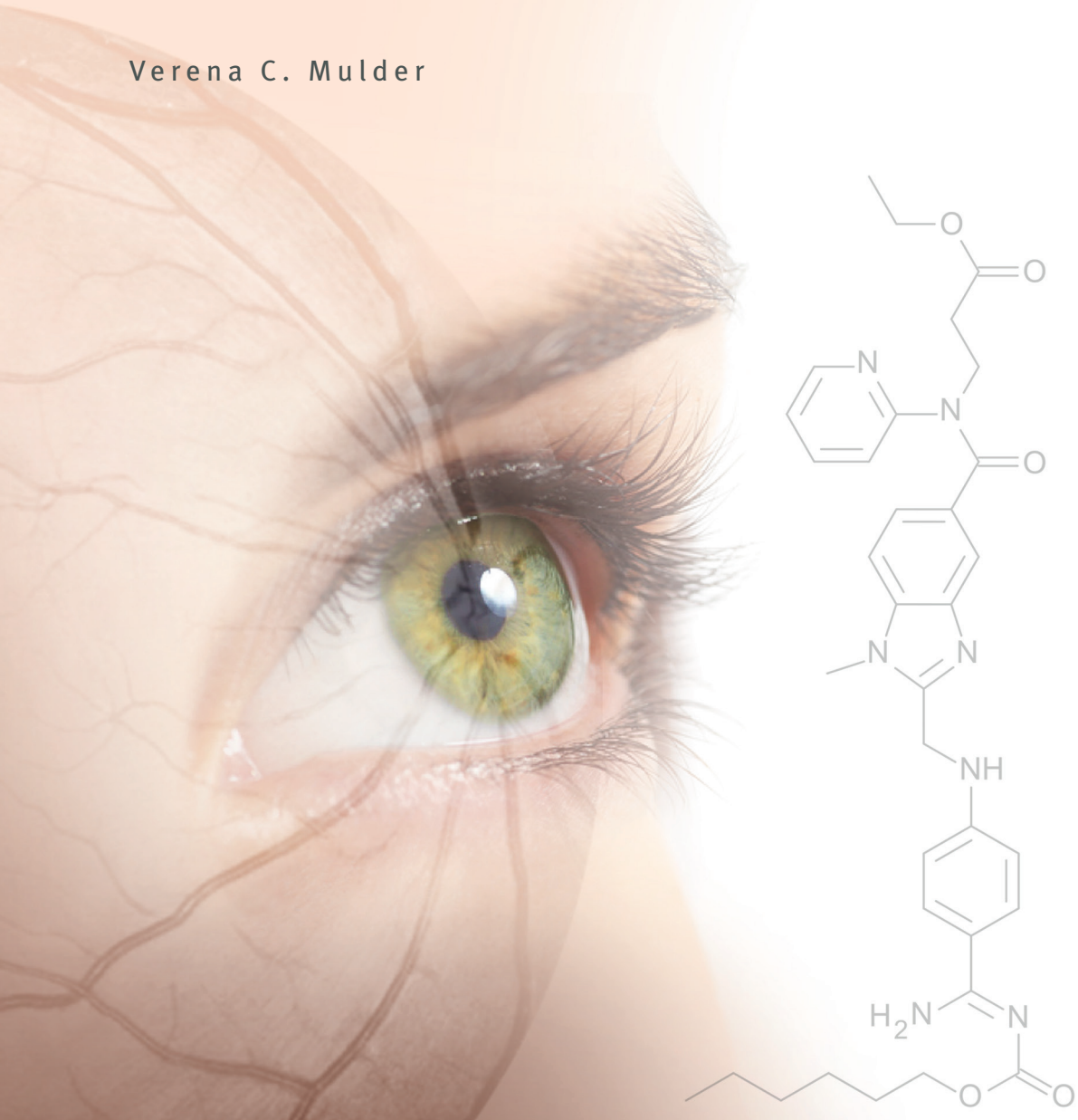
Verena C. Mulder



Proliferative Vitreoretinopathy

Steps towards prevention

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PROLIFERATIVE VITREORETINOPATHY
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Proliferative Vitreoretinopathy

Steps towards prevention

Proliferatieve vitreoretinopathie

Op weg naar preventie

Proefschrift

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"You can, you should, and if you're brave enough to start, you will."

Stephen King

Voor pap en mam

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Introduction and outline of this thesis

1

INTRODUCTION

One of the most delicate and specialised structures of the eye is the retina. The retina is a light-sensitive layer that converts the light that falls onto it into an electrical signal. Subsequently, the electrical signal travels through the optic nerve and optical radiation to the visual cortex where it is translated into an image.

In the retinal structure we can distinguish ten layers. The innermost layer is the internal limiting membrane (ILM), followed by the nerve fibre layer, ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer, external limiting membrane, the photoreceptor layer and the retinal pigment epithelium (RPE). The first nine layers are collectively called the neurosensory retina. Although the neurosensory retina is strongly attached at the edges of the optic nerve head and the ora serrata region (the anterior edge of the neurosensory layer), the adhesion to the underlying RPE is weaker. The nutrition of the inner layers of the retina is supplied by the central retinal artery and its branches, which enter the eye through the optic nerve. The metabolism of the outer layers of the retina is further supported by the underlying choroid vessels and the sclera (see **Figure 1**).

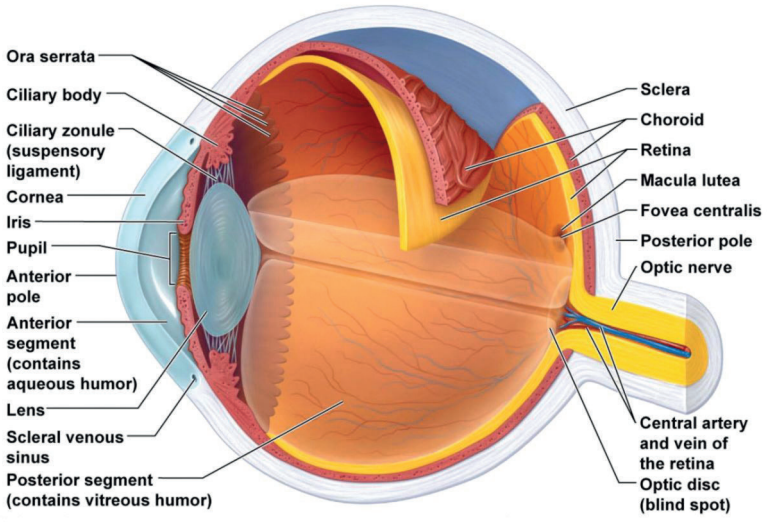

Figure 1. Anatomy of the eye. | © Pearson Education

THE VITREOUS

Approximately 80% of the volume of the eye contains the vitreous. The vitreous is a clear matrix composed of collagen, hyaluronic acid, and water. The vitreous is most firmly attached to the vitreous base but it is also firmly attached to retinal vessels, the optic nerve, and the macula. With ageing (starting already at the age of 2 years), the vitreous humour

starts to liquefy. While it first filled the whole cavity, the vitreous gel eventually starts to shrink, putting various portions of the retina under tractional stress. When the posterior vitreous starts to detach from the retina – called a posterior vitreous detachment or PVD – the tractional stress can become too much and produce a tear or hole in the neurosensory layer.[1]

RHEGMATOGENOUS RETINAL DETACHMENT

The separation of the neurosensory layer from the underlying RPE is called a retinal detachment. In the case of a rhegmatogenous retinal detachment (RRD), the cause of the detachment is a tear or hole in the retina, implied by the Greek word rhegma, which allows fluid from the vitreous cavity to flow into the subretinal space resulting in the separation of the layers (**Figure 2**).[2, 3]

Patients with a retinal detachment usually experience floaters (mouches volantes), light flashes and – dependent on the extent of the detachment – visual field loss and/or loss of vision. Nearly all patients with symptomatic RRD will progressively lose vision and will eventually become blind when left untreated.[2, 3]

The incidence of RRD is approximately 18 per 100 000 people and increases significantly with age, with the mean age being 60 years.[3-5] Besides age, other risk factors include high myopia, male gender, trauma, cataract surgery, a retinal detachment in the other eye or a family history of retinal detachment.[2]

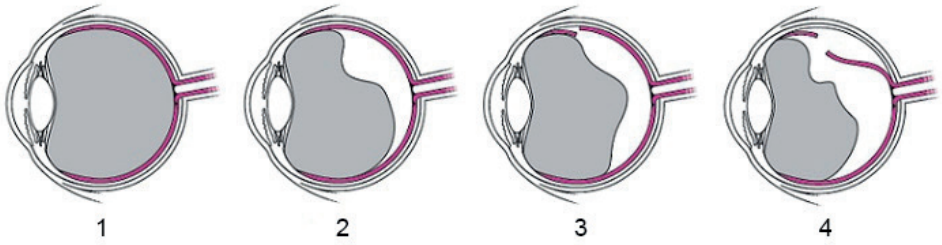

Figure 2. Schematic representation of a rhegmatogenous retinal detachment. Normally the posterior segment is completely filled with vitreous (grey) (1). With ageing the vitreous liquefies and shrinks slowly (2), it detaches from the retina (pink). This posterior vitreous detachment (PVD) sometimes leads to a tear in the retina (3). When fluid flows into the subretinal space the retina detaches (4). Adapted from: www.oogziekenhuis.nl

SURGICAL TREATMENT

Treatment of RRD consists of closing the retinal break and relieving vitreous traction on the retina. To accomplish this, the surgeon can either choose an external or internal approach.

The external approach consists of suturing a silicone encircling band and/ or segmental silicone explant onto the sclera. By using mattress sutures wider than the explant material the resulting indentation causes relief of traction internally at that exact point. This procedure can be combined with external drainage of subretinal fluid and the injection of air or gas

to temporarily close the retinal tear and approximate the neurosensory layer to the RPE. Laser photocoagulation or cryopexy can be added to create a chorioretinal burn to induce adhesion by reactive scarring.

The internal approach is by pars plana vitrectomy (PPV). This approach involves the surgical removal of the vitreous gel as a source of retinal traction. The surgical instruments enter the eye through three ports in the pars plana. After removal of the vitreous gel up to its adhesion in the vitreous base, the eye is usually filled with a tamponade to close retinal breaks and approximate the neurosensory layer to the RPE and to maintain intraocular pressure (IOP). Frequently used tamponades are gas (SF_6, C_3F_8) or silicone oil (1000 or 5000 centistoke viscosity).

COMPLICATIONS

Both scleral buckling (SB) and vitrectomy have their advantages and disadvantages. The scleral buckling procedure can lead to a change in refractive error and is associated with complications including diplopia, choroidal detachment, and perforation of the sclera when suturing the explants or draining subretinal fluid. Vitrectomy may avoid some of these complications, but it carries a higher risk of endophthalmitis and glaucoma, and leads to cataract formation.[6]

Anatomical reattachment is accomplished with a single surgery in 80-90% of patients.[6] In patients where the primary surgery fails, reoperation results in final reattachment rates around 96%.[6] The most frequent cause of the need for reoperation – due to persisting or recurrent detachment – is a missed or new retinal break which in general can be treated successfully. The primary cause of failure of reattachment despite multiple interventions is the development of proliferative vitreoretinopathy.

PROLIFERATIVE VITREORETINOPATHY

Proliferative vitreoretinopathy (PVR) is characterised by the growth of contractile membranes on or under the retina, or fibrosis within the retina that causes detachment of the neurosensory layer from the underlying RPE (**Figure 3**). PVR develops in 5-10% of patients and is still the most severe and most difficult complication of RD to treat because these membranes are very difficult to remove completely without further damaging the neurosensory layer and moreover have the tendency to recur.[7] PVR can develop in eyes with RRD if the detachment remains untreated for a period of weeks to months but it more typically occurs in eyes that have undergone retinal reattachment surgery. Its onset is usually 2 weeks to 6 months after surgery, with a median of 2 months.[8]

PATHOPHYSIOLOGY

After detachment of the neurosensory layer from the RPE, the outer retinal layers become ischemic. Due to activation of intrinsic protective mechanisms, this does not lead to immediate neurone death but after cessation of the initial stress response will.[7] Subsequently, glial cells initiate a nonspecific tissue repair response that involves inflammation and proliferation that finally leads to remodelling of the retina.[7]

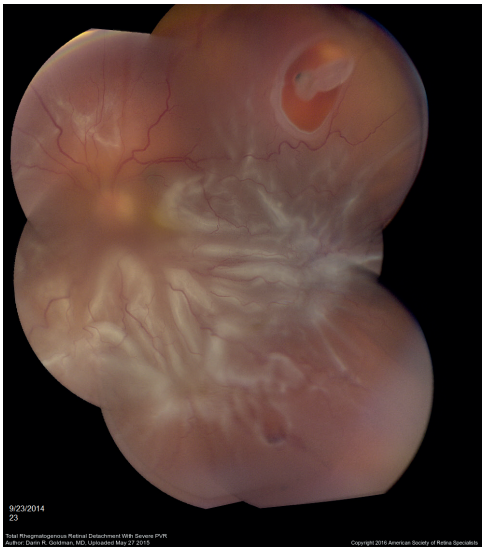

Figure 3. Total RRD with severe PVR. This image was originally published in the Retina Image Bank by Darin R. Goldmann. 2015; # 25035.
© the American Society of Retina Specialists.

Although these responses are part of normal tissue healing, in 5-10% of patients these responses progress to the development of PVR. What critical distinctive difference is present to direct these events towards PVR is yet unclear.

Under physiologic conditions, RPE is organised as a monolayer of hexagonal shaped cells densely packed together and constitutes an important part of the blood-retinal barrier. However, after tissue injury such as in RD, RPE cells may detach from their normal attachment to Bruch's membrane and start to proliferate while undergoing transformation.[7, 9] This transformation is called epithelial-mesenchymal transition (EMT). The RPE cell loses its epithelial features such as tight junction molecules, and acquires mesenchymal features that include enhanced migratory capacity, invasiveness, resistance to apoptosis, and production of extracellular matrix (ECM) components.[7, 9-11] The now myofibroblast-like cells migrate into the vitreous through breaks in the retina. With the blood-retinal barrier breakdown (see *Blood-Ocular Barriers*) the cells are exposed to inflammatory mediators such as C-C motif chemokine ligand (CCL)2, C-X-C motif chemokine ligand (CXCL)8, granulocyte-macrophage-colony-stimulating factor (GM-CSF), interleukin (IL)-6 and IL-8, growth factors, and ECM. Tissue damage also triggers the recruitment of monocytes and macrophages, which in their turn are able to produce pro-fibrotic mediators such as PDGF, TGFβ, and VEGF. Myofibroblasts exhibit contractile properties and a strong capacity to produce ECM molecules such as collagen, elastin, laminin, fibronectin, and vitronectin.

The interplay of all these factors finally leads to the development of contractile membranes and formation of fixed folds in the retina.

PHARMACOLOGIC PREVENTIVE AND ADJUVANT THERAPY

In general, pharmacological attempts to prevent PVR have focused on either interfering with proliferation or modifying the inflammatory cascade. One of the first types of drugs tested for PVR were corticosteroids. Corticosteroids are widely used for a variety of conditions and exert anti-inflammatory properties. However, despite success in animal models of PVR, studies in patients failed to demonstrate the same beneficial effect. An intravitreal injection of 2-20mg **triamcinolone acetonide** did not improve outcomes in patients undergoing vitrectomy with silicone oil for PVR.[12-15] A preoperative injection of **dexamethasone diphosphate** showed a decrease in laser flare measurements at 1 week postoperatively. This suggested that steroid priming might be useful in reducing BRB breakdown and hence PVR. However, the follow-up period in this study was short.[16, 17] Oral **prednisone** 1mg/kg during 10 days did not improve reattachment rate, visual acuity, PVR, or postoperative complications.[18] A 15-day oral prednisone regimen tapered from 100mg to 12.5mg did significantly reduce the formation of cellophane membranes compared to placebo.[19]

Until recently, proliferation RPE and glial cells were seen as one of the main features of PVR.[7] Therefore, most proposed therapies are aimed at inhibiting cell proliferation. A frequently tested combination in patients is the antimetabolite **5-Fluorouracil** (5-FU) and a Low-Molecular-Weight Heparin (LMWH), usually **dalteparin** or **enoxaparin**.[20-24] These drugs were added to the vitrectomy infusion fluid in concentrations of 200 µg/ml and 5 IU/ml, respectively, and exposure was approximately 1 hour.[25] 5-FU inhibits DNA synthesis and fibroblast proliferation, while LMWHs are thought to inhibit fibronectin and prevent fibrin formation.[26] Although LMWHs have reduced tendency to bind macrophages and plasma proteins – including vitronectin, fibronectin, and fibrinogen – compared to the large negatively charged molecule heparin, effects on fibrin formation and reduction of tractional detachment have been demonstrated.[27, 28] However, randomised controlled trials showed little efficacy.

A second antineoplastic agent tested in patients with advanced preoperative PVR is the anthracycline **daunorubicin**. In a randomised controlled trial of 286 patients it was infused intravitreally over a 10 minute period (7.5 µg/ml) before a tamponade was injected.[29] The authors found no significant difference in reattachment rate at 6 month postop but the daunorubicin group needed fewer reoperations in the first year.

Colchicine, which is normally used in gout treatment, was tested in patients with RRD undergoing SB. Patients were randomly assigned to oral colchicine 1mg twice daily for 7 weeks or placebo.[30] Colchicine had shown an effect in an animal study, but in a double-masked controlled trial of 184 patients, no effect was found on the prevention of retinal detachment due to PVR.[31]

Oral 13-Cis-retinoic acid (**isotretinoin**) has been tested in two different administration schemes in patients undergoing surgery for PVR. In the first retrospective study, isotretinoin was used for 4 weeks in a dosage of 40mg twice daily in combination with oral prednisone 1mg/kg for 3 weeks followed by a taper over the subsequent 3 weeks. Although the study population was small (n = 20) it showed some encouraging results.[32] In the second prospective randomised controlled study (n = 16/19), the treatment period was prolonged

to 8 weeks and the isotretinoin dose was lowered to 10mg twice daily, no prednisone was used.[33] The results showed a significantly higher initial and final repair rate with isotretinoin. Neither of the two studies was placebo controlled.
A different type of drug tested in humans was the DNA-RNA chimeric ribozyme VIT100, which targets proliferating cell nuclear antigen (PCNA). Inhibition of this cell cycle controlling gene that inhibits cell division was not effective in preventing PVR recurrence in a double-masked, placebo-controlled, randomised clinical trial that enrolled 154 patients with established PVR.[17]

Other drugs tested *in vitro* or in experimental PVR are the antineoplastic agents etoposide, tacrolimus, paclitaxel, vincristine, cisplatin, doxorubicin, mitomycin, and actinomycin D, the kinase inhibitors hypericin, herbimycin A, alkyl phosphocholine, fasudil and AG1295, the TGFβ inhibitor tranilast, the TNFα blocker adalimumab, the anti-fibrotic drug pirfenidone, the antiprotozoal suramin, the antioxidant N-acetylcysteine, the cholesterol-lowering drug simvastatin, and glucosamine.[7, 17] More recently, the antiangiogenic drugs ranibizumab and aflibercept have been added to the list.[7]
Most drug therapies have been tested in patients in combination with membrane removing surgery. In these patients, the processes involved in PVR development are fully active and possibly difficult to reverse. Ideally, one would like to have a prophylactic treatment and interfere in this process before it even starts.

COAGULATION PROTEINS

Activation of the coagulation cascade after tissue injury is crucial in facilitating the healing process. However, uncontrolled activation of the coagulation cascade has been recognised to contribute to fibrosis.[34, 35] In the case of RD, RPE cells are exposed to serum factors as shown by the procoagulant activity in subretinal fluid from patients with RRD because of the damage in the blood-retinal barriers (see *Blood-Ocular Barriers*).[36] In his thesis, *"A novel role for coagulation proteins in the development of proliferative vitreoretinopath*y", Bastiaans *et al* showed that the cellular processes activated by the coagulation factor thrombin contribute to the development of PVR and that thrombin is a possible new target for therapy.

BLOOD-OCULAR BARRIERS

Nutrients and other substances – either endogenous or administered – can enter the eye through the vessels in the stroma of the iris and pass into the newly formed aqueous humour. The cornea is exposed to substances from the limbal capillaries and the aqueous humour as well as from the tear layer. The retina and vitreous body are served by either the choroidal or retinal circulation, and to a lesser extent by the aqueous in the posterior chamber, the space behind the lens.
To protect the eye from potentially damaging agents and to maintain homeostasis, the eye exploits a variety of mechanisms including tear secretion, an active transport system pumping potentially damaging agents from the retina back into the bloodstream, and several

barriers to prevent diffusion from the bloodstream into the eye (see **Figure 4).**[37]
The two most important barriers are the blood-aqueous barrier (BAB) and the blood-retinal barrier (BRB), collectively called the blood-ocular barriers (BOB). As the name implies, the blood-aqueous barrier regulates exchanges between the blood and aqueous humour. It is comprised of a non-pigmented epithelial layer of the ciliary body and an endothelial layer in the blood vessel walls of the iris.[38] These cell layers exclude blood proteins that would impair transparency and disturb the osmotic and chemical equilibrium.
The blood-retinal barrier consists of tight junctions between the endothelial cells of the retinal vessels (the inner blood-retinal barrier) and of tight junctions between the RPE cells (the outer blood-retinal barrier). In addition, certain enzymes reside in the endothelial and epithelial cells which contribute to the protective function. These enzymes include Angiotensin Converting Enzyme (ACE), dopa decarboxylase, γ-GTP, pseudocholinesterase, and monoamine oxidases.[38]

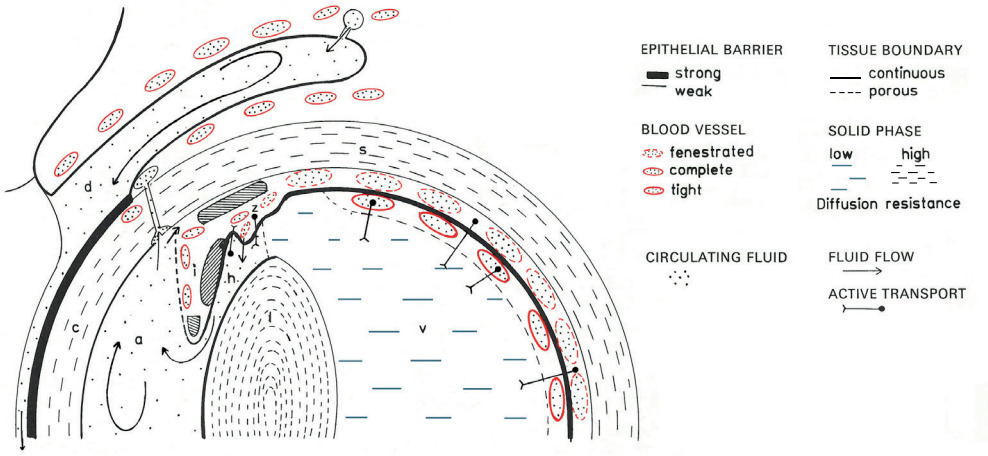

Figure 4. Schematic representation of the Blood-Ocular Barriers. A= Aqueous humour; C= Cornea; D= tear fluid; H= posterior chamber; I= Iris; L= Lens; S= Sclera; V= Vitreous; Z= Ciliary body
Adapted from Maurice DM, Mishima S. Pharmacology of the Eye; Handbook of Experimental Pharmacology. Vol.69 ed. Germany: Springer-Verlag Berlin Heidelberg New York Tokyo; 1984:736-21.

BLOOD-OCULAR BARRIERS AND DRUG DELIVERY

The presence of the BOBs greatly influences the delivery of drugs into the eye. In addition, the clearing mechanisms complicate maintaining an adequate drug level. For example, the cytochrome p-450 drug metabolising system and the efflux pump P-gp have been identified in RPE.[37, 39]
The properties of the drug are therefore of importance. Molecules with higher lipid solubility penetrate cell membranes more easily and this is also true for cellular layers. In addition, a smaller molecular size and a charge aid in diffusion.

Repeated topical application of drops and ointments is usually adequate for most external conditions and those of the anterior segment. Local injections are an option for reaching the posterior segment. They have the advantage of circumventing the eye's natural barriers but carry the risk of endophthalmitis, especially with repeated administration. Administration via the systemic route is easier but suffers from the disadvantage that all the organs of the body are exposed to the drug when only a small volume of tissue in the eye needs the treatment.[37]

BLOOD-OCULAR BARRIER BREAKDOWN ASSOCIATED WITH DISEASE

In disease, inflammatory mediators cause tight junctions to disappear leading to increased vascular permeability. It has been shown by anterior segment fluorophotometry (ASFM) that the BAB is significantly more permeable in RRD.[40]

As described earlier, RD also leads to disruption of the BRB. This breakdown of the BOB barriers makes the eye more susceptible to systemic substances and opens up the possibilities for systemic drug treatment.

It was also shown however that the BAB permeability returned to normal within two months of successful reattachment of the retina.[40]

LASER FLARE PHOTOMETRY

Under normal conditions, the anterior chamber is an optically empty space which facilitates optimal visual function. When the eye becomes inflamed, however, disruption of the BAB leads to leakage of proteins and inflammatory cells into the anterior chamber.[41] Increased protein content in the anterior chamber produces an optical phenomenon called flare (or Tyndall effect).[42] Flare can be compared to the effect produced by a narrow beam of light crossing a dark smoky room.[42]

Clinicians examine the severity of inflammation by slit-lamp biomicroscopy of the aqueous humour, and they grade the amount of flare on a scale from "faint" to "intense".[43, 44] This type of examination gives at best semi-quantitative values, because it highly relies on light conditions, the experience of the examiner, and contrast sensitivity of the examiner's eye.[45]

In 1989 Kowa company marketed their first laser flare cell meter (FC-1000). This instrument was able to quantify flare and cells by using a laser. Laser flare offered an objective, reproducible and non-invasive measurement technique to assess inflammation.

WORKING PRINCIPLE (FM-500)

Laser flare photometry quantifies aqueous humour protein by measuring the amount of light scattered by the proteins in the anterior chamber. The light source is a semiconductor diode laser (650nm) which is placed at a 90 angle from the light receiving device (a photomultiplier).[46] During the measurement, a small window of 0.3mm x 0.5mm is scanned over 0.5 seconds. In addition, it records two background measurements from above and below this window, which is regarded as noise from surrounding ocular tissues. The flare count is then obtained by subtracting the average of the two background readings from the main signal (see **Figure 5**).

CLINICAL APPLICATION

Since the introduction, laser flare measurements have mainly been used to monitor disease activity in diseases such as uveitis and retinitis pigmentosa.[44, 47-51] In addition, laser flare has been used to follow up on inflammation after surgical procedures such as cataract extraction, vitrectomy, and scleral buckling.[16, 52, 53] It was found not only to be useful in monitoring the anterior segment but also of the posterior segment.[44, 45, 49, 54-56]

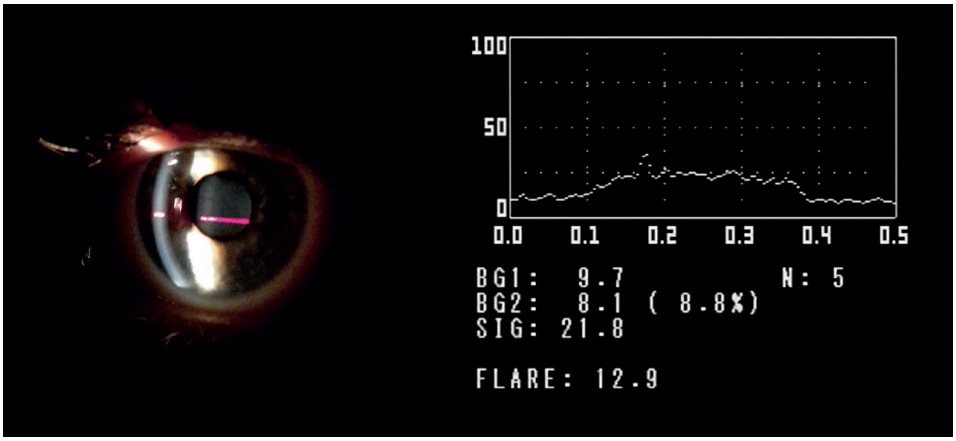

Figure 5. On the left: the laser beam passing the cornea and anterior chamber. On the right: the output of a typical flare measurement.

THIS THESIS

It is still not completely understood why some patients develop PVR and others do not despite extensive research on cytokine biomarkers, genetic profiles and ocular clinical risk factors. Recently, a new possible treatment target was proposed based on studies on the role of coagulation proteins in the development of PVR.

While several pharmaceutical drug therapies have been proposed for PVR – targeting inflammation, proliferation and growth factors – these drugs all have potential side effects. Therefore, to optimise the benefit/risk ratio of drug therapies, it is crucial to select only those patients at high risk of developing PVR

In **chapter 2** we tested whether the oral direct thrombin inhibitor dabigatran would be a potential drug candidate for the treatment of PVR. In this respect, we tested whether oral administration would lead to measurable levels in the eye (**2.1** and **2.2**) and whether dabigatran was able to oppose the effects of thrombin in an in vitro model (**2.3**). In chapter **2.4** we tested the amount of endogenous thrombin generation and inhibition in vitreous and subretinal fluid.

In **chapter 3** we tested the applicability of aqueous humour laser flare measurements in distinguishing between patients at high and low risk of developing PVR. Firstly, we looked at preoperative flare values (**3.1**) and secondly at postoperative flare values (**3.2**). Chapter **3.3** shows that the choice of surgery might also influence postoperative inflammation and thus influences the risk of developing PVR.

An aspect that has gained less attention in research is the possible influence of concomitant systemic drug use. As mentioned earlier, the prevalence of RRD increases with age with a mean age around 60 years. In this age group, systemic drug use is not uncommon but little is known about the possible impact of these drugs on the eye. Of particular interest would be systemic drugs known to affect inflammation or fibrosis that could potentially have a stimulating or a protective effect on the course and the occurrence of PVR. In **chapter 4**, we describe our research into whether medication use around the time of surgery for RRD was of importance for the development of PVR.

After the summaries in **chapters 5** and **6**, **chapter 7** will discuss our findings and future perspectives.

