |
| DAPI | 4´, 6-Diamidin-2-phenylindol |
| Ed | Endothelium |
| ELISA | Enzyme-linked immunosorbent assay |
| Ep | Epithelium |
| GCL | Ganglion cell layer |
| HSK | Herpetic stromal keratitis |
| IHC | Immunohistochemistry |
| IL | Interleukin |
| INL | Inner nuclear layer |
| IOD | Integrated optical density |
| IPL | Inner plexiform layer |
| MCP-1 | Monocyte Chemoattractant Protein-1 |
| mRNA | Messenger RNA |
| $MTD_{2.3:16}$ | Maximum tolerable dose |
| NaCl | Natrium chloride |
| NKR-1 | Neurokinin receptor 1 |
| ONL | Outer nuclear layer |
| OPL | Outer plexiform layer |
| Pc | Posterior chamber |
| RC | Rods and cones |
| RLB | Retinal laser burn |
| Sp | Sphincter muscle |
| SP | Substance P |
| Str | Stroma |

| | |
|---|---|
| TNF-α | Tumour necrosis factor α |
| UVR-B | Ultraviolet radiation type B |
| VEGF | Vascular endothelial growth factor |

# 1. Summary

## 1.1 Brief summary

The presented study investigates the influence of exposure of one eye to UVR-B on the unexposed, partner eye in vivo in a mouse model. Furthermore, we try to answer the question whether this sympathizing reaction of the partner eye is transmitted via a neurokinin-dependent signaling pathway of substance P (SP) and its neurokinin-1 receptor (NKR-1). In all experiments mice were unilaterally exposed in vivo to UVR-B. The unexposed eyes were completely shielded during exposure time. NKR-1 protein levels in ocular tissues were quantified by immunohistochemical staining and enzyme-linked immunosorbent assay (ELISA). In a second experiment, NKR-1 was blocked with NKR-1 antagonists. Ocular tissues were extracted for the detection of pro-inflammatory cytokine and chemokine expression by ProcartaPlex™ Multiplex Immunoassay. The UVR-B radiation induces the expression and upregulates NKR-1 in the exposed and unexposed, partner eye. Pretreatment with Fosaprepitant decreased NKR-1 expression in all exposed ocular tissues as well as in the unexposed lens epithelium. Overall, UVR-B exposure triggers NKR-1 upregulation not only in the exposed but also in the partner eye in various ocular tissues. In addition, the clinical approved NKR-1 antagonist Fosaprepitant effectively reduces NKR-1 expression in both mouse eyes.

## 1.2 Introduction and purpose

Sunlight consists of radiant energy, including wavelengths of UVR-B which reaches the eyes directly (Rosenthal *et al.*, 1985; Young, 2006). Acute high-dose exposure to UVR-B has an impact on the physiology of the eye and induces inflammation in the anterior segment (Chen *et al.*, 2011; Meyer *et al.*, 2013; Newkirk *et al.*, 2007; Norval, 2011; Sliney, 2002). Ocular implications of UVR-B have been studied in a wide spectrum of eye diseases, including ocular pathologies such as pterygium (Hill and Maske, 1989; Threlfall and English, 1999), photokeratitis (Cullen, 2002; Delic *et al.*, 2017) or cataract formation (Hightower, 1994; Ivanov *et al.*, 2018; Roberts, 2011; Zhang *et al.*, 2012).

In addition to its pro-inflammatory properties, UVR-B exposure is considered as the main environmental risk factor for cataract disease in humans and animals (Cruickshanks *et al.*, 1992; Dolin, 1994; Pascolini and Mariotti, 2012). Cataract is one of the most prevalent eye disease and still the leading cause of visual impairment worldwide (Delcourt *et al.*,

2000; Wu *et al.*, 2004). While cataract development is a common global health problem in aging populations with surgery as the only therapeutic treatment option (Delcourt *et al.*, 2000; Riaz *et al.*, 2006), it is essential to find novel strategies to prevent or slow down cataract development.

Inflammatory processes in the eye, induced by acute exposure to UVR-B often include an involvement of pro-inflammatory molecules, such as SP. The neuropeptide SP exerts its actions by interacting with neurokinin receptors, among which NKR-1 displays the preferential affinity for SP (Leal *et al.*, 2015; Mashaghi *et al.*, 2016; O'Connor *et al.*, 2004). The interaction of SP and NKR-1 have been involved in several inflammatory reactions in the eye, including apoptosis (Ou *et al.*, 2019; Wang *et al.*, 2019; Yoo *et al.*, 2017; Zhou *et al.*, 2008) and increased production of pro-inflammatory cytokines and chemokines (Foldenauer *et al.*, 2012; Lucas *et al.*, 2012; Sun *et al.*, 2008; Twardy *et al.*, 2011). Previous experimental studies have focused on the induction of pro-inflammatory cytokine and chemokine synthesis via the SP/NKR-1 signaling pathway, including the upregulation of interleukin (IL)-6 and TNF-α in the infected mouse cornea (Hazlett *et al.*, 2007; McClellan *et al.*, 2008; Twardy *et al.*, 2011). Additionally, treatment with NKR-1 antagonists significantly reduced the amount of pro-inflammatory cytokines and chemokines and improved disease outcome, by inhibition of NKR-1 signaling (Bignami *et al.*, 2014; Bignami *et al.*, 2017; Hazlett *et al.*, 2007; McClellan *et al.*, 2008; Twardy *et al.*, 2011). For example, intraperitoneal injection with the selective NKR-1 antagonist Spantide I resulted in a significant reduction of IL-1β mRNA (Messenger RNA) levels and protein levels in corneas infected with *Pseudomonas aeruginosa* (Hazlett *et al.*, 2007). Some reports indicated that NKR-1 antagonists decrease the inflammatory response in ocular diseases (Bignami *et al.*, 2014; Bignami *et al.*, 2017; Hazlett *et al.*, 2007; McClellan *et al.*, 2008; Twardy *et al.*, 2011). However, experimental evidence showing that SP receptor antagonists are capable of inhibiting inflammation in the eye by reducing pro-inflammatory molecules after acute high-dose exposure to UVR-B remained unknown so far.

In addition to the well-studied phototoxic effects of UVR-B exposure in the eye, such as apoptosis (Galichanin, 2017; Kronschläger *et al.*, 2015) and epithelial cell damage (Jiang *et al.*, 2014; Meyer *et al.*, 2014), there is growing evidence that UVR-B exposure is capable to induce inflammatory processes that also affects the contralateral, partner eye in a sympathizing reaction (Lucas *et al.*, 2012; Meyer *et al.*, 2013; Paunicka *et al.*, 2015). A

previous study found indications that UVR-B exposure to only one eye also affects the unexposed, partner eye in a sympathetic manner (Meyer *et al.*, 2013). This study showed a significant UVR-B dose-dependent increase in lens light scattering as well as an inflammatory infiltration with monocytes and macrophages also in the unexposed, partner eye (Meyer *et al.*, 2013). Furthermore, earlier reports demonstrated that damage to one eye induces NKR-1 expression and abrogation of the immune privilege in both eyes (Guzmán *et al.*, 2018; Lucas *et al.*, 2012; Mo *et al.*, 2017; Paunicka *et al.*, 2015). A study by Lucas and co-workers showed that unilateral retinal laser burns in a mouse model abrogated the immune privilege bilaterally (Lucas *et al.*, 2012). Immunohistological analysis showed a significant increase of NKR-1 in both the burned and contralateral retina after retinal laser burn to only one eye (Lucas *et al.*, 2012). Based on these findings, the present thesis hypothesized that the neuropeptide SP and its receptor NKR-1 might be the signaling molecules involved in the immunological ocular cross-talk to the partner eye following an acute insult from UVR-B exposure to one eye. The effect of unilateral UVR-B exposure was investigated on the expression and upregulation of NKR-1 in ocular tissues of the exposed and contralateral eyes. The current PhD thesis further examined whether treatment with NKR-1 antagonists (Spantide I and Fosaprepitant) reduces the inflammation in both eyes after unilateral UVR-B exposure. In addition, there was a suspicion that protein levels of NKR-1 and pro-inflammatory cytokines and chemokines decreased in both eyes. Detailed knowledge of the signaling pathway of SP and NKR-1 as well as an extensive understanding of NKR-1 antagonists, like the clinical approved NKR-1 antagonist Fosaprepitant could open new treatment options and clinical preventive strategies for bilateral eye diseases, including UVR-B-induced cataract formation.

## 1.3 Material and methods

### 1.3.1 Experimental design and UVR-B exposure

Experiments were performed with six-week-old C57Bl/6 mice (Charles River Laboratories) maintained in the laboratory at the Department of Ophthalmology, University of Bonn. For all experiments, mice were anesthetized with a mixture of ketamine and xylazine injected intraperitoneally (i.p.) (Gross *et al.*, 2018; Gross *et al.*, 2019; Gross *et al.*, 2020) followed by a topical administration of tropicamide eye drops in both eyes to induce mydriasis. Prior to UVR-B exposure mice were examined with a slit lamp (Coherent) to exclude congenital

cataract or other ocular lesions. Afterwards, one eye of each mouse was exposed in vivo to UVR-B radiation with a defined wavelength peak at 312 nm using a microprocessor-controlled UV irradiation system (Vilber Lourmat©). The unexposed eye was completely shielded with aluminium foil during exposure time. Radiation exposure is expressed as cataract threshold dose equivalents (maximum tolerable dose – $MTD_{2.3:16}$) for UVR-B and was defined in detail by Söderberg and co-workers (Söderberg *et al.*, 2002). One MTD equivalent complies to 2.9 kJ/m² in the pigmented mouse(Meyer *et al.*, 2007). Following the first experiments with the UVR-B dose of 2.9 kJ/m² no suitable NKR-1 upregulation in ocular tissues was found. Hence, for all continuing experiments the UVR-B dose was increased to 14.5 kJ/m².

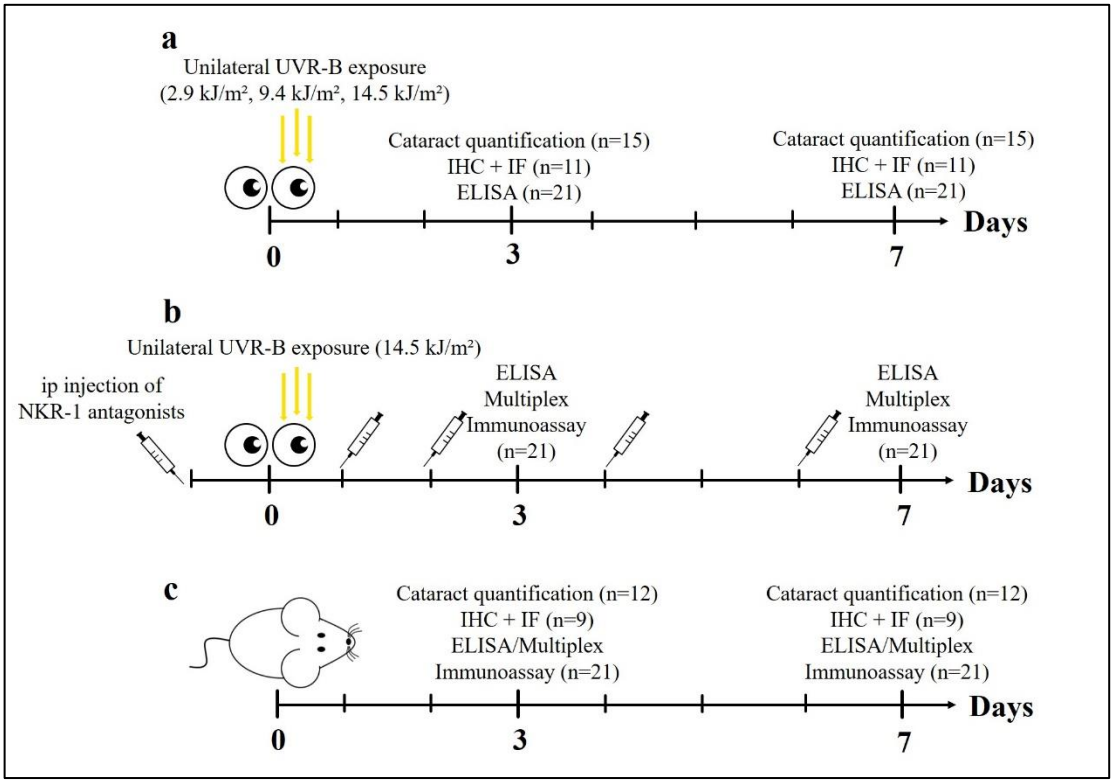


**Figure 1:** Experimental setup. Mice were divided into different experimental groups: (a) Unilateral UVR-B exposure to different intensities (2.9 kJ/m², 9.4 kJ/m², 14.5 kJ/m²), (b) unilateral UVR-B exposure with pretreatment (Fosaprepitant, Spantide I, saline) and (c) control animals.

### 1.3.2 Drug administration

Mice were randomly assigned into different experimental groups, including **(a)** unilateral UVR-B exposure with various irradiation intensities (2.9 kJ/m², 9.4 kJ/m², 14.5 kJ/m²), **(b)** unilateral UVR-B exposure (14.5 kJ/m²) with pretreatment (Fosaprepitant: 10 mg/kg, MSD Merck Sharp & Dohme Ltd.; Spantide I: 72 µg/mouse, Bachem; saline) and **(c)** control animals received only anesthesia but no UVR-B exposure and no pretreatment (Fig. 1). The volume per day amounts to 0.2 ml/mouse of NKR-1 antagonists and saline. After predefined time points of 3 and 7 days following UVR-B exposure, animals of all experiments were sacrificed, eyes enucleated and prepared for immunohistochemistry or molecular biological methods. The NKR-1 antagonists, Fosaprepitant and Spantide I, were used for inhibiting of the SP/NKR-1 signaling pathway in mice which obtained unilateral UVR-B exposure (14.5 kJ/m²). Mice received repeated i.p. injections of Fosaprepitant, Spantide I or saline on day -1, 0 (day of UVR-B exposure), 1 and 2 for a latency period time of 3 days post-exposure, as well as on day -1, 0, 1, 2, 4 and 6 for a time period of 7 days post-exposure (Fig. 1).

### 1.3.3 Cataract morphology and cataract quantification

The lens was microsurgical extracted by careful dissection. Cataract morphology was documented and visualized in light- and dark field illumination photography (Leica Microsystems GmbH). Cataract morphology was quantified by measuring the integrated optical density (IOD) of the lens photographs as a quantification for experimentally induced cataract. IOD measurements were taken with Image J/FIJI 1.46, based on a standardized protocol with fixed illumination settings.

### 1.3.4 Immunohistochemistry

For immunohistochemical staining, eyes were embedded in paraffin and sections were cut, dewaxed and rehydrated. Thereafter, sections were incubated overnight with a polyclonal anti-SP receptor antibody. The following day, slides were washed before incubating with a secondary antibody. Counter-staining of the nuclei was performed with hematoxylin for immunohistochemical AEC (3-amino-9-ethylcarbazole) staining and DAPI (4´,6-Diamidin-2-phenylindol) for immunofluorescence staining respectively. Specificity of staining was confirmed with an isotype control (Chemicon) prepared under the same conditions.

NKR-1 expression was quantified as immunofluorescence in stained ocular tissues by IOD.

### 1.3.5 ELISA and ProcartaPlex™ Multiplex Immunoassay

NKR-1 protein levels following the different treatments was quantified using a SP receptor ELISA kit (Cusabio Biotech). To investigate if the pro-inflammatory chemokine MCP-1/CCL2 is affected by UVR-B exposure in the exposed or in the partner eye ELISA analysis (R&D systems) was conducted after unilateral UVR-B exposure. In all experiments (Fig.1), ocular tissues including aqueous humor (AqH), cornea, lens epithelium, iris/ciliary body, retina and choroid were analyzed on day 3 or 7 following unilateral UVR-B exposure (14.5 kJ/m$^2$). Ocular tissue samples were homogenized in phosphate-buffered solution containing protease inhibitor and centrifuged. Furthermore, to examine whether pro-inflammatory cytokines and chemokines (IL-α, IL-6, IL10, IL-12p70, MCP-1/CCL2, VEGF-A, TNF-α, Gro-α/CXCL1) have an effect on unilateral UVR-B exposure in mice treated with Fosaprepitant, a ProcartaPlex™ Multiplex Immunoassay (Thermo Fisher Scientific) was performed.

### 1.3.6 Statistical analysis

All data were analyzed with SPSS Statistics 23. One-way analysis of variance (ANOVA) with post-hoc comparison was used to evaluate cataract quantification, NKR-1 immunoreactivity, NKR-1 and MCP-1 protein levels as well as cytokine and chemokine protein concentration. A paired sample t-test was used to quantify differences between unexposed and exposed eyes within latency period groups or treatment groups. P value ≤ 0.05 was considered as statistically significant.

## 1.4 Results

### 1.4.1 Cataract morphology and cataract quantification

All lenses irradiated with UVR-B exposure developed an anterior subcapsular cataract, while fine granular opacities were observed throughout the anterior surface of the lens at day 3 post-exposure. At day 7 following UVR-B exposure, a dense triangular shaped cataract developed in the center of the exposed lens. Control lenses which received no UVR-B exposure remained clear. IOD for the examination of cataract intensity showed signifi-

cant increased IOD levels in exposed lenses at day 3 ($P \leq 0.001$) and 7 ($P \leq 0.001$) post-exposure, compared to the controls. The unexposed, contralateral lenses were mostly transparent as seen through the microscope. However, in one mouse a slight clouding were also detected in the contralateral lens.

### 1.4.2 Analysis of NKR-1 immunostaining

In vivo UVR-B irradiation induced NKR-1 expression and upregulation in ocular tissues not only in the exposed but also in the unexposed, partner eye. After unilateral UVR-B exposure, exposed and contralateral eyes displayed a strong NKR-1 immunostaining in the corneal epithelium and endothelium, in the lens epithelium and nuclear bow region of the lens at the membrane level, in nerve fibers at the end of the sphincter muscle in the iris, in the pigmented epithelium of the ciliary processes as well as in the inner plexiform layer (IPL) and in the inner nuclear layer (INL) of the retina in cross sections (Gross *et al.*, 2018; Gross *et al.*, 2019). Control eyes which received no UVR-B exposure showed a mild staining for NKR-1 in all ocular tissues, but the staining was less prevalent than in the exposed and contralateral eyes. Morphologically, the corneal epithelium and endothelium of the exposed eye was damaged with a loss of cell layers after UVR-B exposure. Irradiated eyes were partially damaged in the lens epithelium and a multilayered accumulation of cells in the disrupted lens epithelium. The contralateral and control cornea and lens were regularly structured with no structural changes. The remaining ocular tissues showed no morphological alterations after unilateral UVR-B exposure. NKR-1 quantification as IOD showed a significant upregulation in the exposed cornea (3+7 days: $P = 0.009$), nuclear bow region of the lens (3 days: $P = 0.024$; 7 days: $P = 0.002$) and retina (3 days: $P = 0.021$; 7 days: $P = 0.002$) at day 3 and 7 post-exposure compared to control. The unexposed eye demonstrated a significant increase of NKR-1 immunoreactivity in the cornea (3 days: $P = 0.019$; 7 days: $P = 0.033$), nuclear bow region of the lens (3 days: $P = 0.006$; 7 days: $P = 0.002$) and iris (3 days: $P = 0.012$) after UVR-B exposure compared to the control group.

### 1.4.3 Enzyme linked immunosorbent assay

ELISA analysis was performed to quantify NKR-1 protein expression in ocular tissues of exposed, unexposed and control mouse eyes following unilateral UVR-B exposure. In ad-

dition, ELISA assay was also implemented to examine NKR-1 expression in mice pre-treated with the NKR-1 antagonists, Fosaprepitant and Spantide I. NKR-1 ELISA of untreated mouse eyes showed a significant post-exposure upregulation of NKR-1 levels in the exposed cornea (3 days: $P = 0.006$; 7 days: $P \leq 0.001$), iris/ciliary body (3 days: $P \leq 0.001$; 7 days: $P \leq 0.001$) and choroid (3 days: $P = 0.024$; 7 days: $P = 0.008$), when compared to the control group (Gross *et al.*, 2019). Significantly higher NKR-1 protein levels were also observed in the unexposed iris/ciliary body (3 days: $P = 0.034$), retina (7 days: $P = 0.05$) and choroid (7 days: $P = 0.030$) of untreated mouse eyes after UVR-B exposure to only one eye.

In mice treated with the NKR-1 antagonist Fosaprepitant, a significant reduction of NKR-1 protein levels was examined in the exposed cornea (3 days: $P = 0.004$; 7 days: $P = 0.006$), lens epithelium (3 days: $P = 0.001$; 7 days: $P = 0.034$), AqH (7 days: $P = 0.028$), iris/ciliary body ( 3 days: $P = 0.035$) as well as in the retina (3 days: $P = 0.035$; 7 days: $P = 0.011$) and choroid (7 days: $P = 0.005$) in comparison to the saline-treated group (Gross *et al.*, 2020). In the unexposed lens epithelium (3 days: $P = 0.028$; 7 days: $P = 0.034$) a significant reduction of NKR-1 was also observed in the Fosaprepitant group compared to the saline group. By contrast, in the exposed cornea (3 days: $P = 0.002$; 7 days: $P = 0.044$), lens epithelium (3 days: $P \leq 0.000$), retina (7 days: $P = 0.001$) and choroid (3 days: $P = 0.022$) as well as in the unexposed cornea (3 days: $P = 0.002$) and lens epithelium (3 days: $P = 0.018$; 7 days: $P = 0.010$), a significant increase of NKR-1 protein levels were displayed in mice treated with Spantide I compared to mice treated with Fosaprepitant. UVR-B exposure induced a significant MCP-1 elevation in the exposed cornea ($P \leq 0.001$), iris/ciliary body ($P = 0.014$) as well as in the AqH ($P = 0.004$) at day 3 post-exposure compared to the control group (Gross *et al.*, 2019). MCP-1 protein levels of the unexposed partner eyes revealed no significant differences, when compared to the control group.

### 1.4.4 ProcartaPlex™ Multiplex Immunoassay

In exposed corneas VEGF-A ($P = 0.001$) and Gro-α/CXCL1 ($P \leq 0.001$) protein levels were increased significantly in mice treated with saline, compared to the unexposed control group (Gross *et al.*, 2020). In the unexposed lens epithelium, a significant upregulation of VEGF-A expression ($P = 0.040$) was observed in mice treated with saline, compared to

the control group which received no UVR-B exposure. In Fosaprepitant treated mice, the level of Gro-α/CXCL1 (P = 0.001) of the exposed cornea was statistically reduced compared to the saline group. Interestingly to note, mice treated with Spantide I showed a strong upregulation of various pro-inflammatory cytokines and chemokines, compared to the saline or Fosaprepitant group.

## 1.5 Discussion

### 1.5.1 NKR-1 immunostaining and protein expression in ocular tissues of the mouse eye

In the current PhD thesis, UVR-B exposure to only one eye induced an intraocular inflammatory reaction in both the exposed and the unexposed, partner eye in vivo. These results are similar to the investigations of a previous study by Meyer and coworkers, finding indications that after unilateral UVR-B irradiation also the contralateral eye might react in a sympathetic manner (Meyer *et al.*, 2013). The present study demonstrated an UVR-B-induced expression and upregulation of the SP receptor, NKR-1 in ocular tissues of the exposed and unexposed, partner eye. In addition, NKR-1 upregulation not only in the exposed but also in the unexposed eye was effectively reduced by the selective and protective NKR-1 antagonist Fosaprepitant after unilateral UVR-B exposure. Thus, the clinical approved NKR-1 antagonist Fosaprepitant is capable to inhibit the sympathizing pro-inflammatory signaling to the contralateral eye in the established UVR-B cataract mouse model. The neuropeptide SP has a short half-life time in tissues and is thus experimentally very difficult to measure. Since SP persists in tissues only seconds to tens of minutes (Mashaghi *et al.*, 2016; McGregor and Bloom, 1983; Skidgel *et al.*, 1984) the SP receptor, NKR-1 was measured to indirectly quantify SP.

In the current study, NKR-1 expression was present in ocular tissues of the naïve, exposed and unexposed of untreated mouse eyes after unilateral exposure to UVR-B. The PhD thesis demonstrate for the first time that NKR-1 is located in the lens epithelium and nuclear bow region of the lens of mouse eyes after UVR-B exposure to only one eye. These results implicated that additionally to a direct phototoxic effect of UVR-B, an inflammatory reaction, possibly regulated by inflammatory molecules in the AqH, is involved in UVR-B-induced cataract formation. A contralateral upregulation of NKR-1 in several ocular tissues of the unexposed, partner eye was observed after high-dose UVR-B exposure to only one

eye, suggesting NKR-1 as a potential key player in the communication between both eyes. One possible explanation for the sympathizing reaction after unilateral exposure to UVR-B might be the transmission of signals by pro-inflammatory molecules such as SP from the exposed to the unexposed eye. A conceivable signaling pathway for the neuropeptide SP could be via the optic nerve or through the adjacent tissue. A sympathizing contralateral response to the partner eye through the SP signaling pathway is described by a bilateral loss of immune privilege after severing corneal nerves of one eye that occurs during corneal graft surgery in a mouse model (Lucas *et al.*, 2012; Paunicka *et al.*, 2015). The authors showed that the unilateral severing of corneal nerves induced SP secretion in both eyes, including a disability of T regulatory cells that are essential for allograft survival.

The expression and upregulation of SP, and its receptor NKR-1 has been described in several inflammatory eye diseases, such as herpes stromal keratitis (Gaddipati *et al.*, 2016; Twardy *et al.*, 2011; Yun *et al.*, 2016), pterygium (Chui *et al.*, 2007), corneal neovascularization (CNV) (Bignami *et al.*, 2014; Marco *et al.*, 2018) and proliferative vitreoretinopathy (Lorenz *et al.*, 2008; Troger *et al.*, 1998). Moreover, experimental studies have revealed a correlation between SP and NKR-1 in UVR-B-induced inflammation in the skin (Eschenfelder *et al.*, 1995; Legat *et al.*, 2002). An experimental study demonstrated significant higher concentrations of SP in the skin of rats irradiated with repeated sub-inflammatory doses of UVR-B, suggesting that UVR-B irradiation increases SP locally (Legat *et al.*, 2002). These results are also in accordance with a report indicating a significant higher concentration of SP in psoriasis patients after UVR-B therapy (Narbutt *et al.*, 2013). Although the presence of SP was documented in skin diseases after irradiation to UVR-B, the link between SP/NKR-1 and UVR-B in eye diseases is not known.

### 1.5.2 Treatment with NKR-1 antagonists, Fosaprepitant and Spantide I

In the present study, the effect of two NKR-1 antagonists, Fosaprepitant and Spantide I, were evaluated in the established UVR-B-induced cataract mouse model.

Fosaprepitant is a water-soluble prodrug of Aprepitant, a selective antagonist of NKR-1 (Muñoz *et al.*, 2017; Robinson *et al.*, 2008). The prodrug is rapidly converted to Aprepitant via the action of phosphatase enzymes (Navari, 2007, 2008; Van Belle and Cocquyt, 2008). Aprepitant is clinically approved for the prevention of chemotherapy-induced or

post-operative nausea and vomiting (Bayati *et al.*, 2016; Pojawa-Gołąb *et al.*, 2019). It is well known that Aprepitant crosses the blood-brain barrier and has anti-inflammatory properties (Bignami *et al.*, 2017; Lönndahl *et al.*, 2018; Muñoz *et al.*, 2017; Robinson *et al.*, 2008). Aprepitant was also shown to exert a broad-spectrum antitumor activity implicating a potent growth inhibition on human neuroblastoma or glioma (Berger *et al.*, 2014; Harford-Wright *et al.*, 2014; Muñoz *et al.*, 2010).

In the current thesis, ELISA investigations of Fosaprepitant-treated mice showed a significant decrease of NKR-1 protein levels in all examined exposed ocular tissues as well as in the unexposed, contralateral lens epithelium after unilateral UVR-B exposure, compared to the saline group. These results suggest that SP and its receptor play an essential role in signaling the inflammatory insult to the unexposed, contralateral eye. In accordance to the present results, an earlier study demonstrated that topical administration of Fosaprepitant significantly reduced corneal inflammation in a CNV mouse model (Bignami *et al.*, 2017). These results are in concordance to the presented findings, indicating an alleviated inflammation on the ocular surface by Fosaprepitant, suggesting a protective effect of Fosaprepitant in the cornea. The selective high-affinity NKR-1 antagonist Aprepitant and its prodrug Fosaprepitant effectively reduce inflammation in various diseases (Bignami *et al.*, 2017; Lönndahl *et al.*, 2018; Martinez *et al.*, 2017; Robinson *et al.*, 2008; Robinson *et al.*, 2016). However, a link among Fosaprepitant and the immunological connection between both eyes in sympathizing ocular diseases must be established. The current study demonstrated for the first time that Fos-aprepitant inhibits NKR-1 expression effectively in the eye. Thus, Fosaprepitant is clinically capable to inhibit the sympathizing pro-inflammatory signaling to the contralateral eye in the present UVR-B-induced cataract mouse model. Aprepitant and Fosaprepitant are generally well tolerated and no significant toxic side effects have been described for the NKR-1 antagonists (Bignami *et al.*, 2017; Dando and Perry, 2004; Kramer *et al.*, 1998). Consistent with these reports, in the current study Fosaprepitant was well tolerated by the animals and no visible side effect was observed. The positive characteristics of Aprepitant and Fosaprepitant make this drug interesting for other ocular diseases such as age-related macular degeneration (AMD) and CNV. Furthermore, the clinical approved SP receptor antagonists Aprepitant and Fosaprepitant are directly applicable and could offer a broad application spectrum for new ther-

apeutic and preventive treatment options for various eye diseases, such as cataract formation.

The blocking of NKR-1 with Spantide I demonstrated in the present study a significant reduction of NKR-1 expression only in the cornea. This finding is in line with previous studies, indicating a protective effect of Spantide I in the cornea after induced impairment (Hazlett *et al.*, 2007; Twardy *et al.*, 2011; Zhou *et al.*, 2008). The effect of inhibition SP/NKR-1 signal transduction by Spantide is supported by a study demonstrating decreased perforated cornea and bacterial counts in mice infected with *Pseudomonas aeruginosa* (Hazlett *et al.*, 2007). In the current study, ELISA analysis showed a tendency of an NKR-1 overexpression after Spantide I administration in various ocular tissues of both eyes. Interestingly, in a preliminary experiment a massive opacity on the cornea was determined after topical administration of Spantide I within a few minutes. Confirming the pro-inflammatory feature of Spantide I, treatment with Spantide I repeatedly resulted in a strong overexpression of various pro-inflammatory cytokines and chemokines. These observations, together with the known drawbacks of Spantide I (e.g. its high neurotoxicity) (Quartara and Maggi, 1997; Rosso *et al.*, 2012) suggest that Spantide I is no effective NKR-1 antagonist in the presented UVR-B-induced cataract model but rather has a toxic effect. Altogether, these findings are of importance for the elucidation of the molecular mechanism of SP/NKR-1 in sympathizing eye diseases and support the contention that the clinical approved NKR-1 antagonist Fosaprepitant constitutes a selective and effective antagonist to inhibit UVR-B-induced inflammation in the mouse eye.

### 1.5.3 Pro-inflammatory cytokine and chemokine expression in ocular tissues

The present study investigated whether unilateral UVR-B exposure induced an upregulation of pro-inflammatory cytokines and chemokines in exposed and unexposed ocular tissues after two different time periods. SP/NKR-1 interaction modulates the secretion of pro-inflammatory molecules (Fiebich *et al.*, 2000; Mashaghi *et al.*, 2016), thus we postulated a reduction of pro-inflammatory cytokines and chemokines after administration of Fosaprepitant.

ELISA analysis of the current study showed an upregulation of MCP-1 expression in the exposed cornea, iris/ciliary body and AqH after 3 days post UVR-B exposure. Interestingly, MCP-1 levels were decreased in exposed mouse eyes on day 7 post-exposure. The

finding suggests that MCP-1 levels increase fast and are not involved in UVR-B-induced inflammation after a time period of 7 days post-exposure. This observation is congruent with an earlier study by Wolf and co-workers (Wolf *et al.*, 2019) showing that elevated MCP-1 expression in injured mouse corneas returned to near basal levels within a few days after injury. Previous reports are in accordance with the present results and demonstrated links between elevated MCP-1 level in the cornea and aqueous in various ocular diseases, including cataract (Aketa *et al.*, 2017; Kawai *et al.*, 2012; Sauer *et al.*, 2016), CNV (Mukwaya *et al.*, 2019; Nakano *et al.*, 2018; Wolf *et al.*, 2019) and uveitis (Bauer *et al.*, 2019; Pei *et al.*, 2019; Tang *et al.*, 2018). The increase in MCP-1 in exposed eyes might be indicative of a pro-inflammatory ocular environment. It is well known that MCP-1 is secreted by a wide variety of cells that mediate the cell influx to inflammation, such as monocytes and macrophages (Conrady *et al.*, 2013; Kim *et al.*, 2017; Melgarejo *et al.*, 2009; Zhu *et al.*, 2015). MCP-1 expression in ocular tissues of the contralateral eyes were not increased, suggesting that MCP-1 is not involved in the immunological cross-talk between both eyes.

In the current thesis, a UVR-B-induced increase of Gro-α/CXCL1 and VEGF-A expression in the exposed cornea as well as a significant increase of TNF-α in the exposed lens epithelium was determined in the saline group. These results are in accordance with experimental studies which indicated an upregulation of pro-inflammatory cytokines and chemokines in the cornea (Chen *et al.*, 2014; Foldenauer *et al.*, 2012; McClellan *et al.*, 2008; Twardy *et al.*, 2011). Interestingly, a significant reduction of Gro-α/CXCL1 in the cornea after treatment with Fosaprepitant was also observed. This finding is in agreement with experimental and clinical studies, showing a significant reduction of pro-inflammatory molecules after administration of the clinical approved NKR-1 antagonist, Aprepitant (Barrett *et al.*, 2016; Liu *et al.*, 2019; Martinez *et al.*, 2017; Spitsin *et al.*, 2017). A few reports implying an increased expression of pro-inflammatory molecules after induced inflammation (Chen *et al.*, 2014; Foldenauer *et al.*, 2012; McClellan *et al.*, 2008; Twardy *et al.*, 2011) as well as effective inhibition by NKR-1 antagonists (Barrett *et al.*, 2016; Liu *et al.*, 2019; Martinez *et al.*, 2017; Spitsin *et al.*, 2017). However, the present study is the first report that demonstrated an upregulation of pro-inflammatory cytokines in the cornea and lens epithelium after exposure to UVR-B. These results represent the first investigations that Fosaprepitant inhibited the upregulated Gro-α/CXCL1 protein concentration in the

cornea, assuming that Fosaprepitant is a potent NKR-1 antagonist with a protective effect. The positive properties of Aprepitant and Fosaprepitant, such as no toxic side effects (Berger *et al.*, 2014; Bignami *et al.*, 2017; Robinson *et al.*, 2008) makes this drug interesting for many inflammatory eye diseases.

### 1.5.4 Conclusion

In conclusion, unilateral exposure to UVR-B triggers the expression and upregulation of NKR-1 in various ocular tissues of the exposed and unexposed partner eye. In addition, the clinical approved NKR-1 antagonist Fosaprepitant reduces the inflammation in UVR-B exposed ocular tissues, including a significant decrease of NKR-1 protein levels in exposed and partner eyes. SP and its receptor, NKR-1 play a major role in the signaling pathway to the partner eye in UVR-B-induced inflammation after exposure of only one eye to UVR-B. Therefore, SP and NKR-1 might be candidate molecules in other sympathizing eye diseases, like sympathetic ophthalmitis. Most age-related eye diseases, such as AMD, are of bilateral nature. Thus, SP and NKR-1 and their antagonist Fosaprepitant might play an important role for new treatment targets and strategies in various inflammatory ocular diseases. Further experiments may enable an overall better understanding of pathophysiological changes as well as redefined pharmaceutical interventions in the eye.

## 1.6 References

Aketa N, Yamaguchi T, Asato T, Yagi-Yaguchi Y, Suzuki T, Higa K, Kurihara T, Satake Y, Tsubota K, Shimazaki J. Elevated Aqueous Cytokine Levels in Eyes With Ocular Surface Diseases. Am J Ophthalmol 2017; 184: 42–51

Barrett JS, Spitsin S, Moorthy G, Barrett K, Baker K, Lackner A, Tulic F, Winters A, Evans DL, Douglas SD. Pharmacologic rationale for the NK1R antagonist, aprepitant as adjunctive therapy in HIV. J Transl Med 2016; 14: 148

Bauer D, Kasper M, Walscheid K, Koch JM, Müther PS, Kirchhof B, Heiligenhaus A, Heinz C. Alteration of MCP-1 and MMP-9 in aqueous humor is associated with secondary glaucoma in Fuchs uveitis syndrome. Ocul Immunol Inflamm 2019: 1–11

Bayati S, Bashash D, Ahmadian S, Safaroghli-Azar A, Alimoghaddam K, Ghavamzadeh A, Ghaffari SH. Inhibition of tachykinin NK1 receptor using aprepitant induces apoptotic cell death and G1 arrest through Akt/p53 axis in pre-B acute lymphoblastic leukemia cells. Eur J Pharmacol 2016; 791: 274–283

Berger M, Neth O, Ilmer M, Garnier A, Salinas-Martín MV, Agustín Asencio JC de, Schweinitz D von, Kappler R, Muñoz M. Hepatoblastoma cells express truncated neurokinin-1 receptor and can be growth inhibited by aprepitant in vitro and in vivo. J Hepatol 2014; 60: 985–994

Bignami F, Giacomini C, Lorusso A, Aramini A, Rama P, Ferrari G. NK1 receptor antagonists as a new treatment for corneal neovascularization. Invest Ophthalmol Vis Sci 2014; 55: 6783–6794

Bignami F, Lorusso A, Rama P, Ferrari G. Growth inhibition of formed corneal neovascularization following Fosaprepitant treatment. Acta Ophthalmol 2017; 95: e641-e648

Chen B-Y, Lin DP-C, Wu C-Y, Teng M-C, Sun C-Y, Tsai Y-T, Su K-C, Wang S-R, Chang H-H. Dietary zerumbone prevents mouse cornea from UVB-induced photokeratitis through inhibition of NF-κB, iNOS, and TNF-α expression and reduction of MDA accumulation. Mol Vis 2011; 17: 854–863

Chen W, Lin H, Zhong X, Liu Z, Geng Y, Xie C, Chen W. Discrepant expression of cytokines in inflammation- and age-related cataract patients. PLoS ONE 2014; 9: 1-7

Chui J, Di Girolamo N, Coroneo MT, Wakefield D. The role of substance P in the pathogenesis of pterygia. Invest Ophthalmol Vis Sci 2007; 48: 4482–4489

Conrady CD, Zheng M, Mandal NA, van Rooijen N, Carr DJJ. IFN-α-driven CCL2 production recruits inflammatory monocytes to infection site in mice. Mucosal Immunol 2013; 6: 45–55

Cruickshanks KJ, Klein BE, Klein R. Ultraviolet light exposure and lens opacities: the Beaver Dam Eye Study. Am J Public Health 1992; 82: 1658–1662

Cullen AP. Photokeratitis and other phototoxic effects on the cornea and conjunctiva. Int J Toxicol 2002; 21: 455–464

Dando TM, Perry CM. Aprepitant: a review of its use in the prevention of chemotherapy-induced nausea and vomiting. Drugs 2004; 64: 777–794

Delcourt C, Carrière I, Ponton-Sanchez A, Lacroux A, Covacho MJ, Papoz L. Light exposure and the risk of cortical, nuclear, and posterior subcapsular cataracts: the Pathologies Oculaires Liées à l'Age (POLA) study. Arch Ophthalmol 2000; 118: 385–392

Delic NC, Lyons JG, Di Girolamo N, Halliday GM. Damaging Effects of Ultraviolet Radiation on the Cornea. Photochem Photobiol 2017; 93: 920–929

Dolin PJ. Ultraviolet radiation and cataract: a review of the epidemiological evidence. Br J Ophthalmol 1994; 78: 478–482

Eschenfelder CC, Benrath J, Zimmermann M, Gillardon F. Involvement of substance P in ultraviolet irradiation-induced inflammation in rat skin. Eur J Neurosci 1995; 7: 1520–1526

Fiebich BL, Schleicher S, Butcher RD, Craig A, Lieb K. The neuropeptide substance P activates p38 mitogen-activated protein kinase resulting in IL-6 expression independently from NF-kappa B. J Immunol 2000; 165: 5606–5611

Foldenauer MEB, McClellan SA, Barrett RP, Zhang Y, Hazlett LD. Substance P affects growth factors in Pseudomonas aeruginosa-infected mouse cornea. Cornea 2012; 31: 1176–1188

Gaddipati S, Rao P, Jerome AD, Burugula BB, Gerard NP, Suvas S. Loss of Neurokinin-1 Receptor Alters Ocular Surface Homeostasis and Promotes an Early Development of Herpes Stromal Keratitis. J Immunol 2016; 197: 4021–4033

Galichanin K. Exposure to subthreshold dose of UVR-B induces apoptosis in the lens epithelial cells and does not in the lens cortical fibre cells. Acta Ophthalmol 2017; 95: 834–838

Gross J, Wegener AR, Kronschlaeger M, Holz FG, Schönfeld C-L, Meyer LM. Ultraviolet radiation exposure triggers neurokinin-1 receptor upregulation in ocular tissues in vivo. Exp Eye Res 2018; 174: 70-79

Gross J, Wegener AR, Kronschläger M, Schönfeld C-L, Holz FG, Meyer LM. UVR-B-in duced NKR-1 Expression in Ocular Tissues is blocked by Substance P Receptor Antagonist Fosaprepitant in the Exposed as well as Unexposed Partner Eye. Ocul Immunol Inflamm 2020: 1–13

Gross J, Willimsky E, Wegener AR, Kronschläger M, Schönfeld C-L, Holz FG, Meyer LM. Ultraviolet Radiation Exposure of One Eye Stimulates Sympathizing Expression of Neurokinin-1 Receptor but Not Monocyte Chemoattractant Protein-1 in the Partner Eye. Ophthalmic Res 2019: 1–13

Guzmán M, Miglio MS, Zgajnar NR, Colado A, Almejún MB, Keitelman IA, Sabbione F, Fuentes F, Trevani AS, Giordano MN et al. The mucosal surfaces of both eyes are immunologically linked by a neurogenic inflammatory reflex involving TRPV1 and substance P. Mucosal Immunol 2018; 11: 1441–1453

Harford-Wright E, Lewis KM, Ghabriel MN, Vink R. Treatment with the NK1 antagonist emend reduces blood brain barrier dysfunction and edema formation in an experimental model of brain tumors. PLoS ONE 2014; 9: 1-7

Hazlett LD, McClellan SA, Barrett RP, Liu J, Zhang Y, Lighvani S. Spantide I decreases type I cytokines, enhances IL-10, and reduces corneal perforation in susceptible mice after Pseudomonas aeruginosa infection. Invest Ophthalmol Vis Sci 2007; 48: 797–807

Hightower KR. A review of the evidence that ultraviolet irradiation is a risk factor in cataractogenesis. Doc Ophthalmol 1994; 88: 205–220

Hill JC, Maske R. Pathogenesis of pterygium. Eye (Lond) 1989; 3 (Pt 2): 218–226

Ivanov IV, Mappes T, Schaupp P, Lappe C, Wahl S. Ultraviolet radiation oxidative stress affects eye health. J Biophotonics 2018; 11: e201700377

Jiang X, McClellan SA, Barrett R, Foldenauer M, Hazlett LD. HGF signaling impacts severity of Pseudomonas aeruginosa keratitis. Invest Ophthalmol Vis Sci 2014; 55: 2180–2190

Kawai M, Inoue T, Inatani M, Tsuboi N, Shobayashi K, Matsukawa A, Yoshida A, Tanihara H. Elevated levels of monocyte chemoattractant protein-1 in the aqueous humor after phacoemulsification. Invest Ophthalmol Vis Sci 2012; 53: 7951–7960

Kim JW, Jeong H, Yang M-S, Lim CW, Kim B. Therapeutic effects of zerumbone in an alkali-burned corneal wound healing model. Int Immunopharmacol 2017; 48: 126–134

Kramer MS, Cutler N, Feighner J, Shrivastava R, Carman J, Sramek JJ, Reines SA, Liu G, Snavely D, Wyatt-Knowles E et al. Distinct mechanism for antidepressant activity by blockade of central substance P receptors. Science 1998; 281: 1640–1645

Kronschläger M, Talebizadeh N, Yu Z, Meyer LM, Löfgren S. Apoptosis in Rat Cornea After In Vivo Exposure to Ultraviolet Radiation at 300 nm. Cornea 2015; 34: 945–949

Leal EC, Carvalho E, Tellechea A, Kafanas A, Tecilazich F, Kearney C, Kuchibhotla S, Auster ME, Kokkotou E, Mooney DJ et al. Substance P promotes wound healing in diabetes by modulating inflammation and macrophage phenotype. Am J Pathol 2015; 185: 1638–1648

Legat FJ, Griesbacher T, Schicho R, Althuber P, Schuligoi R, Kerl H, Wolf P. Repeated subinflammatory ultraviolet B irradiation increases substance P and calcitonin gene-related peptide content and augments mustard oil-induced neurogenic inflammation in the skin of rats. Neurosci Lett 2002; 329: 309–313

Liu X, Zhu Y, Zheng W, Qian T, Wang H, Hou X. Antagonism of NK-1R using aprepitant suppresses inflammatory response in rheumatoid arthritis fibroblast-like synoviocytes. Artif Cells Nanomed Biotechnol 2019; 47: 1628–1634

Lönndahl L, Holst M, Bradley M, Killasli H, Heilborn J, Hall MA, Theodorsson E, Holmberg J, Nordlind K. Substance P Antagonist Aprepitant Shows no Additive Effect Compared with Standardized Topical Treatment Alone in Patients with Atopic Dermatitis. Acta Derm Venereol 2018; 98: 324–328

Lorenz K, Troger J, Fischer-Colbrie R, Kremser B, Schmid E, Kralinger M, Teuchner B, Bechrakis N, Kieselbach G. Substance P and secretoneurin in vitreous aspirates of patients with various vitreoretinal diseases. Peptides 2008; 29: 1561–1565

Lucas K, Karamichos D, Mathew R, Zieske JD, Stein-Streilein J. Retinal laser burn-induced neuropathy leads to substance P-dependent loss of ocular immune privilege. The Journal of Immunology 2012;189:1237–1242

Marco B, Alessandro R, Philippe F, Fabio B, Paolo R, Giulio F. The Effect of Aging on Nerve Morphology and Substance P Expression in Mouse and Human Corneas. Invest Ophthalmol Vis Sci 2018; 59: 5329–5335

Martinez AN, Burmeister AR, Ramesh G, Doyle-Meyers L, Marriott I, Philipp MT. Aprepitant limits in vivo neuroinflammatory responses in a rhesus model of Lyme neuroborreliosis. J Neuroinflammation 2017; 14: 1-9

Mashaghi A, Marmalidou A, Tehrani M, Grace PM, Pothoulakis C, Dana R. Neuropeptide substance P and the immune response. Cell Mol Life Sci 2016; 73: 4249–4264

McClellan SA, Zhang Y, Barrett RP, Hazlett LD. Substance P promotes susceptibility to Pseudomonas aeruginosa keratitis in resistant mice: anti-inflammatory mediators downregulated. Invest Ophthalmol Vis Sci 2008; 49: 1502–1511

McGregor GP, Bloom SR. Radioimmunoassay of substance P and its stability in tissue. Life Sci 1983; 32: 655–662

Melgarejo E, Medina MA, Sánchez-Jiménez F, Urdiales JL. Monocyte chemoattractant protein-1: a key mediator in inflammatory processes. Int J Biochem Cell Biol 2009; 41: 998–1001

Meyer LM, Dong X, Wegener A, Söderberg P. Light scattering in the C57BL/6 mouse lens. Acta Ophthalmol Scand 2007; 85: 178–182

Meyer LM, Löfgren S, Holz FG, Wegener A, Söderberg P. Bilateral cataract induced by unilateral UVR-B exposure - evidence for an inflammatory response. Acta Ophthalmol 2013; 91: 236–242

Meyer LM, Wegener AR, Holz FG, Kronschläger M, Bergmanson JP, Soderberg PG. Ultrastructure of UVR-B-induced cataract and repair visualized with electron microscopy. Acta Ophthalmol 2014; 92: 635–643

Mo J, Neelam S, Mellon J, Brown JR, Niederkorn JY. Effect of Corneal Nerve Ablation on Immune Tolerance Induced by Corneal Allografts, Oral Immunization, or Anterior Chamber Injection of Antigens. Invest Ophthalmol Vis Sci 2017; 58: 137–148

Mukwaya A, Mirabelli P, Lennikov A, Thangavelu M, Jensen L, Peebo B, Lagali N. Repeat Corneal Neovascularization is Characterized by More Aggressive Inflammation

and Vessel Invasion Than in the Initial Phase. Invest Ophthalmol Vis Sci 2019; 60: 2990–3001

Muñoz M, Rosso M, Coveñas R. A new frontier in the treatment of cancer: NK-1 receptor antagonists. Curr Med Chem 2010; 17: 504–516

Muñoz M, Rosso M, Coveñas R. The NK-1 receptor antagonist L-732,138 induces apoptosis in human gastrointestinal cancer cell lines. Pharmacol Rep 2017; 69: 696–701

Nakano Y, Uchiyama M, Arima T, Nagasaka S, Igarashi T, Shimizu A, Takahashi H. PPARα Agonist Suppresses Inflammation after Corneal Alkali Burn by Suppressing Proinflammatory Cytokines, MCP-1, and Nuclear Translocation of NF-κB. Molecules 2018; 24: 1-12

Narbutt J, Olejniczak I, Sobolewska-Sztychny D, Sysa-Jedrzejowska A, Słowik-Kwiatkowska I, Hawro T, Lesiak A. Narrow band ultraviolet B irradiations cause alteration in interleukin-31 serum level in psoriatic patients. Arch Dermatol Res 2013; 305: 191–195

Navari RM. Fosaprepitant (MK-0517): a neurokinin-1 receptor antagonist for the prevention of chemotherapy-induced nausea and vomiting. Expert Opin Investig Drugs 2007; 16:1977–1985

Navari RM. Fosaprepitant: a neurokinin-1 receptor antagonist for the prevention of chemotherapy-induced nausea and vomiting. Expert Rev Anticancer Ther 2008; 8: 1733–1742

Newkirk KM, Chandler HL, Parent AE, Young DC, Colitz CMH, Wilkie DA, Kusewitt DF. Ultraviolet radiation-induced corneal degeneration in 129 mice. Toxicol Pathol 2007; 35: 819–826

Norval M. The mechanisms and consequences of ultraviolet-induced immunosuppression in the skin and eye. Eye Contact Lens 2011; 37: 176–184

O'Connor TM, O'Connell J, O'Brien DI, Goode T, Bredin CP, Shanahan F. The role of substance P in inflammatory disease. J Cell Physiol 2004; 201: 167–180

Ou K, Mertsch S, Theodoropoulou S, Wu J, Liu J, Copland DA, Schrader S, Liu L, Dick AD. Restoring retinal neurovascular health via substance P. Exp Cell Res 2019; 380: 115–123

Pascolini D, Mariotti SP. Global estimates of visual impairment: 2010. Br J Ophthalmol 2012; 96: 614–618

Paunicka KJ, Mellon J, Robertson D, Petroll M, Brown JR, Niederkorn JY. Severing corneal nerves in one eye induces sympathetic loss of immune privilege and promotes rejection of future corneal allografts placed in either eye. Am J Transplant 2015; 15: 1490–1501

Pei M, Liu X, Zhao C, Gao F, Tao Y, Zhang M. Chemokine and Adhesion Molecule Profiles in Aqueous Humor of Clinically Quiescent Uveitic Cataracts. Curr Eye Res 2019; 44: 194–199

Pojawa-Gołąb M, Jaworecka K, Reich A. NK-1 Receptor Antagonists and Pruritus: Review of Current Literature. Dermatol Ther (Heidelb) 2019; 9: 391–405

Quartara L, Maggi CA. The tachykinin NK1 receptor. Part I: ligands and mechanisms of cellular activation. Neuropeptides 1997; 31: 537–563

Riaz Y, Mehta JS, Wormald R, Evans JR, Foster A, Ravilla T, Snellingen T. Surgical interventions for age-related cataract. Cochrane Database Syst Rev 2006; 4: 1-45

Roberts JE. Ultraviolet radiation as a risk factor for cataract and macular degeneration. Eye Contact Lens 2011; 37: 246–249

Robinson P, Kasembeli M, Bharadwaj U, Engineer N, Eckols KT, Tweardy DJ. Substance P Receptor Signaling Mediates Doxorubicin-Induced Cardiomyocyte Apoptosis and Triple-Negative Breast Cancer Chemoresistance. Biomed Res Int 2016; 2016: 1-9

Robinson P, Martin P, Garza A, D'Souza M, Mastrangelo M-A, Tweardy D. Substance P receptor antagonism for treatment of cryptosporidiosis in immunosuppressed mice. J Parasitol 2008; 94: 1150–1154

Rosenthal FS, Safran M, Taylor HR. The ocular dose of ultraviolet radiation from sunlight exposure. Photochem Photobiol 1985; 42: 163–171

Rosso M, Muñoz M, Berger M. The role of neurokinin-1 receptor in the microenvironment of inflammation and cancer. Scientific World Journal 2012; 2012: 1-21

Sauer A, Bourcier T, Gaucher D, Candolfi E, Speeg-Schatz C. Intraocular cytokines imbalance in congenital cataract and its impact on posterior capsule opacification. Graefes Arch Clin Exp Ophthalmol 2016; 254: 1013–1018

Skidgel RA, Engelbrecht S, Johnson AR, Erdös EG. Hydrolysis of substance p and neurotensin by converting enzyme and neutral endopeptidase. Peptides 1984; 5: 769–776

Sliney DH. How light reaches the eye and its components. Int J Toxicol 2002; 21: 501–509

Söderberg PG, Löfgren S, Ayala M, Dong X, Kakar M, Mody V. Toxicity of ultraviolet radiation exposure to the lens expressed by maximum tolerable dose. Dev Ophthalmol 2002; 35: 70–75

Spitsin S, Meshki J, Winters A, Tuluc F, Benton TD, Douglas SD. Substance P-mediated chemokine production promotes monocyte migration. J Leukoc Biol 2017; 101: 967–973

Sun J, Ramnath RD, Zhi L, Tamizhselvi R, Bhatia M. Substance P enhances NF-kappaB transactivation and chemokine response in murine macrophages via ERK1/2 and p38 MAPK signaling pathways. Am J Physiol Cell Physiol 2008; 294: 1586-1596

Tang W, Ma J, Gu R, Ding X, Lei B, Wang X, Zhuang H, Xu G. Lipocalin 2 Suppresses Ocular Inflammation by Inhibiting the Activation of NF-κβ Pathway in Endotoxin-Induced Uveitis. Cell Physiol Biochem 2018; 46: 375–388

Threlfall TJ, English DR. Sun exposure and pterygium of the eye: a dose-response curve. Am J Ophthalmol 1999; 128: 280–287

Troger J, Kremser B, Irschick E, Göttinger W, Kieselbach G. Substance P in proliferative vitreoretinopathy: the significance of aqueous humor levels for evolution of the disease. Graefes Arch Clin Exp Ophthalmol 1998; 236: 900–903

Twardy BS, Channappanavar R, Suvas S. Substance P in the corneal stroma regulates the severity of herpetic stromal keratitis lesions. Invest Ophthalmol Vis Sci 2011; 52: 8604–8613

Van Belle SJ, Cocquyt V. Fosaprepitant dimeglumine (MK-0517 or L-785,298), an intravenous neurokinin-1 antagonist for the prevention of chemotherapy induced nausea and vomiting. Expert Opin Pharmacother 2008; 9: 3261–3270

Wang P, Me R, Yuan Y, Yu Y, Li M, Ke B. Substance P inhibits high urea-induced apoptosis through the AKT/GSK-3β pathway in human corneal epithelial cells. J Cell Biochem 2019; 120: 11342-11349

Wolf M, Clay SM, Zheng S, Pan P, Chan MF. MMP12 Inhibits Corneal Neovascularization and Inflammation through Regulation of CCL2. Sci Rep 2019; 9: 11579-11592

Wu K, Kojima M, Shui YB, Sasaki H, Sasaki K. Ultraviolet B-induced corneal and lens damage in guinea pigs on low-ascorbic acid diet. Ophthalmic Res 2004; 36: 277–283

Yoo K, Son BK, Kim S, Son Y, Yu S-Y, Hong HS. Substance P prevents development of proliferative vitreoretinopathy in mice by modulating TNF-α. Mol Vis 2017; 23: 933–943

Young AR. Acute effects of UVR on human eyes and skin. Prog Biophys Mol Biol 2006; 92: 80–85

Yun H, Lathrop KL, Hendricks RL. A Central Role for Sympathetic Nerves in Herpes Stromal Keratitis in Mice. Invest Ophthalmol Vis Sci 2016; 57: 1749–1756

Zhang J, Yan H, Löfgren S, Tian X, Lou MF. Ultraviolet radiation-induced cataract in mice: the effect of age and the potential biochemical mechanism. Invest Ophthalmol Vis Sci 2012; 53: 7276–7285

Zhou Z, Barrett RP, McClellan SA, Zhang Y, Szliter EA, van Rooijen N, Hazlett LD. Substance P delays apoptosis, enhancing keratitis after Pseudomonas aeruginosa infection. Invest Ophthalmol Vis Sci 2008; 49: 4458–4467

Zhu X-J, Wolff D, Zhang K-K, He W-W, Sun X-H, Lu Y, Zhou P. Molecular Inflammation in the Contralateral Eye After Cataract Surgery in the First Eye. Invest Ophthalmol Vis Sci 2015; 56: 5566-5573

# 2. Ultraviolet radiation exposure triggers neurokinin-1 receptor upregulation in ocular tissues *in vivo*

Janine Gross[a], Alfred R. Wegener[a], Martin Kronschläger[b], Frank G. Holz[a], Carl-Ludwig Schönfeld[c,d], Linda M. Meyer[a,c]

[a] Department of Ophthalmology, University of Bonn, Bonn, Germany
[b] Department of Ophthalmology, Hanusch Hospital, Vienna, Austria
[c] Herzog Carl Theodor Eye Clinic, Munich, Germany
[d] Department of Ophthalmology, Ludwig-Maximilians University, Munich, Germany

Experimental Eye Research
Volume 174, September 2018, Pages 70-79
DOI: 10.1016/j.exer.2018.04.016

## 2.1 Abstract

**Purpose:** The purpose of this study was to investigate the neurokinin receptor-1 (NKR-1) protein expression in ocular tissues before and after supra-cataract threshold ultraviolet radiation (UVR-B peak at 312 nm) exposure *in vivo* in a mouse model.

**Methods:** Six-week-old C57Bl/6 mice were unilaterally exposed to a single (2.9 kJ/$m^2$) and an above 3-fold UVR-B cataract threshold dose (9.4 kJ/$m^2$) of UVR. UVR-exposure (λpeak = 312 nm) was performed in mydriasis using a Bio-Spectra exposure system. After latency periods of 3 and 7 days, eyes were fixed in 4 % paraformaldehyde, embedded in paraffin, sectioned and stained with fluorescence coupled antibody for NKR-1 and DAPI for cell nuclei staining. Control animals received only anesthesia but no UVR-exposure. Cataract development was documented with a Leica dark-field microscope and quantified as integrated optical density (IOD).

**Results:** NKR-1 is ubiquitously present in ocular tissues. An above 3-fold cataract threshold dose of UV-radiation induced NKR-1 upregulation after days 3 and 7 in the epithelium and endothelium of the cornea, the endothelial cells of the iris vessels, the pigmented epithelium/stroma of the ciliary body, the lens epithelium, pronounced in the nuclear bow region and the inner plexiform layer of the retina. A significant upregulation of NKR-1 could not be provoked with a single cataract threshold dose (2.9 kJ/$m^2$ UVR-B) ultraviolet irradiation. All exposed eyes developed anterior subcapsular cataracts.

**Conclusion:** Neurokinin-1 receptor is present ubiquitously in ocular tissues including the lens epithelium and the nuclear bow region of the lens. UV-radiation exposure to an above 3-fold UVR-B cataract threshold dose triggers NKR-1 upregulation in the eye *in vivo*. The involvement of inflammation in ultraviolet radiation induced cataract and the role of neuroinflammatory peptides such as substance P and its receptor, NKR-1, might have been underestimated to date.


**Keywords:** Cataract, Ultraviolet radiation B, Substance P, Neurokinin receptor 1, Inflammation

## 2.2 Introduction

In the eye, pro-inflammatory molecules, such as neuropeptides and cytokines regulate inflammatory responses in multiple diseases. Until now, the role of inflammation in the development of UVR-B induced lens opacification, or cataract, is unclear.

Cataract is defined as the opacification of the lens, which impairs the passage of light to the retina. It is still the major cause of blindness in the world (Brian and Taylor, 2001; World Health Organization, 2010; Wu et al., 2013) and there are so far no therapeutic strategies to prevent cataract formation. Apart from other risk factors such as age, trauma, diabetes or the use of corticosteroids, exposure to ultraviolet radiation type B (UVR-B) is considered to be the main risk factor for cataract formation in humans and animals (Delcourt et al., 2000; Hejtmancik, 1998). UV irradiation causes lens epithelial cell damage and disrupts the physiological functions of the lens. It can lead to apoptosis via UV-induced phototoxic DNA damage (Jiang et al., 2015). Furthermore, protein denaturation, protein insolubility of normally water soluble crystallins and inflammatory changes of lens cells due to the activation of pro-inflammatory mediators are attributed to UVB induced lens damage (Hejtmancik and Kantorow, 2004).

Several experimental and epidemiological studies have shown that UVR-B exposure induces cataract development in humans and animals (Cruickshanks et al., 1992; Hightower, 1995; Ji et al., 2015; Jose and Pitts, 1985; Merriam et al., 2000; Pitts et al., 1977; Taylor, 1989; Wu et al., 2013). Recently, an experimental study in rats demonstrated UVR-B dependent corneal damage and found a significant increase of pro-inflammatory cytokines, TNF-α and VEGF in the cornea (Chen et al., 2016). Another correlation to inflammation and cataract disease is the inflammatory response to unilateral UVR-B irradiation in conjunction with an increased IL-1β production in a mouse model (Meyer et al., 2013).

So far it is known that ocular inflammatory reactions to different lesions include the expression and upregulation of several inflammatory chemokines and cytokines, such as substance P (SP), which mediates diverse pathways involved in immune cell proliferation (Castagliuolo et al., 2000; Koon et al., 2004; Mei et al., 2013), cytokine production (Mashaghi et al., 2016), apoptosis (Muñoz et al., 2008; Zhou et al., 2008) and inflammation (O'Connor et al., 2004; Park et al., 2016; Weinstock et al., 2003). SP is a member of the tachykinin family and consists of 11 amino acids (Chang et al., 1971). It is established that SP is encoded by the TAC1 gene and secreted by both, non-neuronal cell types like

epithelial cells, immune cells and specific neuronal cells (Bignami et al., 2014; Hong et al., 2015; O'Connor et al., 2004). The cellular actions of SP are mediated by the neurokinin 1 receptor (NKR-1). The G-protein coupled receptor, NKR-1 exhibits the highest affinity for SP (Monastyrskaya et al., 2005). Previous studies demonstrated the presence of NKR-1 and SP in ocular tissues of different species (Casini et al., 2000, 2002; Catalani et al., 2004; Oyamada et al., 1999): NKR-1 has been localized in rabbit (Casini et al., 2002), rat (Casini et al., 2000; Oyamada et al., 1999) and mouse (Catalani et al., 2004) bipolar and amacrine cells of the retina using immunohistochemical techniques. Furthermore, the role of SP in ocular inflammation has been investigated in different corneal disease models. For example, SP was present in the corneal stroma of mouse eyes in a HSK (herpetic stromal keratitis) lesion model (Twardy et al., 2011). Furthermore, Bignami et al. (2014) showed an increase of SP expression in two different mouse models that verify SP upregulation in the epithelial layer of the infected cornea (Bignami et al., 2014). However, the role of SP and its receptor NKR-1 in association with UV-radiation with peak wavelength at 312 nm and cataract disease is unknown. Here we investigated whether *in vivo* UVR exposure has an effect on the localization and expression of the SP receptor NKR-1 in various ocular tissues, including the lens in a mouse model.

## 2.3 Materials and methods

### 2.3.1 Animals

Six-week-old C57BL/6 mice were obtained from Charles River Laboratories (Germany) and housed in the laboratory at the Department of Ophthalmology, University of Bonn (accession number: 84–02.04.2015.A154). All animals were kept and treated according to the Association of Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

### 2.3.2 Immunohistochemical procedure for NKR-1

Exposed eyes were embedded in paraffin. Sections were cut, dewaxed and rehydrated. For immunohistochemical AEC staining, endogenous peroxidase was blocked using 3 % $H_2O_2$. Thereafter, sections were heated in citrate buffer and incubated overnight with a rabbit polyclonal anti-substance P receptor antibody (1:800, AB5060, Chemicon). Following washing, the slides were incubated with a polyclonal swine anti-rabbit secondary an-

tibody (1:200, E0431, DAKO). AEC (3-amino-9-ethylcarbazole) was applied to enhance the staining of horseradish peroxidase reaction. Counter-staining of the nuclei was performed with hematoxylin. For immunofluorescence staining sections were incubated with the primary anti-substance P receptor antibody. Sections were washed before adding anti-rabbit secondary antibody conjugated to Alexa Fluor® 488 (1:200, Thermo Fisher Scientific). Nuclei were counterstained with DAPI. Specificity of staining was confirmed using equal concentrations of an isotype control. Isotype controls were prepared under same immunohistochemically conditions replacing the primary anti-substance P receptor antibody with an isotype-specific immunoglobulin (NI01, Chemicon, rabbit, polyclonal).

### 2.3.3 Quantification of NKR-1 expression

NKR-1 expression was quantified as immunofluorescence in stained ocular tissues as the integrated optical density (IOD) in IHC samples of exposed, and control eyes (Lucas et al., 2012). Cataract morphology was also quantified by measuring the IOD of the lens photographs. Measurements were analyzed with Image J/FIJI 1.46, based on a standardized protocol with fixed illumination settings.

### 2.3.4 UVR exposure

UV- irradiation was performed in mydriasis using a Bio-Spectra system (Vilber Lourmat©, Marne-La Vallee, France). UVR was generated by 40 W illumination tubes providing a uniform irradiation area at an energy peak at 312 nm in an area of 900×80 mm. The spectral UVR curve of the Vilber Lourmat© tubes includes mainly UVR-B (280–315 nm) with a defined wavelength peak at 312 nm.

A small fraction of UVR-A in a wavelength region from 315 nm to 370 nm is also emitted by the Bio-Spectra system (Fig. 1).

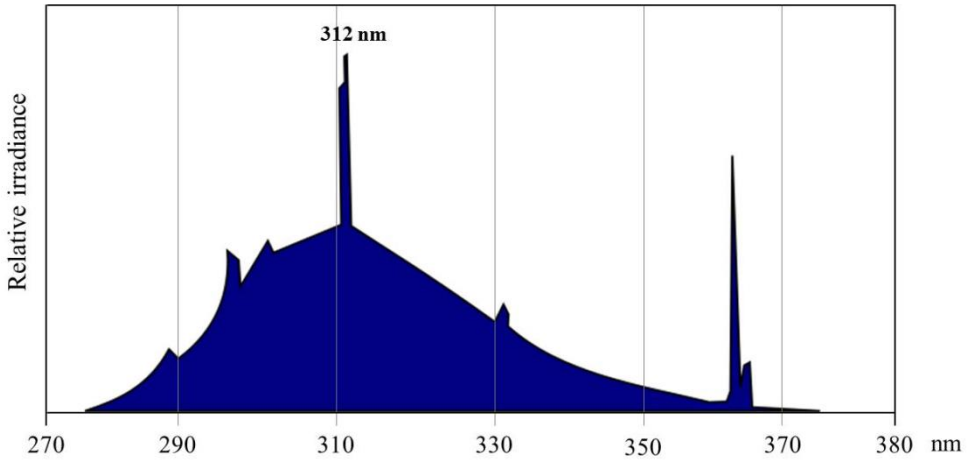


**Figure 1:** Spectral curve of the Vilber Lourmat© UVR tube (312 nm). The UVR spectrum incorporates λ = 280-370 nm (UVR-B 280-315 nm and UVR-A 315-380 nm) with a defined wavelength peak at 312 nm.

Radiation exposure is expressed as cataract threshold dose equivalents (maximum tolerable dose – $MTD_{2.3:16}$) for UVR (312 nm). The $MTD_{2.3:16}$ concept was defined in detail by Soederberg et al. (2002). One MTD in the pigmented mouse equals 2.9 kJ/m$^2$ (Meyer et al., 2007). The comparability to the Soederberg irradiation system is given by adjusting the Bio Spectra system to an energy dose of 9.4 kJ/m$^2$. Mice were unilaterally exposed *in vivo* to a low-dose, single cataract threshold dose of 2.9 kJ/m$^2$ (in the following "low-dose group") and an above 3-fold cataract threshold dose (9.4 kJ/m$^2$) of UVR (in the following "high-dose group"). The exposure time was 3 min. To exclude congenital cataract all animals were examined with a slit lamp prior to UVR exposure (peak at 312 nm). Mice were anaesthetized with a mixture of 40 mg/kg ketamine and 5 mg/kg xylazine injected intraperitoneally and 1 % tropicamide was instilled into both eyes to induce pupil dilation. The non-exposed eye was shielded with a specially designed aluminum foil during exposure. The mice were sacrificed after 3 and 7 days following UVR exposure. Eyes were enucleated and prepared for immunohistochemical staining. To document cataract morphology, lenses were extracted microsurgically and visualized in light and dark field illumination photography.

### 2.3.5 Experimental design

A total of 74 C57BL/6 mice were subdivided into two latency groups (3 and 7 days post UVR irradiation). For immunohistochemical analysis 22 animals were included in each

latency period group for both UVR dose groups. Cataract morphology was analyzed in 15 animals per group. One eye of each animal was exposed to UVR single and above the 3-fold cataract threshold equivalent dose ($MTD_{2.3:16}$). Control mice (n=30) received no UV-radiation. Six mice were excluded from the experiment due to pre-existing cataract and/or dissecting problems.

### 2.3.6 Statistical analysis

The data was analyzed with SPSS Statistics 23. One way ANOVA was performed to analyze the difference in NKR-1 level following UVR exposure. The ANOVA data (F-test) is summarized in Tables 1 and 2. If significant differences were detected according to ANOVA, a post hoc test (Least significant difference: LSD) were performed to indicate the difference in NKR-1 level of the groups. To quantify differences between cataract threshold dose groups of each latency periods, paired sample t-test were used. Regarding sample size the significance levels were set to 0.05 and the confidence coefficients to 0.95, respectively.

## 2.4 Results

### 2.4.1 NKR-1 immunohistochemistry and NKR-1 upregulation

An above 3-fold cataract threshold dose of UV-radiation induced NKR-1 expression and upregulation at day 3 and 7 in the following tissues of the exposed mouse eyes: corneal epithelium and endothelium, endothelial cells of the iris vessels, epithelium and stroma of the ciliary body, the anterior lens epithelium including the nuclear bow region of the lens, and the strongly marked inner plexiform layer as well as the inner nuclear layer of the retina (Figs. 2–9). A low UVR dose of 2.9 $kJ/m^2$ induced no measurable NKR-1 upregulation at day 3 and 7 following exposure in the above mentioned ocular tissues (Figs. 3, 5, 7 and 9). NKR-1 immunostained photomicrographs of these animals were comparable to the control group (data not shown).

*Cornea*

Positive NKR-1 immunostaining was observed in the corneal epithelium and endothelium of the high-dose exposed eyes (Fig. 2 A + D). The corneal epithelium showed a decrease in cell layers and the endothelium showed a loss of endothelial cells 3 days post-exposure.