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Evaluation of dabigatran as a potential drug for the prevention of PVR

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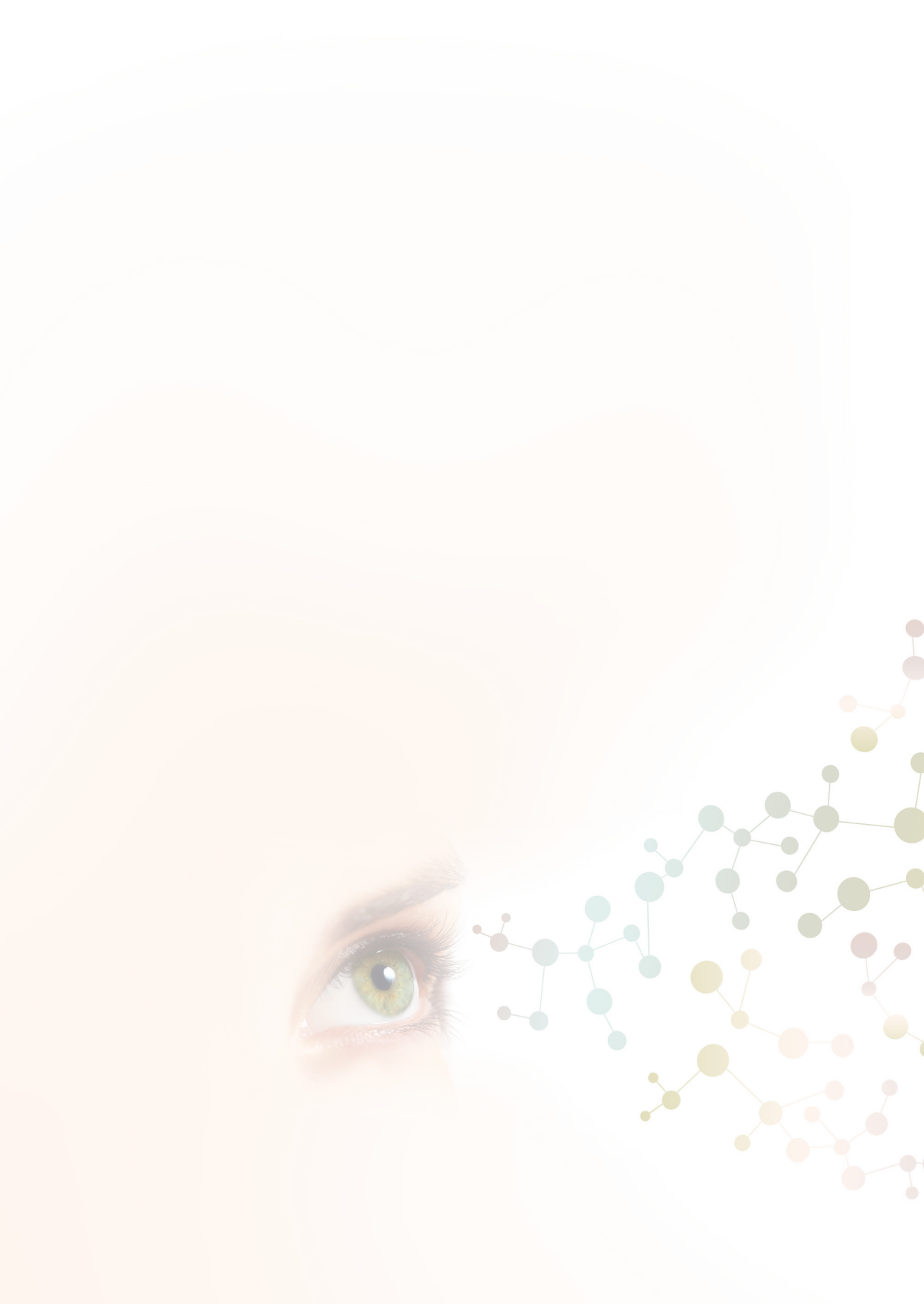

2.1

Vitreous and subretinal fluid concentrations of orally administered dabigatran in patients with rhegmatogenous retinal detachment

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ABSTRACT

PURPOSE

Thrombin appears to play a role in the development of proliferative vitreoretinopathy, a complication of retinal detachment characterized by epiretinal membranes. A specific oral thrombin inhibitor, like dabigatran, might be a possible therapeutic option. It opens the possibility of prolonged administration in contrast to drugs that can only be applied during vitrectomy. We tested if dabigatran reaches the vitreous and subretinal fluid (SRF) after a single oral dose of dabigatran.

METHODS

Twenty-eight patients with a rhegmatogenous retinal detachment received a single dose of 220mg dabigatran etexilate 2 to 8 hours prior to surgery. During surgery, we took a blood sample and depending on the type of surgery, a vitreous or subretinal fluid sample. The concentration of dabigatran was measured using LC-MS/MS.

RESULTS

The dabigatran concentration in SRF between 2 and 9 hours after administration varied up to 8.5ng/mL. The concentration in the vitreous fluid was lower and varied up to 3.8ng/mL. Corresponding plasma concentrations ranged from 15ng/mL to 225ng/mL. There was a significant relationship between SRF levels and plasma levels (r_s= .68, p=.014); the levels in vitreous fluid showed no such relationship (r_s= .20, p=.48). In addition, we measured the vitreous concentration of a non-study patient using 150mg dabigatran BID. The concentration was 25.8ng/mL, approximately 10 times higher than after a single dosage.

CONCLUSION

In conclusion, we demonstrate that oral intake of dabigatran, a candidate drug to modulate PVR, results in potentially relevant intraocular concentrations. We suggest that repeated dosing may lead to higher concentrations, but this should be further explored.

INTRODUCTION

Dabigatran etexilate (Pradaxa®) is an oral prodrug that is metabolised by serum esterases to dabigatran. Dabigatran is a competitive and reversible direct thrombin inhibitor used to decrease the risk of venous thromboembolic events in patients undergoing hip or knee replacement surgery.[1]

Apart from its role in the coagulation cascade, thrombin appears to play a role in the development of proliferative vitreoretinopathy (PVR) – a complication that is seen in approximately 10% of patients after surgery for a retinal detachment – inhibition of thrombin might be a possible therapeutic target for prevention of PVR.[2] It opens the possibility of prolonged administration in contrast to drugs that can only be applied during vitrectomy.

The main challenge of an oral drug is to achieve effective drug levels in the posterior segment of the eye. Ideally over a sustained period of time, as PVR is a process that typically develops during first 6 weeks after a retinal detachment. A healthy eye is protected from potentially damaging agents by barriers to diffusion and an active transport system clearing the retina, but these mechanisms also prevent effective drug concentrations in the ocular tissues when administered through the circulation.[3]

In the past several anti-inflammatory agents, antiproliferative agents and heparin have been clinically tested for their disease-modifying effect on PVR, as single agents as well as in combination. Most of these drugs were administered perioperatively in the vitrectomy infusion fluid or locally by intravitreal injection. Exposure to these drugs was relatively short with at best modest results on PVR modulation.[4-9] Prolonged local exposure by repeated intravitreal injections might be more effective than oral administration but is less attractive in terms of infection risk and convenience for the patient.

In case of a retinal detachment, the blood-retinal barrier might be (partially) disrupted, making the eye more permeable to systemic drugs. Dabigatran is a small polar molecule of approximately 471 Dalton with a great likelihood of passing the blood-retinal barrier in case of a rhegmatogenous retinal detachment. The maximum time-concentration point in vitreous and subretinal fluid is difficult to predict as there is little knowledge about the ratio between serum and eye concentrations. Weijtens et al. conducted a bioavailability study of different administration routes of dexamethasone, which has a similar molecular weight as dabigatran.[10] Although dexamethasone differs in lipophilicity from dabigatran (LogP 1.93 vs -2.4), it is the best comparison available. Weijtens et al. found a 15 times and 7.5 times lower concentration of dexamethasone in vitreous and subretinal fluid respectively than in serum. It has been reported that a single administration of 200 mg dabigatran etexilate resulted in a maximum concentration of 161 ng/mL in plasma.[11] In analogy with dexamethasone, we assume that a concentration of 10ng/mL dabigatran could be achieved. In this study, we investigated whether dabigatran reaches potentially relevant levels in the vitreous and subretinal fluid after oral administration of a single dose of 220 mg.

METHODS

This study was approved by the Medical Ethical Committee of the Erasmus Medical Center (Rotterdam, The Netherlands), and was registered on www.trialregister.nl (NTR4825). The research followed the tenets of the Declaration of Helsinki and all patients signed informed consent.

POPULATION

For this study, we included twenty-eight patients between the age of 18 and 75 years with a rhegmatogenous retinal detachment. Twelve patients who were eligible for scleral buckling surgery and sixteen patients undergoing vitrectomy. Patients using anti-coagulant drugs and drugs that are known to increase the risk of bleeding (e.g. NSAIDs, SSRIs, and corticosteroids) were excluded. Other exclusion criteria were a history of stomach ulcer or bleeding, creatinine clearance < 50 mL/min and elevated liver enzymes (ASAT, ALAT, gamma-GT) > 2 upper limit of normal.

INTERVENTION

Patients received 220 mg of dabigatran etexilate (Pradaxa®) administered as two capsules of 110mg with a glass of water on the morning prior to surgery, supervised by the study coordinator. The time between intake and surgery was varied between 2 and 8 hours to obtain a population-based pharmacokinetic profile. However, exact randomization of the time intervals was not possible, because operation schedules are subject to last-minute changes.

SAMPLE TAKING

Undiluted vitreous (1-1.5 ml) was obtained at the start of vitrectomy, before opening the infusion line. During scleral buckle surgery, undiluted subretinal fluid was obtained by drainage through a 23 gauge needle mounted on 2 ml syringe without a plunger.[10] Vitreous and subretinal fluid were immediately injected into Eppendorf vials and stored at –80 ºC. A blood sample was collected prior to or at the end of surgery in 0.105 mol/L sodium citrate solution. To isolate platelet poor plasma (PPP), the sample was centrifuged for 10 minutes at 2500 g/ 4 C and stored at -80ºC.

ANALYSIS

Blank control and blinded study samples were sent to Boehringer Ingelheim (Biberach, Germany) for analysis. The concentration of dabigatran was measured with liquid chromatography - tandem mass spectrometry (LC-MS/MS) according to its validated method using the Sciex API 3000 (PerkinElmer, Boston) system.[12] The samples were injected onto a precolumn and subsequently transferred to an HPLC column (purospher RP-18 E analytical column [60 × 2 mm, 5 µm]). MS measurements were performed in the positive ionisation mode with [$^{13}C_6$]-labeled BIBR 953 ZW as the internal standard. Monitored ions were 472.2 → 289.5 (dabigatran) and 478.2 → 295.6 (internal standard). The lower limit of quantification (QL) was 1.73 ng/mL.

Thrombin inhibiting activity was measured as diluted thrombin time using the Hemoclot assay which had a detection limit of 30 ng/mL.[13]

RESULTS

Forty-four patients were asked to participate. One patient was excluded because of recent stomach complaints, six patients were excluded due to abnormal lab results according to exclusion criteria and nine patients refrained from participation. Written informed consent was obtained from all participants. We included twelve patients in the scleral buckle group and sixteen patients in the vitrectomy group. For four patients the surgeon changed the initially planned scleral/buckle procedure to vitrectomy right before surgery, hence the larger vitrectomy group. Patient characteristics are shown in **Table 1**.

In addition, we analysed the vitreous fluid of a non-study patient who was on dabigatran therapy – 220 mg twice daily – for atrial fibrillation. This patient had a recurrent retinal detachment due to a missed break after a previous vitrectomy with gas tamponade for a rhegmatogenous retinal detachment.

Patients were followed up at day 1 and approximately two and six weeks after surgery. Dabigatran etexilate was well tolerated by all patients. None of the patients reported any side effects and we did not see excessive bleeding during or after surgery. Due to redetachment – considered unrelated to the treatment with dabigatran – four patients needed additional surgery. Three reoperations in the buckle group were due to insufficiently closed retinal breaks and one reoperation in the vitrectomy group was due to PVR.

Table 1. Patient characteristics

	Scleral/Buckle (n=12)	**Vitrectomy (n=16)**
Age (yr.)		
median (range)	60 (44 - 69)	62 (48 -71)
Weight (kg)		
median (range)	84 (60 -110)	84 (63 -115)
Gender		
male, n (%)	8 (66)	14 (88)
Body mass index		
mean (SD*)	26 (± 4.4)	27 (± 3.6)
Creatinine clearance (mL/min)		
median, range	93 (67 -148)	91 (50 -155)
Anaesthesia		
General, n	11	15
Local, n	1	1
Extent of detachment		
1 quadrant (n, %)	4 (33)	6 (38)
2 quadrants	6 (50)	7 (44)
3 quadrants	2 (17)	3 (19)

DABIGATRAN CONCENTRATION IN VITREOUS AND SUBRETINAL FLUID

Figure 1 shows the semi-logarithmic concentration-time curve of the measured dabigatran concentrations by LC-MS/MS. Samples that were below QL are shown as [QL/2]. The dabigatran concentration in SRF between 2 and 9 hours after administration ranged from below QL to 8.5 ng/mL (mean 4.3ng/mL; n=11). The concentration in vitreous was lower and varied between below QL and 3.8 ng/mL (mean 1.9ng/mL; n=15). The vitreous concentration of dabigatran in the additional patient on dabigatran therapy was 25.8 ng/mL (t = 5 hours after the last dose, data not shown in figure). One SRF value was considered an outlier (t = 11hr, 22.4ng/mL).

Figure 2A displays median dabigatran concentrations and the upper and lower limits of measurements in four time windows; around 2, 4, 6 and 8 hours. The median concentration in SRF showed a peak around 3-5 hours of approximately 8 ng/mL. For vitreous the peak seemed to be outside our sampling window.

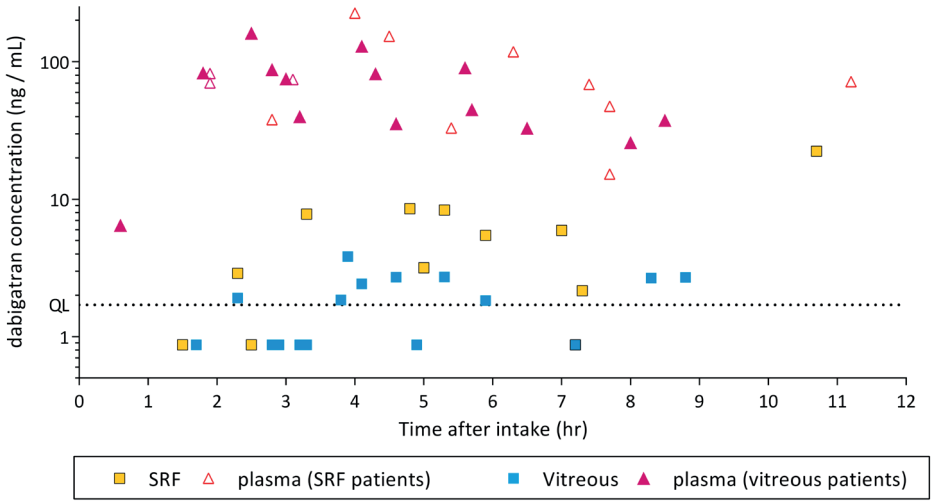

Figure 1. Semi-logarithmic concentration-time curve of dabigatran. The squares represent the concentrations found in vitreous and in subretinal fluid (SRF). The triangles are their respective plasma concentrations. The quantification limit was 1.73 ng/mL. BQL values are shown as [QL / 2].

DABIGATRAN CONCENTRATION IN PLASMA

Plasma concentrations between 2 and 9 hours after oral administration ranged from 15 ng/mL to 225 ng/mL (mean 71.3ng/mL; n=25) (Figure 1). **Figure 2B** shows the median and the upper and lower limits of these values in five time windows. The peak concentration of approximately 80 ng/mL was reached 1-3 hours after oral administration. The blood samples of two patients were unmeasurable due to strong haemolysis; one sample was considered an outlier (t = 11hr, 71.6ng/mL).

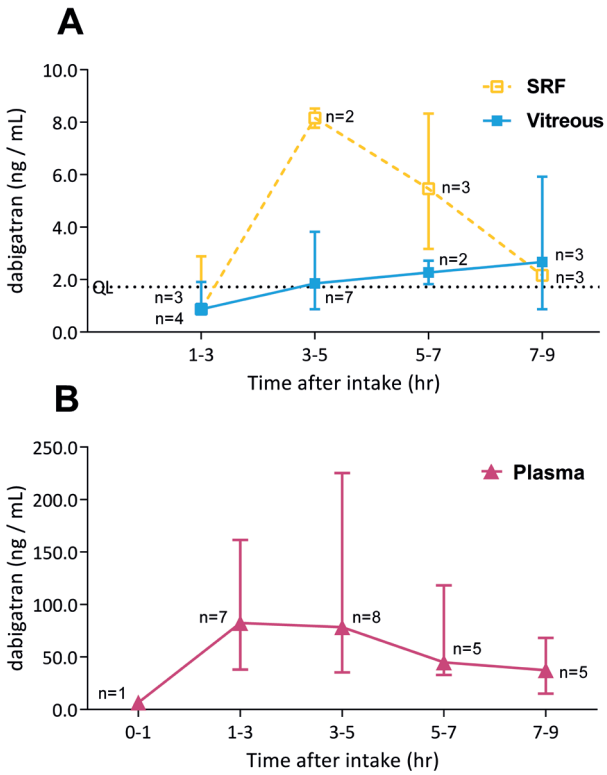

Figure 2. (A) Median concentrations of dabigatran in vitreous and subretinal fluid (SRF) in different time windows. One SRF value was excluded in this graph 22.35ng/mL, 11 hours after intake. **(B)** Median plasma concentrations of dabigatran. One plasma value was excluded in this graph 71.61ng/mL, 11 hours after intake. Whiskers represent upper and lower limit of measurements.

DILUTED THROMBIN TIME (DTT)

Anti-thrombin measurements with the standard diluted thrombin time in vitreous and SRF were not reliable due to concentrations below the quantification limit of 30-50 ng/mL dabigatran. Unfortunately, also the measurements in plasma were not reliable. Partly this could be explained by the assay that was used. Re-measurement of the samples with different diluted thrombin time kits (Technoclot, Hemosil) showed values closer to the LC-MS/MS (gold standard) values (data not shown).

CORRELATION BETWEEN PLASMA AND OCULAR LEVELS

Although time between ocular fluid sampling and blood sampling varied (26 ± 19 minutes; mean ± SD), evaluation of the plasma levels versus the SRF and vitreous levels showed that there was a significant relationship between SRF levels and plasma levels (r_s = .68, p = 0.014); the levels in vitreous fluid showed no such relationship (r_s = .20, p = 0.48).

DISCUSSION

The results of the current study demonstrate that a single oral administration of 220mg dabigatran etexilate leads to measurable levels of dabigatran in subretinal fluid and vitreous. As expected, the dabigatran levels in the eye were significantly lower than in plasma. Based on analogy with dexamethasone, we expected that a vitreous concentration of 10ng/mL could be achieved, especially because of the assumed partially disrupted blood-retinal barrier. In reality, we found concentrations in the range of 2 ng/mL in vitreous. Concentrations in the subretinal fluid were somewhat higher (up to 8 ng/mL), possibly due to the closer proximity to the choroidal and retinal vessels. This discrepancy between expected and actual values might be a result of the difference in lipophilicity between dexamethasone and dabigatran.
Of a single oral administration of moxifloxacin - an antibiotic with a similar molecular weight as dabigatran - only 6.8% of the plasma concentration was found in vitreous (t=±2 hours).[14] Two administrations of the same dose increased the vitreous concentration to 37.5% of the plasma concentration (t=3-4 hours).[15] Repeated dosing of the oral antiviral drugs famciclovir and valacyclovir (prodrugs of penciclovir and acyclovir with comparable lipophilicity as dabigatran) led to vitreous concentrations of 27% and 23% of the plasma concentration, respectively, in patients with a normal blood-retinal barrier.[16, 17] This shows that repeated dosing leads to higher intraocular drug levels.
Extrapolating the above information renders it very likely that repeated dosing of dabigatran also leads to higher concentrations. This is supported by the higher levels we found in the previously mentioned non-study patient on regular dabigatran intake. Five hours after the last dose we found an approximately ten times higher concentration in vitreous (25.8 ng/mL) than in study patients, despite a similar plasma concentration (52.5 ng/mL). It should be noted that this was not a primary vitrectomy. The fluid filling the vitreous cavity after vitrectomy might have been less viscous and less resistant to diffusion than vitreous. This is a possible mechanism that also leads to higher concentrations through faster diffusion.[18]
There is little knowledge about pharmacokinetic profiles in vitreous and subretinal fluid. Maximum drug concentrations in vitreous are likely to occur later than in plasma. In the study of Weijtens *et al.*, the maximum concentration (Cmax) in vitreous and subretinal fluid was reached 5 and 4 hours after the Cmax in serum, respectively.[10] Therefore, we varied the time of intake among patients between 2 and 8 hours prior to surgery to obtain a population-based pharmacokinetic profile. Not uncommon for a single dose administration, we found a large variability in plasma between individuals. Possibly, this was due to predominantly general anaesthesia and its associated need for an empty stomach, which may result in an unpredictable absorption.[19] Also the concomitant use of a proton-pump inhibitor (PPI) – although shown not to interfere with clinical efficacy – was shown to cause variability, a lower C_{max} and a 30% lower overall absorption.[19, 20] However, this appeared not to be a strong modifier, as the five patients in this study that used a PPI had among the highest plasma concentrations. The maximum concentration in SRF appeared around the same time as in plasma. The concentration peak in vitreous fluid seemed to lie outside our sampling window.

To define whether the concentrations we found are therapeutically relevant we calculated the required concentration to inhibit 50 and 80% of thrombin activity. Bastiaans *et al* reported a thrombin activity in vitreous of PVR patients of approximately 39 mU/mL (0.56 nM).[2] With the reported inhibition constant (Ki) for dabigatran being 4.5 nM, we calculated that the concentration required to inhibit 50% and 80% of this thrombin activity is approximately 2 and 9ng/mL, respectively.[21] These concentrations are exactly in the range of concentrations measured in this study, but as PVR has a protracted course it is unlikely that the thrombin concentration has a static value. It is still unclear whether thrombin is supplied from the blood stream or also produced locally by the proliferated RPE cells. It is important to elucidate these mechanisms in order to determine if a high enough concentration is reached to competitively inhibit thrombin.

In patients with a retinal detachment due to PVR, contractile periretinal membranes are formed that cause re-detachment or prevent reattachment. These membranes are formed during an inflammatory and fibrotic process, involving cytokines, growth factors and cells, such as RPE cells. To prevent or treat PVR in patients, anti-inflammatory (steroids) and cell-cycle inhibitors (daunorubicin, retinoic acid, and 5-FU) have been used, the latter particularly to decrease RPE cell proliferation.[7, 8, 22-25] Heparin has also been used, without a clearly described rationale, but likely to prevent fibrin deposition or to bind growth factors. Apart from prednisone, colchicine and retinoic acid, administration of all agents occurred during surgery. Due to the protracted course of PVR, a single intravitreal injection or 1-hour drug exposure during vitrectomy is unlikely to be effective in prevention or modulation. To obtain effective drug levels over a sustained period of time repeated administration is necessary. The most efficient way remains local delivery by intravitreal injection, but these are not only inconvenient for the patient, they also pose a large risk of infection.

Dabigatran has a plausible mode of action to modulate PVR and is a relatively safe and patient-friendly drug for repeated administration. Its use has become even safer since the availability of an antagonist in case of excessive bleeding.[26] Nevertheless, all drugs have potential side effects. Therefore, it remains important to select those patients most at risk of developing PVR.

In conclusion, we have demonstrated that oral intake of dabigatran, a candidate drug to modulate PVR, results in potentially relevant intraocular concentrations. We suggest that repeated dosing may lead to higher concentrations, but this should be further explored.

Acknowledgements

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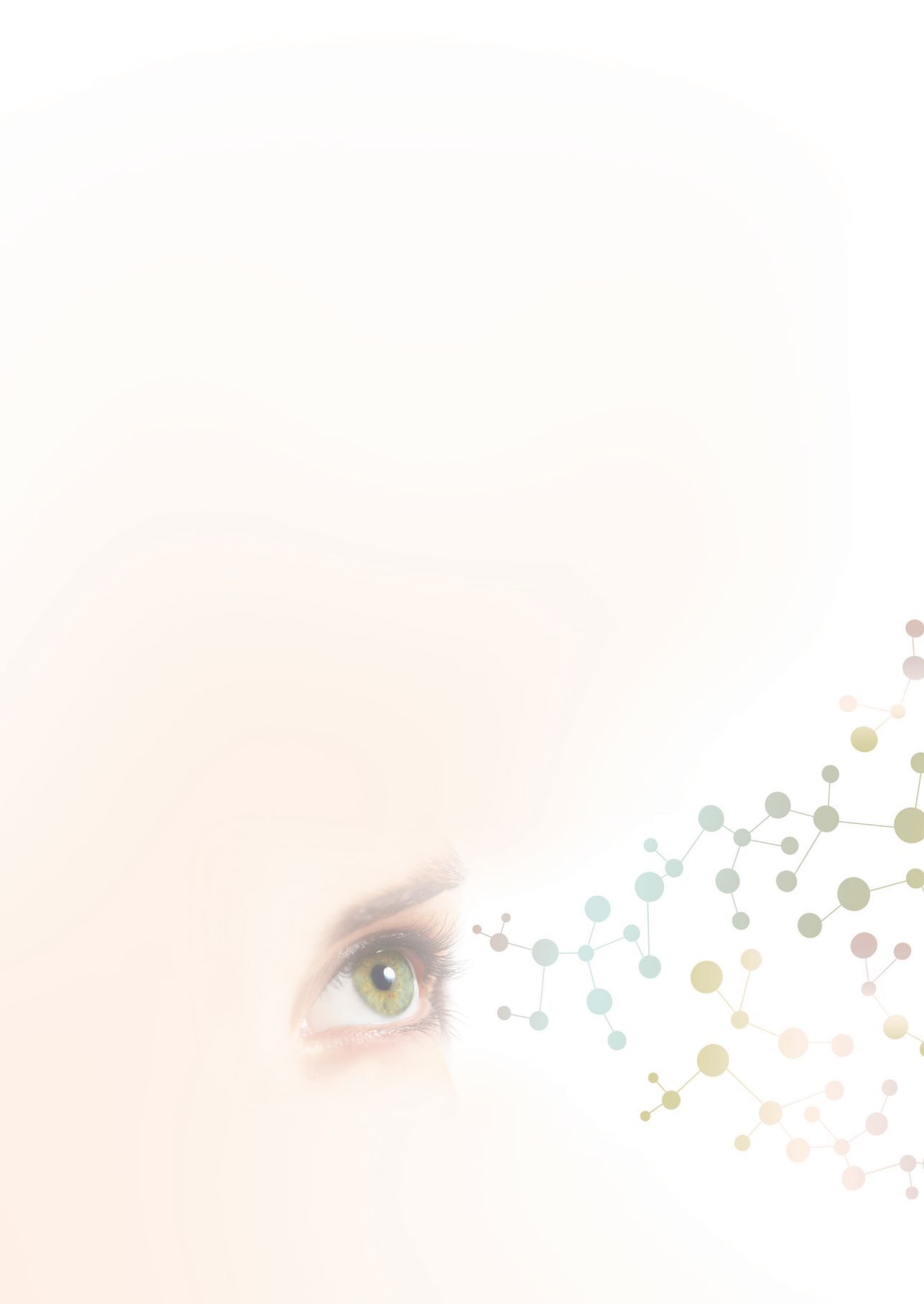

2.2

Higher vitreous concentrations of dabigatran after repeated oral administration

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Cornelis Kluft
Peter G. van Etten
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INTRODUCTION

The oral thrombin inhibitor dabigatran (Pradaxa®) has been shown to be detectable in the vitreous and subretinal fluid after a single oral administration of 220mg.[1] The maximum concentrations that were found were 8.5ng/ml in subretinal fluid and 3.8ng/ml in vitreous. An unexpected finding in this study was the 10 times higher vitreous concentration of a non-study patient, who used 150mg dabigatran twice daily for atrial fibrillation. This finding in combination with the observation that the median vitreous concentration was highest at our last time point and thus possibly still increasing, led to the hypothesis that repeated administration of dabigatran might lead to higher intraocular levels. Therefore, we tested this hypothesis in patients on standard dabigatran therapy who were admitted to the Rotterdam Eye Hospital for retinal surgery.

METHODS AND RESULTS

We were able to include one male and two female patients, who were being treated with standard twice daily dosages of dabigatran (see table 1). One patient underwent surgery for a dropped nucleus after cataract surgery and two patients for a macular hole. One of these patients had a persistent macular hole and underwent repeat surgery after 5.5 weeks during which we collected a second sample. The collection of undiluted vitreous samples and analysis with LC-MS/MS were the same as described in our previous study.[1]

Table 1. Patient characteristics and results

	Patient 1	**Patient 2**	**Patient 3**
Age	89 yr	81 yr	71 yr
Gender	Female	Male	Female
Weight	49 kg	90 kg	65 kg
Body mass index	18	29	21
Anaesthesia	Local	Local	General
Reason for surgery	dropped nucleus	macular hole	macular hole 2. persistent macular hole
Dosage of dabigatran	2 x 110mg	2 x 110mg	2 x 150mg
Concentration in vitreous (time after last intake)	10.1ng/ml (4.5hr)	5.6 ng/ml (6.0hr)	1. 6.0ng/ml (3.6hr) 2. 19.5 ng/ml (3.5hr)
Concentration in plasma (time after last intake)	-	95.8 ng/ml (7.3hr)	1. 159.1 ng/ml (5.0hr) 2. 100.4 ng/ml (3.3hr)
Concomitant medication	bumetanide, metoprolol, perindopril, isosorbide mononitrate, ranitidine, spironolactone	valsartan, hydrochlorthiazide	chlorthalidone, desloratadine, fluticasone, digoxin, pravastatin

DISCUSSION

Due to the relative rarity of patients on dabigatran therapy undergoing vitrectomy, we were able to include only three unique patients in one year of which one on two separate occasions. These were not patients with a rhegmatogenous retinal detachment (RRD) – like the previous study – but patients with a macular hole and a complication after cataract surgery. This difference in patient population might have influenced the measured vitreous concentrations. In contrast to patients with a macular hole, we assume that patients with RRD have a partially disrupted blood-retinal barrier which contributes to the influx of dabigatran in the vitreous. Therefore, these results show that even with an intact blood-retinal barrier dabigatran is able to penetrate the vitreous as long as there is sufficient supply (i.e. repeated administration).

In addition, the presence of formed vitreous seemed to influence the concentration in the vitreous cavity. Patient 3 underwent two consecutive vitrectomy procedures in which we both collected vitreous respectively vitreous cavity fluid samples. The collection time was in both cases 3.5 hours after the last dose, but the concentration of dabigatran in the second sample was much higher than in the first one (6.0 ng/ml vs. 19.5 ng/ml). A possible explanation for this result is the lower viscosity of aqueous humour filling the vitreous cavity after the first vitrectomy.[2, 3] As dabigatran is a hydrophilic compound, lower viscosity possibly results in higher diffusion in this medium than in vitreous.[3] The same mechanism might have contributed to the high concentration we found in our previous study (25.8 ng/ml).

In conclusion, we have demonstrated that repeated use of the oral thrombin inhibitor dabigatran leads to higher vitreous levels of dabigatran than a single administration, even in patients with a supposedly intact blood-retinal barrier. This result increases the potential of dabigatran as a possible therapeutic agent in the modulation of PVR.