The corneal stroma displayed no specific immunostaining for the SP receptor. Control mice showed a mild staining for NKR-1 in the regularly structured corneal epithelium (Fig. 2 B + E), but less than in the high dose exposed eyes. The negative control is demonstrated in Fig. 2 C and F. Fig. 3 summarizes the quantification of NKR-1 immunolabeling as IOD of the low-dose (2.9 kJ/m$^2$) and a high-dose group (9.4 kJ/m$^2$) in the cornea of control and exposed eyes. Three and seven days following high-dose UV-radiation, exposed eyes had a significantly increased IOD for NKR-1 compared to the control group ($P = 0.009$) and the low-dose UV-R group ($P = 0.001$; $P < 0.001$). Corneas in the low-dose group had a tendency to reduced IOD over the period studied compared to the control group, but the difference was not statistically significant.

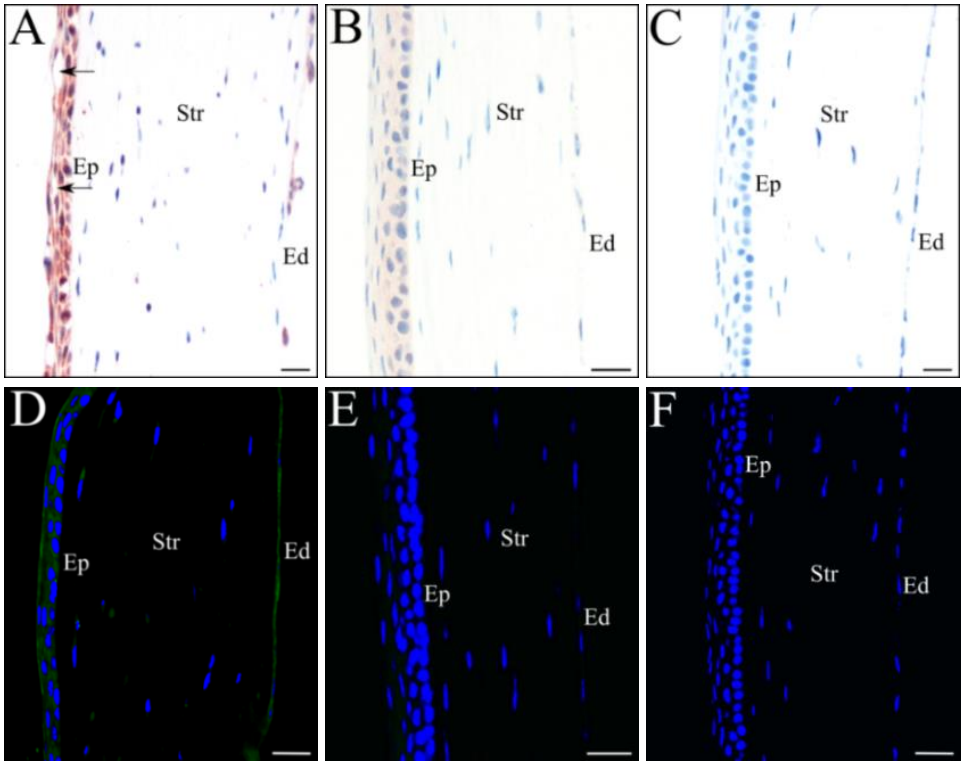


**Figure 2:** NKR-1 immunostaining and NKR-1 fluorescence staining of the exposed and control mouse cornea. Three days post high-dose UVR irradiation, the corneal epithelium is damaged with irregular epithelial cells. NKR-1 is upregulated in the epithelium and endothelium of the exposed cornea **(A, D)**. Control corneas showed a mild epithelial NKR-1 staining in the AEC staining **(B)** but no immunofluorescence staining **(E)**. Low-dose UVR exposed corneas showed mild epithelial damage but no increased immunofluorescent staining compared to control sections (data not shown). Negative controls showed no immunofluorescence staining **(C+F)**. Ep = epithelium, Str = stroma, Ed = endothelium. Red/Green: Substance P receptor (NKR-1). Blue: H&E/DAPI (nuclei staining). Scale bars = 25 µm.

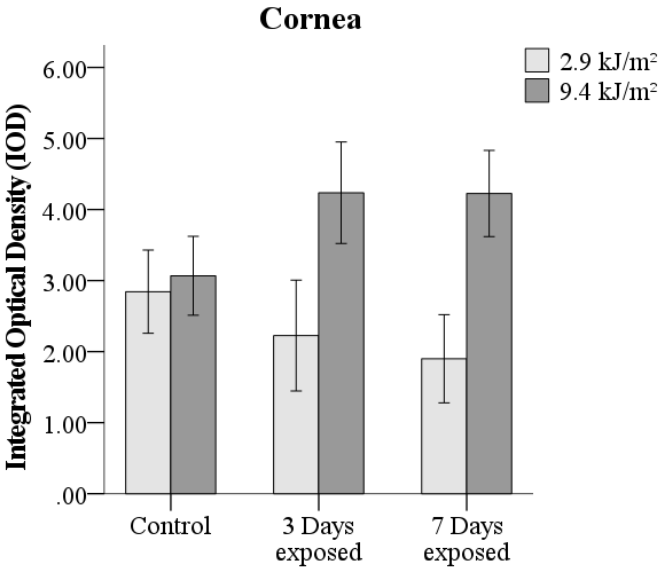


**Figure 3:** NKR-1 quantification of the mouse cornea. Quantification of NKR-1 immunolabeling in the cornea expressed as integrated optical density (IOD) after low-dose, single (2.9 kJ/m²) and an above 3-fold (9.4 kJ/m²) cataract threshold dose of UVR. The cornea revealed a statistically significant increase of NKR-1 after 3 and 7 days post UVR exposure (9.4 kJ/m²), compared to control eyes. Differences are significant between cataract threshold dose groups 3 and 7 days following UVR exposure (**P < 0.01; ***P < 0.001). Bar is 95 % CI for mean.

*Iris and Ciliary body*

NKR-1 immunostained nerve endings were detected at the margin of the iris sphincter muscle of exposed eyes (Fig. 4 A + G). Most NKR-1 positive nerve fibers of exposed eyes were located at the end of the sphincter muscle. Muscle cell membranes also displayed a positive staining for NKR-1. Additionally, examinations with albino mice demonstrated immunoreactive nerve fibers in the stroma of the exposed iris and at the endothelium of iris vessels (Data not shown). In control eyes NKR-1 immunostained nerve endings were only detected sporadically at the margin of the sphincter muscle (Fig. 4 B + H). The NKR-1 quantification of the mouse iris displayed an increase 3 and 7 days after high-dose UVR exposure as compared to the low-dose and the control group (Fig. 5A). A significant difference was observed at day 3 ($P = 0.007$) as well as at day 7 ($P < 0.001$) post UVR exposure between high-dose and low-dose animals but not between high-dose and control eyes. Similar to other ocular tissues NKR-1 in the low-dose group decreased over time.

The pigmented epithelium of the ciliary processes of exposed eyes exhibited an intense NKR-1 immunostaining (Fig. 4 D + J). The control group demonstrated only a mild NKR-1 immunostaining in the pigmented epithelium of the ciliary body (Fig. 4 E + K). The un-pigmented epithelium of the ciliary body illustrated no specific NKR-1 immunoreactivity, neither in exposed eyes nor in the control group. The negative control of the iris and ciliary body is shown in Fig. 4 F + L. Three and seven days post high-dose exposure NKR-1 in the ciliary body showed a slight increase in exposed eyes, compared to the control group (Fig. 5B). Eyes exposed to low-dose UVR demonstrated a slight decrease of IOD over time as compared to the control group. However, a significant difference was found for the high-dose and the low-dose group at day 3 ($P = 0.002$) and day 7 ($P = 0.018$).

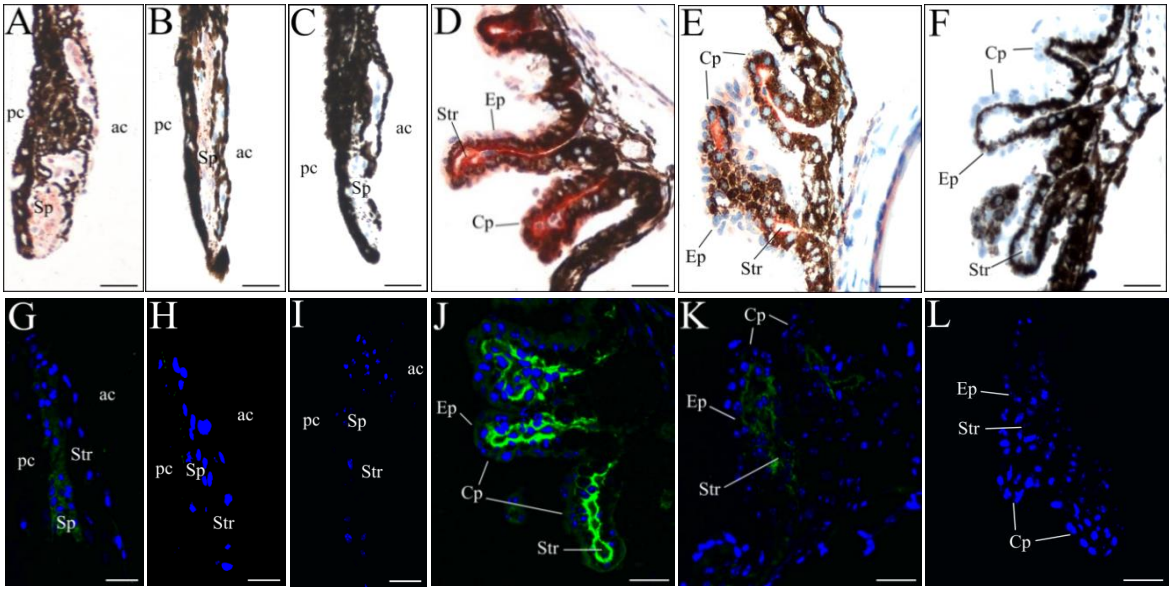


**Figure 4:** NKR-1 expression in the iris and in the ciliary body epithelium of exposed and control mouse eyes. NKR-1 immunoreactivity showed immunohistochemical AEC staining **(A-F)** and immunofluorescence staining **(G-L)** of the iris and ciliary body. Positive NKR-1 staining was detected in the sphincter muscle at day 3 after high-dose UVR **(A+G).** Control eyes showed mild NKR-1 staining in the sphincter muscle of the iris **(B+H).** After high-dose UVR exposure, the pigmented epithelium of the ciliary body displayed strong NKR-1 immunoreactivity **(D+J)**, whereas control eyes showed a weaker NKR-1 expression **(E+K)** similar to eyes exposed to low-dose UVR (data not shown). The negative controls of the fluorescence and AEC staining show no staining **(C+I, F+L)**. Ac = anterior chamber, pc = posterior chamber, Sp = sphincter muscle, Ep = epithelium, Str = stroma, Cp = ciliary processes. Red/Green: Substance P receptor (NKR-1). Blue: H&E/DAPI (nuclei staining). Scale bars = 25 µm.

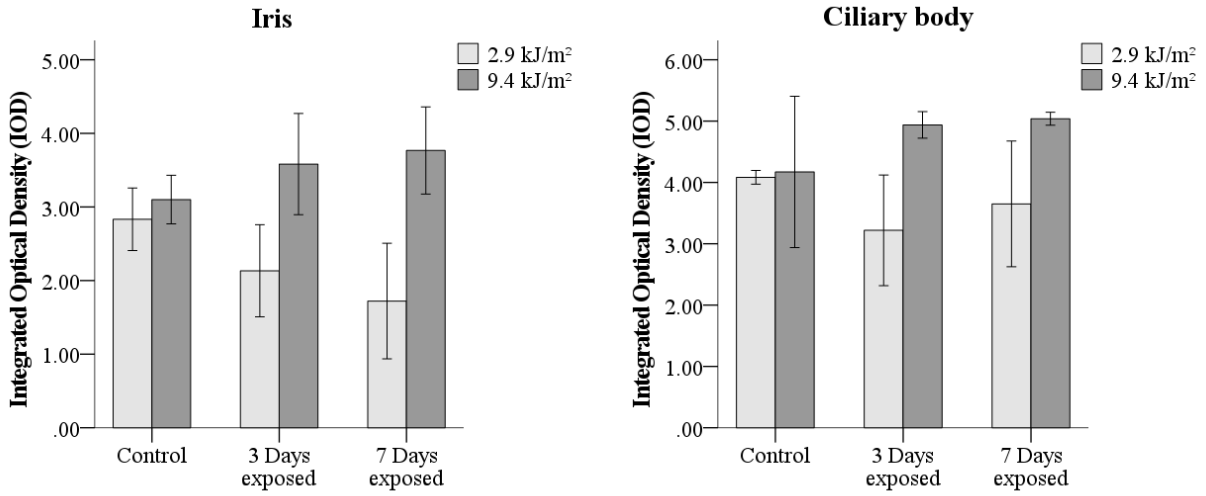


**Figure 5:** NKR-1 quantification of the mouse iris and ciliary body. Quantification of NKR-1 immuno-labeling is expressed as integrated optical density (IOD). IOD of the mouse iris and ciliary body revealed no significant differences between exposed eyes and the control group **(A+B)**. Iris and ciliary body of the high-dose (9.4 kJ/m²) UVR group revealed a significant increase 3 and 7 days post UVR exposure, compared to the low-dose (2.9 kJ/m²) group. Error bars are 95 % confidence interval for the mean. *P <0.05, ** < 0.01, ***P < 0.001.

*Lens*

In the exposed lens epithelium intense NKR-1 staining was found at the membrane level of all epithelial cells including the equatorial region (Fig. 6 A + D + G + J). Irradiated eyes showed a partial loss of epithelium at day 3 and a multilayered accumulation of cells in the disrupted epithelium at day 3 after exposure (Fig. 6A). Lenses of control eyes revealed milder NKR-1 immunostaining (Fig. 6 E + K) and their lens epithelium had a regularly monolayered cuboidal form (Fig. 6 B + H). The negative control of the lens epithelium and the nuclear bow region of the lens are demonstrated in Fig. 6 C + F + I + L.

IOD of the anterior lens epithelium of both exposure groups revealed no significant differences for NKR-1 between latency periods and the control group (Fig. 7A). However, quantification of NKR-1 immunoreactivity of the exposed nuclear bow region showed a significant increase of NKR-1 immunoreactivity after 3 days (Fig. 7B; P = 0.024) as well as 7 days (P = 0.002) in the high-dose exposure group of 9.4 kJ/m$^2$ UVR compared to the control group. NKR-1 quantification of the low-dose group revealed a slight NKR-1 increase over time but no significant difference to control lenses. Furthermore, the low-dose

group displayed a significant decrease compared to the high-dose group after 3 days ($P = 0.015$) and 7 days ($P < 0.001$) post-exposure.

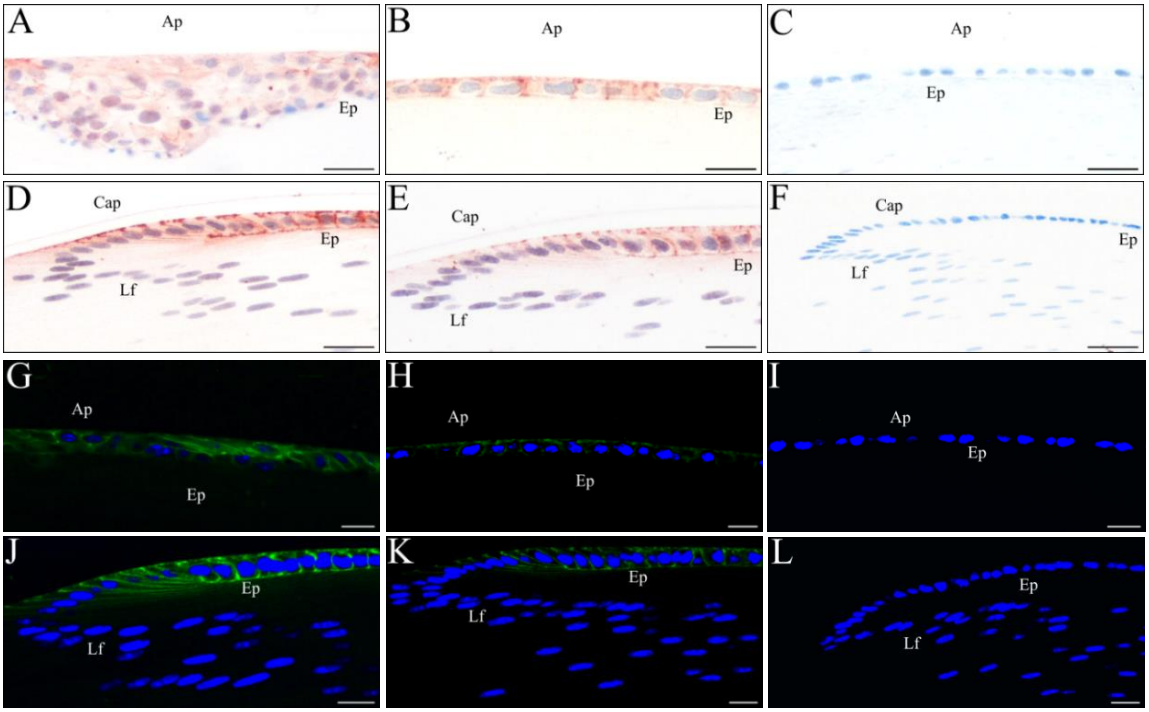


**Figure 6:** Positive NKR-1 immunostaining of exposed and control anterior lens epithelium and nuclear bow region of the lens. Photomicrographs show immunohistochemical AEC staining **(A-F)** and immunofluorescence staining **(G-L)** for NKR-1. NKR-1 stained and disrupted epithelial cells in the anterior polar region were observed in exposed lenses, 3 days post-exposure to both dose groups UVR **(A+G)**. Control lenses showed a light NKR-1 staining and a regular epithelial cell layer in the anterior polar region of the lens **(B+H)**. Intense NKR-1 immunostaining was detected in the nuclear bow region (membrane labeling), following high-dose UVR exposure **(D+J)**. The nuclear bow region of control lenses exhibited a mild NKR-1 staining in the epithelial cell membrane **(E+K)**. The negative controls are shown in **C+F** and **I+L**. Ap = anterior pole, Ep = epithelium, Cap = lens capsule, Lf = lens fibers. Red/Green: Substance P receptor (NKR-1). Blue: H&E/DAPI (nuclei staining). Scale bars = 25 µm.

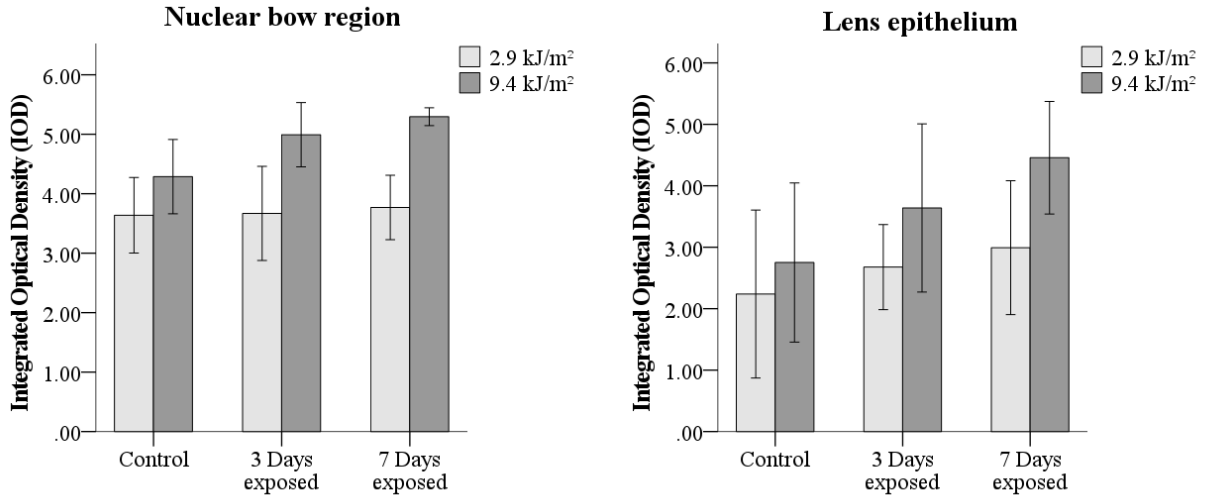


**Figure 7:** NKR-1 quantification of the anterior lens epithelium and nuclear bow region of the lens. Quantification of NKR-1 immunolabeling is expressed as integrated optical density (IOD). In the anterior lens epithelium IOD is slightly increased in the high-dose group and decreased in the low-dose group throughout days 3 and 7 compared to control but no statistical difference between is found **(A)**. The nuclear bow region of the lens displays a significant increase of IOD at 3 as well as 7 days following high-dose (9.4 kJ/m²) UVR exposure as compared to the low-dose (2.9 kJ/m²) and the control group **(B)**. Error bars are 95 % confidence interval for the mean. *P<0.05, **P<0.01, ***P<0.001.

*Retina*

NKR-1 immunostaining of exposed and control mouse retinae are shown in Fig. 8. After three days exposed eyes of the high-dose UVR group (9.4 kJ/m$^2$) demonstrated a high density of NKR-1 immunoreactivity in the cell bodies of the inner plexiform layer (IPL) while the outer plexiform layer (OPL) and in the proximal INL of these retinae exhibited only a mild NKR-1 immunoreactivity (Fig. 8 A + D + G). In control eyes positive NKR-1 immunostaining was found in the IPL and INL, but less than in exposed eyes (Fig. 8 B + E + H) with laminae 2 of the IPL containing the highest density. The negative control of the retina revealed no NKR-1 staining (Fig. 8 C + F). In exposed and control eyes, the ganglion cell layer (GCL) and outer nuclear layer (ONL) revealed no specific NKR-1 immunostaining.

The classification of the IPL was adapted as suggested by Cajal (1893) and subdivided into laminae 1–5 (Fig. 8 G + H) (Cajal, 1893). High power photomicrographs demonstrated the localization of NKR-1 immunostaining in bipolar and amacrine cells in the IPL of the exposed retinae (Fig. 8G). Lamina 2 contained the highest density of NKR-1 immunostain-

ing, whereas lamina 1 and 3 illustrated a medium density. Sparsely immunolabeled processes were visible in lamina 4 and the lowest density was determined in lamina 5. NKR-1 immunoreactivity in the high-dose UVR exposed eyes was significantly higher after day 3 and 7 compared to the control group ($P = 0.021$) and ($P = 0.002$) respectively, as well as the low-dose exposure group ($P < 0.001$) (Fig. 9). In the low-dose exposure group NKR-1 slightly decreased over time compared to the control group but no statistical significance was found.

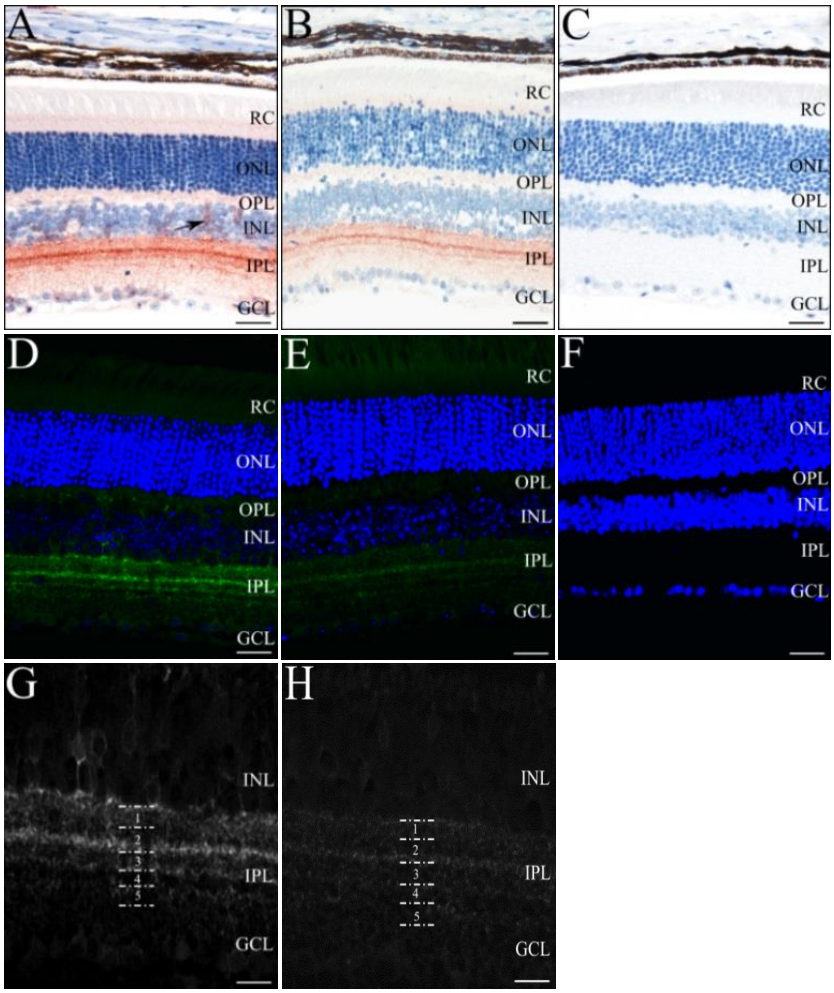


**Figure 8:** Substance P receptor immunostaining pattern in a coronal section of exposed and control mouse retina. Photomicrographs demonstrated NKR-1 staining with immunohistochemical AEC staining **(A-C)** and immunofluorescence staining **(D-H)**. Three days following high-dose UV-radiation, NKR-1 immunostaining is localized in the inner plexiform layer and the inner nuclear layer of the exposed retina**.** In the outer plexiform layer and proximal INL slightly NKR-1 immunolabeling was found **(A+D).** In the control retina, mild NKR-1 immunostaining was observed in the inner plexiform layer **(B+E)**. Negative controls of AEC and fluorescence staining showed no staining **(C+F)**. Higher power photomicrographs demonstrated intense NKR-1 expression in the IPL laminae, especially in

the laminae 2, of the exposed retina **(G).** Control eyes showed mild NKR-1 immunostaining in the IPL laminae, with the highest density in laminae 2 **(H).** IPL laminae are subdivided into laminae 1-5 (Cajal et al., 1893). RC = rods and cones, ONL = outer nuclear layer, OPL = outer plexiform layer, INL = inner nuclear layer, IPL = inner plexiform layer, GCL = ganglion cell layer. Red/Green: Substance P receptor (NKR-1). Blue: H&E/DAPI (nuclei staining). Scale bars = 25 µm.

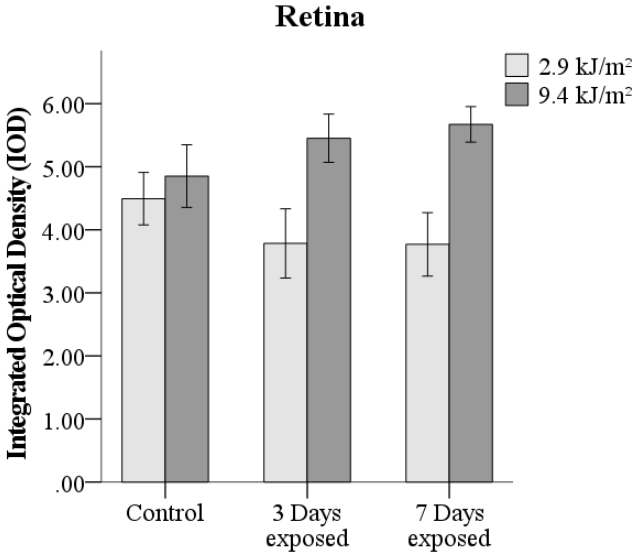


**Figure 9:** Quantification of NKR-1 staining in the retina of exposed and control eyes. NKR-1 immunoreactivity expressed as integrated optical density (IOD). A single and an above 3-fold cataract threshold dose of UVR are evaluated. A significant increase of IOD was observed between exposed and control eyes following a high-dose exposure to UVR (9.4 kJ/m²) and between the high-dose and low-dose (2.9 kJ/m²) group. Error bars show 95 % confidence interval for the mean. *P < 0.05, **P < 0.01, ***P < 0.001.

### 2.4.2 Cataract morphology and quantification

All UVR (peak wavelength 312 nm) exposed lenses developed an anterior subcapsular cataract but no cortical and/or nuclear cataract. Control lenses were clear. At day 3 following exposure, dose-dependent fine granular opacities were noted throughout the anterior surface of the lens (Fig. 10A) resembling the damaged epithelium. At day 7 post UVR exposure a dense triangular shaped cataract developed in the center of the lens (Fig. 10B).

To quantify cataract intensity in exposed and control lenses IOD of lens photographs was measured. IOD levels of exposed lenses at day 3 ($P < 0.001$) and at day 7 ($P < 0.001$) post-exposure were significantly increased in comparison to the control lenses (Fig. 11).

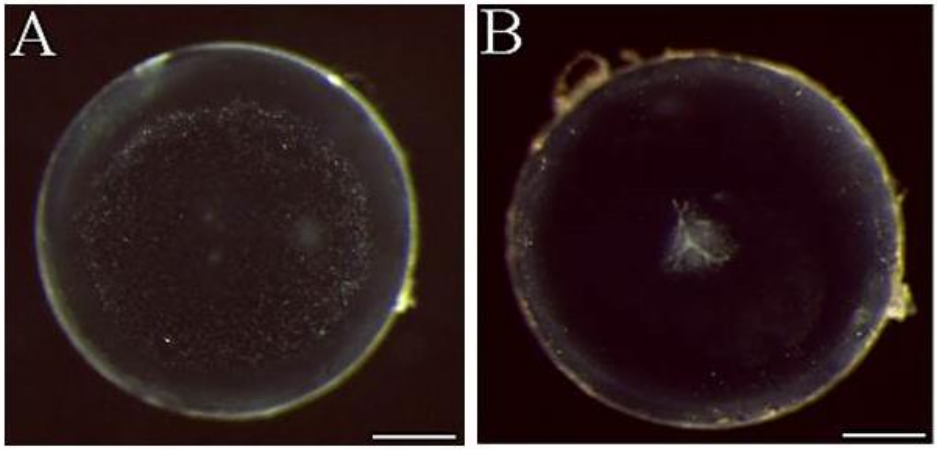


**Figure 10:** Cataract development following UVR exposure. Dose dependent anterior sub-capsular cataract developed 3 and 7 days post UVR exposure. Fine granular opacities were visible throughout the anterior surface of the lens, 3 days following UVR exposure **(A)**. 7 days post-exposure a triangular-shaped opacity had developed around the anterior lens sutures **(B)**. Scale bars = 0.5 mm.

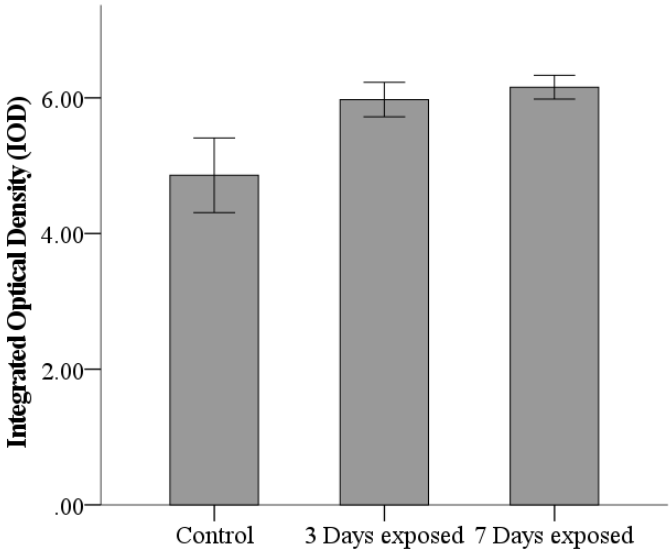


**Figure 11:** Cataract quantification of exposed and control lenses. IOD of the lenses showed a significant difference between exposed latency period groups in relation to the control group (n=15/latency period, control mice, n=12). Error bars represent 95 % CI of the mean. ***P < 0.001.

## 2.5 Discussion

We investigated the effect of UVR exposure with a UV peak at 312 nm on the SP receptor, NKR-1, protein expression in different ocular tissues in an *in vivo* mouse model.

### 2.5.1 NKR-1 immunostaining and NKR-1 upregulation after UVR exposure

The presence of SP, a neuroinflammatory peptide, and its receptor, NKR-1, had been described for ocular tissues of several mammalian species (Bignami et al., 2014; Catalani et al., 2006; Cole et al., 1983). It is well known that the SP protein has a short half-life and is thus very difficult to measure. The neuropeptide persists in tissues only seconds to tens of minutes, while it is stable in plasma over hours (Matsas et al., 1984; McGregor and Bloom, 1983). Thus, in this study the SP receptor protein expression was investigated to quantify SP expression and its upregulation in ocular tissues following UVR-irradiation. To minimize potential drawbacks in measuring and quantifying fluorescence intensity we used constant technical parameters as well as background subtraction to control for artefacts. Furthermore, we standardized the measuring area of interest using a specific template. With these above mentioned parameters we are able to minimize potential drawbacks of the technique.

In summary we demonstrate that a high-dose UVR exposure (9.4 kJ/m$^2$) induces a significant NKR-1 upregulation up to one week post-exposure in different ocular tissues in contrast to a low-dose, or cataract threshold dose of UVR (2.9 kJ/m$^2$). One possible explanation for this might be the capacity of the ocular immune response to suppress an intraocular inflammatory response after a mild but not severe phototoxic insult via a suppression of SP or downregulation of NKR-1, respectively.

*Cornea*

The cornea is a highly innervated tissue comprising sensory and autonomic nerve fibers (Mueller et al., 2003). Corneal nerve endings contain several neuropeptides like SP (Keen et al., 1982; Tervo et al., 1981). Bignami et al. (2014) investigated SP expression in the epithelial layer of avascular corneas and a co-localization of SP in corneal nerve endings (Bignami et al., 2014). Therefore we expected the cornea to be a suitable target tissue to explore the effects of UVR-B 312 nm on SP since UVR-B is partially absorbed by the cornea. An upregulation of NKR-1 in the cornea following an experimental insult is in ac-

cordance with other disease models. In two different CNV (corneal neovascularization) mouse models SP was significantly increased after injury (Bignami et al., 2014). A recent study using a HSK (herpetic stromal keratitis) mouse model demonstrated that corneas with severe HSK lesions exhibited a significantly higher amount of SP compared to those with mild lesions (Twardy et al., 2011). Positive NKR-1 immunostaining in the corneal stroma could not be demonstrated in our study as UVR at a relatively mild exposure does not induce deep corneal lesions.

An experimental study hypothesized the role of SP and NKR-1 in directing cell migration in pterygia that may explain the centripetal growth pattern (Chui et al., 2007). Pterygium is an ocular surface disease and is caused by UVR-B exposure. Interestingly, the work group demonstrated for the first time the presence and upregulation of NKR-1 in pterygia as well as SP as a potent chemoattractant for pterygium fibroblasts and vascular endothelial cells. This indicates that SP and its receptor, NKR-1 play also an important role in pterygia disease and is involved in ocular surface inflammation.

The finding that UVR with a peak at 312 nm leads to an NKR-1 upregulation following an above 3-fold cataract threshold dose (9.4 $kJ/m^2$) in the cornea, 3 and 7 days following exposure suggests that UVR has not only a phototoxic effect but also provokes dose-dependent an unspecific inflammatory reaction in the cornea. The corneal epithelium showed the highest expression of NKR-1. This is most likely due to it being the first tissue being struck by UVR. Interestingly, the corneal endothelium also demonstrated an upregulation of NKR-1. Endothelial cells play an important role in the maintenance of corneal transparency and also act as a barrier as well as contact tissue towards the aqueous humor. It is conceivable that a SP mediated inflammatory signal might reach the aqueous humor also via the endothelium.

*Iris and Ciliary body*

The SP receptor was detected immunohistochemically at the margin of the sphincter muscle as well as sporadically in the iris stroma of control as well as exposed mouse eyes. Similar findings of NKR-1 immunoreactivity were reported in rabbits (He and Bazan, 2015; Tervo et al., 1981; Tornqvist et al., 1982). Cole et al. even demonstrated that SP positive nerve fibers run circumferentially in the sphincter muscle of the normal rabbit iris (Cole et al., 1983).

In our study *in vivo* UVR exposure to an above 3-fold UVR-B cataract threshold dose (9.4 kJ/m$^2$) induced a visible upregulation of NKR-1 around the sphincter muscle and the membranes of the sphincter muscle cells. The eye reacts to a strong inflammatory stimulus with the constriction of the pupil by contracting the sphincter muscle inducing miosis. The findings underscore the view that UV-radiation with a peak wavelength at 312 nm acts as a strong stimulus on the anterior segment and is capable of inducing inflammatory miosis transmitted possibly via substance P.