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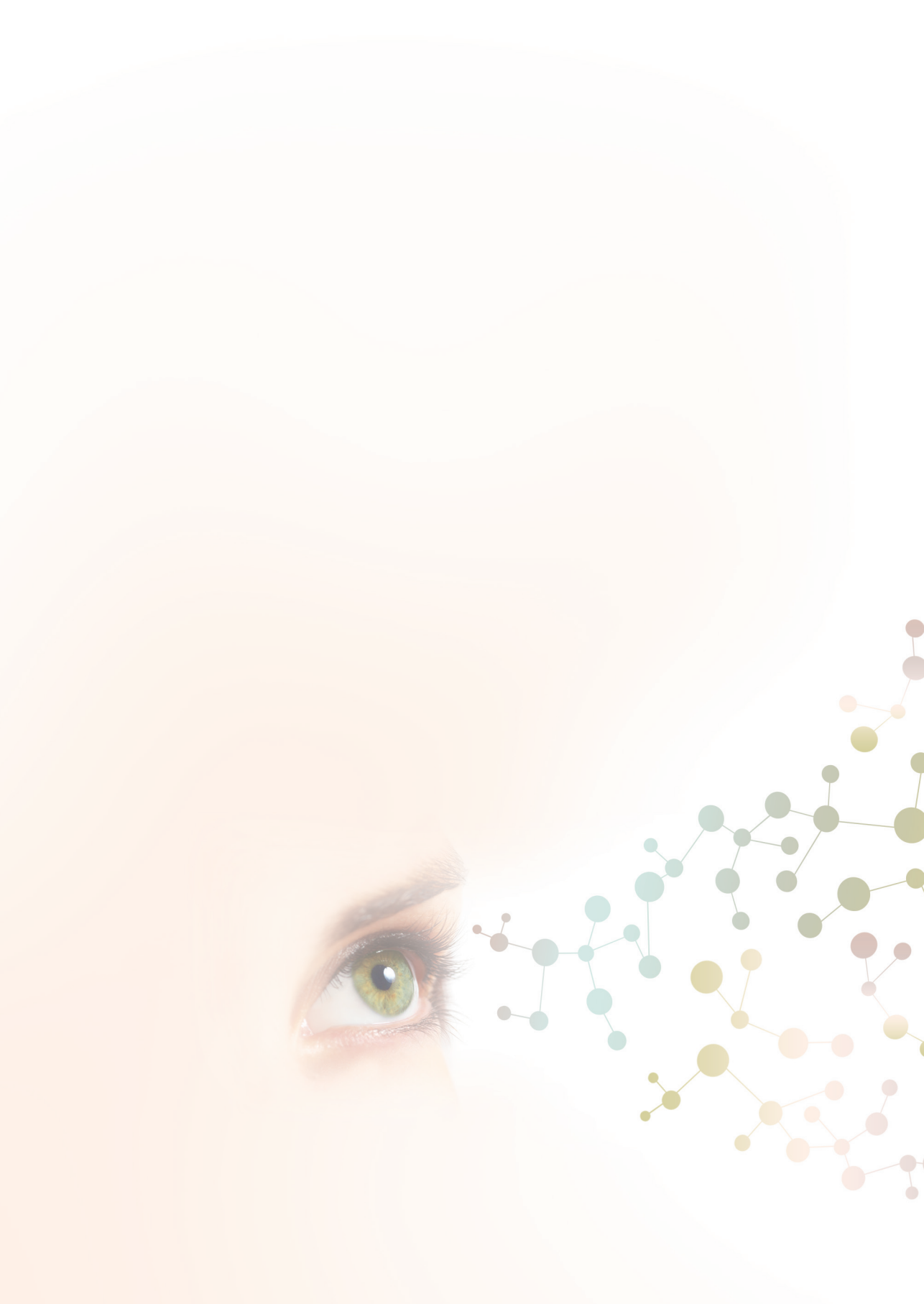

2.3

Dabigatran inhibits intravitreal thrombin activity

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ABSTRACT

PURPOSE

Proliferative vitreoretinopathy (PVR) is a vitreoretinal disorder in which retinal pigment epithelial (RPE) cell activation contributes to both formation of fibrotic retinal membranes and inflammation. Vitreous of PVR patients contains increased thrombin activity which induces pro-fibrotic and pro-inflammatory programs in RPE cells. Inhibition of intravitreal thrombin activity may thus represent a therapeutic option for PVR. In this study, we examined the capacity of the clinically available direct thrombin inhibitor dabigatran to inhibit thrombin activity in vitreous fluids.

METHODS

ARPE-19 cells were cultured with 1) thrombin, 2) vitreous without thrombin activity and 3) vitreous with elevated thrombin activity (PVR samples and thrombin spiked vitreous) either in the presence or absence of dabigatran (range: 10^{-5} - 10^{-7} M). Subsequently, *CCL2, CXCL8, GM-CSF, IL6,* and *PDGF-B* mRNA expression levels were determined by RQ-PCR and protein levels of 27 cytokines, chemokines and growth factors were detected in culture supernatants using a multiplex approach. In addition, the capacity of vitreous fluids obtained from patients after oral dabigatran intake was tested in an in vitro thrombin activity assay.

RESULTS

Thrombin and vitreous fluids containing thrombin activity induced CCL2, CXCL8, GM-CSF, IL-6 and PDGF-BB expression by ARPE-19 cells, which was inhibited by dabigatran. In addition, dabigatran that reached the vitreous after repeated oral intake did inhibit thrombin activity in the in vitro activity assay.

CONCLUSION

PVR is associated with increased intravitreal thrombin activity that activates pro-fibrotic and pro-inflammatory pathways in RPE cells. Our findings provide evidence that this activation pathway can potentially be inhibited by dabigatran.

INTRODUCTION

Thrombin is a key serine protease of blood coagulation that converts soluble fibrinogen into insoluble fibrin.[1] In addition to its role in coagulation, thrombin induces cellular responses that have important roles in inflammation and tissue repair, but also contribute to the development of fibrosis.[2-4] Thrombin, for instance, stimulates the production of inflammatory mediators by several cell types, promotes chemotaxis of inflammatory cells, stimulates the proliferation of fibroblasts and smooth muscle cells, and induces myofibroblast differentiation and the production of extracellular matrix (ECM) components such as collagen.[5] These cellular responses to thrombin are mostly mediated via the G protein-coupled protease-activated receptor (PAR)-1.[6]

Proliferative vitreoretinopathy (PVR) is a complication of retinal detachment – caused by a retinal tear (rhegmatogenous retinal detachment (RRD)) or trauma – which is characterized by the formation of subretinal, intraretinal, and/or epiretinal fibrotic membranes, as well as an inflammatory component.[7, 8] Retinal pigment epithelial (RPE) cells dispersed during retinal detachment are important contributors to PVR pathogenesis as they dedifferentiate into collagen-producing myofibroblasts, which is a key event in the development of the contractile fibrotic membranes.[7] Moreover, the activated RPE cells produce cytokines and chemokines that recruit and activate immune cells.[8] Although these pathobiological processes are well recognized to contribute to PVR, medical treatment options are limited so far, and treatment mostly still depends on (recurrent) surgical intervention.[7]

Activation of the coagulation cascade, as evidenced by intraocular fibrin deposition and higher intravitreal thrombin activity, occurs in PVR and likely contributes to both retinal fibrotic membrane formation and inflammation.[9-11] Previously, we demonstrated that thrombin stimulated dedifferentiation of RPE cells into α-smooth-muscle-actin-expressing contractile, collagen-producing myofibroblasts. This involved production of the profibrotic mediator platelet-derived growth factor (PDGF)-BB and subsequent autocrine activation of the PDGF-receptor.[9, 12] In line with this, we also showed that thrombin activity in vitreous from patients with PVR is a main contributing component of vitreous-induced production of PDGF-BB by RPE.[9]

Elevated thrombin activity in vitreous of patients with PVR was also identified as an important stimulator of the production of pro-inflammatory mediators such as C-C motif chemokine ligand (CCL)2, C-X-C motif chemokine ligand (CXCL)8, granulocyte-macrophage-colony-stimulating factor (GM-CSF) and interleukin (IL)-6 by RPE. CCL2, CXCL8, GM-CSF and IL-6 are potent chemoattractants and activators of immune cells such as monocytes, macrophages, neutrophils and B-lymphocytes which have been identified in PVR membranes and vitreous. GM-CSF also stimulates differentiation of monocytes into macrophages.[7,8,13-17] These immune cells, especially monocytes/macrophages, are considered an important source of pro-fibrotic mediators in PVR, including PDGF and transforming growth factor (TGF)-β which are elevated in vitreous and membranes of patient with PVR.[7, 8, 18]

Altogether, this data points to an important role for thrombin in stimulating and prolonging pro-fibrotic and pro-inflammatory responses by RPE in PVR. Thrombin may, therefore, represent an attractive treatment target for this complication. This consideration, however, is not new. Peroperative intravitreal low-molecular-weight heparin was used in 3 randomized controlled trials on PVR modulation, with an inhibiting effect only seen in patients at high risk of developing PVR.[19-21] As PVR is a protracted process, a more prolonged treatment by an oral drug may be more appropriate.

Dabigatran-etexilate is a small molecule oral prodrug that is hydrolysed to its active compound dabigatran. Dabigatran is a selective, competitive and reversible direct thrombin inhibitor (DTI) that binds to the active site of the thrombin molecule, thereby blocking the interaction between thrombin and its substrates.[22] Dabigatran prevents cleavage of fibrinogen by thrombin (at particular Arg-Gly bonds) thereby preventing the formation of fibrin and thus negatively affects clotting. Dabigatran also inhibits thrombin-induced cleavage of PAR-1 at the peptide bond between residues Arg-41 and Ser-42, thereby preventing cells from being activated.[23, 24] Recently, Mulder et al demonstrated that dabigatran is able to reach the vitreous and subretinal fluid after oral intake, making dabigatran an interesting drug candidate to modulate PVR development.[25, 26] Together these data suggest that dabigatran may be considered as a potential drug for the treatment of disorders associated with elevated intravitreal thrombin activity, including PVR. Therefore the aim of the present study was to investigate whether dabigatran exhibits the capacity to inhibit thrombin activity within the vitreous environment.

MATERIALS AND METHODS

VITREOUS FLUIDS

Vitreous fluid samples were selected from our previous studies. [9, 25] Vitreous fluid samples without thrombin activity were collected from patients during their primary vitrectomy procedure for RRD, who had not developed PVR afterwards (n=5). Vitreous fluid samples with thrombin activity were collected from patients with established PVR (mean thrombin activity = 56.19 ± 83.62 mU/ml) (n = 5). In these vitreous fluid samples, thrombin activity was determined by using the thrombin-specific substrate Tos-Gly-Pro-Arg-pNA (Sigma-Aldrich, St. Louis, MO) as described previously.[9]

Vitreous fluid samples containing dabigatran were collected from patients during their primary vitrectomy procedure for RRD which received dabigatran before surgery (mean measured dabigatran = 3.23 ± 6.89 ng/ml) (n = 13).

All subjects gave their consent for the use of rest material for research; storage and use of the vitreous for further studies were according to the guidelines of the Medical Ethics Committee of the Erasmus MC, University Medical Center, Rotterdam and were performed in accordance with guidelines established by the Declaration of Helsinki.

CELL CULTURES

The human retinal pigment epithelial cell line ARPE-19 was obtained from American Type Culture Collection (ATCC; Manassas, VA, US). ARPE-19 cells were cultured in RPE medium (Dulbecco's modified Eagle's medium /F-12 (HyClone, Logan, UT, US)), containing 10% heat-inactivated fetal calf serum (FCS) and penicillin/streptomycin (all from BioWhittaker, Verviers, Belgium)) and were between passage 23-30 when used for experiments. ARPE-19 cells were analysed by short tandem repeat (STR) analysis (DSMZ, Braunschweig, Germany) for cell line authentication and were mycoplasma free. ARPE-19 cells were maintained under standard cell culture conditions at 37 ^{o}C in humidified air with 5% CO_2.

DABIGATRAN TITRATION

ARPE-19 cells were seeded at a density of 3 x 10^5 cells/well in RPE medium containing 10% FCS in 12-well plates and were allowed to adhere overnight. Subsequently, the medium was refreshed twice a week till the cells were grown 100% confluent. Prior to stimulation, the cells were serum starved in RPE medium containing 1% FCS for 24 hours, followed by an additional 24 hours in serum-free RPE medium. Hereafter, cells were pre-incubated with fresh serum-free RPE medium with or without active dabigatran for 60 minutes (BIBR 953, range: 10^{-5}-10^{-7}M: kindly provided by Boehringer Ingelheim Pharma GmbH & Co. KG (Ingelheim am Rhein, Germany)). Subsequently, thrombin (Calbiochem, La Jolla, CA) was added to a concentration of 5 U/ml (concentration based on previous studies [9, 12, 27]) or cells remained unstimulated for a period of 2 hours. Hereafter cells were harvested for RNA isolation. The dabigatran concentrations that were used were non-toxic to ARPE-19 cells as was determined by lactate dehydrogenase (LDH) release (Roche, Mannheim, Germany) and microscopic appearance of the cells.

THE ABILITY OF DABIGATRAN TO INHIBIT PVR-VITREOUS-INDUCED CYTOKINE, CHEMOKINE AND GROWTH FACTOR EXPRESSION LEVELS

ARPE-19 cells were seeded in 12-well plates at a density of 3 x 10^5 cells/well in RPE medium containing 10% FCS and allowed to adhere overnight. Subsequently, the medium was refreshed twice a week till the cells were grown 100% confluent. Prior to stimulation, the cells were serum starved in RPE medium containing 1% FCS for 24 hours followed by an additional 24 hours in serum-free RPE medium. Hereafter, cells were pre-incubated with serum-free RPE medium with or without 10^{-5} M dabigatran or 7.5 U/ml hirudin for 60 minutes and subsequently stimulated for 2 hours with serum-free medium containing 1/8 diluted vitreous (dilution based on previous study [9]) with or without 10^{-5} M dabigatran or 7.5 U/ml hirudin. Hereafter cells were harvested for RNA isolation.

MESSENGER RNA EXPRESSION ANALYSIS BY REAL-TIME QUANTITATIVE PCR

RNA was isolated using a GenEluteTM Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) and reverse transcribed into cDNA.[9] Transcript levels of CCL2, CXCL8, GM-CSF, IL6 and PDGF-B mRNA were determined by real-time quantitative (RQ)-PCR (7700 PCR system;

Applied Biosystems [ABI], Foster City, CA, US) using primer and probe combinations as previously described.[9] Expression levels of the analysed gene transcripts were normalized to the control gene ABL (Abelson).[9]

MULTIPLEX DETECTION OF THROMBIN AND VITREOUS-INDUCED CYTOKINE, CHEMOKINE AND GROWTH FACTOR EXPRESSION

ARPE-19 cells were seeded in 12-well plates at a density of 3 x 10^5 cells/well in RPE medium containing 10% FCS and allowed to adhere overnight. Subsequently, the medium was refreshed twice a week till the cells were grown 100% confluent. Prior to stimulation, the cells were serum starved in RPE medium containing 1% FCS for 24 hours followed by an additional 24 hours in serum-free RPE medium without FCS. Hereafter cells were pre-incubated with fresh serum-free RPE medium with or without or 10^{-5} M dabigatran or 7.5 U/ml hirudin (Sigma, St Louis, MO, US) for 60 minutes. Subsequently, the cells were stimulated with serum-free medium containing 1/8 diluted thrombin-free vitreous with or without 5 U/ml thrombin, with or without 10^{-5} M dabigatran or 7.5 U/ml hirudin for 24 hours. As a control, cells were stimulated in serum-free medium with or without 5 U/ml thrombin for 24 hours. Following the stimulation period of 24 hours, culture supernatants were harvested and analysed with a Bio-Plex Pro™ Human Cytokine, Chemokine, and Growth Factor Assay (Bio-Rad, Hercules, CA, US) for simultaneous detection of the following cytokines, chemokines and growth factors: basic fibroblast growth factor (FGFb), Eotaxin, CCL2, CCL3, CCL4, CCL5, CXCL8, CXCL10, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, G-CSF, GM-CSF, interferon (IFN)γ, PDGF-BB, tumour necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF)-A (see supplemental table 1 for the detection limits). The assay was performed according manufacturer's instructions. Because CXCL8 levels in a few culture supernatants exceeded the upper detection limit of the assay all culture supernatants were re-analysed for CXCL8 by ELISA (Invitrogen) according manufacturer's instructions.

DABIGATRAN ACTIVITY IN VITREOUS FLUIDS

In previous studies, dabigatran was detected in the vitreous of patients which had either received a single oral dosage 2-9 hours before vitrectomy or were being treated therapeutically with dabigatran.[25, 28] In this study, we measured the activity of dabigatran in those vitreous samples collected during vitrectomy, by using a thrombin-specific substrate as described in our previous study.[9] In short, 50 µl vitreous fluid containing dabigatran was diluted in a 96-well microtiter plate with 50 µl of a 1 mM solution of the thrombin specific substrate Chromozym TH (Sigma) dissolved in 1.5 mM HCl. Thereafter, 5 µl Tris-buffered saline (TBS) pH 8.3 or TBS containing 1 U/ml thrombin was added to each reaction. Vitreous fluids without dabigatran were taken along as controls. A thrombin standard curve ranging from 50 - 0.78 mU/ml was prepared in TBS or TBS containing 0.1 U/ml hirudin. The reaction was performed in an incubator at 37 °C. The optical density (OD) was measured at 405 nm after 4 hours. Thrombin activity in vitreous fluid was quantified based on the difference in OD between vitreous fluid with and without dabigatran, and the reference curve, and was expressed as mU/ml.

STATISTICAL ANALYSIS

Data were analysed using the Kruskal-Wallis (One-way ANOVA) test followed by the Mann-Whitney U test when applicable. A *P*-value < 0.05 was considered significant.

RESULTS

THE CONCENTRATION-DEPENDENT EFFECT OF DABIGATRAN ON THROMBIN-INDUCED CYTOKINE, CHEMOKINE AND GROWTH FACTOR EXPRESSION

Thrombin significantly (*P* < 0.05) induced *CCL2*, *CXCL8*, *GM-CSF*, *IL6,* and *PDGF-B* mRNA expression levels in ARPE-19 (**Figure 1**). This stimulatory effect of thrombin was dose-dependently inhibited by dabigatran for all genes, becoming significant (*P* < 0.05) at a concentration of 10^{-5} M which is approximately 4.7 µg/ml (**Figure 1**). Dabigatran without the addition of thrombin did not induce or inhibit mRNA expression levels of *CCL2*, *CXCL8*, *GM-CSF*, *IL6* and *PDGF-B* (**Figure 1**).

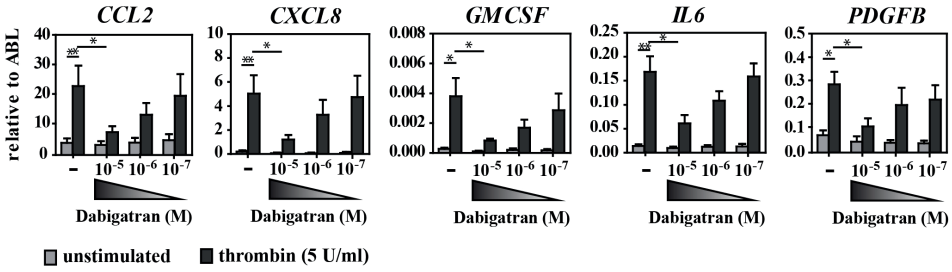

Figure 1. ARPE-19 cells were stimulated for 2 hours with thrombin (5 U/ml) or remained unstimulated in the absence or presence of dabigatran (range: 10-5 - 10-7 M). CCL2, CXCL8, GM-CSF, IL6 and PDGF-B mRNA expression levels were determined by RQ-PCR and normalized against the control gene ABL. Data are presented as the mean value from 4 independent experiments ± SEM. Statistical analysis was performed with the Kruskal-Wallis (One-way ANOVA) test followed by the Mann-Whitney U test when applicable. P < 0.05 was considered significant. * = P < 0.05 and ** = P <0.01.

THE EFFECT OF DABIGATRAN ON VITREOUS-INDUCED CCL2, CXCL8, GM-CSF, IL6 AND PDGFB EXPRESSION

Vitreous fluids from RDD patients that lacked detectable thrombin activity did not induce *CCL2*, *CXCL8*, *GM-CSF*, *IL6* and *PDGF-B* mRNA expression by ARPE-19 cells nor was this affected by dabigatran (**Figure 2A**). Vitreous fluids from patients with PVR that contained thrombin activity clearly induced *CCL2*, *CXCL8*, *GM-CSF*, *IL6,* and *PDGF-B* mRNA expression by ARPE-19 cells. The addition of dabigatran significantly (*P* < 0.05) reduced the capacity of these PVR vitreous samples to induce *CCL2*, *CXCL8*, *IL6* and *PDGF-B* mRNA expression by ARPE-19 (**Figure 2B**).

THE EFFECT OF DABIGATRAN ON THROMBIN-INDUCED CYTOKINE, CHEMOKINE AND GROWTH FACTOR PRODUCTION

Thrombin significantly ($P < 0.05$) induced CCL2, CXCL8, GM-CSF, IL-6 and PDGF-BB protein production by ARPE-19 cells in both serum-free medium and vitreous-diluted medium (Figure 3). This stimulatory effect of thrombin was inhibited by both hirudin ($P < 0.05$) and dabigatran ($P < 0.05$) for all. Hirudin and dabigatran alone did not affect CCL2, CXCL8, GM-CSF, IL-6 and PDGF-BB production (**Figure 3**). Other cytokines, chemokines and growth factors which were upregulated by thrombin in both serum-free medium and vitreous diluted medium are IL-9, IL-10, IL-12(p70), FGFb and VEGF-A (supplemental table 1). For these factors, the stimulatory effect of thrombin was inhibited by both hirudin and dabigatran as well (supplemental table 1).

INTRAVITREAL DABIGATRAN INHIBITS THROMBIN ACTIVITY

Vitreous fluids from patients which were given dabigatran 2-9 hours before surgery contained a concentration of 3.23 ± 6.89 ng/ml dabigatran. Allowing these vitreous fluids to inhibit thrombin activity in a thrombin activity assay with a thrombin-specific chromogenic substrate, showed hardly any effect of the dabigatran in the vitreous fluid on the thrombin activity (**Figure 4**). However, one vitreous fluid of a patient which has been taken dabigatran on a daily basis for a longer time period for atrial fibrillation contained a much higher concentration (25.84 ng/ml, undiluted) of dabigatran and inhibited total thrombin activity (**Figure 4**).

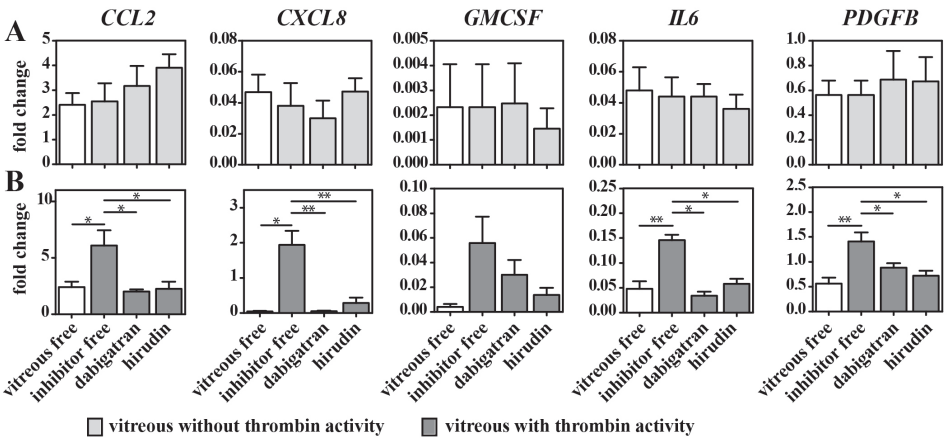

Figure 2. ARPE-19 cells were stimulated for 2 hours with **(A)** 1/8 diluted vitreous without thrombin activity, in the absence or presence of dabigatran (10-5 M) or hirudin (7.5 U/ml) or **(B)** 1/8 diluted vitreous with thrombin activity, in the absence or presence of dabigatran (10-5 M) or hirudin (7.5 U/ml). CCL2, CXCL8, GM-CSF, IL6 and PDGF-B mRNA expression levels were determined by RQ-PCR and normalized against the control gene ABL. Data are presented as the mean value from 4 independent experiments ± SEM. Statistical analysis was performed with the Kruskal-Wallis (One-way ANOVA) test followed by the Mann-Whitney U test when applicable. P < 0.05 was considered significant. * = P < 0.05 and ** = P < 0.01.

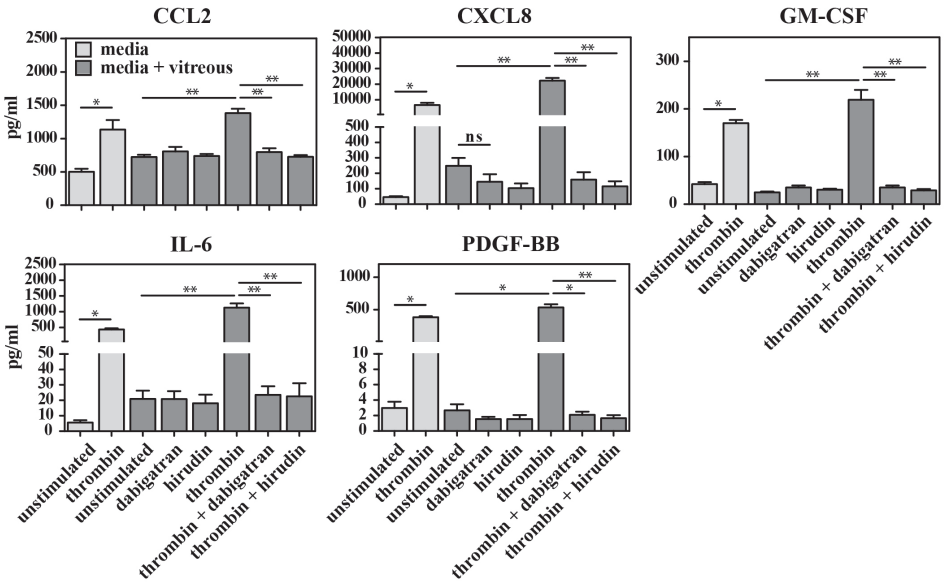

Figure 3. ARPE-19 cells were stimulated with thrombin (5 U/ml) in serum free medium (light grey bars) or 1/8 diluted vitreous fluids in medium (dark grey bars) in the absence or presence of dabigatran (10-5 M) or hirudin (7.5 U/ml) for 24 hours. Culture supernatants were analysed by a Bio-Plex Pro™ Human Cytokine, Chemokine, and Growth Factor Assay; allowing the detection of 27 cytokines, chemokines and growth factors simultaneously. CCL2, CXCL8, GM-CSF, IL-6 and PDGF-BB data are presented as the mean value from 6 individual vitreous fluids per group ± SEM and from 4 individual serum free and vitreous free medium samples per group ± SEM. Statistical analysis was performed with the Kruskal-Wallis (One-way ANOVA) test followed by the Mann-Whitney U test when applicable. $P < 0.05$ was considered significant.
* = $P < 0.05$, ** = $P < 0.01$ and ns = no significant difference.

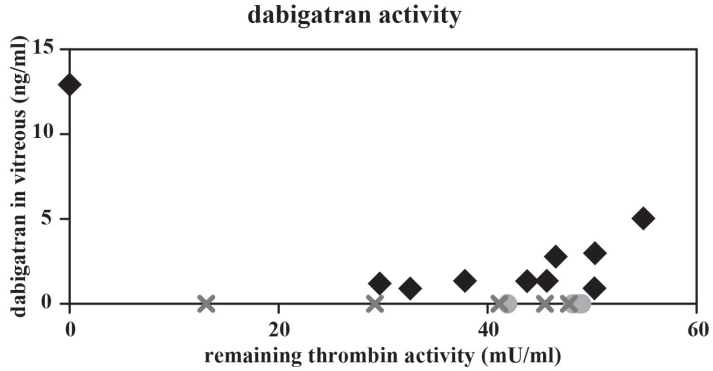

Figure 4. Dabigatran activity in vitreous fluids (2-9 hours after oral intake) was determined with a thrombin activity assay by using the thrombin-specific chromogenic substrate Chromozym TH in the absence and presence of thrombin (50 mU/ml). Black diamonds indicate vitreous fluids with dabigatran concentrations > detection limit 1.73 ng/ml and dark grey crosses indicate vitreous fluids with dabigatran concentrations < detection limit 1.73 ng/ml as previously determined.[1] Light grey circles indicate vitreous fluids without dabigatran.

DISCUSSION

There is accumulating evidence that elevated intravitreal thrombin activity is involved in the pathogenesis of PVR and that its contribution goes beyond fibrin formation. In PVR, thrombin activates PAR-1 signalling in RPE cells which results in the production of numerous cytokines, chemokines and growth factors including the proinflammatory cytokines CCL2, CXCL8, GM-CSF, IL-6 and the pro-fibrotic mediator PDGF-BB. Inhibition of thrombin may thus represent an attractive therapeutic option for PVR treatment. Two recent studies by Mulder et al demonstrated that the clinically available DTI dabigatran can be detected in vitreous fluid and subretinal fluid of patients [25], and in higher concentration after repeated dosing[28]. The question remains whether dabigatran is able to prevent thrombin from activating RPE cells in a vitreous enriched environment which is rich in salts, sugars, collagens, hyaluronic acid and a wide array of proteins which may affect dabigatran's efficiency to inhibit thrombin activity.[29-31]

To answer our research question we conducted four experiments. First, we tested the ability of dabigatran to prevent the induction of five factors that were previously shown to be significantly induced by thrombin (*CCL2, CXCL8, GM-CSF, IL6* and *PDGF-B*) on mRNA level (Figure 1). Second, we tested whether dabigatran was also able to prevent the induction of these factors in a more realistic environment containing vitreous with endogenous thrombin activity (Figure 2). Third, we wanted to confirm whether the effects we see on mRNA level also translate into similar effects on protein level (Figure 3). Lastly, we tested the thrombin-inhibiting effect of dabigatran present in vitreous after oral administration to confirm that this dabigatran (Figure 4) has a comparable thrombin-inhibitory effect as the active compound BIBR 953 which was used in the in vitro experiments with ARPE-19 cells (Figure 1-3). In this study, we demonstrate that dabigatran etexilate, a candidate drug to modulate PVR development, is able to inhibit thrombin-induced expression of cytokines, chemokines and growth factors in a vitreous environment.

For this study, there was little availability of vitreous fluids with increased thrombin activity. The available volumes were just sufficient to perform our mRNA experiments, which required smaller amounts of the fluids. Therefore vitreous fluids lacking endogenous thrombin activity were used for the experiments involving the simultaneous detection of 27 cytokines, chemokines and growth factors via a multiplex approach. For these experiments, when required, these vitreous fluids were spiked with thrombin purified from human plasma to resemble PVR vitreous fluids. This may affect expression levels of various cytokines, chemokines and growth factors, which is taken into consideration. Furthermore, we and others have demonstrated that when studying the thrombin-induced expression of cytokines, chemokines and growth factors, ARPE-19 cells and primary RPE respond in a similar matter.[9, 12, 27, 32-36] Therefore only ARPE-19 cells were used in this study.

Recently we demonstrated that thrombin activity is increased in vitreous from PVR patients where it is a major factor contributing to vitreous-induced production of CCL2, CXCL8, GM-CSF and IL-6 by ARPE-19 cells.[9] Here we demonstrate that dabigatran inhibits the PVR vitreous-induced *CCL2, CXCL8, GM-CSF* and *IL6* mRNA expression and thrombin-induced CCL2, CXCL8, GM-CSF and IL-6 protein expression in ARPE-19 cells (Figure 2 and 3). The

concentration of dabigatran in our first three experiments was approximately 1000x higher compared to the concentrations found in the vitreous after oral intake.[25] However, these high concentrations were required to inhibit thrombin activity in our in vitro experiments (Figure 1) and have been demonstrated to be non-toxic to the cells.