In exposed eyes endothelial cells of iris vessels showed an increased NKR-1 expression as compared to the controls. This could be due to the crucial barrier function the endothelial cells execute towards the blood aqueous barrier.

In the ciliary body, high-dose but not low-dose UVR exposure induced a significant upregulation of NKR-1 immunostaining in the pigmented epithelium at day 3. The ciliary body is involved in the ocular immune response and represents a main part of the blood-aqueous barrier (Cunha-Vaz, 1979). Hereby, the epithelium of the ciliary processes produces aqueous humor and supplies the cornea, lens and anterior vitreous with nutrients and oxygen. From previous studies it is known that inflammation of the anterior segment alters the ocular immune privilege (Gery and Streilein, 1994). Such an inflammation is regulated in the aqueous humor by immunosuppressive neuropeptides and cytokines. Thus, pro-inflammatory cytokines can reach areas that are not directly affected by UVR exposure (peak wavelength 312 nm) via the aqueous humor explaining our results.

*Lens*

Based on results from previous experimental and epidemiological studies it was expected to detect increased SP-receptor immunoreactivity in the cornea following UVR exposure with a wavelength peak at 312 nm. Even though this has not been investigated so far the observed NKR-1 immunostaining in the exposed and control iris and ciliary body had been somewhat expected. However, to the best of our knowledge, we demonstrated here for the first time that the NKR-1 receptor is located in the lens epithelium and nuclear bow region of the lens of naïve and exposed mouse eyes. Interestingly, an above 3-fold cataract threshold dose (9.4 kJ/m$^2$) already led to a significant NKR-1 upregulation in the nuclear bow region of the lens, after 3 and 7 days post-exposure. This finding underlines that additionally to a direct phototoxic effect of UVR, an inflammatory response that pos-

sibly regulated by inflammatory peptides in the aqueous humor, is involved in UVR induced cataract development. Therefore, it is comprehensible that the lens and the nuclear bow region of the lens demonstrated NKR- 1 upregulation after UVR exposure (9.4 kJ/m$^2$), since pro-inflammatory molecules can reach the lens via the aqueous humor.

*Cataract quantification*

A previous study established that, unilateral UVR-B exposure with various threshold dose equivalents leads to anterior subcapsular cataract in exposed mice lenses (Meyer et al., 2013). Galichanin et al. (2010) showed an exponential increase of lens light scattering after UVR-B exposure in rats. The group measured higher lens light scattering with a higher UVR-B dose (Galichanin et al., 2010). Our results are in line with previous studies that demonstrated the characteristic anterior subcapsular cataract development after low-dose or threshold UV-radiation (312 nm) dose of 2.9 kJ/m$^2$, showing granular opacities throughout the anterior surface of the exposed lens (Fig. 10B). However, the cataract development at this low-dose UVR threshold is not associated with a significant upregulation of NKR-1 in the lens epithelium. Furthermore, we established integrated optical density (IOD) as a quantification for experimentally induced cataract. Artefacts such as residues of zonules or the ciliary body were avoided through careful dissection techniques as well as using a specific template to mask the lens periphery and minimize potential light scattering from the lens equator. We verified that the Bio-Spectra exposure system induces similar cataracts than the exposure system used in previous studies (Meyer et al., 2013; Pitts et al., 1983; Soederberg et al., 2002). However, since the Bio-Spectra exposure system also partially emits UVR-A (315 – 380 nm) we cannot exclude that to some extend the demonstrated effects might be attributed to the biological action spectrum of UVR-A.

*Retina*

A previous study demonstrated the localization of the substance P receptor in amacrine cells of the IPL (Haverkamp and Wassle, 2000) and NKR-1 immunolabeling was confined at or near the plasma membrane of cell bodies in INL, OPL and IPL (Catalani et al., 2004). The results of these investigations are in line with our findings, observing a positive NKR-1 immunostaining in the IPL of the naïve mouse retina and a statistically significant NKR-

1 upregulation 3 and 7 days following an above 3-fold cataract threshold dose of UV-radiation, as compared to the control group.

Even though UVR-B does not reach the retina directly, since it is absorbed by the cornea and lens, we expect that the UV-radiation had an effect on retinal NKR-1 expression. We hypothesize, that an inflammatory signal is transmitted through the anterior uveal tissue towards the posterior segment since the pigmented and unpigmented epithelium of the ciliary body have the same evolutionary origin as the retinal pigment epithelium and the neuroretina. Furthermore, because the UVR spectrum used covers also longer wavelengths in the UVR-A spectrum it is possible that the small fraction of emitted UVR-A (315 - 380 nm) might contribute directly to the observed retinal effects.

## 2.6 Conclusion

In conclusion, SP receptor, NKR-1, is present ubiquitously in ocular tissues including the lens. UV-radiation (312 nm) to an above 3-fold cataract threshold dose of 9.4 kJ/m$^2$ triggers NKR-1 upregulation in ocular tissues of the anterior and posterior segment already at day 3 following exposure. Unilateral UVR irradiation to a low-dose cataract threshold dose of 2.9 kJ/m$^2$ induces no markable NKR-1 upregulation. To our knowledge this is the first proof of NKR-1 immunoreactivity in the lens and the first study to demonstrate SP receptor upregulation in the eye after UVR exposure (peak wavelength 312 nm). We conclude that the involvement of inflammation in ultraviolet radiation induced cataract might have been underestimated so far. The role of SP and its receptor in UVR induced cataractogenesis needs further investigations.

## 2.7 Funding sources

This research was funded by the “Deutsche Forschungsgesellschaft (DFG)” (ME 3298/4-1; WE 1303/6-1).

## 2.8 Author disclosures

Janine Gross: None; Alfred Wegener: None; Martin Kronschläger: None; Frank Holz: Acucela (F), Acucela (C), Alcon (F), Alcon (C), Allergan (F), Allergan (C), Bayer HealthCare(F), Bayer HealthCare (C), Boehringer Ingelheim (C), Carl Zeiss Meditec (F), Genentech (F), Genentech/Roche (C), Heidelberg Engineering (F), Heidelberg Engineer-

ing (C), Heidelberg Engineering (R), Merz (C), Novartis (F), Novartis (C), Optos (F); Carl-Ludwig Schoenfeld: None; Linda Meyer: None.

### 2.9 Acknowledgements

The authors thank Claudine Strack and Parand Widmar for expert help with the immuno-histochemistry. We also thank the laboratory group for helpful discussions.

### 2.10 Appendix

**Table 1:** One-way univariate ANOVA (F-Test) for the 3-fold cataract threshold dose (9.4 kJ/m²) group.

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| **Cornea** | | | | | | |
| | Between Groups | 8.658 | 2 | 4.329 | 5.123 | **.013** |
| | Within Groups | 23.660 | 28 | .845 | | |
| | Total | 32.318 | 30 | | | |
| **Iris** | | | | | | |
| | Between Groups | 2.015 | 2 | 1.007 | 1.837 | .180 |
| | Within Groups | 13.712 | 25 | .548 | | |
| | Total | 15.727 | 27 | | | |
| **Lens epithelium** | | | | | | |
| | Between Groups | 16.250 | 2 | 8.125 | 2.985 | .067 |
| | Within Groups | 76.219 | 28 | 2.722 | | |
| | Total | 92.469 | 30 | | | |
| **Nuclear bow** | | | | | | |
| | Between Groups | 5.283 | 2 | 2.642 | 6.080 | **.006** |
| | Within Groups | 12.165 | 28 | .434 | | |
| | Total | 17.449 | 30 | | | |
| **Ciliary body** | | | | | | |
| | Between Groups | 4.319 | 2 | 2.159 | 2.763 | .080 |
| | Within Groups | 21.886 | 28 | .782 | | |
| | Total | 26.205 | 30 | | | |
| **Retina** | | | | | | |
| | Between Groups | 3.489 | 2 | 1.745 | 5.838 | **.008** |
| | Within Groups | 8.367 | 28 | .299 | | |
| | Total | 11.856 | 30 | | | |

**Table 2:** One-way univariate ANOVA (F-Test) for the single cataract threshold dose (2.9 kJ/m²) group.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| **Cornea** | | | | | |
| Between Groups | 3.012 | 2 | 1.506 | 1.450 | .252 |
| Within Groups | 29.080 | 28 | 1.039 | | |
| Total | 32.092 | 30 | | | |
| **Iris** | | | | | |
| Between Groups | 3.816 | 2 | 1.908 | 2.612 | .092 |
| Within Groups | 19.722 | 27 | .730 | | |
| Total | 23.537 | 29 | | | |
| **Lens epithelium** | | | | | |
| Between Groups | 2.587 | 2 | 1.294 | .647 | .532 |
| Within Groups | 51.983 | 26 | 1.999 | | |
| Total | 54.570 | 28 | | | |
| **Nuclear bow** | | | | | |
| Between Groups | .091 | 2 | .045 | .050 | .951 |
| Within Groups | 24.518 | 27 | .908 | | |
| Total | 24.609 | 29 | | | |
| **Ciliary body** | | | | | |
| Between Groups | 3.706 | 2 | 1.853 | 1.251 | .302 |
| Within Groups | 41.470 | 28 | 1.481 | | |
| Total | 45.176 | 30 | | | |
| **Retina** | | | | | |
| Between Groups | 3.282 | 2 | 1.641 | 3.141 | .059 |
| Within Groups | 14.628 | 28 | .522 | | |
| Total | 17.911 | 30 | | | |

## 2.11 References

**Bignami, F**., Giacomini, C., Lorusso, A., Aramini, A., Rama, P., Ferrari, G., 2014. NK1 receptor antagonists as a new treatment for corneal neovascularization. Investigative ophthalmology & visual science 55 (10), 6783–6794.

**Brian, G**., Taylor, H., 2001. Cataract blindness: challenges for the 21st century. Bulletin of the World Health Organization 79 (3), 249–256.

**Cajal S. R**., 1893. La rétine des vertébrés. Cellule 9.

**Casini, G**., Brecha, N.C., Bosco, L., Rickman, D.W., 2000. Developmental expression of neurokinin-1 and neurokinin-3 receptors in the rat retina. The Journal of comparative neurology 421 (2), 275–287.

**Casini, G.**, Sabatini, A., Catalani, E., Willems, D., Bosco, L., Brecha, N.C., 2002. Expression of the neurokinin 1 receptor in the rabbit retina. Neuroscience 115 (4), 1309–1321.

**Castagliuolo, I.**, Valenick, L., Liu, J., Pothoulakis, C., 2000. Epidermal growth factor receptor transactivation mediates substance P-induced mitogenic responses in U-373 MG cells. The Journal of biological chemistry 275 (34), 26545–26550.

**Catalani, E.**, Dal Monte, M., Gangitano, C., Lucattelli, M., Fineschi, S., Bosco, L., Bagnoli, P., Casini, G., 2006. Expression of substance P, neurokinin 1 receptors (NK1) and neurokinin 3 receptors in the developing mouse retina and in the retina of NK1 knockout mice. Neuroscience 138 (2), 487–499.

**Catalani, E**., Gangitano, C., Bosco, L., Casini, G., 2004. Expression of the neurokinin 1 receptor in the mouse retina. Neuroscience 128 (3), 519–530.

**Chang, M.M.**, Leeman, S.E., NIALL, H.D., 1971. Amino-acid sequence of substance P. Nature 232 (29), 86–87.

**Chen, S.-J.**, Lee, C.-J., Lin, T.-B., Liu, H.-J., Huang, S.-Y., Chen, J.-Z., Tseng, K.-W., 2016. Inhibition of Ultraviolet B-Induced Expression of the Proinflammatory Cytokines TNF-α and VEGF in the Cornea by Fucoxanthin Treatment in a Rat Model. Marine drugs 14 (1), 13.

**Chui, J.**, Di Girolamo, N., Coroneo, M.T., Wakefield, D., 2007. The role of substance P in the pathogenesis of pterygia. Investigative ophthalmology & visual science 48 (10), 4482–4489.

**Cole, D.F.**, Bloom, SR, Burnstock, G., Butler, J.M., McGregor, G.P., Saffrey, M.J., Unger, W.G., Zhang, S.Q., 1983. Increase in SP-like immunoreactivity in nerve fibres of rabbit

iris and ciliary body one to four months following sympathetic denervation. Experimental eye research 37 (2), 191–197.

**Cruickshanks, K.J.**, Klein, B.E., Klein, R., 1992. Ultraviolet light exposure and lens opacities: the Beaver Dam Eye Study. American journal of public health 82 (12), 1658–1662.

**Cunha-Vaz, J.**, 1979. The blood-ocular barriers. Survey of Ophthalmology 23 (5), 279–296.

**Delcourt, C.**, Carriere, I., Ponton-Sanchez, A., Lacroux, A., Covacho, M.J., Papoz, L., 2000. Light exposure and the risk of cortical, nuclear, and posterior subcapsular cataracts: the Pathologies Oculaires Liees a l'Age (POLA) study. Archives of ophthalmology (Chicago, Ill.: 1960) 118 (3), 385–392.

**Galichanin, K.**, Lofgren, S., Bergmanson, J., Soederberg, P.G., 2010. Evolution of damage in the lens after in vivo close to threshold exposure to UV-B radiation: cytomorphological study of apoptosis. Experimental eye research 91 (3), 369–377.

**Gery, I.**, Streilein, J.W., 1994. Autoimmunity in the eye and its regulation. Current opinion in immunology 6 (6), 938–945.

**Haverkamp, S.**, Wassle, H., 2000. Immunocytochemical analysis of the mouse retina. The Journal of comparative neurology 424 (1), 1–23.

**He, J.**, Bazan, H.E.P., 2015. Morphology and neurochemistry of rabbit iris innervation. Experimental eye research 135, 182–191.

**Hejtmancik, J.F.**, 1998. INVITED EDITORIAL The Genetics of Cataract: Our Vision Becomes Clearer. The American Journal of Human Genetics 62 (3), 520–525.

**Hejtmancik, J.F.**, Kantorow, M., 2004. Molecular genetics of age-related cataract. Experimental eye research 79 (1), 3–9.

**Hightower, K.R.**, 1995. The role of the lens epithelium in development of UV cataract. Current eye research 14 (1), 71–78.

**Hong, H.S.**, Kim, S., Nam, S., Um, J., Kim, Y.H., Son, Y., 2015. Effect of substance P on recovery from laser-induced retinal degeneration. Wound repair and regeneration: official publication of the Wound Healing Society [and] the European Tissue Repair Society 23 (2), 268–277.

**Ji, Y.**, Cai, L., Zheng, T., Ye, H., Rong, X., Rao, J., Lu, Y., 2015. The mechanism of UVB irradiation induced-apoptosis in cataract. Molecular and cellular biochemistry 401 (1-2), 87–95.

**Jiang, Y.**, Fu, R., Zhao, J., Di Wu, Qiao, G., Li, R., Zhang, J., 2015. Effects of ELL-associated factor 2 on ultraviolet radiation-induced cataract formation in mice. Molecular medicine reports 12 (5), 6605–6611.

**Jose, J.G.**, Pitts, D.G., 1985. Wavelength dependency of cataracts in albino mice following chronic exposure. Experimental eye research 41 (4), 545–563.

**Keen, P.**, Tullo, A.B., Blyth, W.A., Hill, T.J., 1982. Substance P in the mouse cornea: effects of chemical and surgical denervation. Neuroscience letters 29 (3), 231–235.

**Koon, H.-W.**, Zhao, D., Na, X., Moyer, M.P., Pothoulakis, C., 2004. Metalloproteinases and transforming growth factor-α mediate substance P-induced mitogen-activated protein kinase activation and proliferation in human colonocytes. Journal of Biological Chemistry 279 (44), 45519–45527.

**Lucas, K.**, Karamichos, D., Mathew, R., Zieske, J.D., Stein-Streilein, J., 2012. Retinal laser burn-induced neuropathy leads to substance P-dependent loss of ocular immune privilege. The Journal of Immunology 189 (3), 1237–1242.

**Mashaghi, A.**, Marmalidou, A., Tehrani, M., Grace, P.M., Pothoulakis, C., Dana, R., 2016. Neuropeptide substance P and the immune response. Cellular and molecular life sciences: CMLS 73 (22), 4249–4264.

**Matsas, R.**, Kenny, A.J., Turner, A.J., 1984. The metabolism of neuropeptides. The hydrolysis of peptides, including enkephalins, tachykinins and their analogues, by endopeptidase-24.11. The Biochemical journal 223 (2), 433–440.

**McGregor, G.P.**, Bloom, SR, 1983. Radioimmunoassay of substance P and its stability in tissue. Life sciences 32 (6), 655–662.

**Mei, G.**, Xia, L., Zhou, J., Zhang, Y., Tuo, Y., Fu, S., Zou, Z., Wang, Z., Jin, D., 2013. Neuropeptide SP activates the WNT signal transduction pathway and enhances the proliferation of bone marrow stromal stem cells. Cell biology international 37 (11), 1225–1232.

**Merriam, J.C.**, Loefgren, S., Michael, R., Soederberg, P., Dillon, J., Zheng, L., Ayala, M., 2000. An action spectrum for UV-B radiation and the rat lens. Investigative ophthalmology & visual science 41 (9), 2642–2647.

**Meyer, L.M.**, Dong, X., Wegener, A., Soederberg, P.G., 2007. Light scattering in the C57BL/6 mouse lens. Acta ophthalmologica Scandinavica 85 (2), 178–182.

**Meyer, L.M.**, Loefgren, S., Holz, F.G., Wegener, A., Soederberg, P.G., 2013. Bilateral cataract induced by unilateral UVR-B exposure -- evidence for an inflammatory response. Acta ophthalmologica 91 (3), 236–242.

**Monastyrskaya, K**., Hostettler, A., Buergi, S., Draeger, A., 2005. The NK1 receptor localizes to the plasma membrane microdomains, and its activation is dependent on lipid raft integrity. The Journal of biological chemistry 280 (8), 7135–7146.

**Mueller, L.J.**, Marfurt, C.F., Kruse, F., Tervo, T.M.T., 2003. Corneal nerves: structure, contents and function. Experimental eye research 76 (5), 521–542.

**Muñoz, M.**, Rosso, M., Aguilar, F.J., González-Moles, M.A., Redondo, M., Esteban, F., 2008. NK-1 receptor antagonists induce apoptosis and counteract substance P-related mitogenesis in human laryngeal cancer cell line HEp-2. Investigational new drugs 26 (2), 111–118.

**O'Connor, T.M.**, O'Connell, J., O'Brien, D.I., Goode, T., Bredin, C.P., Shanahan, F., 2004. The role of substance P in inflammatory disease. Journal of cellular physiology 201 (2), 167–180.

**Oyamada, H.,** Takatsuji, K., Senba, E., Mantyh, P.W., Tohyama, M., 1999. Postnatal development of NK1, NK2, and NK3 neurokinin receptors expression in the rat retina. Brain research. Developmental brain research 117 (1), 59–70.

**Park, J.H.**, Kim, S., Hong, H.S., Son, Y., 2016. Substance P promotes diabetic wound healing by modulating inflammation and restoring cellular activity of mesenchymal stem cells. Wound repair and regeneration: official publication of the Wound Healing Society [and] the European Tissue Repair Society 24 (2), 337–348.

**Pitts, D.G.**, Bergmanson, J.P., Chu, L.W., 1983. Rabbit eye exposure to broad-spectrum fluorescent light. Acta ophthalmologica. Supplementum 159, 1–54.

**Pitts, D.G.**, Cullen, A.P., Hacker, P.D., 1977. Ocular effects of ultraviolet radiation from 295 to 365 nm. Investigative ophthalmology & visual science 16 (10), 932–939.

**Soederberg, P.G.,** Loefgren, S., Ayala, M., Dong, X., Kakar, M., Mody, V., 2002. Toxicity of ultraviolet radiation exposure to the lens expressed by maximum tolerable dose. Developments in ophthalmology 35, 70–75.

**Taylor, H.R., 1989**. Ultraviolet radiation and the eye: an epidemiologic study. Transactions of the American Ophthalmological Society 87, 802–853.

**Tervo, K.**, Tervo, T., Eranko, L., Eranko, O., Cuello, A.C., 1981. Immunoreactivity for substance P in the Gasserian ganglion, ophthalmic nerve and anterior segment of the rabbit eye. The Histochemical journal 13 (3), 435–443.

**Tornqvist, K.**, Mandahl, A., Leander, S., Loren, I., Hakanson, R., Sundler, F., 1982. Substance P-immunoreactive nerve fibres in the anterior segment of the rabbit eye. Distribution and possible physiological significance. Cell and tissue research 222 (3), 467–477.

**Twardy, B.S.**, Channappanavar, R., Suvas, S., 2011. Substance P in the corneal stroma regulates the severity of herpetic stromal keratitis lesions. Investigative ophthalmology & visual science 52 (12), 8604–8613.

**Weinstock, J.V.**, Blum, A., Metwali, A., Elliott, D., Bunnett, N., Arsenescu, R., 2003. Substance P regulates Th1-type colitis in IL-10 knockout mice. Journal of immunology (Baltimore, Md.: 1950) 171 (7), 3762–3767.

**World Health Organization**, 2010. Action plan for the prevention of avoidable blindness and visual impairment, 2009-2013.

**Wu, Q.**, Guo, D., Bi, H., Wang, D., Du, Y., 2013. UVB irradiation-induced dysregulation of plasma membrane calcium ATPase1 and intracellular calcium homeostasis in human lens epithelial cells. Molecular and cellular biochemistry 382 (1-2), 263–272.

**Zhou, Z.**, Barrett, R.P., McClellan, S.A., Zhang, Y., Szliter, E.A., van Rooijen, N., Hazlett, L.D., 2008. Substance P delays apoptosis, enhancing keratitis after Pseudomonas aeruginosa infection. Investigative ophthalmology & visual science 49 (10), 4458.

# 3. Ultraviolet radiation exposure of one eye stimulates sympathizing expression of neurokinin-1 receptor but not monocyte chemoattractant protein-1 in the partner eye

Janine Gross[a], Eva Willimsky[a], Alfred R. Wegener[a], Martin Kronschläger[b], Carl-Ludwig Schönfeld[c,d], Frank G. Holz[a], Linda M. Meyer[a,c]

[a] Department of Ophthalmology, University of Bonn, Bonn, Germany
[b] Department of Ophthalmology, Hanusch Hospital, Vienna, Austria
[c] Herzog Carl Theodor Eye Clinic, Munich, Germany
[d] Department of Ophthalmology, Ludwig-Maximilians University, Munich, Germany

Ophthalmic Research
Volume 13, August 2019, Page 1-13
DOI: 10.1159/000501320

### 3.1 Abstract

**Purpose:** To investigate the influence of unilateral ultraviolet radiation (UVR) exposure on the unexposed, partner eye in vivo. To characterize the immunological cross-talk between the eyes and verify a sympathizing reaction of the partner eye via a neurokinin-dependent signaling pathway of substance P and its neurokinin-1 receptor (NKR-1) and/or monocyte chemoattractant protein-1 (MCP-1).

**Methods:** C57BL/6 mice were unilaterally exposed in vivo to UVR-B to a 5-fold cataract threshold equivalent dose of 14.5 kJ/m2 with a UV irradiation Bio-Spectra system. The unexposed contralateral eye was completely shielded during irradiation. After 3 and 7 days post exposure, eyes were stained with fluorescence-coupled antibody for substance P NKR-1. The same was performed in control animals receiving only anesthesia but no UVR-B exposure. NKR-1 and MCP-1 levels in ocular tissue lysates were quantified by enzyme-linked immunosorbent assay.

**Results:** UVR-B induces NKR-1 upregulation after 3 and 7 days in the exposed and in the unexposed, contralateral mouse eye. NKR-1 protein level was upregulated in the exposed and contralateral iris/ciliary body complex, choroidea and in the contralateral retina as well as in the exposed cornea. MCP-1 levels were elevated in the exposed cornea, iris/ciliary body complex, and aqueous humor but not in contralateral ocular tissues.

**Conclusions:** UVR-B exposure triggers NKR-1 upregulation not only in the exposed but also in the unexposed, partner eye in various ocular tissues. Following UVR-B exposure, MCP-1 protein levels are upregulated in the exposed eye, but the contralateral side remains unaffected.

**Keywords:** Ultraviolet radiation B, Neurokinin-1 receptor, Cataract, Monocyte chemoattracttant protein-1, Inflammation

### 3.2 Introduction

The eye is one of the only organs directly exposed to sunlight including short wavelength ultraviolet radiation (UVR). Ocular implications of UV irradiation have been studied in a wide spectrum of eye diseases, including acute and chronic inflammatory disorders, especially in the cornea and lens. UVR exposure correlates with ocular pathologies including pterygium, photokeratitis, and cortical cataracts [1–4].

Acute high-dose exposure to UVR type B (UVR-B) induces inflammation in the cornea and damages the epithelium leading to photokeratitis and pterygium [1–3, 5–8]. Set aside the direct phototoxic effect UVR pro-inflammatory cytokines such as IL-6 and IL-8 are upregulated in the eye after in vivo exposure to UVR [1–3].

In addition to its capacity to induce ocular surface disease, exposure to UVR-B is considered the main environmental risk factor for cataract disease in humans and animals [9, 10]. Cataract is still the leading cause of visual impairment worldwide [10–13]. In addition to the known phototoxic damages of UVR in the lens such as epithelial cell damage [14], inactivation of metabolic enzymes [15], and apoptosis [16], evidence is growing that inflammatory processes are involved in cataract development from UVR that also affect the partner eye in sympathizing reaction [4]. However, the exact role of UVR-B and molecular mechanisms involved in the triggered inflammatory processes and the immunological cross-talk to the partner eye are not well understood.

We know from a previous study that in vivo UVR irradiation induces the upregulation of the substance P neurokinin-1 receptor (NKR-1), in various ocular tissues of the eye [17]. Furthermore, the cytokine monocyte chemoattractant protein-1 (MCP-1) is involved in inflammation- dependent eye diseases, such as uveitis [18], herpetic stromal keratitis [19] and is elevated after cataract surgery even in the aqueous humor (AqH) of the nonoperated partner eye [20–23]. Therefore, we hypothesize that the fast-acting neuropeptide substance P and its receptor NKR-1 and possibly MCP-1 might be the candidate signaling molecules involved in the immunological cross-talk to the partner eye following an acute insult to 1 eye as from high-dose UVR-B exposure. Here we investigate whether substance P signaling pathways are altered in the unexposed, partner eye after in vivo exposure of only 1 eye to UVR. Furthermore, we investigate if the proinflammatory chemokine MCP-1 is affected by UVR exposure in either the exposed or partner eye in vivo. Detailed knowledge on the signaling pathways of these 2 cytokines could open new treatment op-

tions for various ocular diseases, including UVR-B-induced cataract, and offer new clinical preventive strategies.

## 3.3 Materials and methods

### 3.3.1 Animals

Six-week-old C57BL/6 mice were purchased from Charles River Laboratories (Germany) and maintained in a 12-h light and 12-h dark cycle environment in the laboratory at the Department of Ophthalmology, University of Bonn.

In the present study, C57BL/6 mice were subdivided into 2 latency groups of 3 and 7 days post UVR-B exposure (wavelength peak at 312 nm) and a control group. For immunohistochemical experiments $n$ = 11 C57BL/6 mice were utilized for each latency group and $n$ = 9 mice for the control group. Enzyme-linked immunosorbent assay (ELISA) was performed in $n$ = 21 animals per group. Animals were anesthetized with a mixture of ketamine/xylazine (40 mg/kg ketamine and 5 mg/kg xylazine) injected intraperitoneally. Topical 0.5% tropicamide (Mydriaticum Stulln; Pharma Stulln GmbH, Stull, Germany) eye drops were administered for pupillary dilation of both eyes. Prior to UVR-B exposure, mice were examined with a slit lamp to exclude pre-existing cataract. One eye of each animal was exposed in vivo to UVR-B above the 5-fold cataract threshold equivalent dose (MTD2.3: 16) for 5 min. The contralateral, unexposed eye was carefully and completely shielded with aluminum foil during exposure. The mice were sacrificed after 3 and 7 days following UVR-B exposure. Eyes were enucleated and prepared for immunohistochemical staining as well as for ELISA. For ELISA analysis AqH, cornea, iris/ciliary body, lens epithelium as well as retina and choroidea were dissected microsurgically. First, AqH was extracted using a 30 gauge syringe needle to carefully penetrate the cornea and collect AqH. After removing all the muscle tissue, the eye was placed in PBS buffer to avoid drying of the lens and carefully opened along the limbus using a microsurgical scissor. Afterwards, ocular tissues were gently separated with forceps, transferred into Eppendorf tubes on ice and prepared for further ELISA analysis (see section below ELISA for NKR-1 and MCP-1). During dissection of the ocular tissues, lenses were visualized in light- and dark field illumination photography.

### 3.3.2 UVR-B source

Animals were irradiated in a microprocessor-controlled UV irradiation system (Bio-Spectra) for laboratory animals from Vilber Lourmat© (Marne-La Vallee, France), equipped with a UVR-B irradiation source. The UVR-B lamps (3 × 40 W) emit ultraviolet rays between 280 and 315 nm with a defined wavelength peak at 312 nm. The spectral curve of the Vilber Lourmat© UVR-B tubes also includes a small fraction of UVR-A in the range of 315–370 nm [17]. Based on a programmable microprocessor, the Bio-Spectra system constantly monitors the UV light emission providing a uniform irradiation area. Radiation exposure is expressed as cataract threshold dose equivalents (maximum tolerable dose – MTD2.3: 16) and was defined in detail by Soderberg et al. [24]. One MTD equivalent is 2.9 kJ/m2 in the pigmented mouse [25]. The comparability with the UVR source of Soderberg et al. [24] is given, and it was adjusted to the Bio-Spectra system, using an energy dose of 14.5 kJ/$m^2$.

### 3.3.3 Immunohistochemistry

For the immunofluorescence, eyes were embedded in paraffin. Thereafter, sections were cut (5 µm), dewaxed in xylene and rehydrated by passing through graded alcohol. For antigen retrieval, sections were heated using citrate buffer (pH 6.0) and incubated overnight with a rabbit polyclonal anti-substance P receptor antibody (1:800, AB5060, Chemicon). The following day, sections were washed before adding anti-rabbit secondary antibody conjugated to Alexa Fluor® 488 (1:200, Thermo Fisher Scientific). Counter-staining of the nuclei was performed with DAPI. In the final step, slides were cover slipped using Immu-Mount (Thermo Scientific™ 9990402). Specificity of staining was confirmed using an isotype control prepared under the same immunohistochemical conditions replacing the primary anti-SP receptor antibody with an isotype-specific immunoglobulin (NI01, Chemicon, rabbit, polyclonal).

### 3.3.4 Quantification of NKR-1expression

NKR-1 expression was quantified as immunofluorescence in stained ocular tissues by measuring the integrated optical density (IOD), as previously described [17]. Measurements were taken with Image J/FIJI 1.46, based on a standardized protocol with fixed illumination settings.

### 3.3.5 ELISA for NKR-1 and MCP-1

Protein levels of NKR-1 and MCP-1 in unexposed, exposed and control mouse eyes were determined with a mouse substance P receptor ELISA kit (Cusabio Biotech, China, CSB-E08362m) and a mouse CCL2/JE/MCP-1 Quantikine ELISA kit (R&D systems, MJE00). After microsurgical dissection on day 3 and 7 after UVR-B exposure, ocular tissues were homogenized in phosphate-buffered solution containing a protease inhibitor cocktail (Sigma-Aldrich®, P8340) and centrifuged at 10.000 g for 10 min. Then, aliquots were stored at -80° until ELISA measurements, which was carried within a week. An aliquot of each supernatant was assayed in duplicate according to the manufacturer´s instructions. The detection range of the substance P receptor ELISA kit fluctuated between 7.8 pg/ml and 500 pg/ml, with a minimum detectable concentration of 1.95 pg/ml. The assay range for MCP-1 was 7.81 - 500 pg/ml with a sensitivity ranged from 0.151 – 0.666 pg/ml.

### 3.3.6 Statistical analysis

Data are presented as mean ± SD. The normal distribution of the results was tested using the Shapiro-Wilk-Test in combination with a quantile-quantile plot (Q-Q plot). One way analysis of variance (ANOVA) followed by a post hoc comparison (LSD) was performed to analyze the difference in NKR-1 and MCP-1 level between latency period groups (control, 3 and 7 days) within the ocular tissues of the exposed or the unexposed partner eye, following UVR-B exposure. A paired sample t-test was used to quantify differences between unexposed and exposed eyes within each latency period group. Regarding sample size, the significance levels were set to 0.05 and the confidence coefficients to 0.95, respectively. The data were evaluated with SPSS Statistics 23.

## 3.4 Results

### 3.4.1 NKR-1 immunohistochemistry of ocular tissues (IOD)

In vivo UVR-B irradiation to a 5-fold cataract threshold dose induced NKR-1 expression and upregulation in ocular tissues not only in the exposed but also in the unexposed, partner eye in a mouse model.

*Cornea*

Histologically, the unexposed, contralateral mouse eye demonstrated a slightly stronger NKR-1 immunostaining in the regularly structured corneal epithelium and endothelium compared to control (Fig. 1A+B). The corneal stroma showed no specific immunostaining for NKR-1 neither in the unexposed, the exposed or in the control eyes. The negative control of the cornea is shown in Fig. 1C.

The unexposed, contralateral eyes demonstrated a significant increase of IOD at days 3 ($P = 0.019$) and 7 ($P = 0.033$) post UVR-B exposure compared to control (Fig. 1D). NKR-1 levels of the exposed cornea were significantly increased at days 3 ($P = 0.009$) and 7 ($P = 0.009$) compared to control eyes. At day 3, UVR-B exposed eyes had a significantly increased IOD for NKR-1 compared to the contralateral eyes ($P = 0.034$).

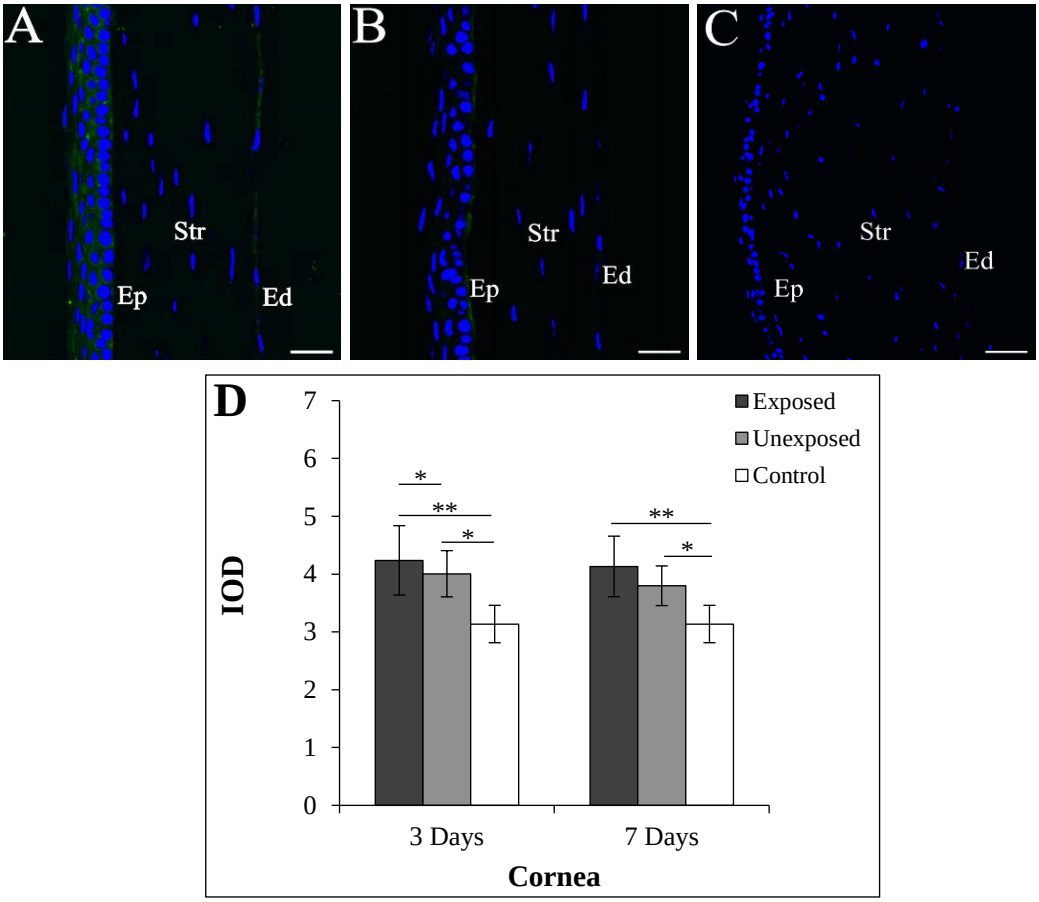


**Figure 1.** NKR-1 fluorescence staining and quantification of the mouse cornea. Three days following UVR-B irradiation, the corneal epithelium of the contralateral mouse eye is regularly structured and showed positive NKR-1 immunostaining (**A**). Control corneas showed a mild epithelial NKR-1 staining (**B**). Negative control showed no immunofluorescence staining (**C**). Quantification of NKR-1 immunoreactivity expressed as IOD after a 5-fold (14.5 kJ/m$^2$) cataract threshold dose of UVR-B (**D**). In the cornea IOD is significantly increased in the unexposed contralateral eye and exposed eye throughout 3 and 7 days compared to control eyes ($n$ = 10 mice per latency period group). * $p < 0.05$, ** $p < 0.01$. Bar is 95% CI for mean. Green: Substance P NKR-1. Blue: DAPI (nuclei staining). Scale bars = 25 µm. Ep, epithelium; Str, stroma; Ed, endothelium; IOD, integrated optical density.