Dedifferentiation of RPE cells into contractile extracellular-matrix-synthesizing myofibroblasts is a key event in the formation of fibrotic membranes in PVR.[7] Thrombin is an important driver of this dedifferentiation process which depends on the autocrine release of PDGF-BB and subsequent PDGF-receptor activation.[27, 37] Here we found that dabigatran inhibits thrombin and PVR vitreous-induced expression of PDGF-BB on both mRNA and protein level in ARPE-19 cells (Figure 2 and 3), suggesting that dabigatran may prevent thrombin-induced fibrotic responses in RPE cells.

In our experiments we used hirudin, a DTI which effectively inhibited thrombin in previous studies, as a control for dabigatran. Although the inhibitory effect of hirudin on thrombin is irreversible as opposed to dabigatran - a competitive and reversible DTI - the effect on cytokines, chemokines and growth factors expression levels were similar.[9]

Available vitreous fluids containing dabigatran from a previous study by Mulder et al were used in a reversed version of a thrombin activity assay.[25] This assay has the elegance to study the effect of intravitreal dabigatran (that is dabigatran which reached the patients' vitreous fluid hours after oral intake) on thrombin activity. Although our findings do not find a strong effect, we find that one of the vitreous fluids, containing 12.92 ng/ml dabigatran after dilution, is able to inhibit all thrombin activity provided (Figure 4). This is in agreement with the previously reported theoretical minimum concentration range of dabigatran required to inhibit the measured thrombin activity in vitreous based on the inhibition constant of dabigatran (4.5nM).[25, 38] Vitreous fluids without dabigatran, which were taken along as controls, had no effect on thrombin activity (light grey circles in Figure 4). However, a few samples with concentrations of dabigatran which were reported to be below the detection limit, were able to partially inhibit thrombin activity (dark grey crosses in Figure 4).[25] In our assay we found increased levels of protease activity in these vitreous fluids (data not shown). Proteases have been found in vitreous before and may have interfered with this assay.[39] The increased levels of protease activity were, however, restricted to these fluids.

Taken together our data suggest that thrombin inhibition with a DTI such as dabigatran may be an interesting therapeutic option in the prevention of PVR development. It should however still be taken into account that the use of a compound such as dabigatran, with potential side effects including haemorrhages, should be introduced with great precaution.[40-42]

Acknowledgements

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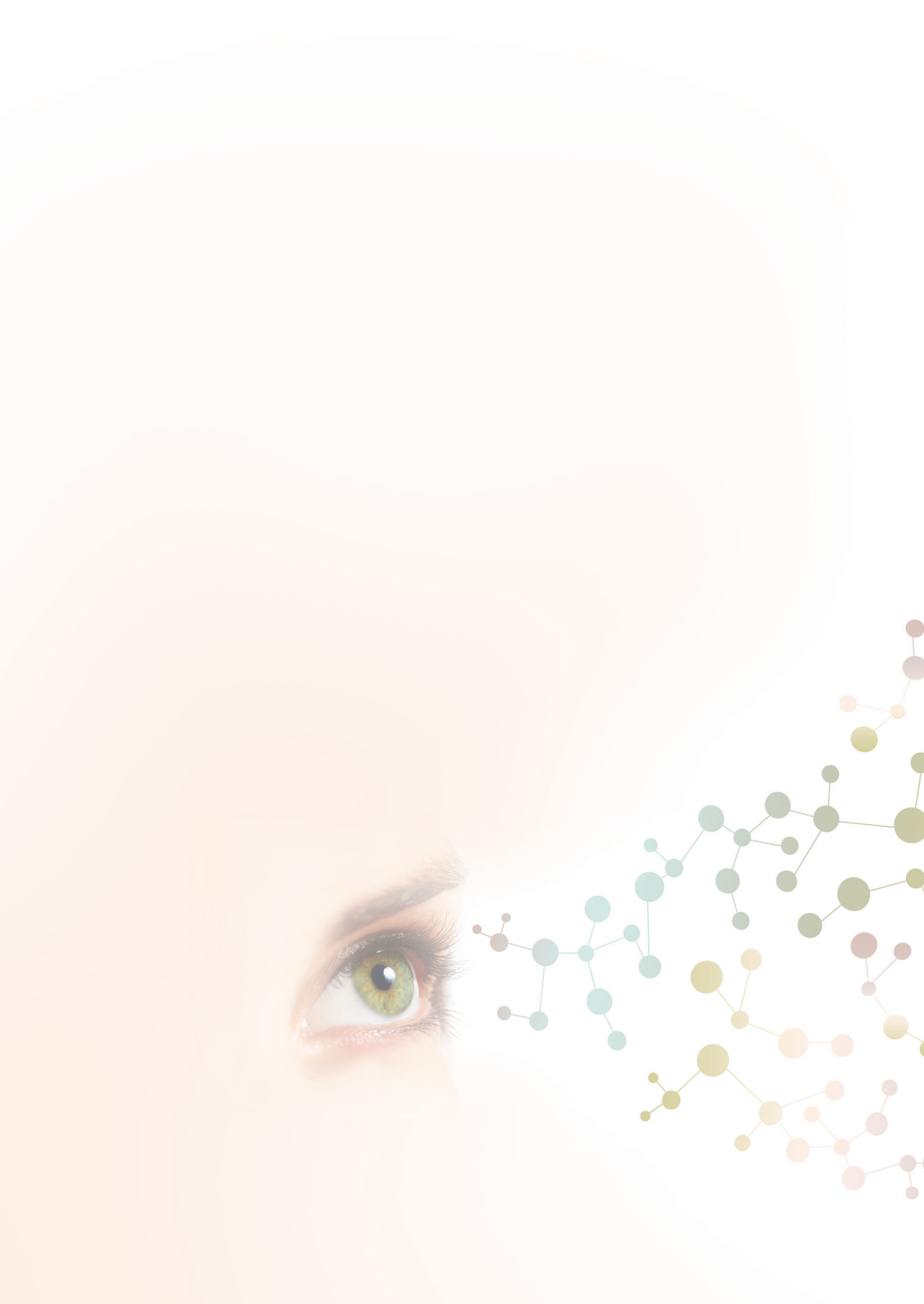
Supplemental table 1. Bio-Plex Pro™ Human Cytokine, Chemokine, and Growth Factor Assay analysis

	culture media	**culture media + thrombin**	**vitreous**	**vitreous + dabigatran**
IL-1β	*0.46 ± 0.8*	*0.84 ± 0.02*	*0.41 ± 0.02*	*0.41 ± 0.04*
IL-1rα	*not detectable*			
IL-2	*not detectable*			
IL-4	*0.39 ± 0.07*	*0.91 ± 0.04*	*0.34 ± 0.02*	*0.34 ± 0.02*
IL-5	*not detectable*			
IL-6	5.54 ± 1.48	430.39 ± 42.72	20.90 ± 5.39	20.87 ± 5.00
IL-7	3.98 ± 0.48	5.47 ± 0.16	3.54 ± 0.30	3.96 ± 0.30
IL-8/CXCL8	46.45 ± 5.52	6634.67 ± 1445.81	247.85 ± 51.70	145.86 ± 48.29
IL-9	10.71 ± 1.45	31.95 ± 1.12	9.05 ± 0.94	8.53 ± 0.98
IL-10	13.95 ± 1.50	26.75 ± 0.97	14.64 ± 0.68	15.53 ± 0.95
IL-12 (p70)	75.45 ± 6.18	143.39 ± 3.41	75.69 ± 3.64	79.81 ± 4.20
IL-13	0.57 ± 0.10	*0.74 ± 0.03*	*0.44 ± 0.03*	*0.46 ± 0.03*
IL-15	*not detectable*			
IL-17	*not detectable*			
FGFb	152.86 ± 45.33	212.11 ± 34.08	40.62 ± 9.65	49.23 ± 16.60
Eotaxin	*not detectable*			
G-CSF	*not detectable*			
GM-CSF	*42.16 ± 4.18*	169.83 ± 6.79	*24.61 ± 1.86*	*34.82 ± 4.50*
IFNγ	*not detectable*			
IP-10/CXCL10	*not detectable*	*not detectable*	*16.86 ± 4.23*	*16.99 ± 4.14*
MCP-1/CCL2	500.92 ± 46.28	1135.27 ± 142.22	*723.72 ± 34.28*	807.93 ± 67.02
MIP-1α/CCL3	*not detectable*			
MIP-1β/CCL4	*not detectable*	*1.07 ± 0.07*	*1.45 ± 0.37*	*1.39 ± 0.22*
PDGF-BB	*2.98 ± 0.80*	381.57 ± 17.80	*2.67 ± 0.45*	*1.54 ± 0.26*
RANTES/CCL5	*not detectable*			
TNF-α	*6.54 ± 1.10*	15.40 ± 0.64	*6.38 ± 0.50*	*6.32 ± 0.38*
VEGF-A	1818.73 ± 244.50	5465.75 ± 179.62	1896.54 ± 116.96	2058.13 ± 195.85

All cytokines/chemokines and growth factors detectable with the Bio-Plex Pro™ Human Cytokine, Chemokine and Growth Factor Assay are given in the first column. The detected concentration of these factors in culture supernatants from ARPE-19 cells in the absence/presence of vitreous, thrombin, hirudin and/or dabigatran is indicated in the second till ninth column. The two last columns indicate the lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ). Cytokines/chemokines and growth factor concentrations are in (pg/ml). Values are the average of 4-6 samples ± SEM. Values in italic are outside limits of quantitation.

vitreous + hirudin	vitreous + thrombin	"vitreous + thrombin + dabigatran"	"vitreous + thrombin + hirudin"	LLOQ	ULOQ
0.34 ± 0.02	*1.44 ± 0.07*	*0.46 ± 0.05*	*0.40 ± 0.05*	3.2	3261
				81.1	70487
				2.1	17772
0.27 ± 0.01	*1.47 ± 0.07*	*0.38 ± 0.05*	*0.33 ± 0.04*	2.2	3467
				3.1	7380
18.03 ± 5.60	1127.75 ± 132.20	23.55 ± 5.52	22.60 ± 8.44	2.3	18880
3.59 ± 0.39	8.93 ± 0.44	3.97 ± 0.31	3.66 ± 0.44	3.1	6001
103.71 ± 30.21	*not detectable*	159.14 ± 48.07	115.76 ± 32.89	1.9	26403
6.70 ± 0.36	40.04 ± 1.29	9.96 ± 2.10	8.09 ± 0.99	2.1	7989
13.82 ± 0.74	31.95 ± 1.19	16.65 ± 1.29	16.08 ± 1.83	2.2	8840
70.65 ± 3.68	165.47 ± 5.11	82.69 ± 5.93	78.67 ± 5.20	3.3	13099
0.39 ± 0.03	*1.11 ± 0.07*	*0.49 ± 0.07*	*0.42 ± 0.03*	3.7	3137
				2.1	2799
				4.9	12235
21.90 ± 1.96	159.56 ± 46.19	101.78 ± 67.76	31.23 ± 5.14	40.9	5824
				27.2	7581
				2.4	11565
30.05 ± 2.56	219.26 ± 20.68	*34.93 ± 4.35*	*28.88 ± 2.74*	63.3	6039
				92.6	52719
15.53 ± 3.68	*20.66 ± 5.27*	*6,03*	*18.83 ± 6.42*	18.8	26867
739.53 ± 29.64	1381.05 ± 66.70	796.69 ± 57.02	726.64 ± 24.38	2.1	1820
				1.4	836
1.22 ± 0.27	*2.22 ± 0.24*	*1.55 ± 0.25*	*1.63 ± 0.49*	2.0	1726
1.56 ± 0.29	538.39 ± 46.15	*2.09 ± 0.39*	*1.67 ± 0.27*	7.0	51933
				2.2	8617
5.05 ± 0.35	21.67 ± 0.66	*7.08 ± 0.54*	*6.32 ± 0.57*	5.8	95484
1691.34 ± 179.39	6662.14 ± 513.77	2173.08 ± 306.62	1908.18 ± 202.61	5.5	56237



2.4

Thrombin generation in vitreous and subretinal fluid of patients with a retinal detachment

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ABSTRACT

PURPOSE

To measure prothrombin fragments (F1+2) and thrombin-antithrombin complex (TAT) in vitreous and subretinal fluid of rhegmatogenous retinal detachment (RRD) patients and to validate and further specify our earlier finding of increased thrombin activity in patients with proliferative vitreoretinopathy (PVR)

METHODS

F+2 and TAT were measured in 31 vitreous and 16 SRF samples using the Enzygnost® immunoassays.

RESULTS

We found significant levels of F1+2 and TAT in the vitreous of all patients with RRD compared to patients with macular hole or macular pucker. However, there was no significant difference between patients who would develop PVR in the future, had established PVR, and patients with uncomplicated RRD both in vitreous concentrations of F1+2 (Kruskal-Wallis p=0.963) and TAT (p=0.516).

CONCLUSION

The analysis of F1+2 and TAT confirmed significant thrombin generation in both vitreous and SRF of patients with RRD. An imbalance between the thrombin regulation mechanisms TAT and α2M possibly explains the difference with our previous findings.

INTRODUCTION

Activation of the coagulation cascade has recently been identified as a potential factor in the development of proliferative vitreoretinopathy (PVR).[2] We found significantly higher intravitreal thrombin concentrations in patients with established PVR and demonstrated that intravitreal thrombin stimulates retinal pigment epithelial (RPE) cells to produce pro-inflammatory and pro-fibrotic mediators.[2, 3]

To validate and further specify our earlier finding of increased thrombin activity in patients with PVR and to gain more insight into the different regulatory mechanism of thrombin in ocular fluids, we explored two additional variables. The first is the concentration of the prothrombin activation fragment (F1+2), which is a measure of the amount of thrombin generated from prothrombin. The second is the concentration of the thrombin-antithrombin complex (TAT) which is a measure of the amount of thrombin inhibited by antithrombin (AT). We further asked the question whether thrombin generation – marked by F1+2 and TAT – was different in patients with RRD who would develop PVR in the future or had established PVR, than in patients with uncomplicated RRD. Lastly, we discuss the implications for intervention with dabigatran, a reversible direct thrombin inhibitor.

METHODS

SAMPLE COLLECTION

For the TAT and F1+2 analyses, we used vitreous fluid and subretinal fluid (SRF) samples from the Rotterdam Eye Hospital Biobank. Vitreous fluid or subretinal fluid are waste materials which are removed during a vitrectomy procedure or scleral buckling procedure, respectively. All patients gave their consent for the use of rest material for research.

Undiluted vitreous (1-1.5 ml) had been obtained at the start of vitrectomy before opening the infusion line. Undiluted subretinal fluid had been obtained by drainage through a 23 gauge needle mounted on a 2ml syringe without a plunger.[11] Vitreous and subretinal fluids were immediately injected into Eppendorf tubes, provided with a unique number and stored at –80 ºC. The location in the freezer and relevant information about the sample were noted on the registration form and later entered into an Access database (Microsoft Office®).

We searched the biobank database for samples from patients who had undergone surgery for one of four conditions: a rhegmatogenous retinal detachment (RRD), proliferative vitreoretinopathy (PVR), a macular hole or a macular pucker. For patients with samples from RRD surgery, we checked their patient file to see whether they had undergone surgery for PVR later on. Samples from patients with a macular hole or macular pucker were included as controls.

F1+2 ANALYSIS

The quantification of the F1+2 prothrombin fragment was performed using the Enzygnost® F1+2 monoclonal immunoassay (Siemens Healthcare Diagnostics Products, Marburg,

Germany). According the manufacturer's instructions, the samples were thawed using a water bath (+37 ºC) for 10min. We diluted the sample 5 times – based on previous experience – using the sample buffer. Processing of samples and standard solution were performed according the manufacturer's instructions.[12] Absorbance was measured at a wavelength of 450nm using a spectrophotometer. The concentrations of F1+2 in the samples were derived from the constructed reference curve.

TAT ANALYSIS

For the quantification of TAT, we used the Enzygnost® TAT micro immunoassay (Siemens Healthcare Diagnostics Products, Marburg, Germany). Based on previous experience the samples were diluted 10 times using the sample buffer. Further processing of samples and standard solution were performed according the manufacturer's instructions.[13] Absorbance was measured at a wavelength of 492nm using a spectrophotometer. The concentrations of TAT in the samples were derived from the constructed reference curve and were converted from µg/L to pmol/L using the molecular weight of the TAT complex of 96 KDa.

STATISTICAL ANALYSIS

Data were separately analysed for vitreous fluid and SRF. The results in vitreous fluid were analysed using the Kruskal-Wallis test. The results in SRF were analysed using the Mann-Whitney U test. A sub-analysis was performed for the three RRD groups. The relationship between F1+2 and TAT was tested using Spearman's correlation coefficient. A P-value < 0.05 was considered significant. Analyses were performed with IBM SPSS statistics version 23 (IBM Corp., Armonk, NY, USA).

RESULTS

A total of 47 samples were analysed. Distribution of samples across groups differed and was subjected to availability. We were able to obtain 31 vitreous samples and 16 SRF samples. SRF was only available for primary RRD surgeries and thus groups 3 and 4.

F1+2 ANALYSIS

Concentrations of F1+2 in vitreous differed significantly between the 5 groups (P= 0.008). The values in the two control groups were consistently low, while the concentration in the three RRD groups showed a large variation (see **Figure 1**). A Kruskal-Wallis sub-analysis in the three RRD groups could not detect a difference (p = 0.963). In addition, the F1+2 concentration in the SRF sample from the one patient who would, later on, develop PVR was not significantly different than the samples from the other RRD patients. The upper limit of quantification (ULQ) was 6000 pmol/L and the lower limit of quantification (LLQ) was 35 pmol/L, both are shown as dotted lines. Values above the ULQ are shown as ULQ, values below the LLQ are shown as LLQ/2.

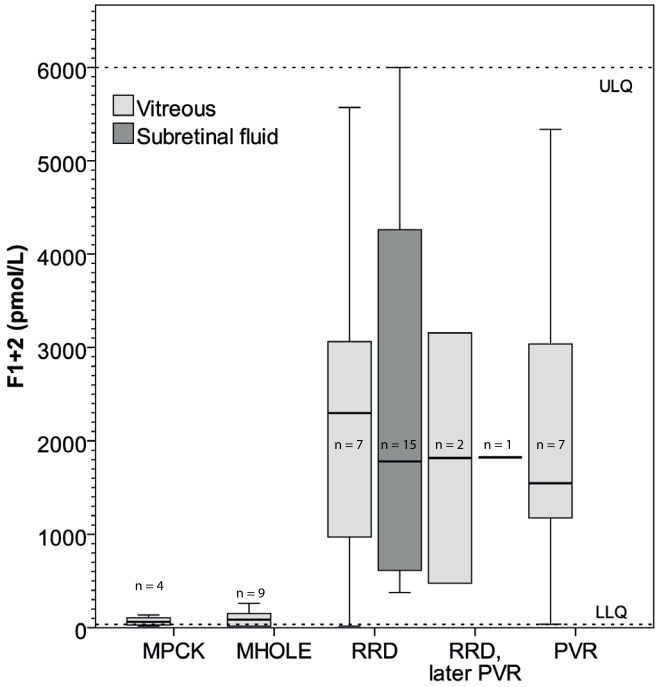

Figure 1. F1+2 measurements for both vitreous and SRF. The upper limit of quantification (ULQ) was 6000 pmol/L and the lower limit of quantification (LLQ) 35 pmol /L, both are shown as dotted lines. Values above the ULQ are shown as ULQ, values below the LLQ are shown as LLQ/2.
MPCK= macular pucker, MHOLE = macular Hole, RRD = rhegmatogenous retinal detachment, PVR = established proliferative vitreoretinopathy, RRD, later PVR = sample is from primary RRD surgery, patient developed PVR in later stage.

TAT ANALYSIS

In **figure 2** the results are shown from the TAT measurements for both vitreous and SRF. Concentrations of TAT in vitreous differed significantly between the 5 groups (P= 0.002). The values in the two control groups were again consistently low. A Kruskal-Wallis sub-analysis in the three RRD groups could not detect a difference (p = 0.516). A difference in the TAT concentration in SRF between the two groups could not be detected. The ULQ was 6250 pmol/L and the LLQ 208 pmol/L, both are shown as dotted lines. Values above the ULQ are shown as ULQ, values below the LLQ are shown as LLQ/2.

CORRELATION BETWEEN F1+2 AND TAT

The graph in **figure 3** shows the relationship between F1+2 and TAT. The production of thrombin marked as F1+2 was significantly related to the concentration of TAT both in vitreous (r_s = 0.84, p <0.001) and SRF (r_s = 0.93, p <0.001). The slope suggests that in vitreous 60% of thrombin was bound to antithrombin and in SRF 70%.

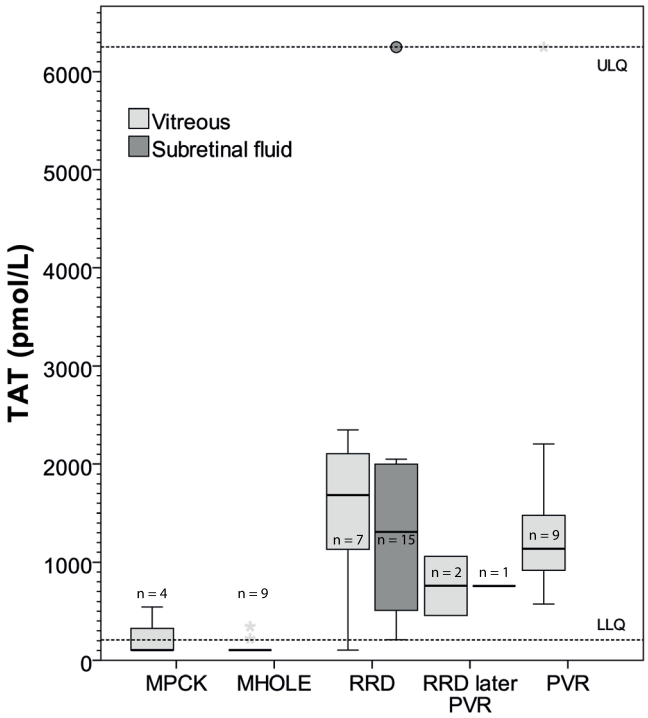

Figure 2. TAT measurements for both vitreous and SRF. The ULQ was 6250 pmol/L and the LLQ 208 pmol /L, both are shown as dotted lines. Values above the ULQ are shown as ULQ, values below the LLQ are shown as LLQ/2. MPCK= macular pucker, MHOLE = macular Hole, RRD = rhegmatogenous retinal detachment, PVR = established proliferative vitreoretinopathy, RRD, later PVR = sample is from primary RRD surgery, patient developed PVR in later stage.

DISCUSSION

These results of two indirect measures of thrombin formation confirm our previous results that significantly more thrombin was generated in vitreous fluids of patients with RRD in contrast to patients with a macular hole or macular pucker (Figure 1 and 2).[2] In contrast to our previous experiment which showed significantly higher thrombin activity in established PVR, we could not detect a difference in F1+2 and TAT values between patients with uncomplicated RRD and patients with established PVR. These findings are important because our more direct measurements of thrombin activity were possibly not accurate and straightforward.

Interestingly, the median concentrations of F1+2 and TAT that we found in vitreous and SRF of RRD patients were much higher than in normal plasma. The median concentration of F1+2 was approximately 10 times higher than in plasma (normal range 69-229 pmol/L) and the median concentration of TAT was approximately 100 times higher than in plasma (normal range 20-40 pmol/L).[4, 5] Our values of TAT were similar to those in vitreous recently reported by Ehrlich et al.[6] These high values argue in favour of local thrombin activation.

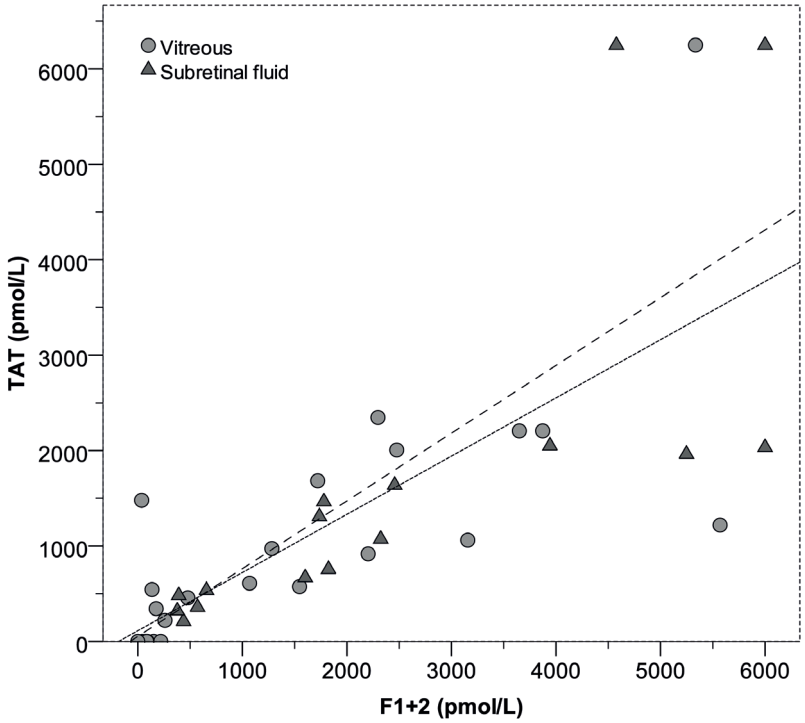

Figure 3 Correlations of F1+2 and TAT values in SRF (triangles) and vitreous (bullets). The dashed line represents the trend line of SRF and the dotted line the trend line in vitreous. LLQ values are included as being 0.

Also the ratio of F1+2 versus TAT conforms with local thrombin activation. In plasma, TAT is cleared about 10 times faster by the liver from the circulation than F1+2 (8 min vs 90min), which results in a difference in molar concentrations and a ratio of approximately 0.1.[7] In the ocular fluids, the molar concentration ratio was closer to 1 (figure 3) indicating the formation of both F1+2 and TAT in the absence of differential clearance related to liver function in the circulation. The general higher levels in vitreous and subretinal fluid may be further explained by accumulation from slower clearance of the molecules from this compartment. When only the production determines the concentration we expect a strong correlation between F1+2 and TAT. The Spearman's correlation coefficient was indeed rather strong (r_s = 0.84 and r_s = 0.93) and the slope suggests that thrombin is 60-70% bound to antithrombin (figure 3). The remaining 30-40% is possibly bound to other inhibitors such as α2M or receptors.

What did we measure previously? In the previous experiments, we used the small thrombin-specific chromogenic substrate Tos-Gly-Pro-Arg-pNA (MW 662.7Da) for the measurements of intravitreal thrombin activity.[2] Although the substrate is very well split by thrombin, other serine proteases such as plasma kallikrein and plasmin are also known

to act on the substrate due to its small molecular weight. Small synthetic substrates do not provide sufficient resemblance to the large natural substrates of thrombin, which was initially articulated by Gaffney.[8] To distinguish between thrombin activity and activity of other enzymes we duplicated the measurements with the addition of a very specific and irreversible inhibitor of thrombin: hirudin (MW 7kDa).[2] Specific thrombin activity in vitreous fluid was identified and quantified based on the difference in activity between vitreous fluid with and without hirudin. We assumed that what we recorded was only free thrombin activity.

At the time of those experiments, we were not aware of the possibility that thrombin bound to one of its inhibitors named α2macroglobulin (α2M) could still interact with small substrates and hirudin. In vivo, thrombin is mainly regulated by two inhibitors. The primary thrombin inhibitor is antithrombin which forms the inactive TAT complex. The action of antithrombin can be increased significantly by the endogenous proteoglycan heparin.[9] The second inhibitor is α2M. Alpha2M is a large plasma protein (725 kDa) that upon binding undergoes a conformational change such that the α2M folds around thrombin and partially shields its active site.[10] This prevents the cleavage of large substrates such as fibrinogen (340kDa) nearly completely but is not much effective for small substrates. The complex of α2M with enzymes is rapidly cleared from the circulation, however, in a relatively enclosed compartment such as the eye or *in vitro* a very slow remaining interaction of the complex with larger substrates (MW up to at least 20KDa) remains possible and may continue to show thrombin activity. [10, 11] Such slow interaction was shown for α2M-bound thrombin with hirudin on a time scale up to 4 hours and for α2M-bound trypsin with soybean trypsin inhibitor (SBTI, MW 20KDa) on a time scale up to 40 hours.[11-13] In the assay we used, we recorded the activity at different time points up to ten hours and based our analysis on the eight-hour values. Re-inspecting the time course of activity showed a progressive inhibition by hirudin similar to what was reported by Pochon et al.[11] We now conclude that the previous report mainly concerned α2M-bound thrombin. The fact that thrombin is bound to α2M does not change the hypothesis that thrombin plays a role in the development of PVR as the presence of the complex shows that thrombin had been formed. In addition, our previous experiment did show a significant increase in the production of CCL2, CXCL8, IL-6, IL-12 in ARPE-19 cells after incubation with vitreous.[2] These effects were abolished by hirudin which makes it unlikely that other enzymes than thrombin in the vitreous played a role.

The total amount of thrombin that has been locally active is probably better described by the concentration of F1+2 than the sum of TAT and our previous measurement of (α2M-bound) thrombin. Although we now know that it is highly likely that all previously measured thrombin activity was bound to α2M the exact quantification from that information is uncertain because of a question about the stoichiometry of the inhibition. We tested the hirudin sensitivity of the complex but α2M possesses two binding sites and binds one or two thrombin molecules. It is unknown whether hirudin is able to interact with both α2M-bound thrombin molecules. For trypsin, it was shown that only one of the two α2M-bound trypsin molecules interacted with SBTI.[13] On retrospect, we also observed residual activity

seen after prolonged hirudin inhibition which might not just be from other serine proteases but partly from α2M-bound thrombin not inhibited by hirudin. Using the hirudin-inhibited activity as a measure of thrombin activity – even after allowing enough incubation time – might therefore underestimate the thrombin activity.

We found significantly higher (what is now known to be) α2M-bound thrombin in patients with established PVR than in patients with uncomplicated RRD and patients who would later develop PVR. Surprisingly, we did not find this difference in F1+2 (formation marker) and TAT (Thrombin portion bound by AT) but found a remarkably broad range of values among the RRD patients. It will be of interest to know whether or not this range in degree of thrombin formation relates further to for example the size of the detachment or overall health of the patient. However, the limited sample size of this study rendered it not feasible to explore this.

Another possible explanation for this difference might be that not only the amount of thrombin generated causes PVR but an imbalance in the different regulation mechanisms of thrombin determines whether or not thrombin causes the development of PVR. We can only speculate that the TAT portion is modulated by local heparins and that the α2M-bound thrombin portion is responsible for the cellular effects. After binding of α2M to a proteinase, the resulting conformational change exposes a receptor recognition site on the α2M molecule. When this site is recognized by an α2M-receptor, mainly found on hepatocytes, macrophages, and fibroblasts, the complex is internalized and degraded.[10, 14] Therefore, it is suggested that α2M may function as a scavenger for different peptide mediators in inflamed tissue and may constitute an important mechanism for the regulation and containment of inflammation.[14] In contrast, it was also demonstrated that α2M binds and modulates the biological activity of several cytokines.[15] Activated α2M enhanced growth responses to TGF- 1 in smooth muscle cells and also Il-6 was shown to retain biological activity after binding to α2M. [16, 17] It may therefore also function as a carrier. An imbalance towards more α2M would possibly show a less steep slope in the correlation graph of F1+2 and TAT, however, there were too few samples in the PVR groups to compare slopes.