*Iris/Ciliary body*

Contralateral eyes displayed NKR-1 positive nerve fibers at the end of the sphincter muscle in the iris similar to exposed eyes and a slight NKR-1 fluorescence staining in the iris stroma (Fig. 2A). The stroma and the sphincter muscle of the control group exhibited a lower immunostaining for the substance P receptor than the contralateral mouse eye (Fig. 2B). NKR-1 was significantly increased in the iris of unexposed, partner eyes 3 days post UVR-B exposure, compared to the control group (Fig. 2D; P = 0.012). NKR-1 quantifica-

tion of partner eyes even exceeded those of exposed eyes at day 3. The unexposed partner eyes showed an intense NKR-1 immunoreactivity in the pigmented epithelium of the ciliary processes (Fig. 2E) compared to the control group (Fig. 2F). The stroma and the unpigmented ciliary epithelium demonstrated no specific NKR-1 immunostaining in none of the experimental groups. In the ciliary body, unexposed and exposed eyes showed a mild trend for an increased IOD 3 and 7 days following exposure compared to the control group (Fig. 2H). The negative control of the iris and ciliary body are demonstrated in Fig. 2C and G.

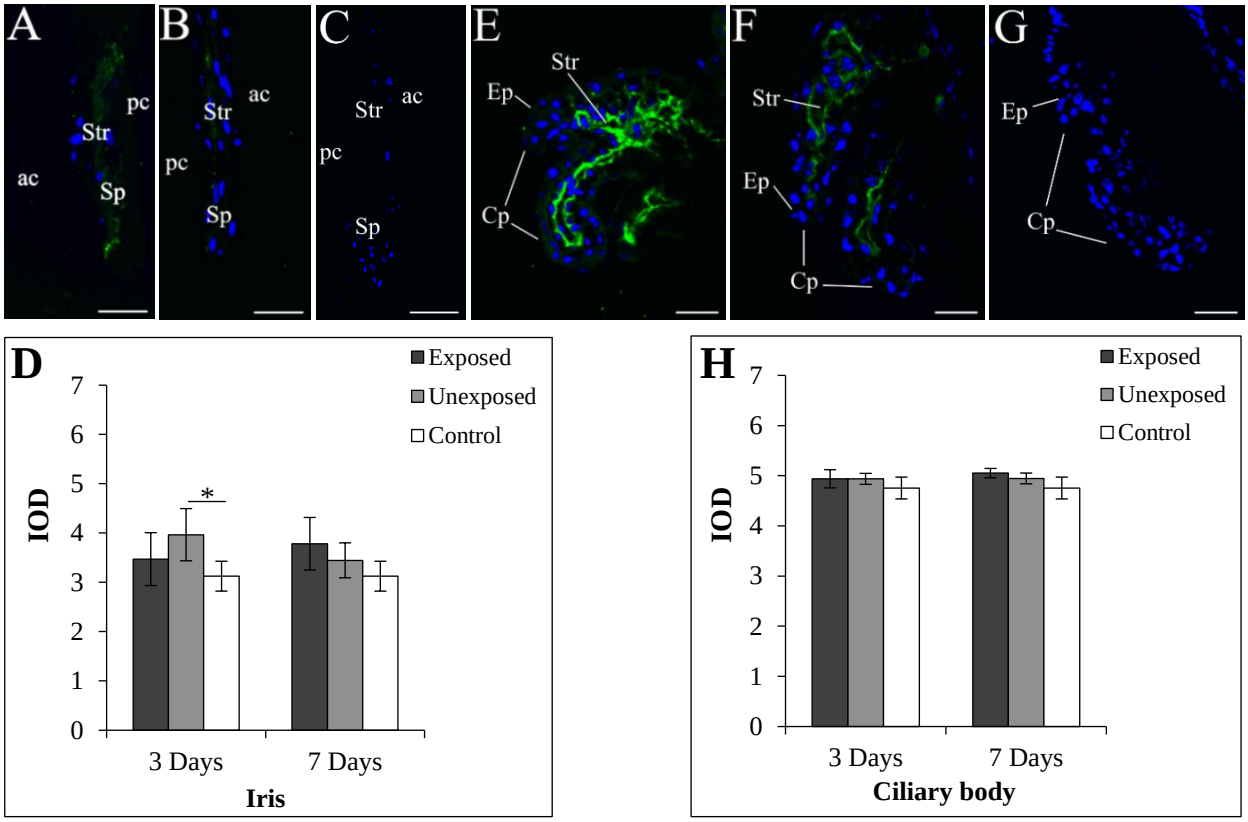


**Figure 2:** NKR-1 immunoreactivity and quantification in the iris and ciliary body of mouse eyes. NKR-1 staining was detected in the sphincter muscle of the contralateral iris at day 3 after UVR-B irradiation (**A**). Control eyes showed a decreased NKR-1 staining in the iris (**B**). The pigmented epithelium of the unexposed contralateral ciliary body displayed a strong NKR-1 staining (**E**), whereas control eyes showed a weaker NKR-1 expression (**F**). The negative controls of the fluorescence staining show no NKR-1 staining (**C**, **G**). Quantification of NKR-1 immunolabeling is expressed as IOD. The contralateral iris displays a significant increase of IOD at day 3 (**D**). IOD of the mouse ciliary body revealed no significant differences between unexposed eyes and the control group (*n* = 10 mice per latency period group; **H**). Error bars are 95% CI for the mean. Green: Substance P receptor (NKR-1). Blue: DAPI (nuclei staining). Scale bars = 25 µm. Ac, anterior chamber; pc, posterior

chamber; Sp, sphincter muscle; Ep, epithelium; Str, stroma; Cp, ciliary processes; IOD, integrated optical density.

*Lens epithelium*

The lens epithelium of the contralateral eyes had a regularly monolayered form, showed no structural abnormalities and a membrane-bound fluorescence staining of NKR-1 different to control eyes (Fig. 3A+B). The negative control of the lens epithelium is shown in Fig. 3C.

NKR-1 quantification in the lens epithelium displayed a slight trend towards an IOD increase of unexposed and exposed eyes at day 3, in comparison to the control group, but the difference was not statistically significant (Fig. 3D). At day 7 post UVR-B exposure, the unexposed eyes displayed almost the same IOD level as the control group, whereas the exposed eyes revealed an IOD increase compared to the control eyes. Both results were not statistically significant. Nevertheless, a significant difference was shown at day 7 between unexposed and exposed eyes ($P = 0.049$).

*Nuclear bow region of the lens*

In the nuclear bow region of the lens, epithelial cells of the unexposed partner eyes displayed an intense fluorescence staining of NKR-1 (Fig. 3E) that was stronger than in control eyes (Fig. 3F). Lens fibers showed no specific NKR-1 immunoreactivity in either group. The negative control of the nuclear bow region of the lens revealed no NKR-1 immunoreactivity (Fig. 3G). After UVR-B exposure the NKR-1 immunoreactivity of the unexposed lenses was significantly higher after 3 ($P = 0.006$) and 7 ($P = 0.002$) days compared to the control group (Fig. 3H). At day 3 and 7 post UVR-B irradiation, exposed eyes had a significantly increased IOD for NKR-1 compared to the control eyes (3 days $P = 0.024$; 7 days $P = 0.002$).

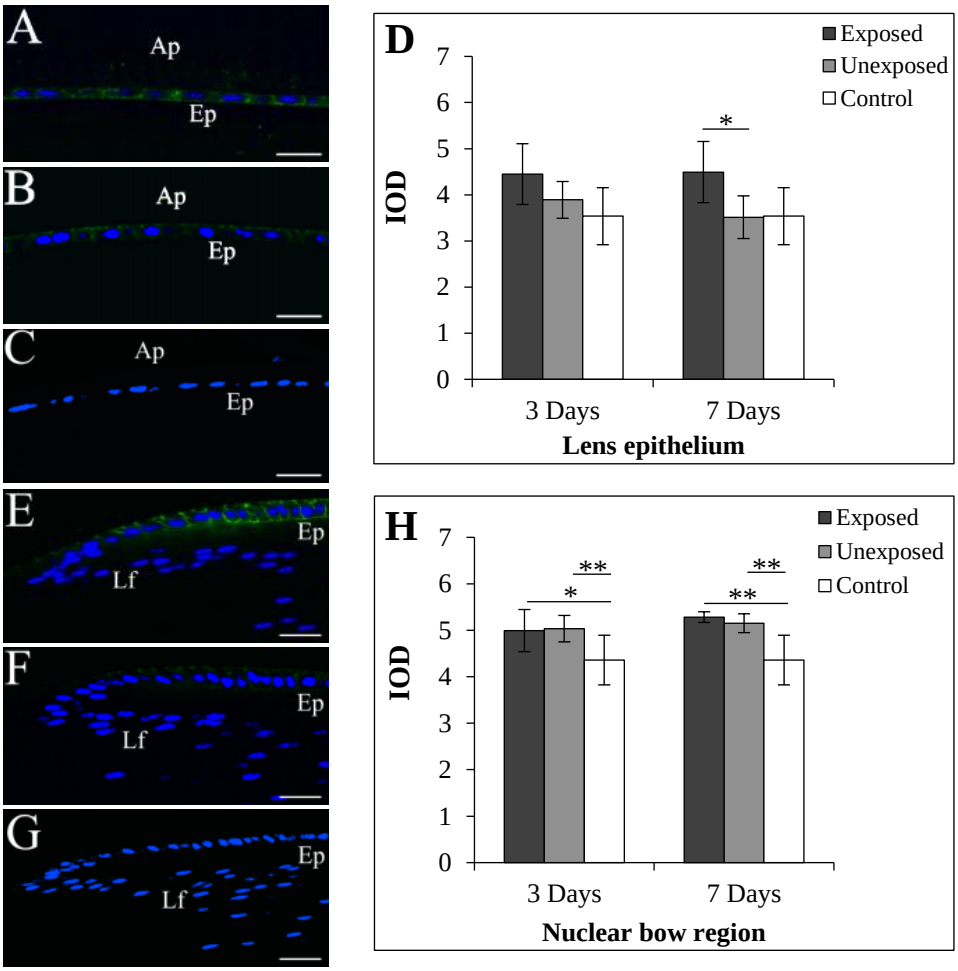


**Figure 3:** Positive NKR-1 immunostaining and quantification of NKR-1 expression in the lens. Photomicrographs show immunofluorescence staining of unexposed and control lens epithelium and nuclear bow region of the lens for NKR-1. Slight NKR-1 immunostaining was observed in the unexposed anterior lens epithelium located in the membrane after unilateral irradiation to UVRB (**A**). Milder NKR-1 immunostaining was detected in the lens epithelium of the control group which received no UVR-B irradiation (**B**). A strong NKR-1 immunostaining was detected in the nuclear bow region (also membrane labeled) of the contralateral eye, following UVR-B exposure (**E**). The nuclear bow region of control lenses exhibited a weak NKR-1 staining in the epithelial cell membrane (**F**). The negative controls are shown in (**C**, **G**). IOD was used to quantify NKR-1 immunolabeling. Three days post exposure IOD in the lens epithelium of contralateral mouse eyes is slightly increased compared to control but not statistically significant. A statistical difference was shown between unexposed and exposed eyes at day 7 (**D**). The nuclear bow region of the unexposed contralateral and exposed lens displays a significant increase of IOD at 3 as well as 7 days following UVR-B exposure as compared to the control group ($n$ = 10 mice per latency period group; **H**). Error bars are 95% CI for the mean. * $p < 0.05$, ** $p < 0.01$. Green: Substance P NKR-1. Blue: DAPI (nuclei staining). Scale bars = 25 µm. Ap, anterior pole; Ep, epithelium; Cap, lens capsule; Lf, lens fibers; IOD, integrated optical density.

*Retina*

In the unexposed retina of the partner eyes intensified NKR-1 fluorescence staining was localized in the inner plexiform layer and in cell bodies of the inner nuclear layer (Fig. 4A), compared to the control group (Fig. 4B). The highest NKR-1 density was found in the laminae 2 of the inner plexiform layer. Furthermore, mild NKR-1 immunostaining was also found in the proximal inner nuclear layer in amacrine cells. The ganglion cell layer revealed no specific NKR-1 immunostaining in none of the examined eyes. The negative control of the retina is demonstrated in Fig. 4C and showed no NKR-1 immunostaining.

As shown in Fig. 4D, NKR-1 expression is slightly increased in the unexposed partner eyes after day 3 and 7 following UVR-B treatment as compared to the control eyes but no statistical significance was observed. The quantitative analysis of NKR-1 immunostaining in the exposed retina revealed a significant increase 3 ($P = 0.021$) and 7 ($P = 0.002$) days after UVR-B exposure compared to the control group.

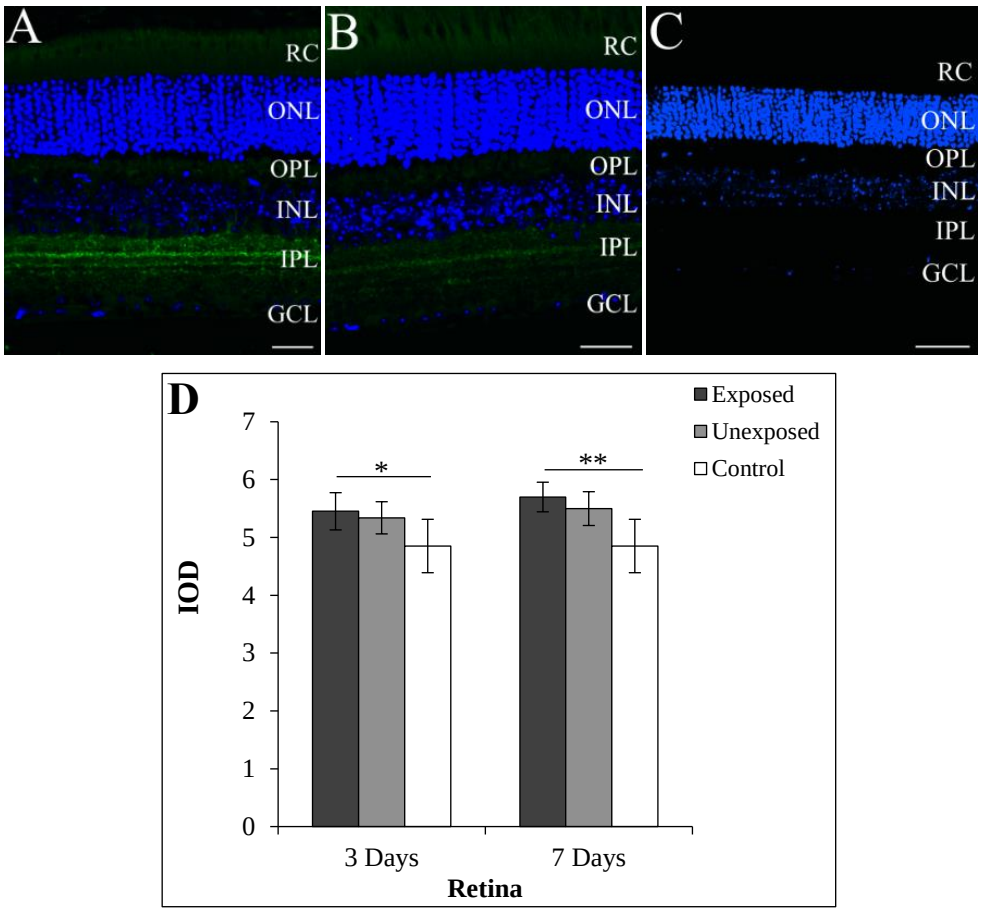


**Figure 4:** NKR-1 immunostaining and NKR-1 quantification of the mouse retina. NKR-1 immunostaining is localized in the inner plexiform layer (IPL) and the inner nuclear layer (INL) of the unexposed contralateral retina. NKR-1 immunolabeling was also found in the proximal INL in amacrine cells (**A**). The control retina showed a mild NKR-1 immunostaining in the IPL (**B**). The negative control revealed no NKR-1 immunostaining (**C**). A slight increase of IOD was observed at days 3 and 7 following UVR-B exposure in the unexposed retina in comparison to the control group. But a significant difference between these groups was not found. Significant differences were found between exposed and control retinae at days 3 and 7 ($n$ = 10 mice per latency period group; **D**). Green: Substance P NKR-1. Blue: DAPI (nuclei staining). Scale bars = 25 µm. Error bars show 95% CI for the mean. * $p < 0.05$, ** $p < 0.01$. RC, rods and cones; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

### 3.4.2 Neurokinin-1 receptor ELISA

To confirm the NKR-1 immunostaining results, and further quantify NKR-1 expression ELISA analysis was conducted to evaluate NKR-1 protein levels after UVR-B exposure in different ocular tissues of unexposed, exposed and control mouse eyes.

*Cornea*

The unexposed cornea revealed a trend of increased NKR-1 level at day 7, in contrast to the control group, but no significant difference was detected (Fig. 5A). Between un-exposed and exposed eyes, significant differences were detected at days 3 ($P = 0.013$) and 7 ($P = 0.032$). In the exposed cornea NKR-1 protein level was significantly increased at days 3 ($P = 0.006$) and 7 ($P \leq 0.000$) days following UVR-B exposure as compared to the control eyes. In addition, exposed eyes showed a significant difference between latency period groups of 3 and 7 days ($P \leq 0.000$).

*Iris/Ciliary body*

In the iris/ciliary body complex, unexposed contralateral eyes showed significantly higher NKR-1 protein levels at day 3 as compared to the control group ($P = 0.034$; Fig. 5B). Comparing unexposed mouse eyes to the exposed eyes, decreased NKR-1 levels at days 3 ($P = 0.005$) and 7 ($P = 0.012$) were shown. Furthermore, NKR-1 expression was significantly increased in the exposed iris/ciliary body complex at 3 ($P \leq 0.000$) and 7 ($P \leq 0.000$) days post UVR-B irradiation, when compared to the control eyes. In the exposed iris/ciliary body complex, significant NKR-1 increases were detected at day 3 post exposure, when compared to the latency period groups of 7 days ($P \leq 0.000$).

*Aqueous humor*

In the unexposed partner eyes and exposed eyes, no significant differences in NKR-1 levels were observed in the AqH at days 3 and 7 as compared to the control group (Fig. 5C).

*Lens epithelium*

Non-significant changes in NKR-1 protein levels were recorded, in unexposed and exposed eyes in comparison to the control group (Fig. 5D).

*Retina and Choroidea*

The unexposed contralateral retinae showed a slight change in NKR-1 protein level at day 7 (P =0.05) post UVR-B exposure as compared to the control group (Fig. 5E). In addition, contralateral retinae showed significant difference between days 3 and 7 (P = 0.014). Exposed retinae displayed no changes in NKR-1 levels compared to the control group. The contralateral choroidea revealed a significant increase of NKR-1 expression 7 days following exposure (P = 0.030) compared to the control as well as a significant difference between latency period groups of 3 and 7 days (P = 0.030; Fig. 5F). Exposed choroidea demonstrated a significantly difference of NKR-1 protein levels at day 3 (P = 0.024) and 7 (P = 0.008), compared to the control eyes.

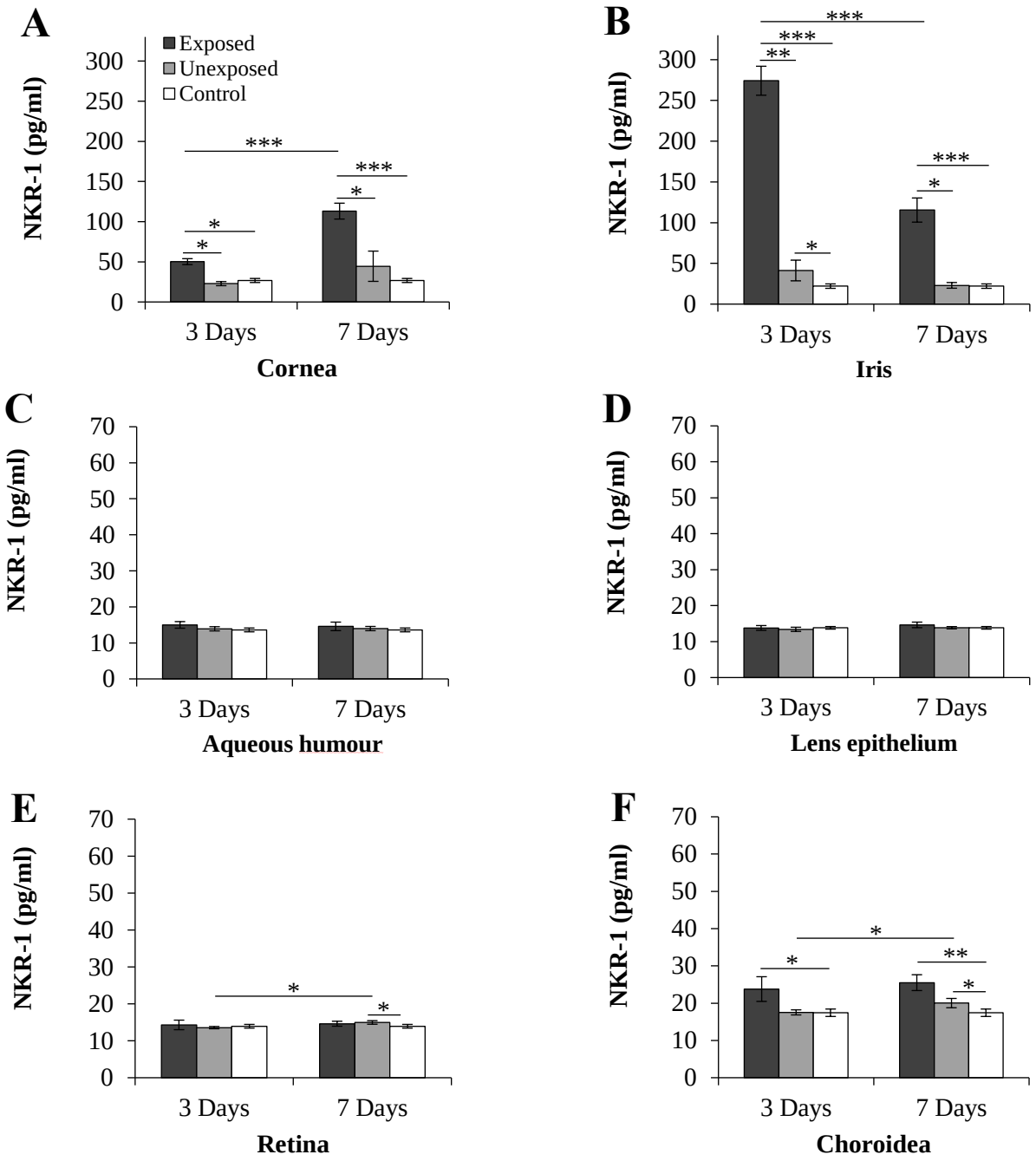


**Figure 5:** NKR-1 protein concentration in ocular tissues after unilateral UVR-B irradiation. Bar graphs demonstrate NKR-1 protein expression of unexposed, exposed, and control ocular tissues at days 3 and 7 post UVR-B exposure (**A–F**). Tissue lysates of the cornea (**a**), iris/ciliary body complex (**B**), AqH (**C**), lens epithelium (**D**), retina (**E**), and choroidea (**F**) were collected and the variations in NKR-1 protein expression were measured by ELISA. Each bar represents the 95% CI for the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n$ = 21 mice per latency period group. NKR-1, neurokinin- 1 receptor.

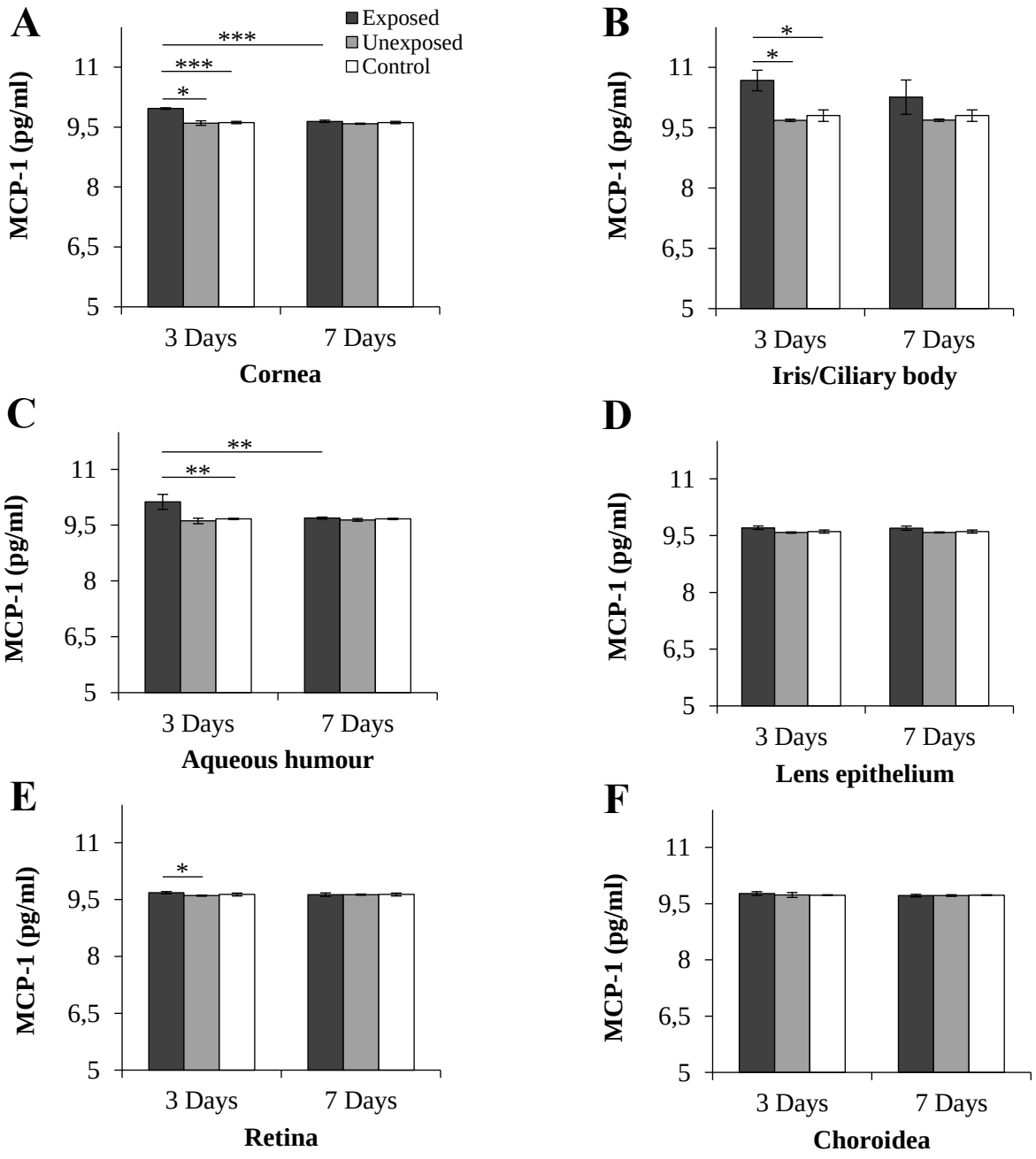


**Figure 6:** MCP-1 protein concentration in various ocular tissues after unilateral UVRB irradiation. Protein expression of MCP-1 is demonstrated in unexposed, exposed, and control ocular tissues at days 3 and 7 following UVR-B irradiation (**A–F**). Tissue lysates of the cornea (**A**), iris/ciliary body complex (**B**), AqH (**C**), lens epithelium (**D**), retina (**E**), and choroidea (**F**) were collected and the variations in MCP-1 protein concentration measured by ELISA. Each bar represents the 95% CI for the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n$ = 21 mice per latency period group. MCP-1, monocyte chemoattractant protein-1.

### 3.4.3 MCP-1 ELISA

To quantify protein expression of the pro-inflammatory chemokine MCP-1 and investigate if MCP-1 is affected by UVR-B exposure in the exposed or in the contralateral partner eye ELISA analysis was performed following unilateral UVR-B exposure.

*Cornea*

The unexposed contralateral cornea showed no difference of MCP-1 protein levels, when compared to the control group (Fig. 6A). A significant difference was demonstrated between unexposed and exposed eyes 3 days following UVR-B exposure ($P = 0.015$). Exposure to UVR-B induced a significant MCP-1 elevation in corneas of exposed eyes at day 3 post-exposure ($P \leq 0.000$) compared to control. A significant difference was also observed between day 3 and 7 of exposed eyes ($P \leq 0.000$).

*Iris/Ciliary body*

In the partner eyes of the iris/ciliary body, no significant difference was found as compared to the control (Fig. 6B). The difference between unexposed and exposed eyes at day 3 post exposure revealed statistical significance ($P = 0.024$). MCP-1 protein levels of the exposed iris/ciliary body complex displayed a significant increase at day 3 ($P = 0.014$) compared to the control eyes.

*Aqueous humor*

MCP-1 levels of the unexposed partner eyes revealed no significant differences compared to the control group (Fig. 6C). Exposed eyes showed significantly higher MCP-1 expression in the AqH at day 3 post exposure ($P = 0.004$) as compared to the control group. In the exposed eyes a significant difference was detected between 3 and 7 days post exposure ($P = 0.006$).

*Lens epithelium*

In the contralateral and exposed lens epithelium, no significant changes in MCP-1 protein levels were observed in comparison the control eyes (Fig. 6D).

*Retina and Choroidea*

Figure 6 E and F summarizes the MCP-1 protein levels of 3 and 7 days post UVR-B irradiation in the unexposed, exposed and control retinae and choroidea. Unexposed and exposed retinae revealed a significant difference at day 3 after UVR-B exposure ($P = 0.027$; Fig. 6E). There were no differences in MCP-1 levels between unexposed, exposed and control choroidea (Fig. 6F).

## 3.5 Discussion

In the present study, we evaluate whether in vivo UVRB irradiation affects NKR-1 and MCP-1 levels not only in the exposed but also in the unexposed, partner eye.

Most eye diseases are of bilateral nature. Age-related macular degeneration and cataract occur predominantly in both eyes. However, very little is known how the right and left eye are immunologically connected. A so far unexplained, but devastating contralateral effect after damage to 1 eye occurs in sympathetic ophthalmitis, a prototypical autoimmune disease in which perforating injury to 1 eye causes sight-threatening uveitic inflammation in the otherwise normal contralateral eye [26]. Unfortunately, the molecular mechanisms underlying sympathizing ocular diseases affecting both eyes in an acute or chronic pattern remain unclear. The verification of a neuropeptide dependent-signaling pathway to the contralateral eye after insult to only 1 eye is thus of high clinical relevance because it could open up new treatment strategies and preventive measures for numerous eye diseases.

### 3.5.1 NKR-1 expression and upregulation in the unexposed, partner eye

Because substance P has a very short half-life in tissues and therefore is very difficult to measure, we examined the SP receptor, NKR-1 in ocular tissues after UVR-B irradiation [27–29]. SP modulates its actions by binding to the G-protein-coupled NK-1 receptor and is secreted by neuronal and non-neuronal inflammatory cells, such as lymphocytes or eosinophils [27]. The interaction of SP with NKR-1 is related to several intracellular pathways, including apoptosis [30–32], cell proliferation [27, 33, 34], inflammation [14, 35–37], stimulation of the production of pro-inflammatory cytokines and chemokines, such as TNF-α or MCP-1 [27, 36, 38].

The expression and upregulation of SP and its receptor, NKR-1, has been described in several inflammatory eye diseases, including pterygium [39], uveitis [40], microbial kera-

titis [14, 32, 38], and herpes stromal keratitis [33, 37]. Furthermore, a few studies have documented a correlation between SP and NKR-1 in UVR-B-induced inflammation in the skin. For example, repeated sub-inflammatory doses of UVR-B irradiation can influence the cutaneous neurosensory system in rats [41]. The authors demonstrated that exposure to UVR-B significantly increased substance P content in the skin, suggesting that UVR-B irradiation locally increases SP. These results are also in line with previous studies concerning the evaluation of narrow-band UVR-B therapy on substance P in psoriasis vulgaris patients [42]. The authors found a significant higher concentration of SP in psoriasis patients compared to the control group. Seike et al. [43] examined the role of calcitonin gene-related peptide in the UVR-B induced proliferation of BALB/c mice keratinocytes. Interestingly, in this experimental study with mice, the expression of substance P was not induced by UVR-B irradiation, indicating that substance P does not take part in the UVR-B-induced epidermal cell proliferation [43]. Although substance P was observed in different skin diseases following irradiation to UVR-B, a link between SP/ NKR-1 and UVR-B in eye diseases remained so far unknown. We observed recently that NKR-1 expression is upregulated in exposed ocular tissues in a UVR-B-induced cataract model [17].

This study is the first report of a contralateral upregulation of NKR-1 in a part of the ocular tissue in the unexposed, partner eye after exposure of only 1 eye to UVR-B, suggesting a role for NKR-1 in the communication between both eyes. One explanation for the bilateral NKR-1 expression after unilateral UVR-B exposure is that proinflammatory peptides such as substance P might relay signals from the UVR-B exposed eye to the unexposed partner eye in a systemic manner. Although both methods are used to detect NKR-1 in ocular tissues after unilateral UVR-B exposure, but they do not always give identical results and can therefore not be equated. The immunofluorescence staining as a subjective method is quantified by the IOD measuring the relative fluorescence intensity of NKR-1 in paraffin sections of the mouse eye. Whereby with the ELISA method the NKR-1 protein concentration could be determined specifically in each ocular tissue, with the dis-advantage that a lower sample volume of the tissue probably also leads to a lower detection of NKR-1 protein level.

Immunohistochemical analysis demonstrated a slight tendency of NKR-1 upregulation in the examined ocular tissues of the unexposed partner eyes. A statistically significant difference could be detected in the cornea, iris, and in the nuclear bow region of the lens.

The involvement of substance P in the contralateral sympathizing reaction is supported by a published bilateral loss of immune privilege after unilateral retinal laser burn in a mouse model [44]. Here the bilateral loss of immune privilege correlated with an increase of NKR-1, which was first seen in the exposed and later in the contralateral eye. Lucas et al. [44] assumed that the loss of immune privilege and the increase of NKR-1 lead to the recruitment of cells from the periphery and the induction of inflammatory changes in the ocular environment of both eyes. The group showed that the occurrence of a contralateral inflammatory effect might depend on the immune privilege disturbing function of SP since antagonizing NKR-1 led to the maintenance of the immune privilege. In this case, no inflammatory signs in the contralateral eye occurred [44]. A third confirmation of a sympathetic response to the partner eye via the SP signaling pathway is the study by Paunicka et al. [45] The authors demonstrated that severing corneal nerves of 1 eye leads to an increase of substance P bilaterally which led to the loss of immune privilege in both eyes. In our model, NKR-1 ELISA investigations showed an immediate response in the exposed iris/ciliary body complex at day 3 post exposure, with an NKR-1 upregulation (Fig. 5b). Although the exposed cornea is the first tissue hit by UVR-B, in this ocular surface tissue NKR-1 upregulation was delayed until day 7 after exposure (Fig. 5a). A possible explanation is the slow metabolism of the vessel-free cornea and the immediate epithelial damage with the loss of epithelial cells following UVR exposure. ELISA quantification also showed a NKR-1 upregulation in the retina and choroidea of the unexposed, contralateral eyes. This may indicate that the uveal tissue is involved in transferring the inflammatory signal to the contralateral eye. Even though we expected but did not find an upregulation of NKR-1 in the exposed as well as in the unexposed AqH and in the central lens epithelium, we assume that this result is related to the low sample volume, impeding the analysis of the aqueous and lens epithelium. After UVR-B irradiation, the lens epithelium is severely damaged with a partial up to subtotal loss of epithelial cells [17]. Therefore, the exposed and unexposed contralateral lens epithelium might have showed less NKR-1 expression due to the small sample volume.