In view of our hypothesis that the α2M-bound thrombin is the active compound in cellular reactions and that an imbalance between regulation mechanism plays a role, the question arises whether or not an artificial inhibitor of thrombin such as dabigatran changes the distribution of thrombin between AT and α2M. In an in vitro experiment in plasma, we observed that the presence of dabigatran actively changed the distribution among inhibitors. With the same concentration of thrombin, we found an increasing concentration of TAT with increasing concentrations of dabigatran (unpublished data). This is considered to be a consequence of the difference in affinity of AT and α2M for thrombin. The addition of dabigatran, a reversible inhibitor that competes with AT and α2M for thrombin, results in favouring the formation of complexes with the inhibitor with the highest affinity (AT). Which would be advantageous if the goal is to have less a2M-bound thrombin.

What are the implications of these results for our proposed therapy with the small direct thrombin inhibitor dabigatran? The median F1+2 concentration of 2000 pmol/L among the RRD groups suggests a four times larger thrombin activity than we previously reported.[2]

A recalculation however of the needed dabigatran concentration to inhibit this amount of thrombin revealed no large increase. For 80% inhibition, a concentration of 9.4 ng/ml would be needed. [1]

At what point in time and how long thrombin has been active remains unclear. However, even when this amount of thrombin would be active all at once, the above-calculated concentration of dabigatran would be able to inhibit both free and α2M-bound thrombin.[18] An additional aim of dabigatran treatment in the prevention of PVR could be reducing α2M-thrombin formation by shifting the balance towards inactive TAT.

In conclusion, the analysis of F1+2 and TAT confirmed significant thrombin generation in both vitreous and SRF of patients with RRD. Vitreous dabigatran concentrations after oral intake would suffice to inhibit this amount of thrombin activity. Although we found a significant difference between uncomplicated RRD and PVR in thrombin activity in our previous study, we could not detect a difference in the formation of thrombin and inactivation via TAT. Possibly additional factors such as overall health and imbalance between the regulatory mechanism of thrombin play a role in steering an RRD towards PVR. A larger study including ASA scores, medication use, peroperative information and analysis of F1+2, TAT, (α2M-bound) thrombin activity and their ratios might give more insight into these factors.

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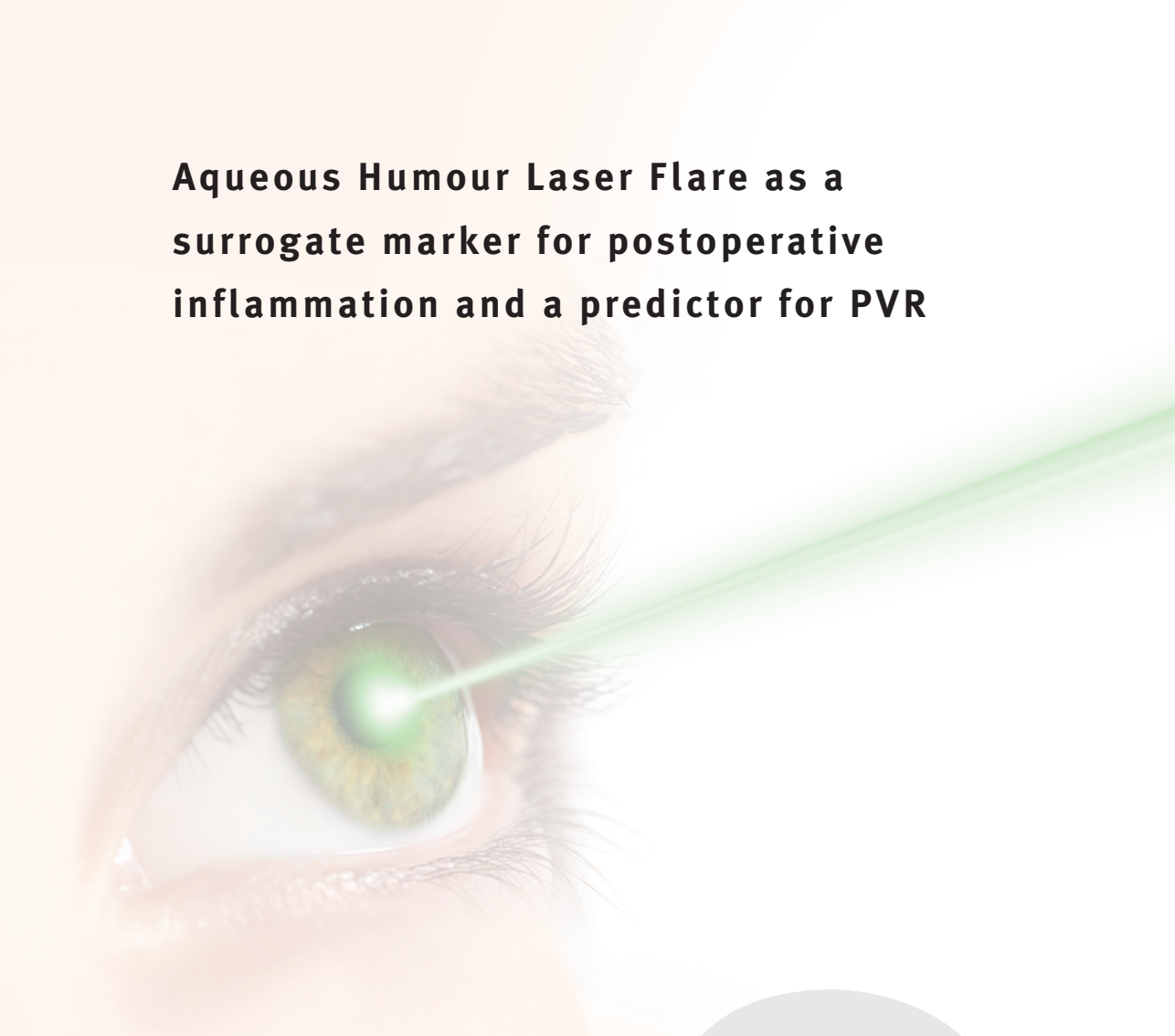
Aqueous Humour Laser Flare as a surrogate marker for postoperative inflammation and a predictor for PVR



3

3.1

Preoperative aqueous humour flare values do not predict proliferative vitreoretinopathy in patients with rhegmatogenous retinal detachment

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ABSTRACT

BACKGROUND/AIMS

Patients with rhegmatogenous retinal detachment (RRD) who develop postoperative proliferative vitreoretinopathy (PVR) have been found to have higher preoperative laser-flare values than RRD patients who do not develop this complication. Measurement of laser flare has therefore been proposed as an objective, rapid and non-invasive method for identifying high-risk patients. The purpose of our study was to validate the use of preoperative flare values as a predictor of PVR risk in two additional patient cohorts, and to confirm the sensitivity and specificity of this method for identifying high-risk patients.

METHODS

We combined data from two independent prospective studies: centre 1 (120 patients) and centre 2 (194 patients). Preoperative aqueous humour flare was measured with a Kowa FM-500 Laser Flare Meter. PVR was defined as redetachment due to the formation of traction membranes that required re-operation within six months of initial surgery. Logistic regression and ROC analysis determined whether higher preoperative flare values were associated with an increased risk of postoperative PVR.

RESULTS

PVR redetachment developed in 21/314 patients (6.7%). Median flare values differed significantly between centres, therefore analyses were done separately. Logistic regression showed a small but statistically significant increase in odds with increasing flare only for centre 2 (OR 1.014; p=.005). Areas under the ROC showed low sensitivity and specificity: centre 1 0.634(95% CI: 0.440 – 0.829) and centre 2 0.731(95% CI: 0.598 – 0.865).

CONCLUSION

Preoperative laser flare measurements are inaccurate in discriminating between those patients with RRD at high and low risk of developing PVR.

INTRODUCTION

Rhegmatogenous retinal detachment (RRD) is a common eye condition whose prevalence increases with age. While surgical repair is effective in most cases, in 5-10% of patients reattachment is complicated by the formation of epiretinal and/or subretinal contracting membranes.[1] This complication is called proliferative vitreoretinopathy (PVR) and often leads to recurrent detachments and a poor prognosis in terms of regaining vision.

While pharmaceutical drug therapies have been proposed for PVR – including anti-inflammatory drugs, anti-proliferative drugs and heparin – all these drugs have potential side effects.[2-7] Therefore, to optimise the benefit/risk ratio of drug therapies, it is crucial to select only those patients at high risk of developing PVR.

Several risk prediction models have been proposed to help identify these high-risk patients. These models are based on clinical characteristics such as aphakia, vitreous haemorrhage, pre-operative PVR, extent and duration of detachment, and high vitreous levels of protein.[8-10] Also the presence of certain gene polymorphisms has been shown to be associated with a higher risk of PVR. [11] However, validation studies have shown the models to have a low sensitivity, specificity, and positive predictive value, making them unsuitable for supporting treatment decisions.[12]

A different potential predictor for PVR development was found by Schröder et al.[13] They found preoperative anterior chamber flare – caused by reflection of laser light by proteins in aqueous humour – to be a strong predictor for PVR development. Patients with a preoperative flare value higher than 15 photon count per millisecond (pc/ms), measured objectively with a laser flare meter, had a 16-fold higher risk of developing PVR.[13]

Based on these findings, measurement of laser flare would provide an objective, rapid and non-invasive method for identifying high-risk patients. The purpose of our study was therefore to validate the use of preoperative laser flare values as a predictor of PVR risk in two additional patient cohorts from Germany and the Netherlands, and to confirm the sensitivity and specificity of this method for identifying high-risk patients.

METHODS

The University of Kiel in Germany (Centre 1) and the Rotterdam Eye Hospital in The Netherlands (Centre 2) both conducted a prospective study on the predictive value of preoperative aqueous humour flare values on the development of PVR. As both studies were set up independent from each other, study protocols differed slightly.

In centre 1 one-hundred and thirty-eight patients with a primary RRD were included in the study between April 2012 and June 2015. From January 2014 until October 2014, two-hundred and eight patients with a primary RRD were included in centre 2. Patients with additional ocular pathologies such as active uveitis, active vasculitis, retinal vein occlusion, diabetic macular oedema, proliferative diabetic retinopathy, exudative age-related macular degeneration, and primary PVR grade C or higher, were excluded. Approval was obtained

from the research ethics committee and institutional review board. All patients gave written informed consent.
We measured aqueous flare in the anterior chamber with a Kowa FM-500 Laser Flare Meter (Kowa Company Ltd. Japan) pre-operatively in both eyes. In centre 1, we made ten measurements per eye on the morning prior to surgery in an undilated eye and recorded the mean. In centre 2, we made seven measurements for each eye and discarded the highest and the lowest value, leaving the average and standard deviation of five measurements. The measurement of the study eye was made 15 minutes after instillation of a drop of 0.5% tropicamide, while the fellow eye remained undilated.
We recorded the lens status, visual acuity, type of surgery, number of retinal tears, the extent of retinal detachment, the presence of rolled over edges, and medication history.
Standard treatment after surgery in centre 1 consisted of the administration of dexamethasone and gentamicin eye drops (Dexamytrex®) five times daily, which was reduced during six weeks. In centre 2 therapy consisted of four times daily prednisolone acetate eye drops (Pred Forte®) which were reduced during four weeks. Deviations from this protocol were also recorded.
Clinically relevant PVR was defined as re-operation for redetachment due to PVR membranes, within six months of initial surgery. This information was extracted from the patient's file or when not conclusive by contacting the patient or his/her referring ophthalmologist.

ANALYSIS

We compared patient characteristics for the two centres using an independent samples t-test and Chi-square tests. To compare the flare values an independent-Samples Kruskal-Wallis test and Mann-Whitney U-tests were used. We performed logistic regression to see to what extent a higher preoperative flare value increased the risk of postoperative PVR development. ROC analysis was used to test the sensitivity and specificity of preoperative flare values in discriminating between PVR and no PVR development. The Wilcoxon signed rank test for related samples was used to compare flare values in undilated versus dilated eyes and with versus without fluorescein administration. Statistical analyses were performed with IBM SPSS statistics version 21.

RESULTS

We recruited a total of 346 patients at the two centres, but we excluded 32 of them from the analysis for various reasons. For details see **Figure 1.** The characteristics of the remaining 314 patients are tabulated in **Table 1.** The two patient populations were comparable and differed only in the type of surgery patients received and in the number of patients who presented with their macula still attached. Out of 314 patients, 46 patients

needed additional surgery within six months. We diagnosed only 21 of these patients with a redetachment due to epiretinal membranes or subretinal strands associated with PVR. Six patients had a persistent detachment (resurgery within one week) due to a missed or insufficiently closed break and the remaining patients had redetachments caused by new breaks, not completely closed old breaks, a macular hole or giant tear, without any signs of traction due to epiretinal membranes or subretinal strands.

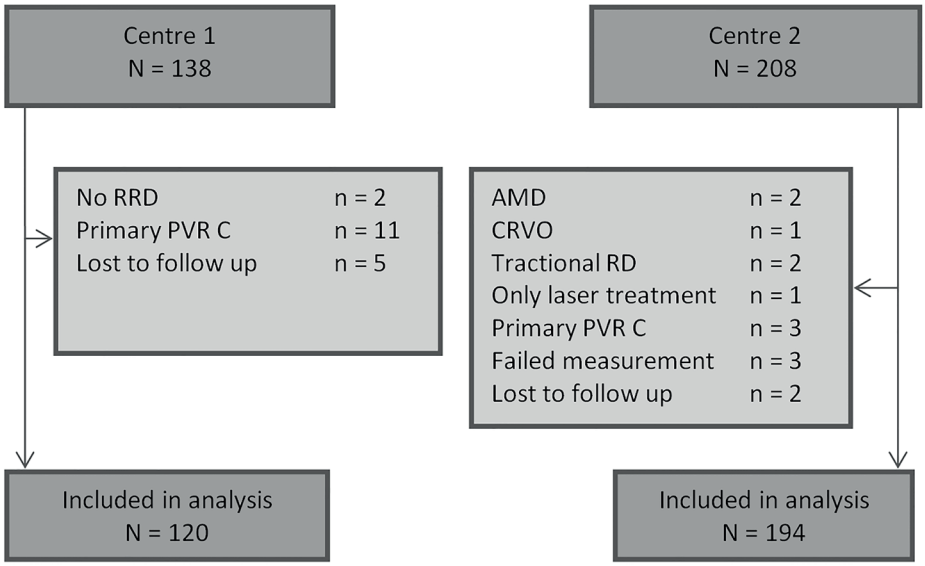

Figure 1. Flowchart of patient selection

PREOPERATIVE FLARE VALUES

The flare values measured in centre 1 were significantly and systematically higher than in centre 2. We therefore decided to perform logistic regression and ROC analysis for each centre separately. **Table 2** shows these median preoperative flare values per centre and the results of the Mann-Whitney U test for the whole patient group and for the different subgroups. Flare values are expressed in pc/ms. **Figure 2** shows the distribution of flare values and their median for the different centres and groups.

For each centre, an independent-Samples Kruskal-Wallis test was performed to compare preoperative flare values of healthy fellow eyes, eyes with no PVR redetachment and eyes with PVR redetachment. For both centres significance was demonstrated ($p < .001$). Post hoc analysis per centre involved pairwise comparisons among the three groups. Statistically significant differences were demonstrated for all comparisons, except for the comparison between patients with and without a PVR redetachment in centre 1 (adjusted significance $p = .843$).

Table 1. Patient characteristics of the two study populations

	Centre 1 (n=120)		Centre 2 (n=194)		
Age (yr.)					
mean (SD*)	61	(11.9)	59	(11.0)	
Gender, *n (%)*					
male	79	(66)	126	(65)	
Lens status preoperatively					
phakic	68	(57)	124	(64)	
pseudophakic	52	(43)	68	(35)	
aphakic	-		2	(1)	
Surgical procedure, *n (%)*					**
Scleral buckle (SB)	17	(14)	70	(36)	
PPV + gas	31	(26)	104	(54)	
PPV + oil	-		15	(8)	
SB + PPV + gas	52	(43)	-		
SB + PPV + oil	20	(17)	-		
PPV + air	-		5	(3)	
Extent of detachment, *n (%)*					
< 1 quadrant	-		7	(3.6)	
1 quadrant	23	(19.2)	45	(23.2)	
2 quadrants	67	(55.8)	97	(50.0)	
3 quadrants	22	(18.3)	30	(15.5)	
4 quadrants	8	(6.7)	15	(7.7)	
Macula attached, *n* (%)					**
Yes	44	(36.7)	100	(51.5)	
Primary success rate, *n* (%)	104	(87)	164	(85)	
Persistent detachment, *n* (%)	2	(1.6)	4	(2.1)	
PVR development, *n (%)*					
Yes	9	(7.5)	12	(6.2)	

* SD = standard deviation. ** = Significant difference between centres $p < 0.05$, Chi-Square

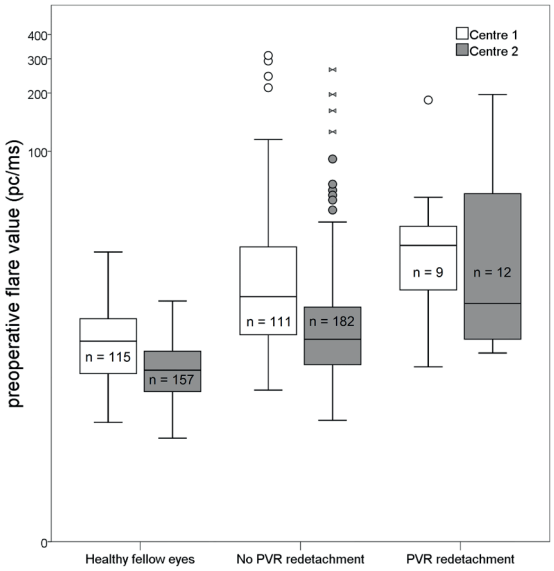

Figure 2 Boxplot showing the distribution of flare values on a logarithmic scale for healthy fellow eyes, patients with no PVR (including patients with redetachments due to other reasons) and patients with PVR.

PREOPERATIVE FLARE VALUES AND PVR REDETACHMENT RISK

The individual logistic regression showed a significant result only for centre 2. An increase of 1 pc/ms of the preoperative flare value increased the odds of PVR redetachment by 1.014 (see **Table 3**). An increase of 10 pc/ms increased the odds by 1.15. When we combined the data from the two centres and controlled for research centre, we found a smaller odds ratio of 1.008. The correct prediction of PVR cases or the correct classification of patients in general did not improve by including preoperative flare. In addition, we tested whether a higher preoperative flare value was a predictor for any redetachment (with or without signs of PVR), this analysis also showed a significant result only for centre 2 and the combined data (see **Table 3**).

SENSITIVITY AND SPECIFICITY

The area under the ROC curve for centre 1 was 0.634 (95% CI: 0.440 – 0.829). At a cut-off value of 10 pc/ms, the accompanying sensitivity and specificity were 89% and 22%. A higher cut-off value of 15 pc/ms, showed higher specificity (37%) and somewhat lower sensitivity (78%). For centre 2 the area under the ROC curve was higher (0.731; 95% CI: 0.598 – 0.865). The cut-off values of 10 and 15 pc/ms showed a sensitivity and specificity of 75% and 50%, and 50% and 76%.

The probability that a patient with a preoperative flare value above 15 pc/ms would develop a PVR redetachment (positive predictive value) was 9 and 12 percent for centre 1 and 2, based on the observed prevalence in these centres.

Table 2. Median flare values (pc/ms) in subgroups and healthy fellow eyes

	Centre 1 (n=120)		Centre 2 (n=194)		P*
	median	**(range)**	**median**	**(range)**	
RD	**17.7**	**(5.0-312.0)**	**10.2**	**(3.2-263.8)**	**< 0.001**
phakic	16.0	(5.0-312.0)	9.1	(3.2-263.8)	
pseudophakic	23.1	(5.4-76.1)	12.4	(4.4-196.4)	
aphakic	-		17.4	(5.9-28.8)	
No PVR redetachment	**17.1**	**(5.0-312.0)**	**10.0**	**(3.2-263.8)**	**< 0.001**
phakic	16.0	(5.0-312.0)	8.5	(3.2-263.8)	
pseudophakic	22.3	(5.4-76.1)	12.3	(4.4-126.0)	
aphakic	-		17.4	(5.9-28.8)	
PVR redetachment	**32.2**	**(6.9-183.9)**	**15.8**	**(8.3-196.4)**	**0.808**
phakic	29.6	(6.9-183.9)	16.7	(9.8-187.8)	
pseudophakic	32.2	(10.6-57.6)	14.8	(8.3-196.4)	
Extent of detachment					
< 1 quadrant	-		7.3	(3.2-9.9)	
1 quadrant	10.0	(5.4-33.0)	7.6	(3.2-37.9)	
2 quadrants	17.8	(5.0-107.0)	10.1	(3.3-67.4)	
3 quadrants	27.0	(7.2-312.0)	11.9	(5.7-196.5)	
4 quadrants	131.9	(17.0-292.1)	62.3	(10.4-263.8)	
Macula On	11.4	(5.0-86.0)	8.5	(3.2-49.4)	0.001
Macula Off	23.2	(6.9-312.0)	12.0	(4.0-263.8)	< 0.001
Healthy fellow eyes	9.7	(3.1-29.7)	6.6	(2.4-16.2)	< 0.001

* independent Samples Mann-Whitney U test between centres. Significance level after Bonferroni correction p = 0.008 Analysis was not performed for extent of detachment

Table 3. Results from logistic regression analyses for redetachment risk.

	Covariate(s)	**P**	**Odds Ratio**	**95% confidence interval**
PVR redetachment				
Centre 1	Preoperative flare	.495	1.004	0.993 – 1.014
Centre 2	Preoperative flare	.005	1.014	1.004 – 1.024
Combined	Preoperative flare	.015	1.008	1.002 – 1.015
	Research centre	.948	1.031	0.408 – 2.607
Any redetachment				
Centre 1	Preoperative flare	.233	1.005	0.997 – 1.013
Centre 2	Preoperative flare	.012	1.012	1.003 – 1.021
Combined	Preoperative flare	.008	1.008	1.002 – 1.014
	Research centre	.342	0.719	0.364 – 1.419

INFLUENCE OF DILATING EYE DROPS ON THE FLARE VALUE

As flare values were systematically higher in centre 1 compared with centre 2, we performed additional analyses in an attempt to explain these differences.
First, we compared 27 study patients from centre 1 in whom measurements were performed in both eyes during the study in undilated and dilated state 30 minutes apart. The Wilcoxon signed rank test for related samples revealed a significantly lower flare value in the dilated state (OD p=.002; OS p < .0001). The median differences were -2.3 pc/ms (Interquartile range(IQR): -4.3 – -0.3) and -2.7 pc/ms (-4.1 – -0.6).
Second, we compared 147 healthy fellow eyes from study patients in centre 2. Fellow eyes were measured in undilated and dilated state at two different standard visits, six weeks apart. Also here we found a significantly lower value in the dilated state (p <.001). The median difference was -0.6 pc/ms (IQR -2.4 – 0.4), less than the values found in centre 1.

INFLUENCE OF FLUORESCEIN

To test the possible influence of fluorescein on the height of the flare value we measured 10 healthy volunteers with undilated pupils. Next, we used a fluorescein strip and oxybuprocaine 0.4% to instil fluorescein in the eye. After an hour we measured flare again. The related samples Wilcoxon signed rank test showed no significant difference (p = .359).

DISCUSSION

The results of our study suggest that preoperative flare values are a poor predictor of postoperative PVR development. Although the logistic regression analysis showed a significant result for one of the two centres (OR 1.014), this did not improve classification of patients into their respective groups (PVR vs. no PVR development). In addition, the sensitivity and specificity of preoperative flare at different cut-off values were too low to adequately filter out the high-risk patients. As a consequence, we were unable to validate the findings of Schröder et al, although they used the same flare meter type and calibration protocol.[13]
During the analysis of the data, we discovered discrepancies between the two centres. One such discrepancy was a statistically significant difference in median flare values; the median value in patients with no PVR was higher for centre 1 (17.7 pc/ms) than for centre 2 (10.2 pc/ms). Also, median flare values of healthy fellow eyes were significantly higher. A comparison of the values with those of previous studies using flare measurement suggests that centre 1 is the one whose values are higher.[13-16] Previously reported mean values range from 3.7 to 6.5 pc/ms in healthy individuals and a median of 10 pc/ms in patients with a rhegmatogenous retinal detachment. We, therefore, decided to analyse the results separately for each centre.
Although the two centres conducted their study independently from each other, the study protocols differed only slightly. When we compared inclusion and exclusion criteria, measurement method and conditions, and primary outcome, we found that the centres

had similar levels of background lighting, both used the same Kowa Laser Flare Meter type and calibrated it monthly. There were however two apparent differences.
The first difference was the number of measurements made per eye. Centre 1 used the mean of ten measurements per eye, excluding any measurements deviating more than two standard deviations from this mean. Centre 2 made seven measurements and excluded the highest and the lowest value, leaving a mean of five measurements. However, we do not expect this difference to add to the large difference in median flare values that we observed.
The second difference was that while centre 2 used dilating eye drops, centre 1 did not. From the literature, it is known that dilating eye drops reduce flare during the first one to two hours after instillation.[17-19] The mechanism is not completely understood, but it seems to be a pharmacological effect rather than a reduction of background noise by a larger pupil size.[20] Tropicamide was reported to decrease the flare value by approximately 10-30% in healthy eyes.[19, 20] To determine whether this was likely to have had a major influence on our flare results, we did two additional analyses. Both analyses showed a significant lower flare value in dilated state, but the absolute median difference was less than 3 pc/ms in centre 1 and 0.6 pc/ms in centre 2. Although the use of dilating eye drops does not explain the entire difference found between the two centres, it might have contributed significantly.
What stood out in both centres was the large range of values and a large number of outliers (3.2 – 312 pc/ms). This large number of outliers in the no PVR group versus the low number of PVR cases makes a distinction between these groups difficult. Possibly the variation in values is more dependent on the timepoint at which flare is measured, rather than on a difference in PVR status. When patients present at the emergency department, they are subjected to various examinations that require the use of multiple eye drops and undergo various manipulations of the eye. Manipulations such as gonioscopy and scleral indentation might cause an inflammatory reaction leading to a rise in flare. In centre 1 examination with scleral indentation was often performed one day before the flare measurements. In contrast, centre 2 did not routinely perform scleral indentation at admission. This might be another reason explaining the higher flare values in centre 1. However, we did not find any literature on this topic and this explanation remains speculative.
Another possibility might be that the presence of fluorescein in the anterior chamber might increase flare. We therefore tested this by measuring flare before and after the administration of fluorescein in ten volunteers, but we could not detect a significant difference (p=.359). As the intensity of scattered light is proportional to particle diameter and fluorescein is approximately 200 times smaller in weight than albumin, the influence of the small concentration fluorescein on the flare value is likely to be negligible compared to the influence of proteins.
In conclusion, the wide variation and overlap in flare values between patients with and without PVR implicate that the measurement of aqueous humour flare with a Kowa Laser Flare Meter is inaccurate in discriminating between those patients with RRD at high and low risk of developing PVR. It should be further explored whether the addition of aqueous flare as a parameter to existing risk formulae would increase their predictive value.

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3.2

Postoperative aqueous humour flare as a surrogate marker for proliferative vitreoretinopathy development

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ABSTRACT

BACKGROUND/AIMS

As some surgical procedures have been shown to increase postoperative flare values and thus contribute to blood-ocular barrier breakdown, retinal reattachment surgery might influence the risk of developing proliferative vitreoretinopathy (PVR). Therefore, we investigated whether postoperative aqueous flare values are a surrogate marker for the development of postoperative PVR.

METHODS

We prospectively included 195 patients with primary rhegmatogenous retinal detachment (RRD) and measured aqueous laser flare preoperatively, and at two and six weeks postoperatively (Kowa FM-500 Laser Flare Meter). Postoperative PVR was defined as reoperation for redetachment due to PVR membranes, within six months of initial surgery. Logistic regression and ROC analysis determined whether higher postoperative flare values were associated with an increased risk of developing PVR later on.

RESULTS

Reoperation for postoperative PVR was needed in 12 (6.2%) patients; in 18(9.2%) reoperation was not related to PVR. The median flare value for patients who would develop PVR was significantly higher than that of patients who would not develop PVR, both at two weeks ($p=0.001$) and six weeks ($p<0.001$) postoperatively. Logistic regression analyses showed that a higher flare value significantly increased the odds of developing PVR, either at two weeks (odds ratio (OR) 1.027; 95%CI: 1.010-1.044) or six weeks (OR 1.076; 95%CI: 1.038-1.115).

CONCLUSION

Flare values both at two and six weeks postoperatively seem a good surrogate marker in terms of sensitivity and specificity for the development of postoperative PVR but have only a modest PPV. The two-week value would be more useful in terms of early recognition of high-risk patients and hence give the possibility to better study effects of treatment methods.

INTRODUCTION

Anterior chamber aqueous flare – a surrogate marker for inflammation – has been proposed as a predictor for proliferative vitreoretinopathy (PVR) in patients with a rhegmatogenous retinal detachment (RRD).[1-3] In contrast to previous reports, we recently reported that the preoperative aqueous flare value is not a strong predictor for the development of PVR postoperatively.[4]

Although a minority of patients without a history of ocular disease presents with PVR prior to retinal reattachment surgery, in industrialised countries 5-10% of patients typically develop PVR in two weeks to six months after surgery. Some of these surgical procedures have been shown to increase postoperative flare values and thus contribute to blood-ocular barrier breakdown.[5-8] Therefore, retinal reattachment surgery is thought to possibly influence the risk of developing postoperative PVR.[9]

The flare value after surgery might therefore be a better indication of the development of postoperative PVR than the preoperative flare value. We investigated whether postoperative aqueous flare values or a change in aqueous flare values from preoperative to postoperative are a surrogate marker for the development of postoperative PVR.

METHODS

PATIENTS

From January 2014 until October 2014 we included 208 patients with RRD admitted to the Rotterdam Eye Hospital, the Netherlands. Patients with additional ocular pathologies such as active uveitis, active vasculitis, retinal vein occlusion, diabetic macular oedema, proliferative diabetic retinopathy, exudative age-related macular degeneration, and primary PVR grade C or higher, were excluded. Postoperative PVR was defined as reoperation for redetachment due to PVR membranes, within six months of initial surgery. This information was extracted from the patient's file or, when not conclusive, by contacting either the patient or his/her current physician. The standardised surgical reports of the reoperations and the patient's file were evaluated by one vitreoretinal surgeon (masked to flare values) who scored each reoperation as either not PVR related or PVR related.