Our results, demonstrating a NKR-1 protein expression after unilateral UVR-B exposure not only in the exposed eye but also in the unexposed partner eye, are in line with a study showing a bilateral NKR-1 expression in a muscle injury and inflammation model [46]. Herein, the authors demonstrated a bilateral upregulation of NKR-1 as well as a bilateral

increase in SP levels measured with EIA analysis, suggesting that the system has cross-over effects [46].

### 3.5.2 MCP-1 expression and upregulation after UVR-B exposure

We investigated MCP-1 protein expression in unexposed and exposed eyes following unilateral UVR-B irradiation using ELISA in a mouse model. We observed a significant MCP-1 upregulation in the exposed cornea, iris/ciliary body complex and AqH after UVR-B irradiation (Fig. 6a–c). However, an upregulation of MCP-1 protein levels could not be determined in the unexposed partner eyes. The small pooled sample volume of the AqH and lens epithelium must be considered in both the NKR- 1 and MCP-1 analysis. The fact that we were able to show a significant increase of MCP-1 in the relatively small aqueous sample but not for NKR-1 indicates a stronger increase of MCP-1 following UVR-B in relation to NKR- 1 in the aqueous. Therefore, we assume that the pro-inflammatory chemokine, MCP-1 is upregulated stronger than the substance P receptor NK-1 after UVR-B-induced inflammation in the AqH and becomes detectable even in a small sample volume. The possible increase of the mainly tissue-bound NKR-1 in the aqueous sample remains below the detection level.

Similar MCP-1 amounts in different ocular compartments can be explained by the relatively low absolute values of MCP-1, which is close to the minimum detection level. In addition, to verify our data we conducted a second MCP-1 ELISA which provided identical MCP-1 values as in the first experiment (data not shown). The only markable difference was a significant MCP-1 upregulation in the choroidea of exposed eyes following 3 days after unilateral UVR-B exposure. The contralateral unexposed choroidea remained as in the current experiment unaffected.

Our results are in line with previous studies on the inflammatory effect of UVR-B focused on cytokines, including increased MCP-1 expression following UVR-B irradiation in human keratinocytes of mice skin [47–49]. For example, Matsui et al. [49] demonstrated increased MCP-1 mRNA levels in ovarian adipose tissues as well as in the serum after UVR-B irradiation in a mouse model. In general, there are few reports on UVR-B-induced expression of pro-inflammatory cytokines, such as MCP- 1. However, there is no evidence of UVR-B-induced MCP-1 expression in ocular tissues. Thus, to our knowledge this is the

first study illustrating MCP-1 expression and upregulation in the exposed mouse eye following different time points after UVR-B irradiation.

Previous studies have demonstrated an association of elevated MCP-1 level in the aqueous of various ocular diseases, including ocular surface diseases, cataract [20, 50–52], and uveitis [53–55]. For example, in a rat model MCP-1 protein levels were significantly elevated in experimental autoimmune uveitis of the AqH [18, 53]. Recently, in a clinical study diverse cytokine levels were investigated in aqueous of eyes with ocular surface diseases, comprising exposure keratitis case as well as chemical and thermal burn [20]. Cytokine levels, such as MCP- 1, in the aqueous were significantly increased in eyes with ocular surface diseases in comparison to the controls. The authors proposed that the elevated cytokine levels in the aqueous are linked with the increase of immune cells in the cornea and conjunctiva in the ocular surface diseases [20]. This suggestion is supported by the findings of several studies showing that elevated MCP-1 levels in the aqueous are associated with an increased number of monocytes in ocular tissue and an aggravation of inflammatory processes in different types of uveitis and retinal ischemia [56–58]. These reports are in line with our results detecting increased MCP-1 expression in the aqueous, cornea, and iris/ciliary body complex of exposed mouse eyes 3 days post UVR-B exposure. The increase of MCP-1 in exposed eyes might be indicative of a pro-inflammatory ocular environment. MCP-1 is secreted in a great variety of cells, including endothelial cells, monocytes, macrophages, and other cell types mediate the cell influx to inflammation [59]. There are some reports indicating that SP stimulates MCP-1 secretion from mast cells, assuming that MCP-1 might be secreted by inflammatory cell types such as mast cells after unilateral UVRB exposure [60, 61].

However, on day 7 after UVR-B exposure MCP-1 levels had decreased on the level of the control group, suggesting that MCP-1 increases fast and is not involved in UVR-B induced inflammation after a time period of 7 days post exposure. Kawai et al. [21] showed for the aqueous in human and rabbit eyes that increased MCP-1 levels occur within 24 h after cataract surgery (phacoemulsification) and then return to near basal levels within several days. This observation is in line with our results, finding no increase of MCP-1 in ocular tissues 7 days post exposure. This study provides evidence that UVR-B-induced lens opacities correlate with increased MCP-1 protein levels in exposed ocular tissues after a time period of 3 days post exposure. Further objective of this study was to investigate if

MCP-1 is involved in UVR-B-induced contralateral, inflammatory effects. In relation to MCP-1, a sympathetic effect could be observed in a clinical study, detecting a significant increase of MCP-1 in the AqH in the contralateral non-operated eye of patients that underwent cataract surgery in the first eye [22]. The authors suggested an MCP-1-mediated sympathetic ophthalmic type uveitis in the contralateral eye and indicated the induction of an inflammatory status in the contralateral eye by cataract surgery in the first eye [22]. However, in the present study a significant increase of MCP-1 levels in the unexposed, contralateral eyes could not be detected in the examined ocular tissues. This suggests that MCP-1 is not involved in the immunological cross-talk between exposed and unexposed eyes following UVR-B irradiation in our cataract model. However, little is known about the functions of many other pro-inflammatory cytokines such as IL-1, IL-6 or TNF-α so that further cytokines might play a role in the ocular cross-talk.

### 3.6 Conclusion

In conclusion, pro-inflammatory neuropeptides and cytokines, such as substance P and MCP-1, play key roles in regulating different ocular diseases. We demonstrated for the first time a sympathizing upregulation of NKR-1 in various ocular tissues of the unexposed partner eye after unilateral UVR-B irradiation. These new results should be considered in relation to the mentioned ocular pathologies, especially with regard to cataractogenesis and other eye diseases such as sympathizing ophthalmitis but also age-related macular degeneration.

### 3.7 Acknowledgement

The authors thank Claudine Strack and Parand Widmar for expert help with the immunohistochemistry. We also thank the laboratory group for helpful discussions.

### 3.8 Statement of Ethics

All animal experiments adhered to the Association of Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by local and national official regulations (Landesamt fur Natur, Umwelt und Verbraucherschutz, North Rhine-Westphalia, Germany; accession number: 84-02.04.2015.A 154).

### 3.9 Disclosure Statement

The author, Prof. Frank G. Holz, received financial support from Acucela, Allergan, Bayer, Bioeq/Formycon, CenterVue, Roche/Genentech, Heidelberg Engineering, NightStarX, Novartis, Optos, Pixium Vision and Zeiss. Furthermore, the author consults for Acucela, Apellis, Bayer, Boehringer-Ingelheim, Bioeq/Formy-con, Galimedix, Roche/Genentech, Geuder, Grayburg Vision, Heidelberg Engineering, LinBioscience, Novartis, Pixium Vision, Oxurion and Stealth Bio Therapeutics. The author is also a recipient of gifts, honoraria, travel reimbursement, patent royalties, or any other financial compensation of Acucela, Allergan, Apellis, Bayer, Ellex, Roche/Genentech, Grayburg Vision, Heidelberg Engineering, LinBioscience, Novartis, Pixium Vision, Oxurion, Stealth Bio Therapeutics and Zeiss. The other authors of this publication have no conflicts of interest to declare.

### 3.10 Funding Sources

This work was supported by the German Research Foundation “DFG” (ME 3298/4-1), (WE 1303/6-1).

### 3.11 Author Contributions

J.G., E.W., A.R.W., M.K., C.-L.S., F.G. H., and L.M.M.: substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work. J.G., E.W., A.R.W., M.K., C.-L.S., F.G.H., and L.M.M.: drafting the article. J.G., E.W., A.R.W., M.K., C.-L.S., F.G.H., and L.M.M.: final approval of the version to be published. J.G., E.W., A.R.W., M.K., C.-L.S., F.G.H., and L.M.M.: agreement to be accountable for all aspects of the work including questions related to the accuracy.

### 3.12 References

1 Di Girolamo N, Wakefield D, Coroneo MT. UVB-mediated induction of cytokines and growth factors in pterygium epithelial cells involves cell surface receptors and intracellular signaling. Invest Ophthalmol Vis Sci. 2006 Jun; 47(6): 2430–7.

2 Zidi S, Bediar-Boulaneb F, Belguendouz H, Belkhelfa M, Medjeber O, Laouar O, et al. Local pro-inflammatory cytokine and nitric oxide responses are elevated in patients with pterygium. Int J Immunopathol Pharmacol. 2017 Dec; 30(4): 395–405.

3 Cejkova J, Ardan T, Cejka C, Luyckx J. Favorable effects of trehalose on the development of UVB-mediated antioxidant/pro-oxidant imbalance in the corneal epithelium, proinflammatory cytokine and matrix metalloproteinase induction, and heat shock protein 70 expression. Graefes Arch Clin Exp Ophthalmol. 2011 Aug; 249(8): 1185–94.

4 Meyer LM, Lofgren S, Holz FG, Wegener A, Soderberg P. Bilateral cataract induced by unilateral UVR-B exposure – evidence for an inflammatory response. Acta Ophthalmol. 2013 May; 91(3): 236–42.

5 Nolan TM, DiGirolamo N, Sachdev NH, Hampartzoumian T, Coroneo MT, Wakefield D. The role of ultraviolet irradiation and heparin- binding epidermal growth factor-like growth factor in the pathogenesis of pterygium. Am J Pathol. 2003 Feb; 162(2): 567–74.

6 Di Girolamo N, Kumar RK, Coroneo MT, Wakefield D. UVB-mediated induction of interleukin- 6 and -8 in pterygia and cultured human pterygium epithelial cells. Invest Ophthalmol Vis Sci. 2002 Nov; 43(11): 3430–7.

7 Kennedy M, Kim KH, Harten B, Brown J, Planck S, Meshul C, et al. Ultraviolet irradiation induces the production of multiple cytokines by human corneal cells. Invest Ophthalmol Vis Sci. 1997 Nov; 38(12): 2483–91.

8 Pauloin T, Dutot M, Joly F, Warnet JM, Rat P. High molecular weight hyaluronan decreases UVB-induced apoptosis and inflammation in human epithelial corneal cells. Mol Vis. 2009; 15: 577–83.

9 Tessem MB, Bathen TF, Lofgren S, Saether O, Mody V, Meyer L, et al. Biological response in various compartments of the rat lens after in vivo exposure to UVR-B analyzed by HRMAS 1H NMR spectroscopy. Invest Ophthalmol Vis Sci. 2006 Dec; 47(12): 5404–11.

10 Wang Y, Li F, Zhang G, Kang L, Guan H. Ultraviolet- B induces ERCC6 repression in lens epithelium cells of age-related nuclear cataract through coordinated DNA hypermethylation and histone deacetylation. Clin Epigenetics. 2016 May; 8(1): 62.

11 Galichanin K. Exposure to subthreshold dose of UVR-B induces apoptosis in the lens epithelial cells and does not in the lens cortical fibre cells. Acta Ophthalmol. 2017 Dec; 95(8): 834–8.

12 Ogasawara S, Hashizume K, Okuno T, Imaizumi T, Inomata Y, Tezuka Y, et al. Effect of Geranylgeranylacetone on Ultraviolet Radiation Type B-Induced Cataract in Heat-Shock Transcription Factor 1 Heterozygous Mouse. Curr Eye Res. 2017 May; 42(5): 732–7.

13 Pascolini D, Mariotti SP. Global estimates of visual impairment: 2010. Br J Ophthalmol. 2012 May; 96(5): 614–8.

14 Jiang X, McClellan SA, Barrett R, Foldenauer M, Hazlett LD. HGF signaling impacts severity of Pseudomonas aeruginosa keratitis. Invest Ophthalmol Vis Sci. 2014 Apr; 55(4): 2180–90.

15 Ji Y, Cai L, Zheng T, Ye H, Rong X, Rao J, et al. The mechanism of UVB irradiation induced- apoptosis in cataract. Mol Cell Biochem. 2015 Mar; 401(1-2): 87–95.

16 Kronschlager M, Talebizadeh N, Yu Z, Meyer LM, Lofgren S. Apoptosis in Rat Cornea After In Vivo Exposure to Ultraviolet Radiation at 300 nm. Cornea. 2015 Aug; 34(8): 945–9.

17 Gross J, Wegener AR, Kronschlager M, Holz FG, Schonfeld CL, Meyer LM. Ultraviolet radiation exposure triggers neurokinin-1 receptor upregulation in ocular tissues in vivo. Exp Eye Res. 2018 Sep; 174: 70–9.

18 Fang IM, Yang CH, Lin CP, Yang CM, Chen MS. Expression of chemokine and receptors in Lewis rats with experimental autoimmune anterior uveitis. Exp Eye Res. 2004 Jun; 78(6): 1043–55.

19 Lee SK, Choi BK, Kang WJ, Kim YH, Park HY, Kim KH, et al. MCP-1 derived from stromal keratocyte induces corneal infiltration of CD4+ T cells in herpetic stromal keratitis. Mol Cells. 2008 Jul; 26(1): 67–73.

20 Aketa N, Yamaguchi T, Asato T, Yagi-Yaguchi Y, Suzuki T, Higa K, et al. Elevated Aqueous Cytokine Levels in Eyes With Ocular Surface Diseases. Am J Ophthalmol. 2017 Dec; 184: 42–51.

21 Kawai M, Inoue T, Inatani M, Tsuboi N, Shobayashi K, Matsukawa A, et al. Elevated levels of monocyte chemoattractant protein-1 in the aqueous humor after phacoemulsification. Invest Ophthalmol Vis Sci. 2012 Dec; 53(13): 7951–60.

22 Zhu XJ, Wolff D, Zhang KK, He WW, Sun XH, Lu Y, et al. Molecular Inflammation in the Contralateral Eye After Cataract Surgery in the First Eye. Invest Ophthalmol Vis Sci. 2015 Aug; 56(9): 5566–73.

23 Zhu X, Zhang K, He W, Yang J, Sun X, Jiang C, et al. Proinflammatory status in the aqueous humor of high myopic cataract eyes. Exp Eye Res. 2016 Jan; 142: 13–8.

24 Soderberg PG, Lofgren S, Ayala M, Dong X, Kakar M, Mody V. Toxicity of ultraviolet radiation exposure to the lens expressed by maximum tolerable dose. Dev Ophthalmol. 2002; 35: 70–5.

25 Meyer LM, Dong X, Wegener A, Soderberg P. Light scattering in the C57BL/6 mouse lens. Acta Ophthalmol Scand. 2007 Mar; 85(2): 178–82.

26 Damico FM, Kiss S, Young LH. Sympathetic ophthalmia. Semin Ophthalmol. 2005 Jul- Sep; 20(3): 191–7.

27 Mashaghi A, Marmalidou A, Tehrani M, Grace PM, Pothoulakis C, Dana R. Neuropeptide substance P and the immune response. Cell Mol Life Sci. 2016 Nov; 73(22): 4249–64.

28 McGregor GP, Bloom SR. Radioimmunoassay of substance P and its stability in tissue. Life Sci. 1983 Feb; 32(6): 655–62.

29 Skidgel RA, Engelbrecht S, Johnson AR, Erdos EG. Hydrolysis of substance p and neurotensin by converting enzyme and neutral endopeptidase. Peptides. 1984 Jul-Aug; 5(4): 769–76.

30 Baek SM, Yu SY, Son Y, Hong HS. Substance P promotes the recovery of oxidative stress damaged retinal pigmented epithelial cells by modulating Akt/GSK-3β signaling. Mol Vis. 2016 Aug; 22: 1015–23.

31 Yang L, Sui W, Li Y, Qi X, Wang Y, Zhou Q, et al. Substance P Inhibits Hyperosmotic Stress-Induced Apoptosis in Corneal Epithelial Cells through the Mechanism of Akt Activation and Reactive Oxygen Species Scavenging via the Neurokinin-1 Receptor. PLoS One. 2016 Feb; 11(2):e0149865.

32 Zhou Z, Barrett RP, McClellan SA, Zhang Y, Szliter EA, van Rooijen N, et al. Substance P delays apoptosis, enhancing keratitis after Pseudomonas aeruginosa infection. Invest Ophthalmol Vis Sci. 2008 Oct; 49(10): 4458– 67.

33 Gaddipati S, Rao P, Jerome AD, Burugula BB, Gerard NP, Suvas S. Loss of Neurokinin-1 Receptor Alters Ocular Surface Homeostasis and Promotes an Early Development of Herpes Stromal Keratitis. J Immunol. 2016 Nov; 197(10): 4021–33.

34 Mei G, Xia L, Zhou J, Zhang Y, Tuo Y, Fu S, et al. Neuropeptide SP activates the WNT signal transduction pathway and enhances the proliferation of bone marrow stromal stem cells. Cell Biol Int. 2013 Nov; 37(11): 1225–32.

35 Hong HS, Kim S, Nam S, Um J, Kim YH, Son Y. Effect of substance P on recovery from laser- induced retinal degeneration. Wound Repair Regen. 2015 Mar-Apr; 23(2): 268–77.

36 Suvas S. Role of Substance P Neuropeptide in Inflammation, Wound Healing, and Tissue Homeostasis. J Immunol. 2017 Sep; 199(5): 1543–52.

37 Yun H, Lathrop KL, Hendricks RL. A Central Role for Sympathetic Nerves in Herpes Stromal Keratitis in Mice. Invest Ophthalmol Vis Sci. 2016 Apr; 57(4): 1749–56.

38 Foldenauer ME, McClellan SA, Barrett RP, Zhang Y, Hazlett LD. Substance P affects growth factors in Pseudomonas aeruginosa infected mouse cornea. Cornea. 2012 Oct; 31(10): 1176–88.

39 Chui J, Di Girolamo N, Coroneo MT, Wakefield D. The role of substance P in the pathogenesis of pterygia. Invest Ophthalmol Vis Sci. 2007 Oct; 48(10): 4482–9.

40 Aki T, Karincaoglu Y, Seyhan M, Batcioglu K. Serum substance P and calcitonin gene-related peptide levels in Behcet's disease and their association with disease activity. Clin Exp Dermatol. 2006 Jul; 31(4): 583–7.

41 Legat FJ, Griesbacher T, Schicho R, Althuber P, Schuligoi R, Kerl H, et al. Repeated subinflammatory ultraviolet B irradiation increases substance P and calcitonin gene-related peptide content and augments mustard oil-induced neurogenic inflammation in the skin of rats. Neurosci Lett. 2002 Sep; 329(3): 309–13.

42 Narbutt J, Olejniczak I, Sobolewska-Sztychny D, Sysa-Jedrzejowska A, Słowik-Kwiatkowska I, Hawro T, et al. Narrow band ultraviolet B irradiations cause alteration in interleukin-31 serum level in psoriatic patients. Arch Dermatol Res. 2013 Apr; 305(3): 191–5.

43 Seike M, Ikeda M, Morimoto A, Matsumoto M, Kodama H. Increased synthesis of calcitonin gene-related peptide stimulates keratinocyte proliferation in murine UVB-irradiated skin. J Dermatol Sci. 2002 Feb; 28(2): 135–43.

44 Lucas K, Karamichos D, Mathew R, Zieske JD, Stein-Streilein J. Retinal laser burn-induced neuropathy leads to substance P-dependent loss of ocular immune privilege. J Immunol. 2012 Aug; 189(3): 1237–42.

45 Paunicka KJ, Mellon J, Robertson D, Petroll M, Brown JR, Niederkorn JY. Severing corneal nerves in one eye induces sympathetic loss of immune privilege and promotes rejection of future corneal allografts placed in either eye. Am J Transplant. 2015 Jun; 15(6): 1490–501.

46 Song Y, Stal PS, Yu J, Forsgren S. Marked Effects of Tachykinin in Myositis Both in the Experimental Side and Contralaterally: Studies on NK-1 Receptor Expressions in an Animal Model. ISRN Inflamm. 2013 Jan; 2013: 907821.

47 Kang JS, Kim HN, Jung DJ, Kim JE, Mun GH, Kim YS, et al. Regulation of UVB-induced IL-8 and MCP-1 production in skin keratinocytes by increasing vitamin C uptake via the redistribution of SVCT-1 from the cytosol to the membrane. J Invest Dermatol. 2007 Mar; 127(3): 698–706.

48 Tang SC, Liao PY, Hung SJ, Ge JS, Chen SM, Lai JC, et al. Topical application of glycolic acid suppresses the UVB induced IL-6, IL-8, MCP-1 and COX-2 inflammation by modulating NF-κB signaling pathway in keratinocytes and mice skin. J Dermatol Sci. 2017 Jun; 86(3): 238–48.

49 Matsui S, Yamane T, Kobayashi-Hattori K, Oishi Y. Ultraviolet B irradiation reduces the expression of adiponectin in ovarial adipose tissues through endocrine actions of calcitonin gene-related peptide-induced serum amyloid A. PLoS One. 2014 May; 9(5):e98040.

50 Chen W, Lin H, Zhong X, Liu Z, Geng Y, Xie C, et al. Discrepant expression of cytokines in inflammation- and age-related cataract patients. PLoS One. 2014 Oct; 9(10):e109647.

51 Chu L, Wang B, Xu B, Dong N. Aqueous cytokines as predictors of macular edema in non-diabetic patients following uncomplicated phacoemulsification cataract surgery. Mol Vis. 2013 Nov; 19: 2418–25.

52 Sauer A, Bourcier T, Gaucher D, Candolfi E, Speeg-Schatz C. Intraocular cytokines imbalance in congenital cataract and its impact on posterior capsule opacification. Graefes Arch Clin Exp Ophthalmol. 2016 May; 254(5): 1013–8.

53 Pepple KL, Rotkis L, Van Grol J, Wilson L, Sandt A, Lam DL, et al. Primed Mycobacterial Uveitis (PMU): Histologic and Cytokine Characterization of a Model of Uveitis in Rats. Invest Ophthalmol Vis Sci. 2015 Dec; 56(13): 8438–48.

54 Fazil Z, Ten Berge JC, Langerak AW, Rothova A, Dik WA. An Intraocular Inflammatory Profile of Rubella Associated Uveitis. Ocul Immunol Inflamm. 2019; 27(3): 418–23.

55 Ohira S, Inoue T, Iwao K, Takahashi E, Tanihara H. Factors Influencing Aqueous Proinflammatory Cytokines and Growth Factors in Uveitic Glaucoma. PLoS One. 2016 Jan; 11(1):e0147080.

56 Adamus G, Manczak M, Machnicki M. Expression of CC chemokines and their receptors in the eye in autoimmune anterior uveitis associated with EAE. Invest Ophthalmol Vis Sci. 2001 Nov; 42(12): 2894–903.

57 Yoshida S, Yoshida A, Ishibashi T, Elner SG, Elner VM. Role of MCP-1 and MIP-1alpha in retinal neovascularization during postischemic inflammation in a mouse model of retinal neovascularization. J Leukoc Biol. 2003 Jan; 73(1): 137–44.

58 Yoshida Y, Yamagishi S, Matsui T, Nakamura K, Imaizumi T, Yoshimura K, et al. Positive correlation between pigment epithelium-derived factor and monocyte chemoattractant protein-1 levels in the aqueous humour of patients with uveitis. Br J Ophthalmol. 2007 Jun; 91(6): 737–8.

59 Melgarejo E, Medina MA, Sanchez-Jimenez F, Urdiales JL. Monocyte chemoattractant protein-1: a key mediator in inflammatory processes. Int J Biochem Cell Biol. 2009 May; 41(5): 998–1001.

60 Spitsin S, Meshki J, Winters A, Tuluc F, Benton TD, Douglas SD. Substance P-mediated chemokine production promotes monocyte migration. J Leukoc Biol. 2017 Apr; 101(4): 967–73.

61 Castellani ML, Vecchiet J, Salini V, Conti P, Theoharides TC, Caraffa A, et al. Stimulation of CCL2 (MCP-1) and CCL2 mRNA by substance P in LAD2 human mast cells. Transl Res. 2009 Jul; 154(1): 27–33.

# 4. UVR-B-induced NKR-1 expression in ocular tissues is blocked by substance P receptor antagonist Fosaprepitant in the exposed as well as unexposed partner eye

Janine Gross[a], Alfred R. Wegener[a], Martin Kronschläger[b], Carl-Ludwig Schönfeld[c,d], Frank G. Holz[a], Linda M. Meyer[a,c]

[a] Department of Ophthalmology, University of Bonn, Bonn, Germany
[b] Department of Ophthalmology, Hanusch Hospital, Vienna, Austria
[c] Herzog Carl Theodor Eye Clinic, Munich, Germany
[d] Department of Ophthalmology, Ludwig-Maximilians University, Munich, Germany

Ocular immunology and inflammation
February 2020, Page 1-13
DOI:10.1080/09273948.2019.1708414

### 4.1 Abstract

**Purpose:** To investigate the effect of NKR-1 antagonists in an established UVR-B-induced cataract mouse model. Furthermore, to examine the expression of pro-inflammatory cytokines/chemokines in mouse eyes following unilateral UVR-B exposure.

**Methods**: Mice received intraperitoneally injections of Fosaprepitant and Spantide I, before and after unilateral exposure to UVR-B. After day 3 and 7 post-exposure, ocular tissues were extracted for the detection of NKR-1 protein level by ELISA.

**Results:** Pretreatment with Fosaprepitant decreases NKR-1 expression in exposed ocular tissues as well as in the unexposed lens epithelium compared to the saline group. Spantide I treatment showed a tendency of NKR-1 overexpression in ocular tissues.

**Conclusion:** The clinically approved NKR-1 receptor antagonist Fosaprepitant decreases NKR-1 protein expression effectively not only in the exposed but also in the unexposed partner eye in a UVR-B irradiation mouse model. No effect was seen on the protein concentration of pro-inflammatory cytokines/ chemokines in either eye.


**Keywords:** Ultraviolet radiation B, Spantide I, Fosaprepitant (IVEMEND®), Neurokinin receptor 1, Cytokines/Chemokines

## 4.2 Introduction

Exposure to acute ultraviolet radiation type B (UVR-B) has an impact on the physiology of the eye and induces an inflammatory reaction in the anterior segment [1-3]. In addition to its pro-inflammatory effect, UVR-B exposure is the major environmental risk factor for cataract development. Cataract disease is still the leading cause of blindness worldwide[4-6]. While the pathology of cataract is a common global health problem with surgery as the only available treatment option the need is being spent to find novel strategies to prevent or slow down cataract formation[7].

Inflammation in the eye can be initiated by exposure to UVR-B leading to ocular pathologies such as pterygium[8-10], cataract[11-14] and photokeratitis[15,16]. Inflammatory pro-cesses often include an involvement of neuropeptides such as substance P (SP). SP is an 11-amino acid neuropeptide of the tachykinin family, expressed on both neuronal and non-neuronal cells. Its actions modulate SP through the interaction with neurokinin receptors, members of G-protein-coupled receptors, among which the neurokinin receptor- 1 (NKR-1) displays the preferential affinity for SP[17,18]. Various cell populations including neurons, epithelial, endothelial and muscle cells as well as some immune cells (macrophages, monocytes, etc.) express NKR-1[17]. SP and NKR-1 have been implicated in a number of inflammatory reactions in the eye including chemoattraction and activation of neutrophils, and induction of cytokine synthesis[19,20]. Previous experimental studies demonstrated that activation of SP/NKR-1 signaling contributes to promoting pro-inflammatory cytokines and chemokine production, including the upregulation of IL-6 and IFN-γ in herpes simplex virus-1 (HSV-1) as well as in *Pseudomonas aeruginosa* infected corneas[20,21]. This inflammatory response could be alleviated by blocking of NKR-1 signaling, while using NKR-1 antagonists. Therefore, treatment with Spantide I, a selective NKR-1 antagonist, resulted in a significant reduction in the amount of IL-6 protein in HSV-1-infected corneas[21]. Recently, evidence is known that NKR-1 antagonists reduce the inflammatory reactions relating to inflammation dependent eye diseases[20,22].

We previously found evidence that inflammatory processes are involved in UVR-B-induced cataract formation including an NKR-1 upregulation not only in the exposed eye but also in the unexposed partner eye in vivo[23]. In order to comprehend the interrelationship between selective NKR-1 antagonists and unilateral UVR-B exposure, in the current project, we hypothesize that the treatment with NKR-1 antagonists reduces the UVR-B-in-

duced inflammation in the exposed as well as in the contralateral partner eye leading to a decreased protein level of substance P receptor and pro-inflammatory cytokines/chemokines in both eyes. Here, we investigated Fosaprepitant/IVEMEND® and Spantide I as the NKR-1 antagonists in the established UVR-B-induced cataract mouse model. The clinical approved Fosaprepitant is the water-soluble, intravenously administered prodrug of Aprepitant[24,25] rapidly converted to the active form, Aprepitant by phosphatase enzymes[26,27]. Fosaprepitant is approved by the Food and Drug Ad-ministration (FDA), indicated for the prevention of nausea and vomiting after chemotherapy or postoperative surgery[28]. To understand the signaling pathway of substance P and its receptor NKR-1 to the partner eye, including detailed knowledge of the nonpeptide NKR-1 antagonist Fosaprepitant could offer new therapeutic treatment options and clinical preventive strategies for various eye diseases, such as UVR-B-induced cataract formation but also sympathetic ophthalmitis.

## 4.3 Materials and Methods

### 4.3.1 Animals

In all experiments, six-week-old C57BL/6 male and female mice were used. Mice were purchased from Charles River Laboratories (Germany) and maintained in a 12-hour light and 12-hour dark cycle environment in the laboratory at the Department of Ophthalmology, University of Bonn. The mice were supplied with normal rodent chow and water ad libitum. All experimental animal protocols were approved by the Association of Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

### 4.3.2 Chemicals and reagents

The following reagents were purchased: peptide NKR-1 antagonist Spantide I (Bachem; H-1925), non-peptide NKR-1 antagonist Fosaprepitant (IVEMEND®, MSD Merck Sharp & Dohme Ltd., Hoddesdon, UK), phosphate buffered saline (PBS) solution (0.1 M, pH 7.4), sodium chloride NaCl (B. Braun Melsungen AG, 0.9 %, 500 ml), mouse substance P receptor ELISA kit (CSB-E08362m, Cusabio Biotech), protease inhibitor cocktail (1:10, P8340, Sigma-Aldrich®) and a ProcartaPlex™ Multiplex Immunoassay (Thermo Fisher Scientific) to measure protein concentration of pro-inflammatory cytokines and chemo-

kines in ocular tissue lysates (IL-α, Il-6, IL-10, IL-12p70, MCP-1/CCL2, VEGF-A, TNF-α and Gro-α/CXCL1).

### 4.3.3 UVR-B exposure and experimental design

Mice were subdivided into three treatment groups (Fosaprepitant, Spantide I, saline), whereas each treatment group consisted of two latency period groups of 3 and 7 days following unilateral UVR-B exposure (wavelength peak at 312 nm), and a control group. Control groups received the same treatment but no UVR-B irradiation.

The first step of the experimental protocol implied the anesthesia of the mice with a mixture of ketamine/xylazine, injected intraperitoneally (i.p.). For mydriasis, topical 0.5 % tropicamide (Mydriaticum Stulln; Pharma Stulln GmbH, Stull, Germany) were administered as eye drops in both eyes. To exclude congenital cataract, mice were examined with a slit lamp. One eye of each mouse was exposed in vivo to a 5-fold cataract threshold equivalent dose ($MTD_{2.3:16}$) of UVR-B, complies to an energy dose of 14.5 kJ/m². The contralateral, partner eye was carefully and completely shielded with aluminum foil during UVR-B exposure. UVR-B irradiation was performed using a microprocessor-controlled UV irradiation system (Bio-Spectra system; Vilber Lourmat©; Marne-La Vallee, France), designed for laboratory animals. The spectral region of the UVR-B lamps emits ultraviolet rays between 280 nm and 315 nm with a wavelength peak at 312 nm. After a time period of 3 and 7 days following unilateral UVR-B exposure, eyes were enucleated and prepared for individual experiments including immunofluorescence staining, enzyme-linked immunosorbent assay (ELISA) and measurements of pro-inflammatory cytokines and chemokines.

### 4.3.4 NKR-1 antagonist treatment

For ELISA analysis and ProcartaPlex™ Multiplex Immunoassay mice received i.p. injection of 72 µg/mouse Spantide I and 10 mg/kg of the non-peptide NKR-1 antagonist Fosaprepitant. The average body weight of a six-week-old C57BL/6 mouse is approximately 20 g, which is equivalent to a volume of 0.2 mg/mouse of the NKR-1 antagonist Fosaprepitant. The volume per day amounts to 0.2 ml/mouse of NKR-1 antagonists. Both NKR-1 antagonists existed in powdered form and were dissolved in saline. NKR-1 antagonists were administered daily (Figure 1) on days -1, 0 (day of UVR-B exposure), 1 and 2 for a

latency period time of 3 days post-exposure, as well as every day on days -1, 0, 1, 2, 4 and 6 for a time period of 7 days after unilateral UVR-B irradiation. Control mice were similarly injected with saline (natrium chloride).

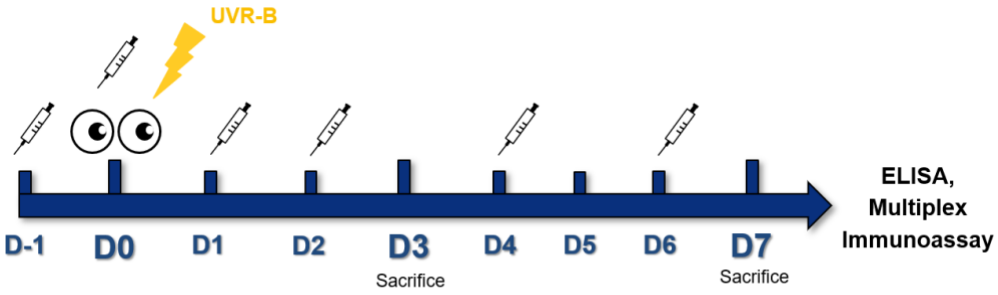


**Figure 1:** Experimental design. Mice were unilaterally exposed to UVR-B (14.5 kJ/m²) in vivo. The contralateral, partner eye was shielded during exposure time. Animals were sacrificed on day 3 and day 7 following UVR-B exposure to only one eye. For a latency period of 3 days following UVR-B exposure, pretreatment of NKR-1 antagonists (Fosaprepitant and Spantide I) and saline were injected intraperitoneally daily on days (D) -1, 0, 1 and 2 as well as once a day on days -1, 0, 1, 2, 4 and 6 for a time period of 7 days post-exposure. Thereafter, eyes were enucleated and ocular tissues microsurgical dissected for ELISA analysis and ProcartaPlex™ Multiplex Immunoassay.

### 4.3.5 Ocular tissue extraction

Ocular tissues (i.e. aqueous humor/AqH, cornea, lens epithelium, iris/ciliary body, retina, and choroid) from mice of all treatment groups (Fosaprepitant, Spantide I, saline) were carefully dissected and pooled for NKR-1 ELISA analysis and ProcartaPlex™ Multiplex Immunoassay. First, eyeballs were removed and immediately placed on filter paper soaked with saline to remove the muscle tissues around the eyeball. Afterwards, a 30 gauge syringe needle was used to gently penetrate the cornea and collect the AqH. To avoid drying of the crystalline lens, the eyeball was placed in PBS buffer and a microsurgical scissor was used to carefully open the eye just behind the limbus. The cornea, iris/ciliary body as well as the retina and choroid were peeled from the lens with forceps and transferred separately into Eppendorf tubes on ice for further analysis (ELISA and ProcartaPlex™ Multiplex Immunoassay). During the dissection process, it is very important to carefully separate specific ocular tissues from a contamination by other ocular tissue residues.