The study followed the tenets of the Declaration of Helsinki and was approved by the institutional review board. All patients gave written informed consent. This patient cohort has been part of a previously published report with a different research question exclusively on preoperative measurements.[4]

FLARE MEASUREMENTS

Aqueous laser flare of the anterior chamber was measured preoperatively and during regular postoperative visits at two and six weeks with a Kowa FM-500 Laser Flare Meter (Kowa Company Ltd. Tokyo, Japan). We performed seven measurements 15 minutes after instillation of 0.5% tropicamide eye drops. The highest and the lowest value were discarded,

leaving an average of five measurements. In addition, we recorded the preoperative and postoperative lens status, the extent of retinal detachment, number of horseshoe tears, presence of curled edges during surgery, type of surgery, and medication history. In all patients undergoing a vitrectomy procedure triamcinolone (Kenacort®) was used to visualize the vitreous during vitreous removal. Standard treatment after surgery consisted of a subconjunctival injection of betamethasone (Celestone® 4mg) and of four times daily prednisolone acetate eye drops (Pred Forte®) which were tapered over four weeks. Deviations from this protocol were also recorded.

SAMPLE SIZE

From previous measurements and other studies, it was known that flare values do not follow a normal distribution and that non-inflamed healthy eyes have (10log-transformed) flare values of 0.7 ± 0.3.[6, 7, 10] For the purpose of the sample size calculation of the original study it was assumed that the standard deviation (SD) would be slightly higher (SD=0.4).[4] The incidence of PVR was estimated at 10%, the two-sided significance level was set at =0.05, power at P=0.80, and a factor two increase in flare value was thought to be clinically relevant. This led to a sample size of 176 eyes of which at least 16 eyes were expected to develop postoperative PVR.

STATISTICAL ANALYSIS

Since aqueous flare values are not normally distributed we looked at median flare values and used non-parametric tests. Patients who required reoperation for another indication than PVR were displayed as a separate group but for the logistic regression and ROC analysis they were included in the uncomplicated RRD group. Median flare values of the three groups were compared using a Kruskal-Wallis test with pairwise comparisons for both time points (two and six weeks).

We performed logistic regression to assess to what extent a higher postoperative flare value at either two or six weeks increased the risk of postoperative PVR development. ROC analysis was used to test the sensitivity and specificity of postoperative flare values in discriminating between PVR and no PVR development, and to define the optimal cut-off point. A Mann-Whitney U-test was used to compare the individual changes in flare values from preoperative to two weeks postoperatively between the two groups. Statistical analyses were performed with IBM SPSS statistics version 21 (IBM Corp., Armonk, NY, USA).

RESULTS

We included 208 patients of which five patients were excluded due to other ocular pathology than RRD, three had preoperative PVR, two patients were lost to follow-up, two had multiple failed flare measurements and one received only laser treatment. The characteristics of the remaining 195 patients are shown in **Table 1**. Thirty patients (15%) underwent reoperation, out of whom four patients had a persistent detachment (reoperation

Table 1. Patient characteristics of 195 patients with a rhegmatogenous retinal detachment

Characteristic	Uncomplicated RRD (n=165)	Reoperation, no PVR (n=18)	Postoperative PVR (n=12)
Age (year)			
mean (SD)	59 ± 11	55 ± 9	69 ± 9
Gender, *n (%)*			
male	106 (64)	14 (78)	7 (58)
Lens status before surgery, *n (%)*			
phakic	102 (61.8)	16 (88.9)	7 (58.3)
pseudophakic	62 (37.6)	1 (5.6)	5 (41.7)
aphakic	1 (0.6)	1 (5.6)	-
Surgical procedure, *n (%)*			
Scleral buckling	56 (33.9)	13 (72.2)	1 (8.3)
PPV + gas	85 (51.5)	3 (16.7)	5 (41.7)
PPV + gas + CE	12 (7.3)	2 (11.1)	3 (25.0)
PPV + oil	10 (6.1)	-	3 (25.0)
PPV + oil + CE	2 (1.2)	-	-
Extent of detachment, *n (%)*			
< 1 quadrant	6 (3.6)	1 (5.6)	1 (8.3)
1 quadrant	40 (24.2)	4 (22.2)	1 (8.3)
2 quadrants	84 (50.9)	10 (55.6)	2 (16.7)
3 quadrants	25 (15.2)	3 (16.7)	3 (25.0)
4 quadrants	10 (6.1)	-	5 (41.7)
Macula attached, *n (%)*	86 (52.1)	10 (55.6)	4 (33.3)
Preoperative hypotony, *n (%)* (IOP ≤ 4 mmHg)	2 (1.2)	-	1 (8.3)
Primary success, *n (%)*	165 (85)		
Persistent detachment, *n (%)*	4 (2.1)		

CE = cataract extraction, IOP = intraocular pressure, PPV = pars plana vitrectomy, RRD = Rhegmatogenous retinal detachment, SD = standard deviation

within one week) and 12 patients (6.2%) developed postoperative PVR for which surgery was performed. The remaining 14 patients had redetachments caused by new breaks (n= 8), not completely closed old breaks (n= 4), a macular hole (n= 1) or giant tear (n= 1), without any signs of traction due to epiretinal membranes or subretinal strands. The median time until reoperation was 49 days (range 12-183 days) for patients who had developed PVR and 20 days (range 2-139 days) for reoperation due to other reasons.

POSTOPERATIVE FLARE VALUES

At two weeks postoperatively, the pairwise comparisons showed a significant difference between patients who would develop PVR postoperatively and patients with uncomplicated RRD (adjusted p=0.001; n=10 vs. n=162). The median flare value of patients with a reoperation due to other reasons (n=12) did not differ significantly from the two other groups (adjusted p= 0.526 and p= 0.176), at two weeks postoperatively. At six weeks, the flare values of patients who would develop PVR (n= 8) remained higher than those of patients who received a reoperation for another reason (adjusted p = 0.002; n= 5) and of patients with uncomplicated RRD (adjusted p < 0.001; n= 164).
Since eight patients required reoperation before their evaluation visit at two weeks and nine patients required reoperation before their evaluation visit at six weeks, the flare values of those visits are missing. **Figure 1** shows the median flare values over time for the three mentioned groups. The whiskers represent the interquartile ranges.

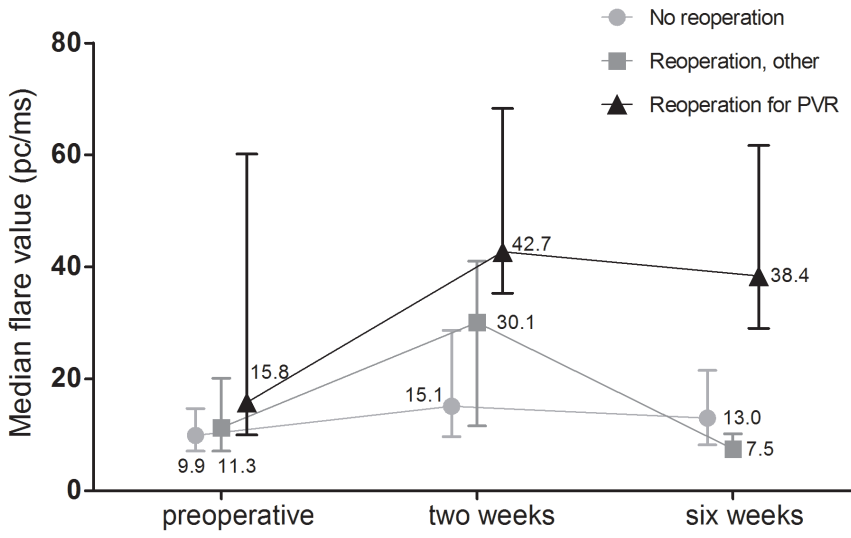

Figure 1 Median flare values over time for the three different groups of patients with a rhegmatogenous retinal detachment. The whiskers represent the interquartile ranges (25-75%)

POSTOPERATIVE FLARE VALUE AND RISK OF PVR DEVELOPMENT

We tested whether a higher flare value at either two or six weeks after surgery was a surrogate marker for a future PVR redetachment. Patients requiring reoperation due to other reasons than PVR were included in the uncomplicated group. The logistic regression analysis showed a significant result for both time points (see **Table 2**).

Table 2. Results from logistic regression comparing uncomplicated rhegmatogenous retinal detachment patients with patients who developed proliferative vitreoretinopathy.

	P	Odds Ratio	95% confidence interval	N
Flare value 2 weeks postoperatively	0.002	1.027	1.010-1.044	10/174
Flare value 6 weeks postoperatively	<0.001	1.076	1.038-1.115	8/169

SENSITIVITY, SPECIFICITY, AND POSITIVE PREDICTIVE VALUE

The ROC analysis showed high area under the ROC curves for both the two-week postoperative values (0.84; 95% CI: 0.76 – 0.93) and the six-week postoperative values (0.92; 95% CI: 0.86 – 0.97). A cut-off value of 34 pc/ms two weeks postoperatively led to both a sensitivity and specificity of 80% (see **Figure 2**). For the values obtained at six weeks, the optimal cut-off value was 27 pc/ms, with an accompanying sensitivity and specificity of 100% and 83%. A cut-off of 27 pc/ms for the two-week postoperative values, showed 85% sensitivity and 69% specificity. The probability that a patient with a flare value above 27 pc/ms at two or six weeks postoperatively developed a PVR redetachment – the positive predictive value – was 14.5 and 22 percent. The positive predictive value with a cut-off of 34 pc/ms two weeks postoperatively was 18%.

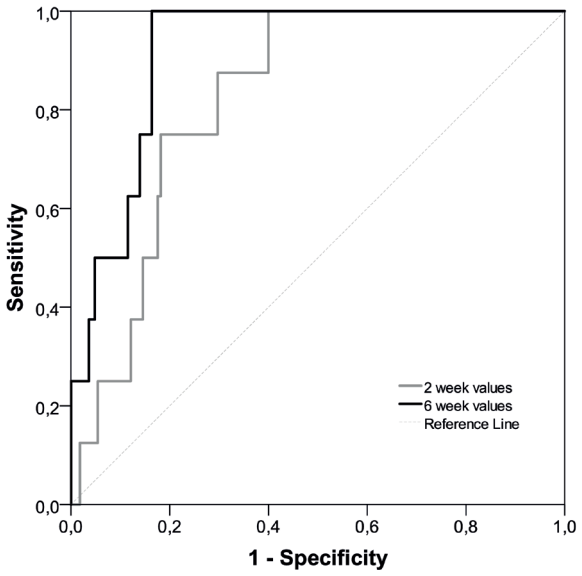

Figure 2. ROC analyses of flare values at two and six weeks postoperatively and postoperative proliferative vitreoretinopathy (PVR) development. Patients requiring reoperation due to other reasons than PVR are included in the uncomplicated group

ABSOLUTE CHANGE IN FLARE VALUES FROM PREOPERATIVELY TO TWO WEEKS POSTOPERATIVELY

We calculated the individual absolute change in pc/ms from the preoperative value to the two-week flare value for patients who would not develop PVR (uncomplicated RRD) - including patients with reoperations due to other reasons - and for patients who would later develop postoperative PVR. A positive value in **Figure 3** means that the flare value increased after surgery and a negative value means a decrease in flare value. **Figure 3** shows that the flare value in patients who would later develop postoperative PVR increased in half of the patients and decreased in the other half. In patients who would not develop PVR, the flare value increased slightly in most cases. The distribution of differences was not significantly different between the two groups (Mann-Whitney U p= 0.672).

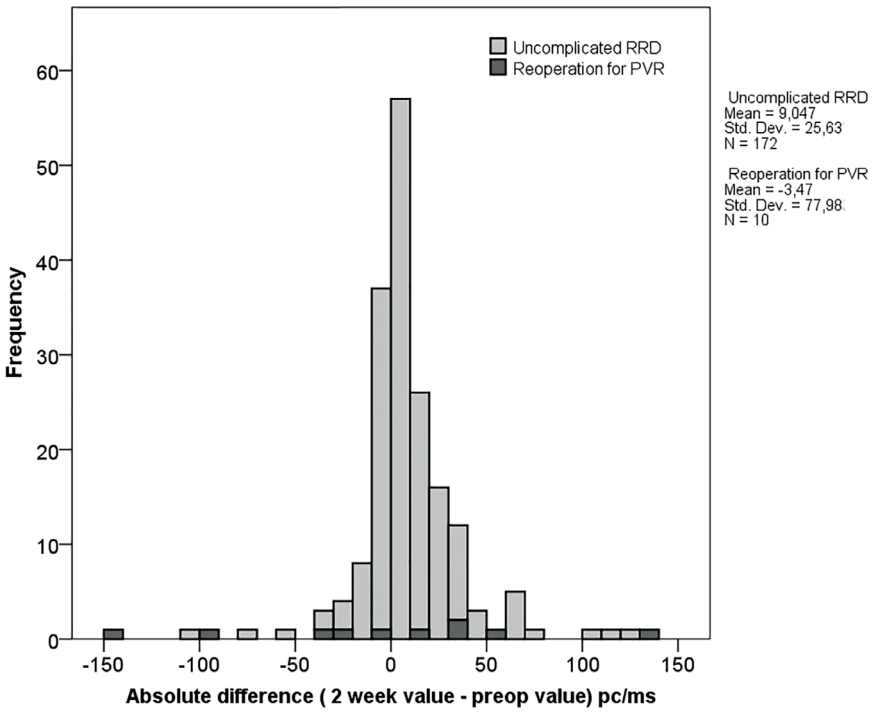

Figure 3. Histogram of absolute differences between the preoperative value and postoperative value at 2 weeks. A positive value means an increase after surgery; a negative value a decrease after surgery.

DISCUSSION

The logistic regression analysis showed that a higher postoperative flare value at either two or six weeks increased the chance of developing postoperative PVR (**Table 2**). The ROC analysis provided insight into the optimal cut-off values, which is a balance between not missing any patients who will develop postoperative PVR (sensitivity) and not labelling too many patients incorrectly as high-risk patients (specificity). The area under the ROC curve showed a better result for the six-week values, due to reaching 100% sensitivity and a good specificity. The accompanying cut-off value was 27 pc/ms. None of the eight patients who had not yet developed postoperative PVR at that point had a flare value below this value and 28 out of 183 patients who would not develop PVR had a flare value above 27 pc/ms (specificity 83%). For the two-week values, the optimum was 80% sensitivity with 80% specificity and an accompanying cut-off value of 34 pc/ms.

Although the ROC analyses showed high sensitivity and specificity at a cut-off value of 34 pc/ms and 27 pc/ms at two and six weeks postoperatively, this led to positive predictive values of only 18% and 22%. The main reason for this was the low prevalence of postoperative PVR in our study (6.2%). Previously, we concluded that preoperative flare values are inaccurate in discriminating between high and low risk of developing PVR. While the logistic regression analysis showed a statistically significant value ($p = 0.005$), the accompanying odds ratio was low (1.014) and at a cut-off value of 15 pc/ms the low sensitivity (50%), specificity (76%) led to a positive predictive value of only 12%.[4] The choice of using a test with a relatively low positive predictive value and thus a high false discovery rate depends on the consequences and risks for the patient associated with a positive test, such as possible side-effects of the treatment initiated. However, the use of postoperative flare would improve the selection of patients by three times relative to the prevalence in our unselected cohort, and would miss fewer patients that would later develop PVR than when using the preoperative value. In a new study design, this could be of value to improve the ratio between cases and controls.

Although the six-week measurements proved to be a better marker for later PVR than the two-week measurements, the two-week measurements would be more useful in terms of earlier recognition of high risk for PVR and subsequently the possibility to start a treatment. Moreover, at two weeks fewer patients will have already experienced a PVR redetachment. Postoperative therapeutic options would be the administration of oral drugs or injections when such a treatment would be available and effective.[11-17] Both postoperative measurements could be used by ophthalmologists to monitor inflammation and study treatment methods.

The absolute flare value was a better surrogate marker for postoperative PVR development than the change in flare value. While the overall trend in flare values was an increase after surgery followed by a decrease towards six weeks, the individual changes from the preoperative flare value to the postoperative flare value at two weeks did not show a clear trend. This was highlighted in the ten patients who would later develop PVR: flare values increased in five cases but decreased in the five other cases.

The absence of a clear trend might be the result of specific proceedings and choices by the surgeon and/or patient during surgery and the early postoperative phase. Patients who underwent a vitrectomy procedure seemed to have higher postoperative flare values than patients who underwent scleral buckling, independent of the number of quadrants detachment (data not shown). In addition, factors such as the occurrence of complications, duration of surgery, choice of vital dyes, manipulation due to indentation and the use of antibiotics and/or steroids, may influence the inflammatory response. Figuring out the individual importance of these factors would require an extremely large sample size, whereas the postoperative flare value represents the sum of these factors.

Adding the outcome of postoperative aqueous flare measurements to existing risk prediction models could possibly increase its value. The size of a detachment is a well-known risk factor for the development of PVR, but the size of the detachment is also correlated to flare. [1,2] In our study, this association was the strongest for preoperative flare and higher number of quadrants detachment ($r_s = 0.42$, $p < 0.001$), for postoperative flare this association was weak ($r_s = 0.20$, $p = 0.005$). In addition, correcting for the number of quadrants detachment in the logistic regression analysis did not change the odds ratio for the postoperative flare value (data not shown).

These results should, however, be interpreted with caution due to a lower prevalence of postoperative PVR in our sample than anticipated. In conclusion, postoperative flare values two weeks after RRD surgery are a reasonable surrogate marker for the development of postoperative PVR. These results should be validated in other cohorts including more patients with postoperative PVR.

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3·3

More breakdown of blood ocular barriers after vitrectomy than scleral buckling

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INTRODUCTION

In recent years there has been a general tendency towards using pars plana vitrectomy (PPV) for uncomplicated rhegmatogenous retinal detachment (RRD) instead of scleral buckling (SB).
In pseudophakic patients, PPV has become the standard surgical approach but also in phakic patients this trend is observable. With the advances of smaller-gauge PPV techniques, surgical trauma appears to be less, which is thought to result in lower postoperative inflammation and better results.[1] Several studies, however, showed better functional results after SB.[2,3] In addition, a systematic review described a higher risk of proliferative vitreoretinopathy (PVR) after PPV.[4]

METHODS AND RESULTS

In a prospective study in patients with primary RRD to find predictors for the development of PVR, we measured preoperative and postoperative inflammation after SB and PPV by means of laser flare photometry.[5] Mean postoperative flare values at different time-points after SB and PPV have been previously reported by others but have not been directly compared.[6, 7] As the extent of the detachment might influence both the surgical approach as well as postoperative flare, we performed a subanalysis of our phakic patients in our previously published cohort to study whether SB and PPV produce comparable postoperative inflammation. Aqueous laser flare was measured during regular postoperative visits at two and six weeks with a Kowa FM-500 Laser Flare Meter (Kowa Company; Tokyo, Japan). We performed seven measurements 15 minutes after instillation of 0.5% tropicamide eye drops. The highest and the lowest value were discarded, leaving an average of five measurements. Postoperatively, patients received four times daily prednisolone acetate eye drops which were tapered over four weeks.[5]
From the original study population, 115 patients were phakic preoperatively. In this group, 62 patients underwent SB, 29 patients underwent PPV with gas, 6 patients underwent PPV with oil, and 18 patients a combination of PPV, cataract extraction (CE) and IOL implantation. Postoperative flare values after SB and PPV were compared using the independent samples Mann-Whitney U test for each quadrant separately. For sample size purposes, all patients that underwent PPV were combined. The results in **Figure 1** show that postoperative flare is significantly higher after PPV than SB, independent of the size of the detachment. Both at two weeks and six weeks postoperatively.

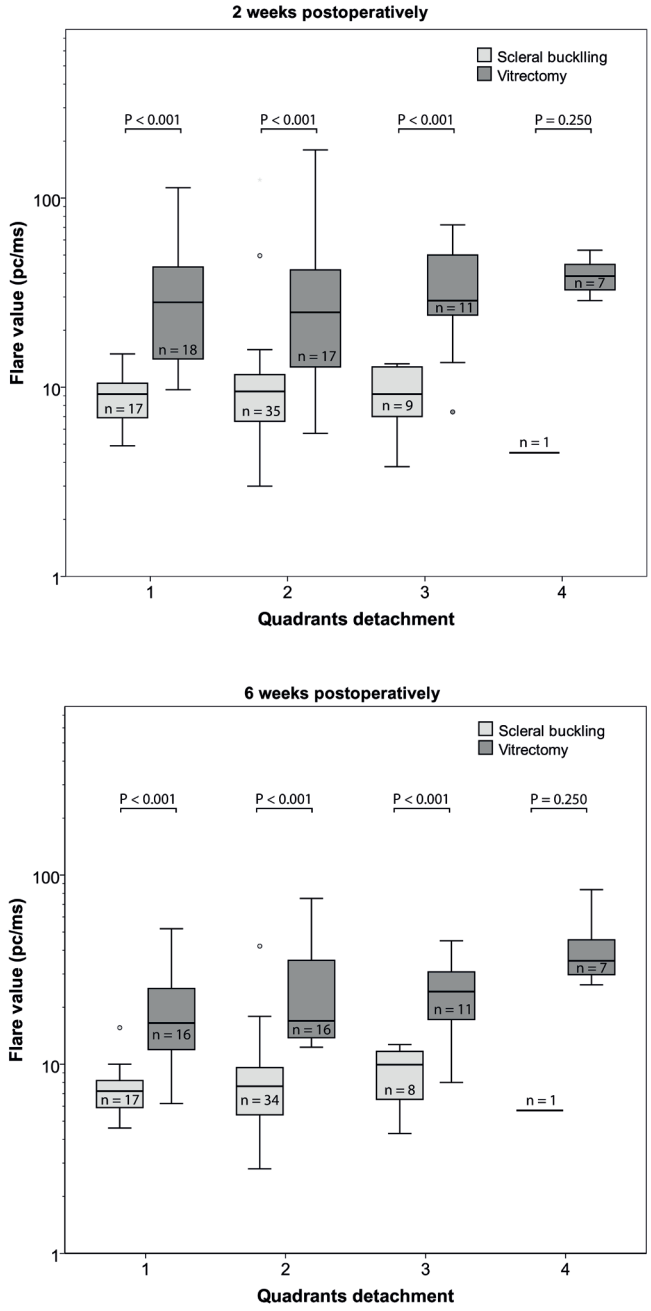

Figure 6.
Boxplot showing postoperative flare values at two **(A)** and six **(B)** weeks, split up by size of detachment. Values are shown as photoncount per millisecond (pc/ms) on a logarithmic scale. P-values refer to the independent samples Mann-Whitney U test.

DISCUSSION

Due to the small sample size, we chose to cluster all patients treated with PPV. Pseudophakic patients, however, often have higher flare values.[4] Therefore, the inclusion of patients with a combined CE surgery might have led to higher postoperative flare values in the PPV group. **Table 1** indeed shows that the median flare value after PPV for two-quadrants detachment might have been higher due to the inclusion of PPV with CE (no statistics performed). Overall, however, the median flare value after PPV is at least two times higher than after SB.

A second factor that might have contributed to higher flare values in the PPV group is age. SB is usually preferred in the younger population and flare is described to increase with age.[8] Although the SB group was somewhat younger than the PPV group (mean 54±12 vs 59±9 years; P = 0.01), this small effect cannot explain the large difference found between SB and PPV. Moreover, 82% of patients in the SB group received cryotherapy, which is known to increase flare.[7]

In conclusion, these results imply a more serious inflammatory reaction and a larger breakdown of the ocular barriers after PPV, which might contribute to better functional results after SB and a higher risk of PVR after PPV.

Table 1. Median postoperative flare values for different surgical procedures split up by preoperative quadrants detachment.

Number of quadrants	Surgical procedure	2 weeks		6 weeks	
		N	Median	N	Median
1	Scleral Buckling	17	9.2	16	6.9
	Vitrectomy	13	27.8	11	14.8
	Vitrectomy + CE	5	29.4	5	21.2
2	Scleral Buckling	35	9.5	34	7.7
	Vitrectomy	12	20.0	12	14.6
	Vitrectomy + CE	5	41.7	3	37.0
3	Scleral Buckling	9	9.2	8	10.0
	Vitrectomy	8	34.8	8	23.6
	Vitrectomy + CE	3	26.9	3	24.2
4	Scleral Buckling	1	4.5	1	5.7
	Vitrectomy	2	47.6	2	58.0
	Vitrectomy + CE	5	35.3	5	35.2

CE = cataract extraction

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In preparation

Medication use in patients with proliferative vitreoretinopathy; an alternative approach for identifying risk factors

4

ABSTRACT

BACKGROUND/AIMS

This study explored whether medication use around the time of surgery for primary rhegmatogenous retinal detachment (RRD) differs between patients who developed postoperative proliferative vitreoretinopathy (PVR) and those who did not. Special emphasis was put on antithrombotic agents, cholesterol lowering agents and ace inhibitors.

METHODS

Patients who had been operated for a retinal detachment in the Rotterdam Eye Hospital between 2006 and 2013 were included. Based on the detailed information in the surgery database patients were categorized as having uncomplicated RRD, postoperative PVR and primary PVR. Medication records were obtained from the health insurance company or the patients' pharmacy. The percentage of users of specific agents in the three groups were compared using a Chi-square test. After controlling for age, gender and first surgery the risk of developing PR was not associated with use of these drug classes.

RESULTS

We obtained medication information for 180 patients with postoperative PVR and 812 patients with uncomplicated RRD. We found no differences in the percentage of users of antithrombotic agents, cholesterol lowering agents or ace inhibitors. The logistic regression revealed no change in risk of developing postoperative PVR when using these drug groups.

CONCLUSION

In conclusion, our alternative approach researching systemic drug use in patients with RRD did not confirm nor identify new risk factors for the development of postoperative PVR. Systemic drug use or underlying conditions, nevertheless, deserve more attention in future research into the development of PVR.

INTRODUCTION

Proliferative vitreoretinopathy (PVR) is a complication that occurs in 5-10% of patients with a rhegmatogenous retinal detachment (RRD). It is characterized by the formation of epiretinal and/or subretinal contracting membranes in an pro-inflammatory environment leading to recurrent retinal detachments with a bad prognosis in terms of regaining vision.[1] Why some patients develop PVR and others do not is still not completely understood despite research on cytokine biomarkers, genetic profile and preoperative ocular clinical risk factors.

An aspect that has gained less attention is the possible influence of concomitant systemic drug use. The prevalence of RRD increases with age with a mean age around 60 years.[2, 3] In this age group systemic drug use is not uncommon. The use of systemic drugs that are known to affect inflammation or fibrosis could potentially have a protective or stimulating effect on the course and the occurrence of PVR.

Three drug groups that are of interest because of their mode of action and frequent use in the Dutch insured population over the age of 25 are the antithrombotic agents (13.7% in 2010[4]), cholesterol lowering agents(13.7% in 2010[4]) and ACE inhibitors (9% in 2010[4]). Recent studies researching the cause of PVR showed that the coagulation factors thrombin and factor Xa might play a role in the development of PVR.[5-7] Therefore, drugs interfering with coagulation might influence the course and the occurrence of PVR.

The cholesterol-lowering drug simvastatin has been shown to prevent PVR in a rabbit model.[8] Statins are being used to reduce endogenous cholesterol synthesis by inhibiting 3-hydroxy-3-methyl-glutaryl (HMG)-CoA reductase. This enzyme blocks the mevalonate pathway which is involved in cholesterol synthesis but it also activates the Rho/Rho-kinase pathway. Rho-kinase plays an important role in smooth muscle contraction and stress fibre formation in nonmuscle cells, a process important in the pathogenesis of PVR.[8, 9]

Angiotensin II, an important peptide in the renin-angiotensin system (RAS) which has long been known for its renal and cardiovascular effects, has now been recognised to both act independently and in synergy with TGF-beta to induce fibrosis in several organ systems.[10-12] Angiotensin II has proinflammatory, profibrotic and vasoconstrictive properties, it regulates cell growth and increases the synthesis of extracellular matrix proteins, such as fibronectin, laminin, and collagens.[11, 13] It was shown that the eye expresses many components of RAS including ACE and the Angiotensin II type 1 receptor.[13] The Angiotensin Converting Enzyme (ACE) inhibitors prevent the formation of angiotensin II and thus potentially interfere with the development of several proliferative eye diseases including PVR.

This retrospective cohort study explored whether there had been a difference—in terms of medication use around the time of primary RRD surgery—between patients who developed postoperative PVR and those who did not. Special emphasis was put on the before mentioned three medication groups.

METHODS

POPULATION

For this study, we used the surgery database of the Rotterdam Eye Hospital, The Netherlands. This database contains prospectively recorded information on consecutive vitreoretinal surgeries since 2003 and is built by means of a standardized surgical report form documenting specific surgical steps such as epiretinal membrane removal, subretinal membrane removal, and retinotomy in clock hours. We sorted the database by patient number, right or left eye and operation date and selected patients with indication codes for any type of retinal detachment surgery. Patients were excluded if their first retinal surgery had been before 2006, was not for RRD, or had been due to trauma or other underlying diseases. The remaining patients were categorized into three groups: Uncomplicated RRD, Postoperative PVR, and primary PVR. Uncomplicated RRD, our control group, was defined as a single intervention or follow-up surgery without entries indicating PVR. Entries indicating PVR were the removal of epiretinal and/or subretinal membranes, the need for retinotomy, or mentioning in the database of PVR grade C or higher (Retina Society Classification 1983[14]) in eyes with RRD.

Postoperative PVR was defined as one intervention for RRD without signs of PVR followed by at least one surgery for a retinal detachment with PVR related entries. Primary PVR was defined as the first surgery for RRD with PVR related entries. Follow-up was at least 6 months. When during this period two eyes underwent surgery for RRD only the first eye was included.

PRESCRIPTION DRUG INFORMATION

We obtained information about medication use in the period around the first surgery in two ways. For a large number of patients we retrieved the prescription information from the health insurance database of the Achmea group, which is one of the main health insurance companies in the Netherlands.[15] We requested an anonymized dataset from Achmea containing all patients operated at the Rotterdam Eye Hospital for a retinal detachment between 2006 and 2013 (code 654). The anonymization procedure was done by a Third Trusted Party (Zorg TTP, Houten, The Netherlands). To be able to match the health insurance dataset (HID) to the eye hospital dataset, they constructed two pseudonyms. The first was based on the national security number and the second was based on the date of birth, gender and postal code. The same anonymization procedure was performed for the eye hospital dataset. Subsequently, we linked the two datasets using Excel (Microsoft Office 2010) and deleted excess data. In the absence of the social security number pseudonym, the second pseudonym could be used.

The group of patients that was not insured by Achmea (n=239) was contacted directly to obtain their informed consent to request the dispensing information from their pharmacy. Patients were first contacted via phone and subsequently received the patient information and informed consent form via mail. We requested them to send the form with their decision back to us. If informed consent was obtained, we contacted their pharmacy to request the information for the time window around their first surgery.

We were mainly interested in three drug classes based on the anatomical therapeutic chemical classification (ATC classification[16]): antithrombotic agents (ATC B01A), cholesterol lowering agents (ATC C10A) and ACE-inhibitors (ATC C09A). For these groups, we recorded if patients used these drugs at any time point within 9 months surrounding their first surgery for RRD. In addition, we recorded whether the medication was used in the 3 months prior to surgery and/or during the 6 months after surgery, and which specific drug was used. For drugs not belonging to the three specified ATC groups only their use somewhere during this timeframe was recorded on a group level (4th ATC level). Combined preparations containing cholesterol lowering drugs or ACE-inhibitors were recorded under the ATC code of the single components.

SAMPLE SIZE

For the sample size we were limited to the number of patients with PVR available in our database (approximately 200) and we aimed for a case to control ratio of 1 to 5. When enough information of more than 1000 suitable controls would be available we would have randomly selected approximately 1000 controls from the same database accordingly. Final selection yielded less than 1000 controls. Patients with primary PVR were not included in the sample size calculation.

ANALYSIS

Age was compared between groups using a one-way ANOVA. We compared categorical variables including the percentage of users between groups using a Chi-square test. The median number of additional drugs was compared using a Kruskal-Wallis test. These tests were performed for two different time periods. Logistic regression was used to determine whether the use of the three specified drug groups (categorical variables) was associated with the risk of developing postoperative PVR, accounting for age, gender and time period of first surgery (before or after 2010). In order to have the variables in the same scale, age was transformed to a Z-score=(age-mean(age)/SD(age)), where SD is the standard deviation of our sample. Interaction effects between the three drug groups and age and gender were also included. Statistical analyses were performed with IBM SPSS statistics version 21 (IBM Corp., Armonk, NY, USA). No correction was performed for multiple testing and we assumed significance at $p < 0.05$.

RESULTS

From the total 5363 patients in the hospital database, we were able to obtain prescription information via the health insurance database from 923 patients. The response rate in the group of patients who were contacted directly (non-HID group) was 79%. Twenty-six of them (13%) refrained from participation (see **Figure 1**). For 4162 patients in our hospital database, we were unable to find a record in the HID because they were not insured by the Achmea group.

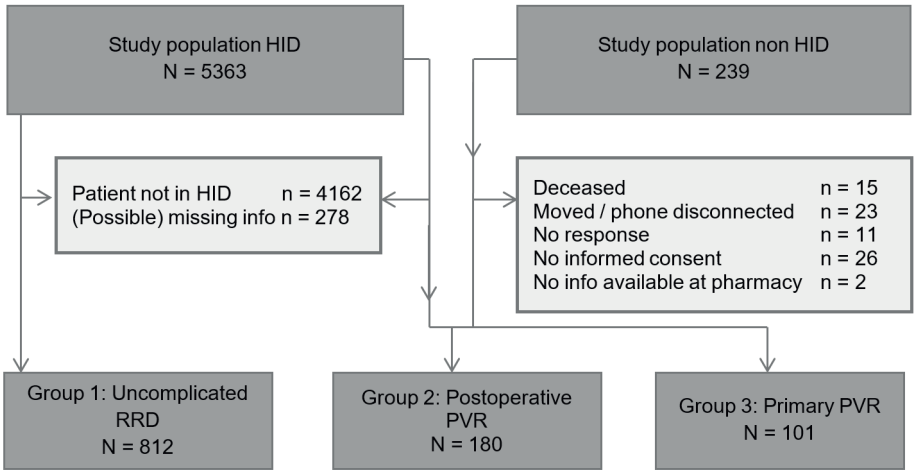

Figure 1. Patient Flow Scheme
HID: Health insurance database, PVR: Proliferative vitreoretinopathy, RRD: rhegmatogenous retinal detachment.

In addition, for 196 patients who had their surgery in the first three months of a year (n = 63) or the last 5 months of a year (n = 133), there were no entries in the HID database for the preceding or following year, respectively. It was impossible to distinguish between patients who had switched from health insurance company and patients who had not used health care in that year, therefore we excluded these patients as well (see **Figure 1**). For 82 patients there were no entries in the HID database for the period around the surgery.

As the study period was 8 years (2006-2013) we explored the patient characteristics in two time periods (before 2010 and after 2010) to check for time-dependent irregularities and found significant differences. Also the distribution of patients over the two time periods differed between groups. Therefore, the dataset was split up for further analyses into patients that had been operated before 2010 and after 2010. **Table 1** shows an overview of the patient characteristics and the percentage of patients that used the specified drug class in the 9-month period around the first surgery. In both time periods, the mean age differed significantly between the three groups (see **Table 1**). The percentage of males differed only after 2010 significantly between the three groups. In general, the percentage of patients that used antithrombotic agents, cholesterol lowering agents and/or ace-inhibitors did not differ between groups (see **Table 1**). The most frequently used antithrombotic and cholesterol lowering agents were the platelet aggregation inhibitors and statins respectively.
The number of additional different drug classes (ATC4th level) that were in use during the 9-month period around the first RRD surgery, excluding opthalmologicals, did not differ between groups (see **Table 1**).

Table 1. Patient characteristics divided by two time periods, primary surgery <2010 and >2010

	Controls (n=812)		Postoperative PVR (n=180)		Primary PVR (n = 101)		P value χ^2	
	<2010	**>2010**	**<2010**	**>2010**	**<2010**	**>2010**	**<2010**	**>2010**
Age (years)								
Mean ±SD	62±12	61±11	61±10	66±11	67±12	68±11	0.02[a]*	<0.001[a]**
Gender								
Male *(%)*	61	65	64	55	68	45	0.651	0.005*
Time period primary surgery *(%)*	45	55	54	46	35	65	0.007*	
Antithrombotic Agents *(%)*	19.0	19.9	19.6	30.1	29.4	25.0	0.342	0.094
Vitamin K antagonists	6.0	5.1	3.1	7.2	0	4.7		
Heparins	1.4	3.3	2.1	2.4	0	1.6		
Platelet aggregation inhibitors	13.5	15.8	16.5	22.9	29.4	20.3		
Thrombin inhibitors	0	0	0	1.2	0	0		
Cholesterol-lowering agents *(%)*	19.5	25.4	21.6	36.1	23.5	35.9	0.791	0.046*
Statins	19.2	25.2	21.6	34.9	23.5	35.9		
Fibrates	0.8	0.7	1.0	0	0	0		
Other	1.4	2.0	1.0	2.4	2.9	1.6		
ACE-inhibitors *(%)*	14.0	15.8	10.3	19.3	20.6	17.2	0.312	0.732
Number of drugs[b]								
Median	3	4	3	4	3	4	0.284[c]	0.835[c]

SD= standard deviation, [a] = ANOVA instead of χ^2, [b] = ATC4[th] level, excluding ophthalmologicals, [c] = Kruskal-Wallis test. Drugs are presented as percentage of users. * = $p < 0.05$, ** = $p < 0.01$

Exposure to the three specified drug groups and the risk of developing postoperative PVR
We performed a logistic regression analysis to see whether the use of the specified drug groups influenced the development of postoperative PVR. In the first analysis we included the use of antithrombotic agents, cholesterol lowering agents and ace-inhibitors somewhere in the 9-month period around the surgery. None of the three groups showed a significant effect on the odds ratio (OR) (see **Table 2**). However, the effect of antithrombotics on the risk of developing postoperative PVR might be different in different age groups as shown by the significant interaction effect. Having primary surgery after 2010 significantly reduced the risk of developing PVR.

To see whether there was a difference between using these drugs before primary surgery or after on the risk of developing PVR we performed a second logistic regression analysis. In this analysis, the use of these drugs during the 9-month period around primary surgery was split up in the 3-month period before surgery and 6-months after surgery (see **Table 3**).

Table 2. Association between use of drugs in 9 months around surgery and risk of postoperative PVR.

Variables	P	Odds Ratio	95% confidence interval
Time period (before/after 2010)	0.012*	0.65	0.47-0.91
Age (Z-transformed)	0.145	1.16	0.95-1.43
Gender	0.517	0.87	0.58-1.32
Ace inhibitors	0.211	0.56	0.23-1.39
Ace inhibitors by age	0.326	1.33	0.75-2.37
Ace inhibitors by gender	0.489	1.44	0.51-4.05
Antithrombotics	0.071	2.19	0.94-5.13
Antithrombotics by age	0.013*	0.51	0.30-0.87
Antithrombotics by gender	0.317	0.61	0.24-1.60
Cholesterol lowering	0.756	0.88	0.40-1.96
Cholesterol lowering by age	0.091	1.58	0.93-2.68
Cholesterol lowering by gender	0.536	1.33	0.54-3.31

* = $p < 0.05$

Table 3. Association between use of drugs before and after surgery and risk of postoperative PVR

Variables	P	Odds Ratio	95% confidence interval
Time period (before/after 2010)	0.010*	0.64	0.46 - 0.90
Age (Z-transformed)	0.154	1.16	0.95 - 1.42
Gender	0.571	0.89	0.59 - 1.34
Ace inhibitors	0.282	0.60	0.24 - 1.51
Ace inhibitors by age	0.414	1.28	0.71 - 2.29
Ace inhibitors by gender	0.634	1.29	0.45 - 3.67
Antithrombotics 3 months before	0.772	1.29	0.24 - 7.05
Antithrombotics 3 months before by age	0.579	0.69	0.19 - 2.52
Antithrombotics 3 months before by gender	0.887	0.86	0.11 - 6.63
Antithrombotics 6 months after	0.581	1.61	0.30 - 8.73
Antithrombotics 6 months after by age	0.701	0.78	0.22 - 2.77
Antithrombotics 6 months after by gender	0.796	0.77	0.10 - 5.70
Cholesterol lowering 3 months before	0.214	4.01	0.45 - 35.79
Cholesterol lowering 3 months before by age	0.059	0.20	0.04 - 1.07
Cholesterol lowering 3 months before by gender	0.190	0.20	0.02 - 2.24
Cholesterol lowering 6 months after	0.216	0.25	0.03 - 2.24
Cholesterol lowering 6 months after by age	0.022*	7.18	1.34 - 38.58
Cholesterol lowering 6 months after by gender	0.153	5.70	0.52 - 62.11

* = $p < 0.05$

For the ace-inhibitors, the correlation between the use before and after surgery was too high (99% identical) and therefore these variables were left out of the analysis and were replaced by use of ace-inhibitors anytime in the 9-month period. Again, surgery after 2010 had a reduced risk of developing postoperative PVR. Use before or after did not significantly influence the risk, only the effect of using cholesterol lowering drugs in the 6-month period after surgery might be different in different age groups.

OTHER DRUGS IN USE AROUND THE TIME OF SURGERY

Drugs other than the three specified groups in use anytime during the 9-months period around primary surgery were scored as well. The low percentage of users however in combination with the relatively small sample size made it unfeasible to compare them or include them in the logistic regression. The numbers and percentages are available in the supplemental tables.

DISCUSSION

The results of this study suggest that there are no significant differences in the percentage of users of antithrombotic agents, cholesterol lowering agents and ace-inhibitors around the time of surgery for RRD between patients who will develop PVR and patients who do not (table 1). Neither did these drug groups significantly influence the risk of developing PVR as suggested by the logistic regression analysis (table 2). However, there seemed to be a significant influence of the time period – before or after 2010 – a patient was operated in.

An advantage of using the health insurance database (HID) was access to information of a large group of patients for whom informed consent was no longer necessary, as the anonymization procedure prevents traceability to a specific patient. This led to less exclusion due to failure of obtaining informed consent as a result of a change of address or passing away. Despite this potential advantage, due to several factors which decreased the number of patient with useful information, groups remained still too small to further explore the subclasses in our main drug groups and the remaining drug classes.

Firstly, we had to exclude a large group of patients because we were unsure whether the HID contained all the drug information we needed. In the Netherlands, everyone is obliged to have basic health insurance. In 2006 the healthcare system was reformed to stimulate competition between health insurers: each person could now choose their own health insurer and switch to another insurance company each new calendar year. The year of introduction approximately 26% switched, the following years approximately 4%.[17, 18] When we had no information on the previous or following year we could not distinguish between patients who had not used any care and patients who had switched from health insurer, we had to exclude such patients.

Secondly, there was a significant difference in the patient characteristics between the time period before and after 2010 and we therefore decided to split the user statistics into two time periods and add the factor time period to the logistic regression. The time period was

a significant factor in the logistic regression analysis, which implied a reduced risk of developing PVR after 2010.

The latter seems unlikely to be a true difference and there are several potential confounding factors. Firstly, it is unsure whether the distribution of controls over the two time periods is real or a result of the unbalanced exclusion of patients with possible missing data. More patients switching from health insurance in the first years after introduction, as described above, might have led to fewer patients in the control group <2010 (table 1). For this reason, we considered using a matched approach including age, gender and year of surgery. However, this yielded only 113 matches and we decided to use all the data.

Secondly, the 2006 introduction and later revision of the cardiovascular risk management guideline might have contributed to differences over the years. This guideline provides a structured risk assessment which standardizes the start of drugs such as platelet aggregation inhibitors, cholesterol lowering drugs (mainly statins), and antihypertensives. This guideline was revised in 2011 recommending a lower threshold for prescribing statins[19], which might have contributed to the difference in the number of users of these specific drugs before and after 2010 in our sample.

These differences before and after 2010, however, seem more pronounced in the group of patients who would develop PVR than in controls. Possibly, patients who developed PVR generally have a worse overall health and more cardiovascular risk factors leading to an altered wound healing response in the eye. This was however not evident from the number of drugs in use and not true for patients with established PVR. Unfortunately, there was no clinical information available about underlying conditions and risk factors. It would be of interest to study whether there are differences in the prevalence of PVR in patients with certain diseases such as for example diabetes, metabolic syndrome or rheumatism.

Lastly, we used two different information routes namely the HID and pharmacy records. While we set out to use only the HID, most of our PVR patients (the relatively rare category we are primarily interested in) were not insured with this insurance company and we thus gathered their information by contacting their pharmacy. However, patients in the Netherlands are generally registered at one specific pharmacy which keeps their complete drug record. Therefore, we only expected a possibly higher number of users of non-prescription drugs such as ibuprofen, some antihistamines and cough medications in this group.

In conclusion, this study set out to find differences in medication use between patients that did and did not develop PVR in the hope to find leads for new drug research and to gain insight into additional pathophysiological risk factors. We focused on three major drug classes for which there had been some evidence. Our alternative approach researching systemic drug use in patients with RRD did not identify a difference in medication use between patients who developed PVR and patients who did not develop PVR. Systemic drug use or underlying conditions should, nevertheless, deserve more attention in future research on the development of PVR.

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Supplemental Table 1. Overview of all drugs in use in the 9 month period around primary surgery. Time period <2010. ATC groups with less or equal than 5 users in all three groups were not reported.

ATC code		Name	Controls (n=364)		Postoperative PVR (n=97)		Primary PVR (n = 34)	
			n	%	n	%	n	%
Alimentary tract and metabolism								
A02	B	Drugs for peptic ulcers and gastro-esophageal reflux disease	84	23.1	13	13.4	8	23.5
A06	A	Drugs for constipation	43	11.8	7	7.2	7	20.6
A10	*Drugs used in diabetes*							
	A	Insulins and analogues	9	2.5	3	3.1	2	5.9
	B	Blood glucose lowering drugs	33	9.1	12	12.4	6	17.6
A12		Mineral supplements	30	8.2	16	16.5	3	8.8
Blood and blood forming organs								
B03		Antianemic preparations	15	4.1	2	2.1	3	8.8
Cardiovascular system								
C01	D	Vasodilators used in cardiac diseases	22	6.0	1	1.0	5	14.7
C03	A	Low ceiling diuretics, Thiazides	35	9.6	6	6.2	3	8.8
	C	High ceiling diuretics (Sulfonamides)	15	4.1	3	3.1	0	0
	E	Diuretics and potassium-sparing agents in combination	8	2.2	3	3.1	2	5.9
C07	A	Beta Blocking agents	66	18.1	11	11.3	10	29.4
C08	C	Selective calcium channel blockers with mainly vascular effects	38	10.4	8	8.2	4	11.8
	D	Selective calcium channel blockers with direct cardiac effects	11	3.0	5	5.2	0	0
C09	C/D	Angiotensin II antagonists or combinations	54	14.8	7	7.2	8	23.5
Dermatologicals								
D01	A	Antifungals for topical use	8	2.2	2	2.1	1	2.9
D02	A	Emollients and protectives	15	4.1	1	1.0	1	2.9
D06	B	Chemotherapeutics for topical use	16	4.4	4	4.1	3	8.8
D07	A	Corticosteroids	55	15.1	13	13.4	5	14.7
	X	Corticosteroids combinations	14	3.8	5	5.2	1	2.9

Supplemental Table 1. *Continued.*

ATC code		Name	Controls (n=364)		Postoperative PVR (n=97)		Primary PVR (n = 34)	
			n	%	n	%	n	%
Genito urinary system and sex hormones								
G03		Sex hormones and modulators of the genital system	24	6.6	2	2.1	0	0
G04	B	Urologicals	8	2.2	3	3.1	0	0
	C	Drugs used in benign prostatic hypertrophy	30	8.2	6	6.2	1	2.9
Systemic hormonal preparations								
H02	A	Corticosteroids for systemic use	17	4.7	5	5.2	3	8.8
H03	A	Thyroid preparations	16	4.4	1	1.0	0	0
Anti-infectives for systemic use								
J01	A	Tetracyclines	37	10.2	4	4.1	1	2.9
	C	Beta-lactam antibacterials, penicillins	49	13.5	10	10.3	5	14.7
	E	Sulfonamides and trimethoprim	11	3.0	1	1.0	1	2.9
	F	Macrolides	16	4.4	0	0	0	0
	M	Fluoroquinolones	21	5.8	3	3.1	1	2.9
	X	Nitrofuran derivatives	18	4.9	5	5.2	0	0
Musculoskeletal system								
M01	A	Anti-inflammatory and antirheumatic products, non-steroids	98	26.9	22	22.7	8	23.5
M05	A/B	Bisphosphonates or combinations	13	3.6	1	1.0	1	2.9
Nervous system								
N02	A	Opioids	27	7.4	6	6.2	1	2.9
	B	Other analgesics	18	4.9	6	6.2	1	2.9
N03	A	Antiepileptics	10	2.7	2	2.1	0	0
N05	A	Antipsychotics	8	2.2	2	2.1	0	0
	B	Anxiolytics	36	9.9	16	16.5	2	5.9
	C	Hypnotics and sedatives	41	11.3	11	11.3	3	8.8
N06	A	Antidepressants	37	10.2	3	3.1	4	11.8
Respiratory system								
R01	A	Decongestives and other nasal preparations for topical use	32	8.8	9	9.3	2	5.9
R03		Drugs for obstructive airway diseases	69	19.0	16	16.5	7	20.6
R05		Cough and cold preparations	29	8.0	10	10.3	2	5.9
R06	A	Antihistamines for systemic use	25	6.9	12	12.4	3	8.8
Sensory organs								
S02		Otologicals	22	6.0	7	7.2	4	11.8

Supplemental Table 2. Overview of all drugs in use in the 9 month period around primary surgery. Time period > 2010. ATC groups with less or equal than 5 users in all three groups were not reported.

ATC code		Name	Controls (n=448)		Postoperative PVR (n=83)		Primary PVR (n = 64)	
			n	%	n	%	n	%
Alimentary tract and metabolism								
A02	B	Drugs for peptic ulcers and gastro-esophageal reflux disease	113	25.2	20	24.1	23	35.9
A03	*Drugs for functional gastrointestinal disorders*							
	A	Drugs for functional gastrointestinal disorders	6	1.3	2	2.4	0	0
	F	Propulsives	9	2.0	0	0	2	3.1
A06	A	Drugs for constipation	47	10.5	8	9.6	10	15.6
A10	*Drugs used in diabetes*							
	A	Insulins and analogues	21	4.7	6	7.2	3	4.7
	B	Blood glucose lowering drugs	54	12.1	5	6.0	15	23.4
A12		Mineral supplements	30	6.7	6	7.2	4	6.3
Blood and blood forming organs								
B03		Antianemic preparations	23	5.1	3	3.6	5	7.8
Cardiovascular system								
C01	D	Vasodilators used in cardiac diseases	20	4.5	6	7.2	5	7.8
C03	A	Low ceiling diuretics, Thiazides	46	10.3	7	8.4	5	7.8
	C	High ceiling diuretics (Sulfonamides)	22	4.9	3	3.6	6	9.4
	E	Diuretics and potassium-sparing agents in combination						
C07	A	Beta Blocking agents	86	19.2	14	16.9	15	23.4
C08	C	Selective calcium channel blockers with mainly vascular effects	54	12.1	8	9.6	8	12.5
	D	Selective calcium channel blockers with direct cardiac effects						
C09	C/D	Angiotensin II antagonists or combinations	26	5.8	4	4.8	3	4.7
Dermatologicals								
D01	A	Antifungals for topical use	13	2.9	4	4.8	1	1.6
D02	A	Emollients and protectives	32	7.1	1	1.2	3	4.7
D06	B	Chemotherapeutics for topical use	31	6.9	0	0	2	3.1
D07	A	Corticosteroids	76	17.0	10	12.0	12	18.8
	X	Corticosteroids combinations	24	5.4	0	0	2	3.1
Genito urinary system and sex hormones								
G03		Sex hormones and modulators of the genital system	23	5.1	6	7.2	2	3.1
G04	C	Drugs used in benign prostatic hypertrophy	30	6.7	5	6.0	2	3.1

Supplemental Table 2. *Continued.*

ATC code		Name	Controls (n=448)		Postoperative PVR (n=83)		Primary PVR (n = 64)	
			n	%	n	%	n	%
Systemic hormonal preparations								
H02	A	Corticosteroids for systemic use	33	7.4	4	4.8	3	4.7
H03	A	Thyroid preparations	15	3.3	5	6.0	3	4.7
Anti-infectives for systemic use								
J01	A	Tetracyclines	33	7.4	3	3.6	2	3.1
	C	Beta-lactam antibacterials, penicillins	77	17.2	13	15.7	15	23.4
	E	Sulfonamides and trimethoprim	10	2.2	4	4.8	5	7.8
	F	Macrolides	26	5.8	5	6.0	1	1.6
	M	Fluoroquinolones	16	3.6	6	7.2	0	0
	X	Nitrofuran derivatives	23	5.1	4	4.8	0	0
J07		Vaccines	8	1.8	5	6.0	3	4.7
Musculoskeletal system								
M01	A	Anti-inflammatory and antirheumatic products, non-steroids	149	33.3	12	14.5	22	34.4
M04	A	Antigout preparations	16	3.6	3	3.6	0	0
M05	A/B	Bisphosphonates or combinations	15	3.3	2	2.4	4	6.3
Nervous system								
N02	A	Opioids	46	10.3	11	13.3	12	18.8
	B	Other analgesics	28	6.3	4	4.8	1	1.6
N03	A	Antiepileptics	15	3.3	1	1.2	1	1.6
N04	B	Anti-Parkinson drugs (dopaminergic)	8	1.8	3	3.6	0	0
N05	A	Antipsychotics	13	2.9	3	3.6	2	3.1
	B	Anxiolytics	10	2.2	2	2.4	6	9.4
	C	Hypnotics and sedatives	10	2.2	5	6.0	7	10.9
N06	A	Antidepressants	48	10.7	5	6.0	7	10.9
Respiratory system								
R01	A	Decongestives and other nasal preparations for topical use	52	11.6	4	4.8	4	6.3
R03		Drugs for obstructive airway diseases	80	17.9	14	16.9	9	14.1
R05		Cough and cold preparations	33	7.4	3	3.6	5	7.8
R06	A	Antihistamines for systemic use	35	7.8	4	4.8	3	4.7
Sensory organs								
S02		Otologicals	17	3.8	4	4.8	4	6.3

Summary and conclusions

5

Because of the potential role of thrombin in the development of PVR we looked into drugs that would be able to inhibit thrombin. One such drug is the direct and reversible thrombin inhibitor dabigatran (Pradaxa®) which has been on the Dutch market since 2010. One of its uses is the prevention of thromboembolic events after knee or hip replacement surgery. It is an oral alternative to the subcutaneous injections with for example dalteparin (Fragmin®). An oral drug has the advantage of being more patient friendly with repeated administration. Disadvantages are the possibility of systemic side effects and the uncertainty whether it reaches its target side in the eye. The latter we investigated in **chapter 2.1**. Twenty-eight patients with RRD who needed surgery received a single administration of 220mg dabigatran two to eight hours prior to their surgery. During surgery, we collected vitreous or subretinal fluid and some blood. The concentrations of dabigatran were analysed using LC-MS/MS. The results showed that dabigatran does reach the eye but in much lower concentrations than in plasma. An unexpected finding was the concentration we measured in vitreous of a patient who was on daily dabigatran therapy. This concentration was approximately 10x higher than after a single administration. This finding piqued our interest on the question whether repeated administration would lead to higher intraocular levels. In **chapter 2.2** we describe three additional patients who used dabigatran on a daily basis but underwent vitreous surgery for different reasons than RRD. Also in these patients the concentration of dabigatran was significantly higher than we measured after a single administration.

However, is dabigatran able to inhibit the effects of thrombin and are these concentrations high enough? In **chapter 2.3** we exposed cultured retinal pigment epithelial cells (ARPE19) to thrombin in combination with dabigatran or without. We found that thrombin and vitreous fluids containing thrombin activity induced CCL2, CXCL8, GM-CSF, IL-6 and PDGF-BB expression by ARPE-19 cells, which was inhibited by dabigatran. In addition, we tested whether the vitreous fluids containing dabigatran from the previous studies were able to inhibit thrombin activity. This appeared to only be the case for the higher dabigatran concentration after repeated oral intake (12.9 ng/ml).

In **chapter 2.4** we tested the formation of thrombin in the form of F1+2 and elimination in the form of thrombin-antithrombin (TAT) complex in vitreous and subretinal fluid. We found that both F1+2 and TAT were significantly higher in patients with a retinal detachment compared to patients with a macular hole or macular pucker. However, between patients with and without PVR we could not detect a difference due to the large variation. Possibly, the balance between the different thrombin regulation mechanisms (TAT and α2M) and the overall health of the patient contribute to differences between patients and the risk of developing PVR. An additional aim of dabigatran treatment in the prevention of PVR could therefore be reducing α2M-thrombin formation by shifting the balance towards inactive TAT.

Taken together the results of chapter 2, dabigatran seems to be able to inhibit intraocular thrombin, reaches the eye in high enough concentrations and is an interesting potential new drug for the prevention of PVR that deserves more research.
However, considering the potential serious side effects that could occur with drugs such as dabigatran one would like to select patients with the highest risk of developing PVR and would thus benefit most from a drug therapy.

Chapter 3 describes the use of aqueous flare measurements as a predictor for the development of postoperative PVR. Flare is the phenomenon that results from the reflection of light on particles such as inflammatory proteins. It can be compared to the effect seen when a light beam shines across a dark smokey room. Laser flare is an objective measurement of inflammation. Previous research found that a high preoperative flare value – measured with a Kowa laser flare meter – was a strong predictor for the development of postoperative PVR. However, we could not confirm these results in a cohort from Rotterdam and Kiel (Germany), as is described in **chapter 3.1**. We combined data from two independent prospective studies: Kiel (120 patients) and Rotterdam (194 patients). PVR was defined as redetachment due to the formation of traction membranes that required re-operation within six months of initial surgery. Logistic regression and ROC analysis determined whether higher preoperative flare values were associated with an increased risk of postoperative PVR.

PVR redetachment developed in 21/314 patients (6.7%). Median flare values differed significantly between centres, therefore analyses were done separately. Logistic regression showed a small but statistically significant increase in odds with increasing flare only for Rotterdam (OR 1.014; p=0.005). Areas under the ROC showed low sensitivity and specificity: Kiel 0.634(95% CI: 0.440 – 0.829) and Rotterdam 0.731(95% CI: 0.598 – 0.865). In addition, using the previous reported cut-off point of 15 pc/ms and our current prevalence led to a positive predictive value of only 12%.
We concluded that preoperative laser flare measurements are inaccurate in discriminating between those patients with RRD at high and low risk of developing PVR.

Because also the surgery itself possibly contributes to inflammation and thus the flare value, we looked at the predictive value of postoperative flare values in **chapter 3.2**. In 195 patients with a primary RRD, flare was measured two and six weeks after surgery. The endpoint was reoperation for PVR within six months of initial surgery. The median flare values of patients who would later develop PVR were significantly higher than those of patients with an uncomplicated course both at two and six weeks after surgery (median 2wks: 43 vs. 15 pc/ms; p =0.001, median 6wks: 38 vs. 13 pc/ms; p < 0.001). In addition, both the logistic regression analysis and the ROC analysis showed a significant increased risk with a higher postoperative flare value.

The individual change of the flare value from preoperative to postoperative did not reveal a clear trend. For most patients with an uncomplicated course the flare value rose less than 10 pc/ms after surgery. In patients who would develop PVR the flare value rose in 50% of patients and declined in the other 50%. The direction of the change seems therefore less important than the actual flare value that remains after surgery. This was further substantiated in **chapter 3.3** as it becomes clear that a vitrectomy procedure induces a stronger inflammatory reaction than a scleral buckling procedure. This was independent of the size of the detachment.
In conclusion, postoperative flare values two weeks after RRD surgery are a reasonable surrogate marker for the development of postoperative PVR. Both postoperative measurements could be used by ophthalmologists to monitor inflammation and study treatment methods.

In **chapter 4** we investigated whether systemic drug use around the time of the RRD influenced the development of PVR. In this retrospective study, 1093 patients were included who had undergone surgery for RRD between 2006 and 2013 at the Rotterdam Eye Hospital. Based on the information in the surgery database patients were divided into three groups: uncomplicated course (control group), postoperative PVR and primary PVR. Information on their drug use was collected in two ways. For the largest group (mainly controls) the information was requested from the Achmea Health Database – one of the largest health insurance groups in the Netherlands – and through an anonymization procedure coupled to the Eye Hospital database. The remaining group of patients who were not insured by Achmea were contacted directly to obtain their permission to request their information from their local pharmacy. We were mainly interested in the use of antithrombotics, cholesterol lowering agents and ACE-inhibitors. The number of users of these drugs was significantly different in the time period before 2010 and after 2010. Therefore, the results were presented separately for each time period and this factor was included in the logistic regression analysis. We could not detect a significant difference in the number of users of these drugs between the different before mentioned patient groups. Neither did the logistic regression analysis reveal a significant influence of these drugs on the risk of developing postoperative PVR.

Samenvatting en conclusies (Nederlands)

6

Proliferatieve vitreoretinopathie (PVR) is een complicatie die in 5-10% van de patienten met netvliesloslating kan ontwikkelen. PVR kan gezien worden als een overdreven littekenreactie en wordt gekarakteriseerd door de vorming van membranen die over en onder het netvlies groeien, samentrekken en zo het netvlies opnieuw lostrekken. Het operatief verwijderen van deze membranen is gecompliceerd en niet altijd mogelijk. Het leidt in de meeste gevallen tot verlies van het zicht in dat oog. Het liefst zouden we dus voorkomen dat PVR ontwikkeld, echter is dit nog niet zo makkelijk als het lijkt.