### 4.3.6 Enzyme-linked immunosorbent assay (ELISA)

NKR-1 protein levels in exposed, unexposed and control mouse eyes of different treatment groups were measured with a mouse substance P receptor ELISA kit (CSB-E08362m, Cusabio Biotech) according to the manufacturer´s guidelines. Ocular tissues, including AqH extraction, cornea, lens epithelium, iris/ciliary body, retina and choroid were microsurgically dissected as described above, after latency periods of 3- and 7-days post UVR-B exposure. Samples were homogenized in phosphate-buffered solution containing a protease inhibitor cocktail and centrifuged for 10 min at 10.000 x g. Each sample was assayed in duplicate.

### 4.3.7 ProcartaPlex™ Multiplex Immunoassay

A ProcartaPlex™ Multiplex Immunoassay was used to measure cytokine and chemokine levels in ocular tissue lysates (AqH, cornea, lens epithelium, iris/ciliary body, retina and choroid) of different treatment groups at day 3 and 7 after unilateral UVR-B irradiation with analyses performed in duplicate. The following cytokines and chemokines were included: IL-α, Il-6, IL-10, IL-12p70, MCP-1/CCL2, VEGF-A, TNF-α and Gro-α/CXCL1. The multiplex assay was performed to the manufacturer´s specifications. Standard curves for each cytokine and chemokine were generated from the reference cytokine and chemokine gradient concentrations supplied by the manufacturer´s. Data were analyzed with the Luminex xPonent for MagPix Version 4.2 software.

### 4.3.8 Statistical analysis

SPSS Statistics 23 was used for statistical analysis. Protein levels of NKR-1 and cytokines/chemokines were expressed as mean standard deviation (SD), and statistical significance among the treatment and latency period groups was determined using one-way analysis of variance (ANOVA) followed by a post hoc comparison (Bonferroni). To compare exposed eyes with unexposed partner eyes within one treatment and latency period group one-sample t-test was performed. Regarding sample size the significance levels were set to 0.05 and the confidence coefficients to 0.95, respectively.

## 4.4 Results

### 4.4.1 NKR-1 ELISA

ELISA analysis was implemented to examine NKR-1 expression in ocular tissue samples pretreated with NKR-1 antagonists (Spantide I and Fosaprepitant) following UVR-B exposure at two different time points, and further to relate NKR-1 protein levels to the saline-treated group (Figures 2-7).

*Cornea*

The NKR-1 ELISA analysis of the exposed cornea showed a significant reduction of NKR-1 levels in the Fosaprepitant-treated group at day 3 (P = .004) and at day 7 (P = .006) post UVR-B exposure, when compared to the saline-treated group (Figure 2). A significant reduction of NKR-1 was also observed in the Fosaprepitant group of the unexposed cornea at day 3 (P= .001) compared to the saline group. A strong upregulation of exposed corneas within the Fosaprepitant group at day 3 (P = .010) and 7 (P = .015) post-exposure, compared to the control group (no UVR-B exposure), was also shown.

Interestingly, the treatment with Fosaprepitant induced a significant NKR-1 decrease in the exposed cornea at day 3 (P = .002) and 7 (P = .044) as well as in the unexposed cornea at day 3 (P = .002) following UVR-B irradiation, when compared to the Spantide I-treated group.

Spantide I treatment showed a significant reduction of NKR-1 expression in the exposed cornea 7 days after exposure to UVR-B (P =.038), when compared to the saline-treated group. In addition, significant differences were observed between the exposed and control cornea at the latency period group of 3 (P = .003) and 7 days (P = .001), as well as the unexposed and control cornea at day 3 (P = .006) within the Spantide I-treated group.

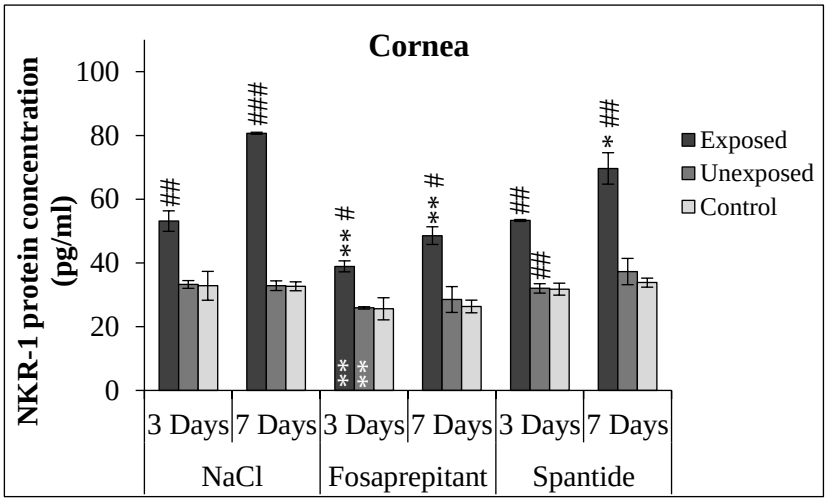


**Figure 2:** ELISA analysis of NKR-1 protein concentration in the cornea. NKR-1 protein expression is displayed as exposed, unexposed and control corneas of three treatment groups (NaCl, Spantide I and Fosaprepitant) at day 3 and 7 after UVR-B exposure of one eye. Exposed corneas of Fosaprepitant-treated mice expressed significantly reduced NKR-1 protein levels compared to NaCl-treated mice at 3 and 7 days post UVR-B exposure. Each bar represents the standard deviation for the mean. Black asterisk indicates a statistically significant difference between Spantide I- /Fosaprepitant-treated mice compared to NaCl-treated mice, whereas white asterisk shows a statistical difference between the Spantide I and Fosaprepitant group. A statistically significant difference between exposed / unexposed corneas to the control cornea within treatment groups is demonstrated as a hash. *P < .05; **P < .01; #P < .05; ##P < .01; ###P < .001. N=21 mice per latency period group/treatment group.

*Lens epithelium*

In the lens epithelium ELISA analysis showed significantly reduced NKR-1 expression in exposed eyes of Fosaprepitant treated mice at day 3 (P = .001) and 7 (P = .034) post-exposure in comparison to the saline group (Figure 3). Remarkably, also the unexposed lens epithelium treated with Fosaprepitant displayed a significant decrease of NKR-1 at day 3 (P = .028) and day 7 (P = .034) after unilateral UVR-B exposure. Significant differences were also observed between Fosaprepitant group and Spantide I group of unexposed eyes (3 days P = .018; 7 days P = .010) as well as of exposed eyes at day 3 following UVR-B irradiation (P ≤ 0.000). Herein, Spantide I treated mice showed higher NKR-1 levels, compared to the mice treated with Fosaprepitant. In the exposed lens epithelium significant NKR-1 increases were detected at day 3 (P = .020) and at day 7 (P = .031) post UVR-B exposure in the Fosaprepitant group as compared to the control eyes.

Furthermore, there was a significant decrease in the amount of NKR-1 expressed in the exposed lens epithelium of the Spantide I-treated group at day 3 post-exposure compared to the vehicle-treated group (P = .001). Exposed lens epithelium of Spantide I-treated mice showed a significant increase of NKR-1 at day 7 when compared to the control mice (P = .022).

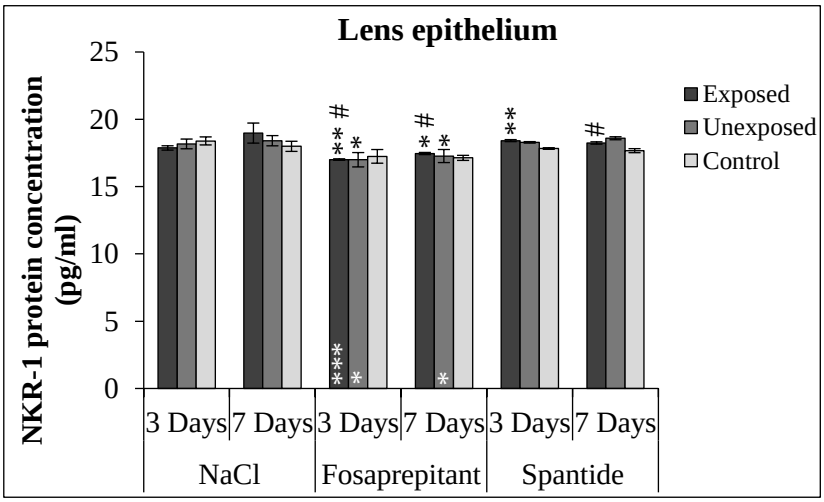


**Figure 3:** ELISA analysis of NKR-1 protein concentration in the lens epithelium. Protein expression of NKR-1 indicates exposed, unexposed and control lens epithelium of NaCl-, Spantide I- and Fosaprepitant-treated mice at day 3 and 7 following unilateral UVR-B exposure. Exposed and unexposed lens epithelium of Fosaprepitant-treated mice expressed significantly reduced NKR-1 protein levels in comparison to the NaCl group at day 3 as well as at day 7 after UVR-B exposure. Error bars reveal the means ± standard deviation. Black asterisk = statistically significant difference between Spantide I- /Fosaprepitant-treated mice compared to NaCl-treated mice. White asterisk = statistical difference between the Spantide I and Fosaprepitant group. Hash = statistically significant difference between exposed / unexposed lens epithelium to the control within treatment groups. *P < .05; **P < .01; #P < .05; ##P < .01; ###P < .001. N=21 mice per latency period group/treatment group.

*Aqueous humor (AqH)*

The NKR-1 levels of the exposed AqH was significantly increased in Fosaprepitant treated mice at day 7 post-exposure as compared to the Spantide I group (Figure 4, P = .028). Unexposed eyes showed also a significantly increase of NKR-1 in Fosaprepitant treated mice at day 3 (P = .030), when compared to the Spantide I group. NKR-1 protein levels of unexposed AqH in mice treated with Fosaprepitant were close to the detection levels of the saline group.

Mice treated with Spantide I revealed significant increase of NKR-1 expression in exposed AqH 3 (P = .003) and 7 (P = .009) days following exposure to UVR-B, compared with the control group of Spantide I-treated mice. Significant differences in exposed and unexposed AqH of the Spantide I-treated mice in comparison to the saline group were not detected.

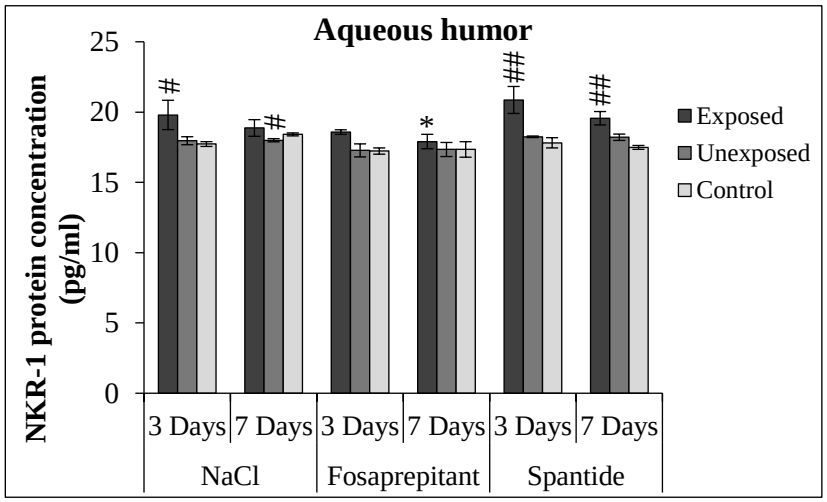


**Figure 4:** ELISA analysis of NKR-1 protein concentration in the lens epithelium. Protein expression of NKR-1 indicates exposed, unexposed and control lens epithelium of NaCl-, Spantide I- and Fosaprepitant-treated mice on days 3 and 7 following unilateral UVR-B exposure. Exposed and unexposed lens epithelium of Fosaprepitant-treated mice expressed significantly reduced NKR-1 protein levels in comparison to the NaCl group on day 3 as well as on day 7 after UVR-B exposure. Error bars reveal the means ± standard deviation. Black asterisk = statistically significant difference between Spantide I-/Fosaprepitant-treated mice compared to NaCl-treated mice. White asterisk = statistical difference between the Spantide I and Fosaprepitant group. Hash = statistically significant difference between exposed/unexposed lens epithelium to the control within treatment groups. *P < .05; **P < .01; #P < .05; ##P < .01; ###P < .001. N = 21 mice per latency period group/treatment group.

*Iris/Ciliary body complex*

ELISA analysis of the iris/ciliary body displayed a reduction of NKR-1levels in exposed eyes of mice treated with NKR-1 antagonist, Fosaprepitant 3 days after UVR-B exposure (Figure 5, P = .035) in comparison to the saline-treated group. The unexposed iris/ciliary body of the NKR-1 antagonist treated group demonstrated almost similar NKR-1 protein levels compared to the vehicle-treated group. An increase of NKR-1 in exposed eyes 3 (P = .012) and 7 (P = .018) days post-exposure was observed in comparison to the control mice within the Fos-aprepitant-treated group. Significant differences were demonstrated

between exposed eyes of the Fosaprepitant and exposed eyes of the Spantide I group at day 3 (P= .042) and 7 (P = .039) post-exposure. Equally, NKR-1 protein levels increased significantly in exposed eyes of the Spantide I-treated group at day 3 (P = .001) as well as at day 7 (P = .001) after UVR-B exposure, when compared to the control mice.

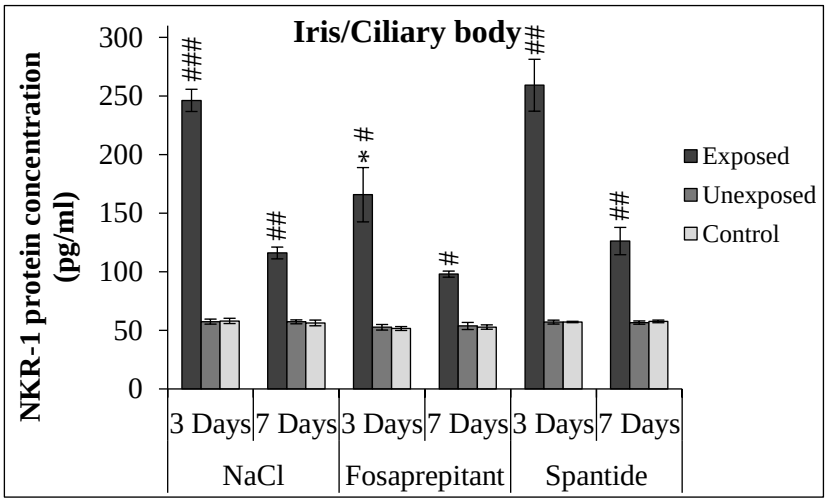


**Figure 5:** ELISA analysis of NKR-1 protein concentration in the iris/ciliary body complex. NKR-1 protein expression is displayed as exposed, unexposed and control iris/ciliary body complex of NaCl-, Spantide I- and Fosaprepitant-treated mice 3 and 7 days following unilateral UVR-B irradiation. Exposed iris/ciliary body complex of Fosaprepitant-treated mice expresses significantly reduced NKR-1 protein levels in comparison to the NaCl group at day 3 after UVR-B exposure. Error bars reveal the means ± standard deviation. Black asterisk indicates a statistically significant difference between Spantide I-/Fosaprepitant-treated mice compared to the NaCl-treated mice. White asterisk shows a statistical difference between exposed/ unexposed eyes of the Spantide I and Fosaprepitant group. Statistically significant difference between exposed/unexposed iris/ciliary body complex to the control within treatment groups is demonstrated as a hash. *P < .05; #P < .05; ##P < .01; ###P < .001. N = 21 mice per latency period group/treatment group.

*Retina*

Exposed retinae demonstrated a significantly difference of NKR-1 protein levels in mice treated with Fosaprepitant at day 3 (P = .035) and 7 (P = .011), compared to the vehicle-treated mice (Figure 6). NKR-1 level of the exposed retina was significantly lower in Fosaprepitant-treated mice at day 7 post-exposure compared with the Spantide I-treated group (P = .001). Exposed and unexposed retina of mice treated with Spantide I displayed a nearly similar NKR-1 protein level as compared with mice treated with saline.

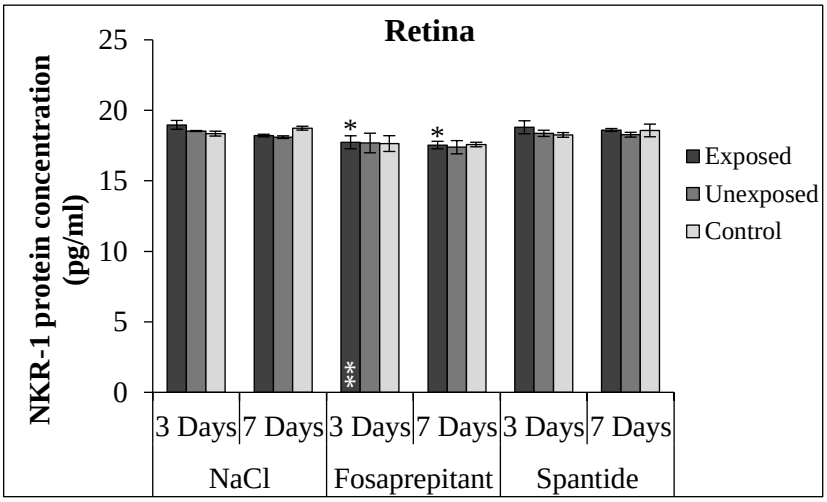


**Figure 6:** ELISA analysis of NKR-1 protein concentration in the retina. Protein expression of NKR-1 is demonstrated in exposed, unexposed and control retina of treatment groups (NaCl, Spantide I, Fosaprepitant) at two different time points after unilateral UVR-B irradiation. Exposed and unexposed retina of the Fosaprepitant-treated mice revealed a significant reduction in NKR-1 protein levels as compared to the NaCl-treated mice on day 3 as well as on day 7 post-exposure. Each bar represents the standard deviation for the mean. Black asterisk indicates the difference between Spantide I/Fosaprepitant group to the NaCl group. White asterisk demonstrates significant differences between Spantide I-treated and Fosaprepitant-treated retinae. *P < .05; **P < .01. N = 21 mice per latency period group/treatment group.

*Choroid*

As it is shown in Figure 7, exposed choroid of Fosaprepitant-treated mice revealed a significant NKR-1 reduction at day 7 following UVR-B exposure when compared to the saline-treated mice (P = .005). Exposed choroid of mice treated with Fosaprepitant showed a significant reduction in NKR-1 expression compared to the exposed choroid treated with Spantide I (P = .022). In addition, Spantide I treated mice revealed significant NKR-1 up-regulation in the unexposed choroid at day 3 post-exposure (P = .022) as well as in the exposed choroid 7 days following UVR-B exposure (P = .043) compared to the saline group. The level of NKR-1 in exposed choroid within the Spantide I-treated mice was statistically different compared with the control group, 3 (P = .004) and 7 (P = .011) days after UVR-B exposure.

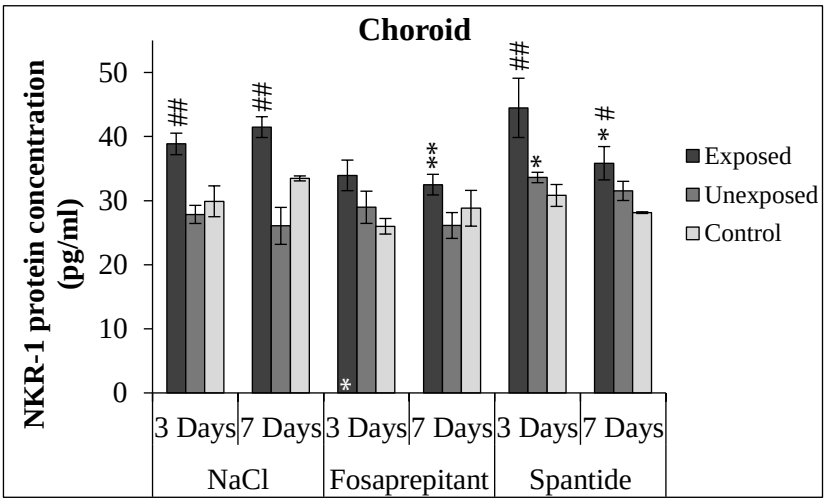


**Figure 7:** ELISA analysis of NKR-1 protein concentration in the choroid. NKR-1 protein levels are demonstrated in exposed, unexposed and control choroid of three different treatment groups (NaCl, Spantide I, Fosaprepitant) at day 3 and 7 following unilateral UVR-B irradiation. Exposed choroid of the Fosaprepitant-treated mice revealed a significant reduction in NKR-1 protein levels as compared to the N-treated mice 7 days after exposure. Each bar represents the standard deviation for the mean. Black asterisk indicates the difference between Spantide I/Fosaprepitant group in comparison to the NaCl group, whereas white asterisk demonstrates significant differences between Spantide I-treated and Fosaprepitant-treated choroid. Differences between exposed and unexposed eyes to the control within the treatment group are showed as a hash. *P < .05; **P < .01; #P < .05; ##P < .01. N = 21 mice per latency period group/treatment group.

### 4.4.2 ProcartaPlex™ Multiplex Immunoassay

To test whether pro-inflammatory cytokines and chemokines has significant impact on unilateral UVR-B exposure in Fosaprepitant- and saline-treated mice, we performed ProcartaPlex™ Multiplex Immunoassay using a MagPix (Luminex Corporation) reader.

*Cornea*

The concentration of TNF-α showed a slight upregulation of the exposed cornea at day 7 post-exposure in the Fosaprepitant group compared to the saline group (Figure 8). But a significant difference was not detected. A significant difference was observed between exposed cornea and control 7 days following UVR-B exposure within the Fosaprepitant-treated group (P = .022).

VEGF-A protein levels was raised significantly in exposed corneas treated with Fosaprepitant (P = .002) and saline (P = .001) 3 days after exposure, when compared with the control group within the treatment groups. The level of Gro-α/CXCL1 of the exposed cor-

nea at day 3 in the Fosaprepitant group was statistically reduced compared with the saline-treated group (P = .001). In the saline group, exposed cornea revealed a significant upregulation at day 3 after UVR-B exposure compared to the control group, which received no UVR-B exposure (P ≤ 0.000).

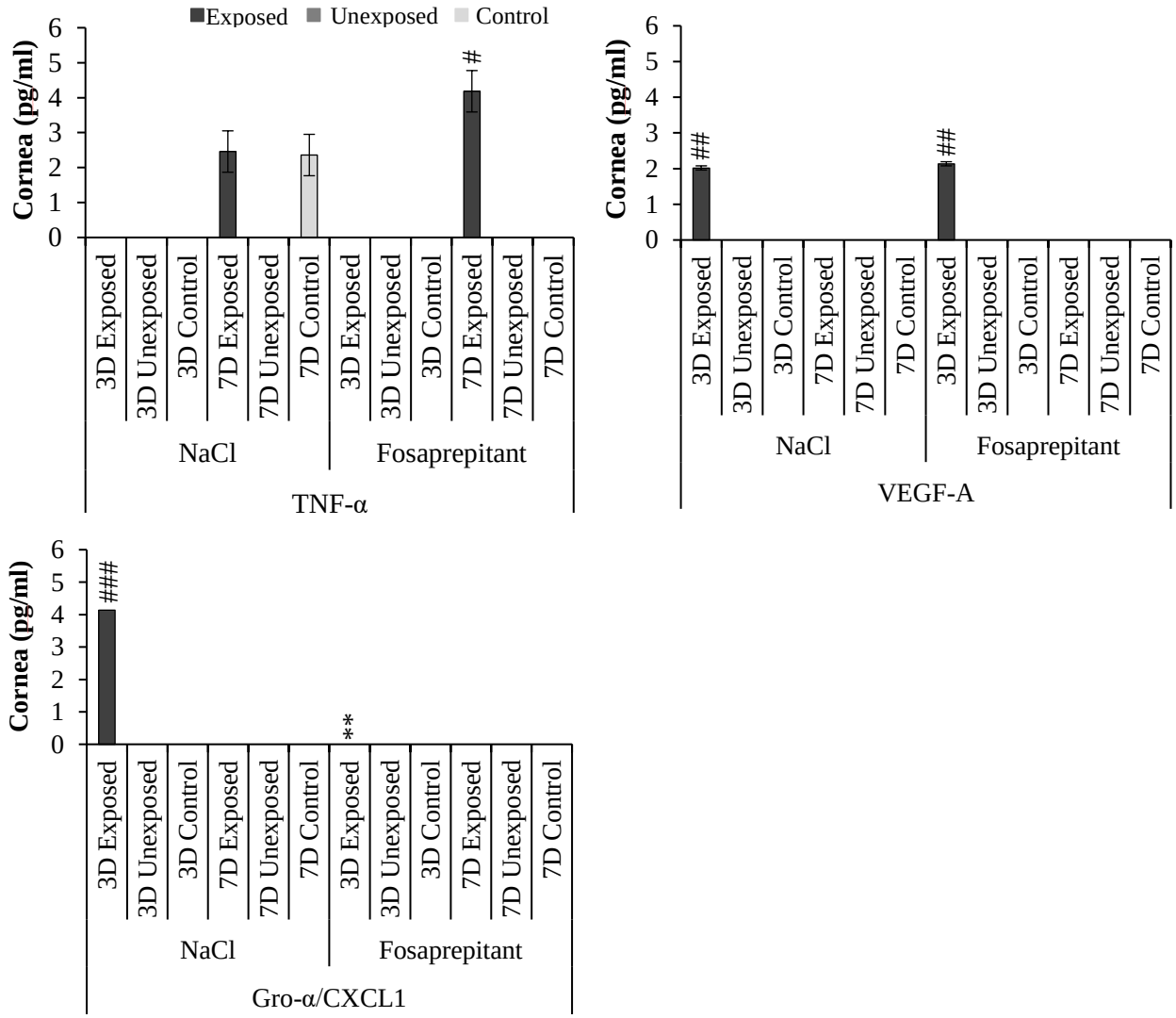


**Figure 8:** Inflammatory cytokine levels in the cornea. Bar graphs demonstrate cytokine protein levels (TNF-α, VEGF-A, Gro-α) of exposed, unexposed and control corneas of NaCl-, Spantide I- and Fosaprepitant-treated mice at day 3 and 7 after unilateral UVR-B irradiation. Each bar reveals the standard deviation for the mean. Black asterisk = significant difference between Spantide I- or Fosaprepitant-treated mice in comparison to the NaCl group. Blue asterisk = statistically significant difference between the Spantide I and Fosaprepitant group. Hash = significant difference between exposed / unexposed corneas compared to the control cornea within treatment groups. *P < .05; **P < .01; ***P < .001; #P <0.05; ##P < .01; ###P < .001. N=21 mice per latency period group/treatment group.

*Lens epithelium*

Analysis of the Multiplex Immunoassay in the lens epithelium showed a strong reduction of VEGF-A in exposed eyes of the Fosaprepitant-treated group at day 7 compared to the control (Figure 9; P ≤ 0.000). Interestingly, levels of VEGF-A in the exposed eyes of the Fosaprepitant group at day 3 were close to the detection levels of the saline group. A significant difference between the Fosaprepitant-treated group and the saline group was observed neither in the exposed eyes nor in the unexposed eyes. The unexposed lens epithelium showed a significant upregulation within the saline group at day 3, when compared with the control group (P = .040).

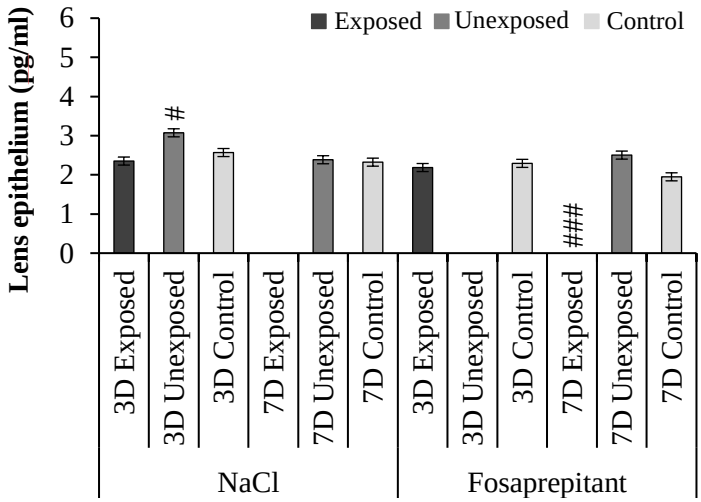


**Figure 9:** Inflammatory cytokine protein level of VEGF-A in the lens epithelium. Cytokine array of exposed, unexposed and control lens epithelium treated with NaCl and NKR-1 Antagonists, Fosaprepitant and Spantide I, post UVR-B exposure at two different time points. Each bar represents the standard deviation for the mean. Black asterisk demonstrates a significant difference between NKR-1 Antagonists compared to the NaCl group, whereas blue asterisk indicates a statistical difference between Spantide I- and Fosaprepitant-treated mice. A statistically significant difference between exposed / unexposed lens epithelium to the control lens epithelium within treatment groups is demonstrated as a hash. *P < .05; **P < .01; ***P < .001; #P < .05; ###P < .001. N=21 mice per latency period group/treatment group.

*Choroid*

For the concentration of VEGF-A, the results showed that the exposed and unexposed choroid of the Fosaprepitant group had slightly higher levels than the saline group, but with no significant differences (Figure 10). Within the Fosaprepitant group exposed choroid at day 3 showed significant higher VEGF-A concentrations compared to the control,

which received no UVR-B exposure (P = .038). Under treatment with Fosaprepitant, levels of Gro-α/CXCL1 as measured by Luminex multiplex assay were downregulated in the exposed (P = .003) and unexposed (P = .003) choroid 7 days after UVR-B exposure, compared to the saline group. Within the Fosaprepitant-treated group Gro-α/CXCL1 levels of exposed choroid revealed a significant difference to the control group at day 3 post-exposure (P ≤ 0.000). Luminex measurements in exposed, unexposed and control choroid at day 7 as well as in unexposed and control choroid at day 3 showed no Gro-α/CXCL1 expression. The unexposed choroid of the saline group displayed a significant decrease of Gro-α/CXCL1 levels in comparison to the control group of the saline group, 7 days after UVR-B exposure (P = .017).

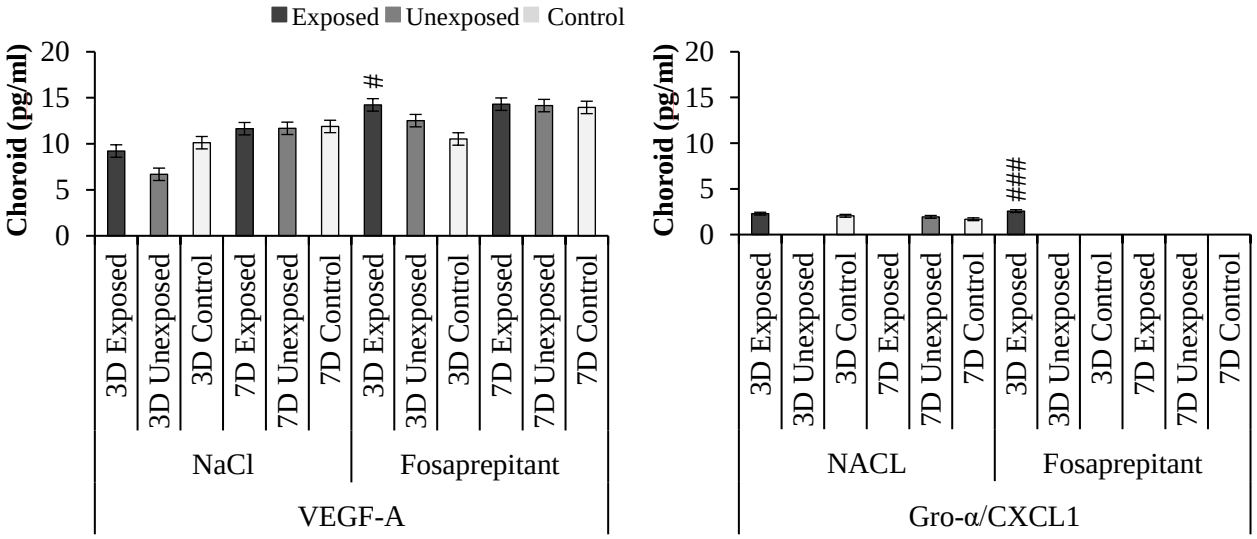


**Figure 10:** Inflammatory cytokine levels in the choroid. Inflammatory cytokines of exposed, unexposed and control choroid treated with NaCl, Spantide I and Fosaprepitant after unilateral UVR-B exposure at two different time points. The following cytokines were investigated: VEGF-A and Gro-α. Error bars reveal the means ± standard deviation. Black asterisk = significant difference between Spantide I- or Fosaprepitant-treated mice compared to the NaCl group. Blue asterisk = significant difference between the Spantide I and Fosaprepitant group. Hash = significant difference between exposed/unexposed choroid to the control choroid within treatment groups. *P < .05; **P < .01; ***P < .001; #P < .05; ##P < .01; ###P < .001. N = 21 mice per latency period group/treatment group.

## 4.5 Discussion

Here we demonstrated for the first time in vivo that Fosaprepitant is a selective and protective NKR-1 antagonist which induces a reduction in NKR-1 expression in the exposed as well as in the unexposed partner eye after ultraviolet exposure to only one eye. Thus, Fosaprepitant is clinically capable to suppress the sympathizing pro-inflammatory signaling to the unexposed partner eye in our in vivo model.

### 4.5.1 NKR-1 protein expression after treatment with NKR-1 antagonists, Fosaprepitant and Spantide I

Aprepitant is a selective high-affinity NKR-1 antagonist commercially available for the prevention of chemotherapy-induced or post-operative nausea and vomiting[29]. It is known that Aprepitant crosses the blood-brain barrier and has immune-stimulatory and anti-inflammatory properties[30,31]. Aprepitant is a non-water soluble NKR-1 antagonist intended for oral administration[32]. Here we used the water-soluble phosphoryl prodrug of Aprepitant, called Fosaprepitant which makes the formulation of the intraperitoneal injection convenient[28]. After administration, Fosaprepitant is rapidly converted to Aprepitant via the action of phosphatase enzymes assuming that the absorbed Fosaprepitant amount complies with an equivalent of Aprepitant[33-35].

Several experimental and clinical reports indicated an inflammatory reduction by Aprepitant or Fosaprepitant in a number of inflammatory diseases[36-38]. It is even known that Aprepitant is a broad-spectrum antitumor drug implicating a potent growth inhibition on human neuroblastoma or glioma[39-41]. However, experimental evidence that Aprepitant is capable of inhibiting inflammation in the eye and in ocular diseases by reducing pro-inflammatory peptides, such as NKR-1 was missing so far.

In the present study, we evaluated the effect of the currently only clinical approved NKR-1 antagonist, Fosaprepitant in an UVR-B-induced cataract mouse model by measuring NKR-1 protein levels in ocular tissues of the exposed as well as of the unexposed partner eyes. Treatment with Fosaprepitant resulted in a decreased NKR-1 protein concentration in all examined exposed ocular tissues, 3 and 7 days after unilateral UVR-B exposure (Figures 2-7). Importantly, also the unexposed contralateral lens epithelium showed a significant decrease of NKR-1 protein level after administration of Fosaprepitant when compared to the saline group (Figure 3). These results provide first evidence that SP and its

receptor play an important role in signaling the inflammatory insult to the contralateral unexposed eye. This transfer process to the partner eye is a valid explanation for the observed sympathizing reaction in multiple previous experiments in our established UVR-B-induced cataract model[42]. Our data are furthermore in agreement with other studies, demonstrating effective substance P receptor inhibition by treatment with the clinical approved NKR-1 antagonist Aprepitant or Fosaprepitant in different inflammation-induced diseases in animal models outside the eye[26,31,36,37]. For example, in immunosuppressed mice infected with the protozoan parasite *Cryptosporidium parvum* increased SP levels in jejunum tissues as well as physiological and structural alterations were significantly inhibited by Aprepitant treatment[36]. Investigations of SP/NKR-1 interactions in damaging CNS inflammation using the NKR-1 antagonist Aprepitant was analyzed in a nonhuman primate model of Lyme neuroborreliosis[37]. In this study Aprepitant treatment resulted in reduced NKR-1 expression in the brain cortex and decreased inflammatory features, associated with *B. burgdorferi* infection. In accordance with our findings, an earlier study by Bignami and co-workers investigated the efficacy of Fosaprepitant in a corneal neovascularization (CNV) mouse model[26]. The authors showed a reduction in opacity as well as a decreased infiltration of neutrophils and macrophages in the cornea after topical Fosaprepitant administration. These results equivalent to our findings indicated that the induced inflammation on the ocular surface is alleviated by Fosaprepitant treatment, assuming a protective effect of Fosaprepitant in the cornea.

Although induced inflammation was effectively reduced by Aprepitant or Fosaprepitant treatment in various diseases[26,36,37], a link among Fosaprepitant and the immunological connection between both eyes in sympathizing ocular diseases remained so far unclear. To our knowledge we show for the first time that Fosaprepitant inhibited NKR-1 expression not only in exposed ocular tissues but also in unexposed lens epithelium following unilateral exposure to UVR-B. Thus, our results are of essential importance for the elucidation of the molecular mechanism of SP/NKR-1 and its signaling properties in sympathizing eye diseases. Most chronic eye diseases are of bilateral nature. Thus, further studies are needed to elucidate the possible involvement of SP signaling in sight-threatening eye diseases such as age-related macular degeneration (AMD). A second but only experimentally used NKR-1 antagonist is Spantide I. After blockade with Spantide I ELISA experiments demonstrated a significant reduction of NKR-1 protein level only in the exposed

cornea at day 7 following UVR-B exposure, compared to the saline group (Figure 2). Interestingly, a tendency of a NKR-1 overexpression was observed in the remaining ocular tissues of Spantide I-treated mice (Figure 3-7). Previous studies together with our current results indicated a protective effect of Spantide I in the cornea after induced impairment[21,22,43]. The involvement of blocking the binding signaling of SP and its receptor, NKR-1 by using Spantide is supported by a study demonstrating reduced development of corneal opacity in a herpetic stromal keratitis (HSK) mouse model[21]. Confirmed with these results, Spantide I treatment decreased perforated corneas and bacterial counts in mice infected with *Pseudomonas aeruginosa*[22]. The immune privilege of mice was abrogated bilaterally and immunohistochemistry analysis showed a bilateral increase of NKR-1 staining in the retina after retinal laser burn (RLB) to one only eye[44]. In this study, early treatment with Spantide I blocked the abrogation of immune privilege in both eyes after unilateral RLB. Contrary to this study, our data showed no effect in blocking SP/NKR-1 signaling pathway with Spantide I in the unexposed partner eye. Interestingly, the treatment with the peptide NKR-1 antagonist Spantide I repeatedly resulted in a strong overexpression of various pro-inflammatory cytokines and chemokines that cannot be explained by a reflectory overexpression after blockade of the receptor alone. It is known that peptide NKR-1 antagonists such as Spantide can show some drawbacks, such as lower binding affinity and neurotoxicity[28,45].

These results are consistent with our ELISA analysis which showed a tendency of an NKR-1 overexpression after Spantide I administration in ocular tissues of both eyes, suggesting that Spantide I is no effective NKR-1 antagonist in our established UVR-B-induced cataract model but rather has a toxic effect. Here further inside on the functional SP levels after NKR-1 blockade with different NKR-1 antagonists is needed.

Taken together, our data support the contention that the clinical approved NKR-1 antagonist Fosaprepitant constitutes a selective and effective antagonist to inhibit UVR-B-induced inflammation in the eye, not only in the exposed side also in the unexposed partner eye. In contrast, treatment with Spantide I induced a tendency of NKR-1 overexpression in ocular tissues of the exposed and unexposed partner eye following unilateral UVR-B exposure.

### 4.5.2 Pro-inflammatory cytokine and chemokine expression in Fosaprepitant- and saline-treated ocular tissues

Additionally, to NKR-1 we investigated whether UVR-B exposure to one eye affected an upregulation of pro-inflammatory cytokines and chemokines in exposed and unexposed ocular tissues after two different time periods. Since it is known that SP/NKR-1 interaction modulates the secretion of pro-inflammatory molecules, we further speculated that the decrease of pro-inflammatory cytokine and chemokine protein levels could be a result of the treatment with the NKR-1 antagonist Fosaprepitant.

UVR-B irradiation can trigger oxidative stress including activation of pro-inflammatory mediators leading to DNA and protein changes[46-48]. Inflammatory reactions by UVR-B exposure comprises the production of several pro-inflammatory cytokines and chemokines, such as interleukin (IL)-6[47-49]. In vitro studies demonstrated that UVR-B irradiation on human epithelial cells and body fibroblasts isolated from normal cornea tissue or pterygium sample promote the expression of pro-inflammatory cytokines including IL-6, IL-1β and tumor necrosis factor (TNF)-α[3,46,49,50]. In human corneal limbus epithelial cells UVR-B irradiation induced a dose-dependent increase of IL-6 and IL-8 secretion levels, suggesting that the UVR-B-induced expression of IL-6 and IL-8 included the mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF-κB) pathways which are inhibited by curcumin[47]. The neuropeptide substance P and its receptor, NKR-1 are involved in many biological processes including inflammation[17,18]. In particular, SP/NKR-1 interaction induce the production of pro-inflammatory cytokines and chemokines through the involvement of intracellular pathways including the activation of second messengers, such as cyclic adenosine monophosphate (cAMP)[17,51]. It has been shown that expression of inflammatory cytokines (IL-1β, IL-6, TNF-α) increases in the mouse cornea infected with *Pseudomonas aeruginosa*[19,20]. In addition, in an HSK mouse model increased protein levels of IL-6 and IFN-γ was determined in corneas with severe disease compared to corneas with a mild disease outcome[21].

In agreement with previous studies, which showed increased pro-inflammatory cytokine and chemokine expression in infected corneas[19,21,48,52], we currently observed a UVR-B-induced upregulation of Gro-α/CXCL1 and VEGF-A protein concentration in the exposed cornea of the saline group when compared to the control group which received no UVR-B exposure (Figure 8). We also found a significant upregulation of TNF-α expression in

the exposed lens epithelium of the saline group at day 7 post-exposure (data not shown) as well as an upregulation of VEGF-A in the unexposed lens epithelium at day 3 following UVR-B exposure when compared to the control group (Figure 9). Interestingly, compared to the saline group, we have determined a significant reduction of Gro-α/CXCL1 in the cornea after treatment with Fosaprepitant (Figure 8). This finding is in accordance with experimental and clinical reports, indicating a significant decrease in various pro-inflammatory molecules, such as TNF-α[31,53,54], MCP-1/CCL2[37,55] and IL-6[31,37] after treatment with the clinical approved NKR-1 antagonist, Aprepitant. Although there are some studies, implying increased expression of pro-inflammatory mediators after induced inflammation as well as effective inhibition by NKR-1 antagonists, the current study is the first report to demonstrate that in the cornea and lens epithelium pro-inflammatory cytokines (Gro-α/CXCL1, VEGF-A and TNF-α) are upregulated after UVR-B exposure. Actually, treatment with Fosaprepitant inhibited the upregulated Gro-α/CXCL1 expression in the cornea, suggesting that Fosaprepitant is a potent NKR-1 antagonist with a protective systemic effect after i.p. administration. Injections of the substances were performed intraperitoneally to control the absorbed dose, since it is difficult to regulate the dose by the oral uptake of the mice through the drinking water.

However, our results including a generally low protein concentration of the remaining tested pro-inflammatory cytokines and chemokines (e.g. IL-1α, MCP-1/CCL2) in the saline group after exposure to UVR-B. One explanation for the low cytokine/chemokine expression would be the relatively mild UVR-B-induced inflammation in the eye, hypothesizing the higher the UVR-B dosage, the higher the expression of pro-inflammatory cytokines and chemokines.

Aprepitant has been used not only successfully for the treatment of chemotherapy- and post-operative-induced nausea and vomiting, but also for the therapy in migraine, pain and emesis[40]. It has recently demonstrated that Aprepitant is an antitumor drug with no known side effects[39,56]. Consistent with previous studies, indicating no toxic side effects of Aprepitant and Fosaprepitant[26,29], in the current study Fosaprepitant was well tolerated by the mice and no externally visible side effect during the days of treatment was observed. These properties of Aprepitant linked to the effectively inhibition of inflammation in several animal models[26,36,40], makes this drug interesting for many ocular diseases such as corneal neovascularization (CNV) or AMD.

In a preliminary experiment, we determined a massive opacity on the cornea after topical administration of Spantide I within a few minutes (data not shown). Confirming the pro-inflammatory feature of Spantide I, we detected a significant increase in various pro-inflammatory cytokines and chemokines after Spantide I treatment compared to the saline group (data not shown). Interestingly, treatment with Spantide I showed a significant reduction of the anti-inflammatory cytokine IL-10 in the retina after UVR-B exposure in comparison to mice treated with saline or Fosaprepitant. These results provide evidence that Spantide I may have a pro-inflammatory effect on the eye disguising its primary function the blockade of the NK-1 receptor.

### 4.6 Conclusion

In conclusion, we demonstrated for the first time that Fosaprepitant reduces inflammation in UVR-B exposed ocular tissues with a significant NKR-1 reduction in the exposed as well as in the unexposed lens epithelium of the partner eye. This finding indicates that substance P and its receptor NKR-1 take part in the contralateral side effect after exposure of only one eye to UVR-B and should be considered in other bilateral as well as sympathizing eye diseases. Most age-related eye diseases such as AMD do have inflammatory pathogeneses and are of bilateral nature and might be candidates for Fosaprepitant treatment. Therefore, further detailed information about the SP/NKR-1 signaling pathway and treatment options with Fosaprepitant is urgently needed in other ocular disease models since it could open new preventive treatment strategies for multiple inflammatory eye diseases and is thus of high clinical relevance.

### 4.7 Acknowledgements

The authors thank Claudine Strack for outstanding expert help. We also thank the laboratory group for helpful discussions.

### 4.8 Declaration of interest

The author, Prof. Frank G. Holz, has financial support from Acucela, Allergan, Bayer, Bioeq/Formycon, CenterVue, Roche/Genentech, Heidelberg Engineering, NightStarX, Novartis, Optos, Pixium Vision and Zeiss. Furthermore, the author consults for Acucela, Apellis, Bayer, Boehringer-Ingelheim, Bioeq/Formycon, Galimedix, Roche/Genentech,

Geuder, Grayburg Vision, Heidelberg Engineering, LinBioscience, Novartis, Pixium Vision, Oxurion and Stealth Bio Therapeutics. The author is also a recipient of gifts, honoraria, travel reimbursement, patent royalties, or any other financial compensation of Acucela, Allergan, Apellis, Bayer, Ellex, Roche/Genentech, Grayburg Vision, Heidelberg Engineering, LinBioscience, Novartis, Pixium Vision, Oxurion, Stealth Bio Therapeutics and Zeiss. The other authors of this publication have no conflicts of interest to declare.

### 4.9 Funding

This work was supported by the German Research Foundation “DFG” (ME 3298/4-1) (WE 1303/6-1); Deutsche Forschungsgemeinschaft [ME 3298/4-1, WE 1303/6-1].

### 4.10 References

1. Nolan TM, DiGirolamo N, Sachdev NH, Hampartzoumian T, Coroneo MT, Wakefield D. The role of ultraviolet irradiation and heparin-binding epidermal growth factor-like growth factor in the pathogenesis of pterygium. Am J Pathol. 2003;162 (2):567–574. doi:10.1016/S0002-9440(10)63850-3.
2. Meyer LM, Löfgren S, Holz FG, Wegener A, Söderberg P. Bilateral cataract induced by unilateral UVR-B exposure – evidence for an inflammatory response. Acta Ophthalmol. 2013;91(3):236–242. doi:10.1111/aos.2013.91.issue-3.
3. Di Girolamo N, Wakefield D, Coroneo MT. UVB-mediated induction of cytokines and growth factors in pterygium epithelial cells involves cell surface receptors and intracellular signaling. Invest Ophthalmol Vis Sci. 2006;47(6):2430–2437. doi:10. 1167/ iovs.05-1130.
4. Hollows F, Moran D. Cataract–the ultraviolet risk factor. Lancet. 1981;2(8258): 1249–1250. doi:10.1016/S0140-6736(81)91490-2.
5. West S. Epidemiology of cataract: accomplishments over 25 years and future directions. Ophthalmic Epidemiol. 2007;14(4):173–178. doi:10.1080/09286580701 423151.
6. Pascolini D, Mariotti SP. Global estimates of visual impairment: 2010. Br J Ophthalmol. 2012;96(5):614–618. doi:10.1136/ bjophthalmol-2011-300539.
7. Cruickshanks KJ, Klein BE, Klein R. Ultraviolet light exposure and lens opacities: the Beaver dam eye study. Am J Public Health. 1992;82(12):1658–1662. doi:10.

2105/AJPH.82.12.1658.

8. Coroneo MT. Pterygium as an early indicator of ultraviolet insolation: a hypothesis. Br J Ophthalmol. 1993;77(11):734–739. doi:10.1136/bjo.77.11.734.
9. Coroneo MT, Di Girolamo N, Wakefield D. The pathogenesis of pterygia. Curr Opin Ophthalmol. 1999;10(4):282–288. doi:10.1097/ 00055735-199908000-00011.
10. Threlfall TJ, English DR. Sun exposure and pterygium of the eye: a dose-response curve. Am J Ophthalmol. 1999;128(3):280–287. doi:10.1016/S0002-9394(99)00161-0.
11. Taylor HR, West SK, Rosenthal FS, et al. Effect of ultraviolet radiation on cataract formation. N Engl J Med. 1988;319 (22):1429–1433. doi:10.1056/NEJM198812013192201.
12. Hightower KR. A review of the evidence that ultraviolet irradiation is a risk factor in cataractogenesis. Documenta Ophthalmologica. 1994;88(3–4):205–220. doi:10.1007/BF01203675.
13. Roberts JE. Ultraviolet radiation as a risk factor for cataract and macular degeneration. Eye Contact Lens. 2011;37(4):246–249. doi:10.1097/ICL.0b013e31821cbcc9.
14. Zhang J, Yan H, Löfgren S, Tian X, Lou MF. Ultraviolet radiation-induced cataract in mice: the effect of age and the potential biochemical mechanism. Invest Ophthalmol Vis Sci. 2012;53(11):7276–7285. doi:10.1167/iovs.12-10482.
15. Delic NC, Lyons JG, Di Girolamo N, Halliday GM. Damaging effects of ultraviolet radiation on the cornea. Photochem Photobiol. 2017;93(4):920–929. doi:10.1111/php.2017.93.issue-4.
16. Mureşan S, Filip A, Mureşan A, et al. Histological findings in the Wistar rat cornea following UVB irradiation. Rom J Morphol Embryol. 2013;54(2):247–252.
17. Mashaghi A, Marmalidou A, Tehrani M, Grace PM, Pothoulakis C, Dana R. Neuropeptide substance P and the immune response. Cell Mol Life Sci. 2016;73 (22):4249–4264. doi:10.1007/s00018-016-2293-z.
18. Suvas S. Role of substance P neuropeptide in inflammation, wound healing, and tissue homeostasis. J Immunol. 2017;199 (5):1543–1552. doi:10.4049/jimmunol.1601751.

19. Foldenauer MEB, McClellan SA, Barrett RP, Zhang Y, Hazlett LD. Substance P affects growth factors in Pseudomonas aeruginosa-infected mouse cornea. Cornea. 2012;31 (10):1176–1188. doi:10.1097/ICO.0b013e31824d6ffd.
20. McClellan SA, Zhang Y, Barrett RP, Hazlett LD. Substance P promotes susceptibility to Pseudomonas aeruginosa keratitis in resistant mice: anti-inflammatory mediators downregulated. Invest Ophthalmol Vis Sci. 2008;49(4):1502–1511. doi:10.1167/iovs.07-1369.
21. Twardy BS, Channappanavar R, Suvas S. Substance P in the corneal stroma regulates the severity of herpetic stromal keratitis lesions. Invest Ophthalmol Vis Sci. 2011;52(12):8604–8613. doi:10.1167/iovs.11-8089.
22. Hazlett LD, McClellan SA, Barrett RP, Liu J, Zhang Y, Lighvani S. Spantide I decreases type I cytokines, enhances IL-10, and reduces corneal perforation in susceptible mice after Pseudomonas aeruginosa infection. Invest Ophthalmol Vis Sci. 2007;48(2):797–807. doi:10.1167/iovs.06-0882.
23. Gross J, Wegener AR, Kronschlaeger M, Holz FG, Schönfeld C-L, Meyer LM. Ultraviolet radiation exposure triggers neurokinin-1 receptor upregulation in ocular tissues in vivo. Exp Eye Res. 2018;174:70–79. doi:10.1016/j.exer.2018.04.016.
24. Saito Y, Kumamoto T, Arima T, et al. Evaluation of aprepitant and Fosaprepitant in pediatric patients. Pediatr Int. 2019;61 (3):235–239. doi:10.1111/ped.2019.61.issue-3.
25. Garnock-Jones KP. Fosaprepitant dimeglumine: a review in the prevention of nausea and vomiting associated with chemotherapy. Drugs. 2016;76(14):1365–1372. doi:10.1007/ s40265-016-0627-7.
26. Bignami F, Lorusso A, Rama P, Ferrari G. Growth inhibition of formed corneal neovascularization following Fosaprepitant treatment. Acta Ophthalmol. 2017;95(7):e641–e648. doi:10.1111/ aos.13304.
27. Lasseter KC, Gambale J, Jin B, et al. Tolerability of Fosaprepitant and bioequivalency to aprepitant in healthy subjects. J Clin Pharmacol. 2007;47(7):834–840. doi:10.1177/0091270007301800.
28. Rosso M, Muñoz M, Berger M. The role of neurokinin-1 receptor in the microenvironment of inflammation and cancer. Scientific World J. 2012;2012:381434.

29. Bayati S, Bashash D, Ahmadian S, et al. Inhibition of tachykinin NK1 receptor using aprepitant induces apoptotic cell death and G1 arrest through Akt/p53 axis in pre-B acute lymphoblastic leukemia cells. Eur J Pharmacol. 2016;791:274–283. doi:10.1016/j. ejphar.2016.09.006.
30. Muñoz M, Coveñas R. Safety of neurokinin-1 receptor antagonists. Expert Opin Drug Saf. 2013;12(5):673–685. doi:10.1517/14740338.2013.804059.
31. Barrett JS, Spitsin S, Moorthy G, et al. Pharmacologic rationale for the NK1R antagonist, aprepitant as adjunctive therapy in HIV. J Transl Med. 2016;14(1):148. doi:10.1186/s12967-016-0904-y.
32. Steinhoff MS, von Mentzer B, Geppetti P, Pothoulakis C, Bunnett NW. Tachykinins and their receptors: contributions to physiological control and the mechanisms of disease. Physiol Rev. 2014;94(1):265–301. doi:10.1152/physrev.00031.2013.
33. Navari RM. Fosaprepitant (MK-0517): a neurokinin-1 receptor antagonist for the prevention of chemotherapy-induced nausea and vomiting. Expert Opin Investig Drugs. 2007;16(12):1977–1985. doi:10.1517/13543784.16.12.1977.
34. Navari RM. Fosaprepitant: a neurokinin-1 receptor antagonist for the prevention of chemotherapy-induced nausea and vomiting. Expert Rev Anticancer Ther. 2008;8 (11):1733–1742. doi:10.1586/ 14737140.8.11.1733.
35. Van Belle SJ, Cocquyt V. Fosaprepitant dimeglumine (MK-0517 or L-785,298), an intravenous neurokinin-1 antagonist for the prevention of chemotherapy induced nausea and vomiting. Expert Opin Pharmacother. 2008;9(18):3261–3270. doi:10.1517/ 14656560802548463.
36. Robinson P, Martin P, Garza A, D'Souza M, Mastrangelo M-A, Tweardy D. Substance P receptor antagonism for treatment of cryptosporidiosis in immunosuppressed mice. J Parasitol. 2008;94:1150–1154.
37. Martinez AN, Burmeister AR, Ramesh G, Doyle-Meyers L, Marriott I, Philipp MT. Aprepitant limits in vivo neuroinflammatory responses in a rhesus model of Lyme neuroborreliosis. J Neuroinflammation. 2017;14(1):37. doi:10.1186/s12974-017-0813-x.
38. Lönndahl L, Holst M, Bradley M, et al. Substance P antagonist aprepitant shows no additive effect compared with standardized topical treatment alone in patients with atopic dermatitis. Acta Derm Venereol. 2018;98(3):324–328. doi:10.2340/000

15555-2852.

39. Muñoz M, Rosso M, Coveñas R. A new frontier in the treatment of cancer: NK-1 receptor antagonists. Curr Med Chem. 2010;17 (6):504–516. doi:10.2174/092986710790416308.
40. Berger M, Neth O, Ilmer M, et al. Hepatoblastoma cells express truncated neurokinin-1 receptor and can be growth inhibited by aprepitant in vitro and in vivo. J Hepatol. 2014;60(5):985–994. doi:10.1016/j.jhep.2013.12.024.
41. Harford-Wright E, Lewis KM, Ghabriel MN, Vink R. Treatment with the NK1 antagonist emend reduces blood brain barrier dysfunction and edema formation in an experimental model of brain tumors. PloS One. 2014;9(5):e97002. doi:10.1371/journal. pone.0097002.
42. Gross J, Willimsky E, Wegener AR, et al. Ultraviolet radiation exposure of one eye stimulates sympathizing expression of neurokinin-1 receptor but not monocyte chemoattractant protein-1 in the partner eye. Ophthalmic Res. 2019:1–13. doi:10.1159/000501320.
43. Zhou Z, Barrett RP, McClellan SA, et al. Substance P delays apoptosis, enhancing keratitis after Pseudomonas aeruginosa infection. Invest Ophthalmol Vis Sci. 2008; 49(10):4458–4467. doi:10.1167/iovs.08-1906.
44. Lucas K, Karamichos D, Mathew R, Zieske JD, Stein-Streilein J. Retinal laser burn-induced neuropathy leads to substance P-dependent loss of ocular immune privilege. J Immunol. 2012;189(3):1237–1242. doi:10.4049/jimmunol.1103264.
45. Quartara L, Maggi CA. The tachykinin NK1 receptor. Part I: ligands and mechanisms of cellular activation. Neuropeptides. 1997;31(6):537–563. doi:10.1016/S0143-4179(97)90001-9.
46. Black AT, Gordon MK, Heck DE, Gallo MA, Laskin DL, Laskin JD. UVB light regulates expression of antioxidants and inflammatory mediators in human corneal epithelial cells. Biochem Pharmacol. 2011;81(7):873–880. doi:10.1016/j. bcp.2011.01.014.
47. Chao S-C, Hu D-N, Roberts J, et al. Inhibition effect of curcumin on UVB-induced secretion of pro-inflammatory cytokines from corneal limbus epithelial cells. Int J Ophthalmol. 2017;10 (6):827–833. doi:10.18240/ijo.2017.06.01.

48. Chen S-J, Lee C-J, Lin T-B, et al. Inhibition of ultraviolet B-induced expression of the proinflammatory cytokines TNF-α and VEGF in the cornea by fucoxanthin treatment in a rat model. Mar Drugs. 2016;14(1):13. doi:10.3390/md14010013.
49. Di Girolamo N, Kumar RK, Coroneo MT, Wakefield D. UVB mediated induction of interleukin-6 and −8 in pterygia and cultured human pterygium epithelial cells. Invest Ophthalmol Vis Sci. 2002;43:3430–3437.
50. Solomon A, Li DQ, Lee SB, Tseng SC. Regulation of collagenase, stromelysin, and urokinase-type plasminogen activator in primary pterygium body fibroblasts by inflammatory cytokines. Invest Ophthalmol Vis Sci. 2000;41:2154–2163.
51. Fiebich BL, Schleicher S, Butcher RD, Craig A, Lieb K. The neuropeptide substance P activates p38 mitogen-activated protein kinase resulting in IL-6 expression independently from NF-kappa B. J Immunol. 2000;165(10):5606–5611. doi:10.4049/ jimmunol.165.10.5606.
52. Chen W, Lin H, Zhong X, et al. Discrepant expression of cytokines in inflammation- and age-related cataract patients. PloS One. 2014;9(10):e109647. doi:10.1371/ journal.pone.0109647.
53. Liu X, Zhu Y, Zheng W, Qian T, Wang H, Hou X. Antagonism of NK-1R using aprepitant suppresses inflammatory response in rheumatoid arthritis fibroblast-like synoviocytes. Artif Cells Nanomed Biotechnol. 2019;47(1):1628–1634. doi:10.10 80/21691401.2019.1573177.
54. Liu B-K, Jin X-W, Lu H-Z, Zhang X, Zhao Z-H, Shao Y. The effects of neurokinin-1 receptor antagonist in an experimental autoimmune cystitis model resembling bladder pain syndrome/interstitial cystitis. Inflammation. 2019;42(1):246–254. doi: 10.1007/s10753-018-0888-2.
55. Spitsin S, Meshki J, Winters A, Tuluc F, Benton TD, Douglas SD. Substance P-mediated chemokine production promotes monocyte migration. J Leukoc Biol. 2017;101(4):967–973. doi:10.1189/jlb.1AB0416- 188RR.
56. Tattersall FD, Rycroft W, Cumberbatch M, et al. The novel NK1 receptor antagonist MK-0869 (L-754,030) and its water soluble phosphoryl prodrug, L-758,298, inhibit acute and delayed cisplatin-induced emesis in ferrets. Neuropharmacology. 2000;39 (4):652–663. doi:10.1016/S0028-3908(99)00172-0.

## List of Publications

The data presented in this PhD thesis were published previously in the following publications:

1. **Janine Gross**, Alfred R. Wegener, Martin Kronschläger, Frank G. Holz, Linda M. Meyer. Ultraviolet radiation exposure triggers neurokinin-1 receptor upregulation in ocular tissues in vivo. Experimental Eye Research 174 (2018) 70-79, DOI: 10.1016/j.exer.2018.04.016

2. **Janine Gross**, Eva Willimsky, Alfred R. Wegener, Martin Kronschläger, Frank G. Holz, Linda M. Meyer. Ultraviolet radiation exposure of one eye stimulates sympathizing expression of neurokinin-1 receptor but not monocyte chemoattractant protein-1 in the partner eye. Ophthalmic Research 13 (2019) 1-13, DOI: 10.1159/000501320

3. **Janine Gross**, Alfred R. Wegener, Martin Kronschläger, Frank G. Holz, Linda M. Meyer. UVR-B-induced NKR-1 expression in ocular tissues is blocked by substance P receptor antagonist Fosaprepitant in the exposed as well as unexposed partner eye. Ocular immunology and inflammation (2020) 1-13, DOI: 10.1080/09273948.2019.1708414

## List of Figures and Tables

# Acknowledgements

**Danksagung**

Bei Herrn Dr. Alfred Wegener möchte ich mich ganz herzlich für die Vergabe der Doktorarbeit und für die Möglichkeit an diesem spannenden Thema arbeiten zu dürfen bedanken.

Mein ganz besonderer Dank geht an Frau Dr. Linda Meyer zum einen für die wissenschaftliche Anleitung und die tatkräftige Unterstützung, zum anderen für die unentwegte Betreuung während des Projektes und die motivierenden Worte, wenn sich die Forschung mal nicht von ihrer besten Seite zeigte.

Prof. Frank Holz danke ich herzlich für die Möglichkeit diese Doktorarbeit an der Universitäts-Augenklinik Bonn anfertigen zu können und darüber hinaus die Präsentation von meinen Forschungsarbeiten auf nationalen und internationalen Kongressen ermöglichte und unterstützte.

Ebenfalls gilt mein Dank Herrn Dr. Eike Latz für die Übernahme der Zweitbegutachtung und Herrn Prof. Per Söderberg und Herrn Prof. Frank Holz danke ich für ihre Bereitschaft als Gutachter dieser Arbeit zur Verfügung zu stehen.

Ein herzlicher Dank geht an Frau Claudine Strack und Frau Parand Widmar für die geduldigen Einweisungen, die Tipps und Tricks in der Immunhistochemie und die Hilfe bei kleinen oder großen Pannen im Labor. Vielen Dank Claudine Strack für die verrückten, lustigen und manchmal sehr sportlichen Momente im Büro!

An dieser Stelle möchte ich mich bei dem gesamten Laborteam der Universitäts-Augenklinik Bonn für das harmonische Arbeitsklima, die hilfreichen Anregungen und die schönen und lustigen Aktivitäten außerhalb des Labors bedanken.

Von ganzem Herzen möchte ich mich bei meiner Familie bedanken. Ich danke Euch, meiner Mutter Bettina Jansen, meinem Stiefpapa Bernhard Jansen und meinem Bruder Nicolai Groß, für das Interesse an meiner Arbeit, die Unterstützung, den Rückhalt und die Liebe an jedem einzelnen Tag. Am Ende möchte ich mich bei meinem Mann Benjamin Marx für seine unermüdliche Unterstützung, sein Verständnis, seine Geduld mit mir und seine aufbauenden Worte an schlechten Tagen bedanken. Danke für Deine Hilfe und Deine Liebe!

## Statement

| |
|---|
| Thesis title: Bilateral inflammation after unilateral UVR-B irradiation. Ocular cross-talk and immunomodulation between exposed and unexposed, contralateral eye. |
| The doctoral student, Janine Marx, has authored the above-mentioned doctoral thesis in the University eye hospital Bonn under the supervision of *PD Dr. rer. nat. Alfred Wegener* . |

| Title of publication: Ultraviolet radiation exposure triggers neurokinin-1 receptor upregulation in ocular tissues in vivo. | |
|---|---|
| Please specify your **own share** in the following areas: | Please specify the **share of others** in the following areas: |
| ***Planning of the academic work:***<br>The doctoral student, Janine Marx, has undertaken the predominant share of planning of the academic work. This includes the planning of the experiments, the time planning and the general procedure of the experiments. | ***Planning of the academic work:***<br>The supervisors, PD Dr. rer. nat. Alfred Wegener and PD Dr. med. Linda Meyer were available to answer question. |
| ***Data collection***<br>The complete data collection was performed by Janine Marx. This includes the housing and care of the laboratory animals, UVR-B irradiation, immunohistochemical staining methods, microscopy as well as microsurgical dissection of ocular tissues. | ***Data collection***<br>The medical-laboratory assistants, Mrs. Claudine Strack and Mrs. Parand Widmar, were available for any immunohistochemical questions and offered helpful advice. |
| ***Evaluation***<br>The data was analyzed with SPSS Statistics 23. The doctoral student, Janine Marx, has carried out the evaluation. A one-way ANOVA with a post-hoc test was performed to indicate the difference of the different groups. Furthermore, a paired sample t-test was per-formed to quantify differences between groups of each latency periods. | ***Evaluation***<br>The doctoral student received support in the selection of statistical test from the statistical consulting of the institute for medical Biometry, Informatics and Epidemiology (IMBIE).<br>The evaluation of the data was also discussed with the super-visors. |
| ***Interpretation***<br>Janine Marx has undertaken the predominant share of the interpretation and has written the first version of the manuscript. | ***Interpretation***<br>The supervisors, PD Dr. rer. nat. Alfred Wegener and PD Dr. med. Linda Meyer support the doctoral student with ques- |

| | |
|---|---|
| | tions. Suggestions for improvement and comments were discussed together with the doctoral student, Janine Marx. |
| ***Please tick the relevant box:*** <br> ☒ *The doctoral student is the lead author and has written the first version of the manuscript himself/herself* <br> ☐ *The doctoral student is a co-author* | |

| Title of publication: Ultraviolet radiation exposure of one eye stimulates sympathizing expression of neurokinin-1 receptor but not monocyte chemoattractant protein-1 in the partner eye. | |
|---|---|
| Please specify your **own share** in the following areas: | Please specify the **share of others** in the following areas: |
| ***Planning of the academic work:*** <br> The planning and implementation of the publication was done by the doctoral student, Janine Marx. This includes the time planning as well as the general procedure planning of the experiments. | ***Planning of the academic work:*** <br> PD Dr. rer. nat. Alfred Wegener and PD Dr. med. Linda Meyer were available for further questions with their scientific and medical know-how. |
| ***Data collection*** <br> The data collection was carried out completely by the doctoral student, Janine Marx. This includes the housing and care of mice, UVR-B irradiation, microscopy, micro-surgical dissection of ocular tissue as well as the performance of the enzyme-linked immunosorbent assay (ELISA) method. | ***Data collection*** <br> The medical-technical assistant Claudine Strack answered questions about immunohistochemical staining. |
| ***Evaluation*** <br> The evaluation of the data was done by the doctoral student, Janine Marx. Data were presented as mean standard deviation (SD) and analyzed with SPSS Statistics 23. One-way analysis of variance followed by a post-hoc comparison was performed to analyze the difference in protein levels between latency periods within the ocular tissues. In addition, a paired sample t-test was used to quantify differences between exposed and unex- | ***Evaluation*** <br> The doctoral student, Janine Marx, received support from the statistical consulting of the institute for medical Biometry, Informatics and Epidemiology (IMBIE). The evaluation of the data was discussed with the supervisors. |

| | |
|---|---|
| posed mouse eyes within a latency period group. | |
| ***Interpretation***<br>The doctoral student, Janine Marx did the interpretation of this publication on her own. | ***Interpretation***<br>Discussion and exchange occurred together with the supervisors. |
| ***Please tick the relevant box:***<br>☒ *The doctoral student is the lead author and has written the first version of the manuscript himself/herself*<br>☐ *The doctoral student is a co-author* | |

| Title of publication: UVR-B-induced NKR-1 expression in ocular tissues is blocked by substance P receptor antagonist Fosaprepitant in the exposed as well as unexposed partner eye. | |
|---|---|
| Please specify your **own share** in the following areas: | Please specify the **share of others** in the following areas: |
| ***Planning of the academic work:***<br>The complete planning of this academic work was done by the doctoral student, Janine Marx. The planning includes the time planning of the experiments as well as the general procedure planning of the experiments. | ***Planning of the academic work:***<br>The supervisors (PD Dr. rer. nat. Alfred Wegener and PD Dr. med. Linda Meyer) were available for any questions. In addi-tion, the final planning of the publication was discussed together with the doctoral student. |
| ***Data collection***<br>The complete data collection was performed by the doctoral student, Janine Marx. This includes the housing and care of the laboratory animals, UVR-B irradiation, microsurgical dissection of ocular tissues as well as the performance of the enzyme-linked immunosorbent assay and multiplex cytokine assay for the detection of various cytokines and chemokines. | ***Data collection***<br>The medical-laboratory assistant Claudine Strack was available for any immunohistochemical questions.<br>The multiplex cytokine assay was performed in the institute of innate immunity by the working group of Prof. Dr. med. Eicke Latz. The doctoral student, Janine Marx received support with the device instruction also by the working group of Prof. Dr. med. Eicke Latz. |
| ***Evaluation***<br>SPSS Statistics 23 was used for the evaluation of data. Statistical significance among the treatment and latency period groups was determined using one-way analysis of variance (ANOVA) followed by a post-hoc | ***Evaluation***<br>The doctoral student, Janine Marx, received support in the selection of statistical test from the statistical consulting of the institute for medical Biometry, Informatics and Epidemiology (IMBIE). The |

| | |
|---|---|
| comparison. To compare eyes within one treatment and latency period group one-sample t-test was performed. The doctoral student, Janine Marx, has undertaken the predominant share of the evaluation. | evaluation of the data was also discussed with the super-visors. |
| ***Interpretation***<br>The doctoral student, Janine Marx, has undertaken the predominant share of the interpretation and has written the first version of the manuscript on her own. | ***Interpretation***<br>The supervisors, PD Dr. rer. nat. Alfred Wegener and PD Dr. med. Linda Meyer, support the doctoral student with ques-tions. Suggestions for improvements were discussed together with the doctoral student. |
| ***Please tick the relevant box:***<br>☒ *The doctoral student is the lead author and has written the first version of the manuscript himself/herself*<br>☐ *The doctoral student is a co-author* | |