Vanwege de potentiele rol van de stollingsfactor trombine in het ontstaan van PVR zijn we gaan kijken naar geneesmiddelen die trombine kunnen remmen. Een geneesmiddel dat specifiek trombine remt en sinds 2010 op de Nederlandse markt is is dabigatran (Pradaxa®). Dit middel wordt onder andere toegepast ter preventie van trombose bij vervanging van een heup of knie en is een oraal alternatief voor de subcutane injecties met bijvoorbeeld dalteparine (Fragmin®). Een oraal toepasbaar geneesmiddel heeft als voordeel dat het patientvriendelijker is voornamelijk bij herhaalde toediening. Nadelen zijn mogelijke systemische bijwerkingen en de vraag of het wel komt op de plek waar het moet werken. Dit laatste hebben we onderzocht in **hoofdstuk 2.1**. Achtentwintig patienten met netvliesloslating die een operatie moesten ondergaan kregen twee tot acht uur voor de operatie eenmalig 220mg dabigatran. Vervolgens werd er tijdens de operatie glasvocht of subretinale vloeistof afgenomen en ter referentie een buisje bloed. De concentratie dabigatran werd bepaald met behulp van LC-MS/MS. De resultaten lieten zien dat dabigatran wel degelijk in het oog terecht komt, maar wel in veel lagere concentraties dan in het bloed. Een onverwachtse bevinding was de concentratie die we vonden in glasvocht van een patient die dabigatran dagelijks als therapie gebruikte. Deze concentratie was ongeveer 10x hoger dan de concentratie na een eenmalige toediening. Dit maakte ons nieuwsgierig naar de concentratie na meermalige toediening. In **hoofdstuk 2.2** beschrijven we drie patienten die allemaal dagelijks dabigatran gebruikten, maar wel voor een andere indicatie geopereerd werden aan het glasvocht. Ook in deze patienten zagen we signficant hogere concentraties dan dat we zagen na een eenmalige toediening. Maar is dabigatran in staat om de effecten van trombine te remmen en zijn de concentraties die we kunnen behalen met orale toediening ook voldoende? In **hoofdstuk 2.3** hebben we dit onderzocht door gekweekte retinaal pigment epitheelcellen (ARPE19) bloot te stellen aan trombine met en zonder dabigatran. We vonden dat trombine en trombine-bevattend glasvocht expressie van de proinflammatoire cytokinen CCL2, CXCL8, GM-CSF, IL-6 en PDGF-BB induceerde wat geremd werd door dabigatran. Daarnaast hebben we gekeken of het glasvocht met dabigatran uit de voorgaande studies de trombine activiteit kon remmen. Dit bleek alleen te lukken met de hogere concentratie verkregen na herhaalde toediening (12.9 ng/ml).

In **hoodstuk 2.4** hebben we gekeken naar de vorming van trombine door het meten van F1+2 en de opruiming van trombine door te kijken naar de vorming van trombine-antitrombine complex (TAT) in glasvochten en subretinale vloeistof. We zagen dat zowel F1+2 en TAT sterk verhoogd waren in patienten met een netvliesloslating ten op zichte

van patienten met maculagat of maculapucker. Tussen patienten met en zonder PVR konden we door de grote spreiding geen verschil aantonen. Mogelijk spelen het evenwicht tussen verschillende regulatiemechanismen van trombine (TAT en α2M) en de algehele gezondheid van de patiënt een rol in het risico op het wel of niet ontwikkelen van PVR. Een aanvullend mechanisme waarmee dabigatran mogelijk kan bijdragen aan het voorkomen van PVR is het verminderen van de vorming van α2M gebonden trombine, door het verplaatsen van het evenwicht naar de vorming van inactief TAT.
De resultaten van hoofdstuk 2 samengevat lijkt dabigatran de potentie te hebben om intraoculair trombine te remmen en is interessant genoeg om verder te onderzoeken als potentieel middel ter voorkoming van PVR. Echter gezien de mogelijk ernstige bijwerkingen die kunnen optreden bij het gebruik van middelen zoals dabigatran is het gewenst die patienten te selecteren die het hoogste risico lopen op het ontwikkelen van PVR en dus ook het meeste baat zouden hebben bij een geneesmiddeltherapie.

Hoofdstuk 3 beschrijft de toepassing van het meten van "flare" in de voorste oogkamer als voorspeller van postoperatief PVR. Flare is een fenomeen dat ontstaat door reflectie van licht op deeltjes, zoals ontstekingseiwitten, en kan vergeleken worden met een lichtstraal in een donkere rokerige kamer waardoor deeltjes zichtbaar worden. Het is een objectieve maat voor inflammatie. Eerder onderzoek vond dat een hoge flare waarde, preoperatief gemeten met behulp van een Kowa laser flare meter, voorspellend was voor het postoperatief ontwikkelen van PVR. Herhaling van deze studie in een cohort uit Rotterdam en een cohort uit Kiel (Duitsland), beschreven in **hoofdstuk 3.1**, kon deze voorspellende waarde echter niet bevestigen. We combineerden de data van twee onafhankelijke prospectieve studies: Kiel (120 patienten) en Rotterdam (194 patienten). PVR werd gedefinieerd als een nieuwe netvliesloslating ten gevolge van tractiemembranen binnen zes maanden na de eerste loslating, waarvoor heroperatie noodzakelijk was. Met behulp van logistische regressie en ROC-analyse bekeken we of een hogere preoperatieve flare waarde geassocieerd was met een hogere kans op het ontwikkelen van PVR.
PVR ontwikkelde in 21/314 patienten (6.7%). De mediane flare waarden verschilde signficant tussen de twee centra waarop besloten is de analyses voor elk centrum apart uit te voeren. De logistische regressieanalyse liet alleen voor Rotterdam een klein maar signficante verhoging van het risico zien bij een hogere flare waarde (OR 1.014; p=.005). De oppervlakte onder de ROC-grafiek liet een lage sensitiviteit en specificiteit zien: Kiel 0.634(95% CI: 0.440 – 0.829) en Rotterdam 0.731(95% CI: 0.598 – 0.865). Daarnaast gaf de gerapporteerde afkapwaarde van 15 pc/ms uit de eerdere studie in combinatie met de lage prevalentie in onze studie slechts een positief voorspellende waarde van 12%. Op basis hiervan concluderen we dat preoperatief gemeten flare niet nauwkeurig genoeg is om onderscheid te kunnen maken tussen patienten met een laag en een hoog risico op het ontwikkelen van postoperatieve PVR.

Aangezien de operatie voor de netvliesloslating zelf mogelijk ook bijdraagt aan inflammatie en dus de hoogte van de flare waarde, is in **hoofdstuk 3.2** gekeken of de postoperatieve

flare waarde een voorspeller of surrogaatmarker was voor het ontwikkelen van PVR. In 195 patiënten met een primaire rhegmatogene netvliesloslating is flare gemeten twee en zes weken na de operatie. Hierbij vonden we dat de mediane flarewaarde in de patiënten die postoperatief PVR zouden gaan ontwikkelen, zowel op twee als zes weken na de operatie, significant hoger was dan bij patiënten met een ongecompliceerd beloop (mediaan 2wkn: 43 vs. 15 pc/ms; p =0.001, mediaan 6wkn: 38 vs. 13 pc/ms; p < 0.001). Ook de logistische regressie en de ROC-analyse lieten een voorspellende waarde zien van de flaremeting.

De individuele verandering van de flarewaarde na de operatie ten opzichte van de flarewaarde voor de operatie liet geen duidelijke trend zien. Voor de meeste patiënten met een ongecompliceerd beloop steeg de flarewaarde na de operatie minder dan 10 pc/ms. Van de patiënten die later PVR zouden ontwikkelen ging in de helft van de patiënten de flare waarde omhoog en in de andere helft de flare waarde omlaag. De richting van de verandering lijkt dus minder van invloed dan de absolute flarewaarde die overblijft na de operatie. De operatie zelf lijkt hier dus ook van invloed te zijn op de uitkomst. Dit wordt verder bevestigd in **hoofdstuk 3.3** waarin duidelijk wordt dat een vitrectomie procedure een hevigere postoperatieve inflammatie tot gevolg heeft dan een cerclage/plombe procedure, onafhankelijk van de grootte van de netvliesloslating.
In conclusie, de postoperatieve flarewaarde van twee weken na de operatie is een redelijke surrogaatmarker voor de ontwikkeling van postoperatieve PVR. Beide metingen, los of in combinatie zouden gebruikt kunnen worden om inflammatie te monitoren en mogelijk het effect van nieuwe therapieën te bestuderen.

In **hoofdstuk 4** hebben we onderzocht of systemisch geneesmiddelgebruik rondom het ontstaan van een netvliesloslating mogelijk van invloed zou kunnen zijn op het wel of niet ontstaan van PVR. In dit retrospectieve onderzoek zijn 1093 patiënten geïncludeerd die tussen 2006 en 2013 geopereerd zijn in het Oogziekenhuis Rotterdam voor een primaire netvliesloslating. Op basis van gegevens uit de operatiedatabase zijn deze patiënten opgedeeld in drie groepen, te weten ongecompliceerd beloop (controlegroep), postoperatief PVR en primair PVR. De geneesmiddelgegevens van deze patiënten zijn vervolgens op twee manieren verzameld. Voor de grootste groep (voornamelijk controles) zijn de gegevens opgevraagd via de Achmea Health Database – een van de grootste zorgverzekeraars in Nederland – en via een pseudonimisatie procedure gekoppeld aan het Oogziekenhuis bestand. De resterende groep patiënten, die niet bij Achmea verzekerd was, is persoonlijk benaderd en gevraagd om toestemming voor het opvragen van hun gegevens bij hun lokale apotheek. We hebben vooral gekeken naar het gebruik van antitrombotica, cholesterolverlagende middelen en ACE-remmers. De resultaten lieten een significant verschil zien tussen het aantal gebruikers van deze geneesmiddelgroepen in de periode voor 2010 en de periode na 2010. De gebruikscijfers zijn daarom uitgesplitst naar tijdvak en het tijdvak is meegenomen als factor in de logistische regressie analyse. We konden echter geen significant verschil aantonen tussen bovengenoemde patiëntgroepen in het

aantal gebruikers van antitrombotica, cholesterolverlagende middelen en ACE-remmers. Ook liet de logistische regressie analyse geen significante invloed zien van deze geneesmiddelgroepen op het ontwikkelen van postoperatieve PVR.

General discussion and future perspectives

7

The development of proliferative vitreoretinopathy is still the major cause of failure of retinal reattachment surgery. While initially there is little difference between patients with uncomplicated RD and patients who later develop PVR, in some patients, something triggers the derangement of the healing process. PVR could be considered an exaggerated response of the active tissue remodelling process triggered by the injured retina. Despite great effort, no relevant advances in clinical management have been made in the last 40 years.[1]
We wished to study a potential new treatment; as any treatment has side-effects, we also wished to find a way to identify only patients at risk.
Recently, coagulation proteins were implicated in the development of PVR.[2] Activation of the coagulation cascade plays a central role in the tissue healing process but dysregulation has been recognised to contribute to fibrosis in lung, liver and kidney.[3-5] Interference with the coagulation cascade, with a long history of drugs in clinical use, would therefore, appear to be a good therapeutic target.

In **chapter 2** we tested whether the oral direct thrombin inhibitor dabigatran would be a potential drug candidate for the treatment of PVR. Our decision to explore the potential of dabigatran instead of other coagulation inhibitors had several reasons. Dalteparin – an LMWH which has been previously tested in clinical use or clinical trials[6-12]– needs the presence of antithrombin (AT) to inactivate factor Xa and/or thrombin. It was unclear whether AT would be present in vitreous and we therefore measured the concentration of AT in a few vitreous samples of patients with RD with and without PVR. The concentrations were much lower than in normal plasma (< 0.10 U/ml vs. 0.40-1.40 U/mL) (unpublished data 2013). In addition, while LMWH's have a weaker inhibitory effect on thrombin than on factor Xa, the results of Bastiaans *et al* revealed that thrombin was a more potent inducer of inflammatory and fibrotic changes than factor Xa.[13] We, therefore, shifted our attention to the direct thrombin inhibitors.
The naturally occurring most potent inhibitor of thrombin is the peptide hirudin which is produced by leeches. Hirudin is not registered for use in humans but there are several drugs that are derived from it using recombinant biotechnology, such as bivalirudin and lepirudin. In addition, small molecule direct thrombin inhibitors have been chemically synthesised such as argatroban and dabigatran. To date, only bivalirudin, argatroban and dabigatran are registered in the Netherlands. The former two are available as intravenous injections/infusion and have a relatively short half-life of 25 and 50 minutes respectively.[14, 15] Dabigatran, on the other hand, is an oral drug with a plasma half-life of approximately 12 hours.[16] As PVR has a protracted course and usually develops between 4 and 12 weeks after surgery[17] a single administration or single exposure during the vitrectomy procedure is probably not sufficient. While local administration is generally preferred, an oral drug is more patient friendly when therapy is extended. An additional advantage of dabigatran is that it is not only registered for treatment purposes but also for prevention. We, therefore, explored dabigatran as a potential drug candidate for the treatment of PVR.

POTENTIAL OF DABIGATRAN

Oral administration, however, has several disadvantages. Firstly, it is questionable whether the drug reaches the target site in the eye via the systemic route and, if it does so, whether it reaches a sufficiently high concentration. We hypothesised however that in the case of a retinal detachment the retinal barrier is disrupted and the eye would, therefore, be more permeable to drugs. Interestingly, diffusion of dabigatran into the vitreous was not limited to a disrupted retinal barrier. As was shown in chapter 2.2 also in patients with a macular hole or dropped nucleus dabigatran reached quantifiable levels after repeated administration.[18] Research by Weijtens et al had already shown this for dexamethasone.[19] Concentration of dabigatran was even higher when formed vitreous had been previously removed and was replaced with aqueous.[18, 20] As dabigatran is a highly hydrophilic compound it is unsure whether this would also be true for other (more hydrophobic) substances.

Although the concentrations we found were low compared to plasma concentrations, the theoretically needed concentration was (almost) met after repeated administration and chapter 2.3 showed that this concentration could diminish thrombin activity. Moreover, the amount of thrombin measured is most likely not active all at once but represents accumulation in the vitreous (chapter 2.4). Therefore, sustained levels of dabigatran might be more effective.

Determining the starting point and optimal duration of therapy is difficult. Ideally, one would like to start as soon as one knows that a patient is at high risk of developing PVR. This is, however, as described in chapter 3, not easy. Previously described oral treatments were prescribed during 10 and 15 days and 7, 4 and 8 weeks.[21-25] The rationale for the chosen duration, however, is not described. The onset of PVR, however, is usually between 2 weeks and 6 months after RD, with a median of 2 months.[26] Earlier research showed that the blood-aqueous barrier returned to normal within 8 weeks and also our own flare measurements suggest that in uncomplicated patients it returns to normal within this period.[27-29] Dabigatran is registered for the prevention of knee and hip replacement surgery in a duration of 10 days and 28-35 days starting 1-4 hours after surgery respectively.[16] The latter duration would be a good starting point for further clinical research.

SAFETY OF DABIGATRAN

A second disadvantage of systemic drug administration is that all organs of the body are exposed to the drug while only a small part of the eye needs the treatment. This increases the risk of side effects and interaction with other systemic drugs. The risk of bleeding is increased when patients have underlying conditions with increased bleeding tendency or when the plasma level of dabigatran increases due to factors such as reduced renal clearance (30-50 ml/min), age > 75 years, body weight < 50kg and/or combination with P-gp inhibitors.[16] Also the concomitant use of other anticoagulant drugs, NSAIDS and SSRI's

increase the risk of bleeding. Dabigatran should therefore not be blindly prescribed to every patient at risk for developing PVR. In our first study, we excluded patients with these risk factors and no adverse events were reported after a single administration. Repeated administration would possibly pose more risk. Most patients, however, are subjected to a preoperative screening by an anesthesiologist whom checks and reports the above-mentioned risk factors. In most cases the dosage could be reduced as is described for knee and hip replacement surgery from 220 mg once daily to 150 mg once daily.
In addition, since 2015 there is – in case of excessive bleeding – an antidote available in the form of a monoclonal antibody named idarucizumab (Praxbind®).[30]

RISK ASSESSMENT

A large part of this thesis focused on the evaluation of aqueous laser flare measurements as a predictor for the development of PVR. Laser flare measurements were reported by Schröder et al to strongly predict the development of PVR.[31] A flare value above 15 pc/ms would increase the risk by 16 times. Several other groups repeated this study, including us, and we therefore decided to combine the results with the research group from Kiel (Germany). As the number of PVR cases was lower than expected in both centres this would be an elegant way to increase statistical power. Unfortunately, the differences in flare values between the two centres were too large and we therefore decided to analyse the results separately. The conclusion for both centres was that flare values of uncomplicated and PVR cases overlapped too much to define a clear cut-off point with high enough sensitivity, specificity and positive predictive value. We were unable to reproduce the previously published results.
A possible explanation for the discrepancy with the previous reports is our low prevalence of postoperative PVR (7.5% and 6.2%) compared to the previous reports.[32] All previous reports included consecutive patients and found prevalences of 10.3%[31], 14.9%[33] and 20% [34]. Both the positive predictive value (PPV) and the odds ratio (OR) are affected by the prevalence. This means that even with comparable sensitivity and specificity the PPV and the OR will be lower when the prevalence is lower. In addition, different reports used different definitions of postoperative PVR. We used reoperation due to epiretinal membranes and/or subretinal strands within six months of initial surgery, as did Schröder *et al.*[31] Hoerster *et al.* based their findings on PVR grade C at 3 months postoperatively. While ten patients developed PVR only four (6%) needed a reoperation.[33] It is unclear from the report whether all four patients had a flare value > 15 pc/ms but if this were the case this would give a PPV of 20% and OR of 8 instead of the reported 40% and 30. Conart *et al.* used PVR grade B and C at 6 months as the outcome. Moreover, 20% of the included patients had preoperative PVR grade C which influenced the postoperative number of patients with PVR.
The predictive value of the flare value thus depends on the patient group that is targeted. Our results indicate that there is a large overlap in flare values which makes selecting those

patients at high risk for developing postoperative PVR according to our definition inaccurate.[32] Dependent on the implications of a positive test result (flare value > 15pc/ms) we should decide whether a false discovery rate of 60-90% is acceptable.
The measurement of postoperative flare values revealed that the type of surgery and events during surgery significantly influence the inflammatory response and thus possibly the risk of developing PVR. Postoperative flare values showed stronger predictive properties than the preoperative values but the positive predictive value remained relatively low. In addition, waiting two to six weeks to start a possible treatment might pass the window of opportunity to intervene in the process. Postoperative flare measurements would be useful in the follow-up of patients both in clinical practice and in studying the effect of treatment methods.

FUTURE PERSPECTIVES

The results in this thesis indicate that dabigatran is an interesting drug candidate that should be further explored for its potential in the prevention of PVR.
However, to initiate a clinical trial to test the efficacy of dabigatran in the prevention of PVR seems premature. As PVR occurs in approximately 6% of patients with RD the number of patients needed to obtain enough statistical power to prove a 50% reduction in the PVR rate is well over 1500 patients. The question is whether the uncertain benefits and unknown optimal dosing regimen at this point outweigh the risks of exposing many patients unnecessarily to dabigatran. This is probably the main reason why previous research often included patients with established PVR. Because the coagulation cascade is activated at an early stage after retinal detachment it is unlikely that intervention with dabigatran will be effective after established PVR.
Another possibility could be to test the effect of dabigatran in an animal model. Over 27 animal models have been described.[1, 35] The most widely used animal models are pigmented rabbits which received an intravitreal injection with a specific cell type such as fibroblasts, RPE-cells or activated macrophages. Other models included surgical manipulation such as lensectomy, vitrectomy, cryotherapy or penetrating trauma.[1, 35] However, positive results found in these animal models did not translate into success in patients.
Possible reasons are the single cell-type/factor nature of the models while new insights have shown other origins of the cells involved and point to a multifactorial pathogenesis. In addition, the difference between humans and rabbits in vascularisation scheme and cell composition of the retina might add to differences in experimental results. Animal models are of value in studying specific features of PVR and help in unravelling the pathogenesis. However, as PVR is a multifactorial disease and is likely to be a result from the interaction of genetic and environmental factors, these models seem unsuitable for testing new drug therapies.[1]

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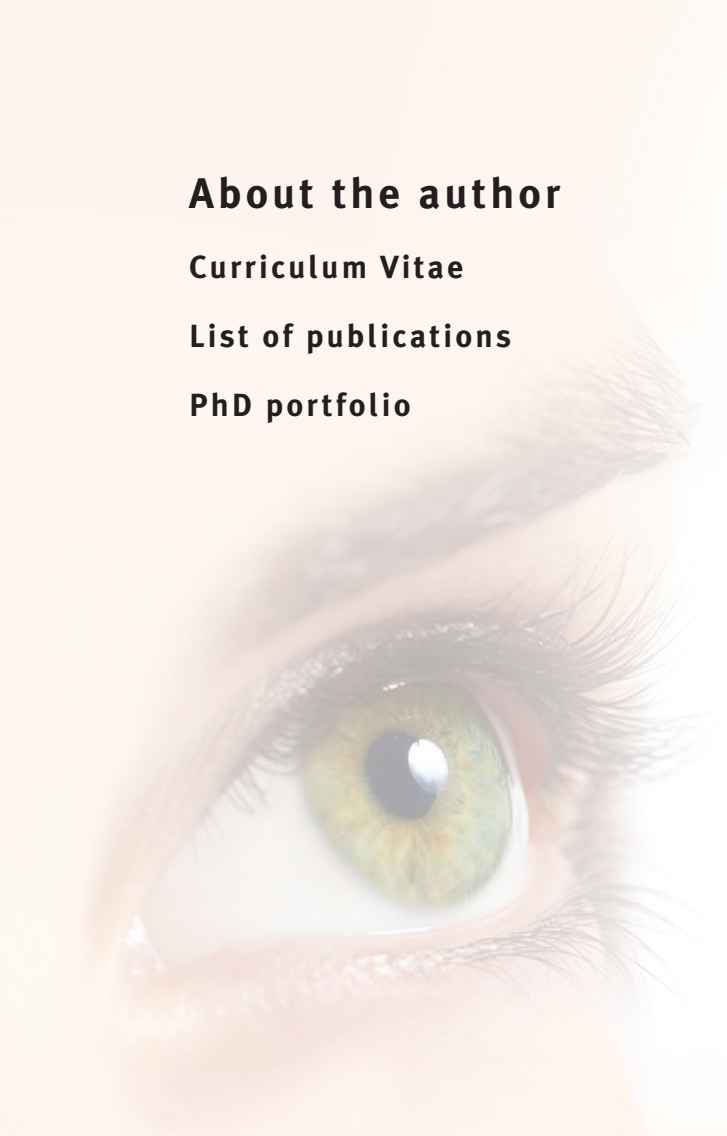
About the author

Curriculum Vitae

List of publications

PhD portfolio



8

CURRICULUM VITAE

Verena Mulder was born on May 15th, 1985 in Almere. After graduating in 2003 from secondary school (VWO) at the Oostvaarders College in Almere, she started a study Pharmaceutical Sciences at the University of Utrecht. During her study, she did a scientific internship at the department of clinical pharmacology at the Western General Hospital and the Royal Infirmary in Edinburgh, Scotland. Here she worked on 'the reproducibility of a one-day protocol for defining salt sensitivity in healthy volunteers and in subjects with high blood pressure'. In addition, she did an internship at the drug registration committee and national health insurance company of Aruba where she worked on an evaluation procedure for drugs which are not FDA or EMA approved. After obtaining her Master's degree, she started working as a pharmacist at Tergooiziekenhuizen in Hilversum and Blaricum. Although she enjoyed this line of work very much, she always had wanted to do something in ophthalmology. In 2012 the opportunity of a PhD-position arose at the Rotterdam Eye Hospital which had both the elements of ophthalmology and pharmacology. So after finishing her projects at Tergooiziekenhuizen, she started her PhD project as described in this thesis in October of 2012.

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Mulder VC, Tode J, van Dijk EHC, Purtskhvanidze K, Roider J, van Meurs JC, Treumer F.
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Thrombin generation in vitreous and subretinal fluid of patients with a retinal detachment
Submitted

Mulder VC, La Heij EC, Klungel OH, van Meurs JC.
Medication use in patients with proliferative vitreoretinopathy; an alternative approach for identifying risk factors

PHD PORTFOLIO

Name PhD student: Verena C. Mulder
Institution: Rotterdam Eye Hospital & Rotterdam Ophthalmic Institute
PhD period: October 2012 – October 2016
Promotor: Prof. dr. J.C. van Meurs
Co-promotor: dr. E.C. La Heij

PhD training	Year	Workload (ECTS)
General courses		
• BROK ('Basiscursus Regelgeving Klinisch Onderzoek')	2012	1
• Courses in the Medical Library	2012	0.5
• Oogheelkundige leerweg	2012-2013	2.5
• Biostatistical Methods I: Basis principles part A	2013	2
• Writing in the sciences (Stanford University)	2013	2
• Data Management for Clinical Research (Vanderbilt University)	2013	0.5
• Design and Interpretation of Clinical Trials (Johns Hopkins University)	2014	0.5
• Biomedical English writing and communication	2016	2
Specific courses		
• Clinical development & clinical trial design (CHDR)	2014	3.6
• Interpreteren van populatiefarmacokinetisch en -dynamisch onderzoek voor beginners	2015	0.3
• Seminars and workshops		
• Scientific day – Rotterdam Ophthalmic Institute	2012	0.3
• Scientific day – Rotterdam Ophthalmic Institute	2014	0.3
• Journal club statistics – Rotterdam Ophthalmic Institute	2015	1.5
• Funding and Grant Writing	2015	0.1
• Pharmacokinetics and pharmacodynamics of intravitreal drugs in vitreoretinal diseases	2015	0.1
• Symposium "The usefulness and necessity of non-human primates in biomedical research" (KNAW)	2015	0.3
• Scientific day – Rotterdam Eye Hospital	2015	0.6
• (Oral presentation)		
• Symposium "Tijd voor kwaliteit" Erasmus MC	2016	0.3
• Monthly scientific seminars, Rotterdam Eye Hospital	2012-2016	2
• Monthly scientific seminars, Rotterdam Ophthalmic institute (4 oral presentations)	2012-2016	3
• Weekly ophthalmology seminars, Rotterdam Eye Hospital	2012-2016	4

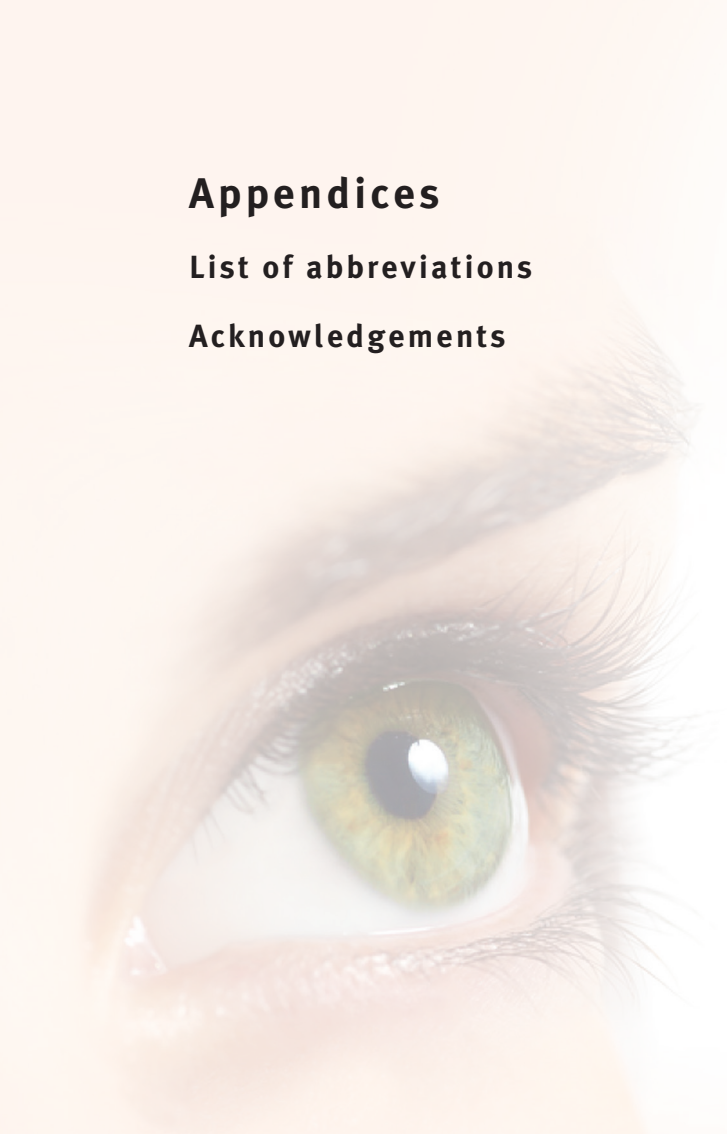
(Inter)national conferences		
Oral presentations/ poster contributions		
• DOPS Nijmegen (Poster contribution)	2015	1
• NOG Groningen (Oral presentation)	2015	1.5
• ARVO Denver (Poster contribution)	2015	3
• Euretina Nice (Poster contribution)	2015	1
• DOPS Nijmegen (Poster contribution)	2016	1
• NOG Maastricht (Oral presentation)	2016	1.5
• Euretina Copenhagen (Oral presentation)	2016	1.5
• WAEH Rotterdam (Poster contribution)	2016	0.5
• NOG Maastricht (Oral presentation)	2017	1.5
Other (inter)national conferences		
• 10th ISOPT clinical Paris	2013	1
• KNMP congress "Immunologie"	2013	0.3
• ARVO Orlando	2014	2
• 17th Rotterdam Glaucoma Symposium	2014	0.3

Teaching	**Year**	**Workload (ECTS)**
Supervising Master's theses Elon van Dijk	2014	1
TOTAL		**44.5**

Appendices

List of abbreviations

Acknowledgements



A

LIST OF ABBREVIATIONS

5-FU	Fluorouracil
a2M	Alpha-2-Macroglublin
ARPE-19	Human retinal pigmented epithelial cell line
ASA	American Standardisation Association
AT	Antithrombin
AUC	Area under de curve
BAB	Blood aqueous barrier
BOB	Blood ocular barriers
BQL	Below quantification limit
BRB	Blood retinal barrier
C3F8	Octafluoropropane
CCL	C-C motif chemokine ligand
Cmax	Maximum concentration
CXCL	C-X-C motif chemokine ligand
Da	Dalton
DTI	Direct thrombin inhibitor
dTT	Diluted thrombin time
ECM	Extracelluar matrix
F1+2	Prothrombin fragments (F1+2)
FCS	Fetal calf serum
FGF	Fibroblast growth factor
GM-CSF	Granulocyte-macrophage-colony-stimulating factor
HPLC	High Performance Liquid Chromotography
IFN	Interferon
IL	Interleukin
ILM	internal limiting membrane
IOP	Intra ocular pressure
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
LDH	Lactate dehydrogenase
LLQ	Lower limit of quantification
MHOLE	Macular hole
MPCK	Macular pucker
MW	Molecular weight
NSAID	Non-steroidal antiinflammatory drug
OD	Optical density
OR	Odds ratio
PAR	Protease-activated receptor
PDGF	Platelet-derived growth factor
PPI	Proton pump inhibitor
PPP	Platelet poor plasma

PPV	Pars plana vitrectomy
ppv	Positive predictive value
PVR	Proliferative vitreoretinopathy
QL	Quantification limit
RD	Retinal detachment
ROC	Receiver operating characteristic
RP	Reversed phase
RPE	Retinal pigment epithelium
RQ-PCR	Real-time quantitative polymerase chain reaction
RRD	Rhegmatogenous retinal detachment
SB	Scleral buckling
SD	Standard deviation
SF6	Sulfur hexafluoride
SRF	Subretinal fluid
SSRI	Selective serotonin reuptake inhibitor
STR	Short tandem repeat
TAT	Thrombin- antithrombin complex
TBS	Tris-buffered saline
TNF	Tumour necrosis factor
ULQ	Upper limit of quantification
VEGF	Vascular endothelial growth factor

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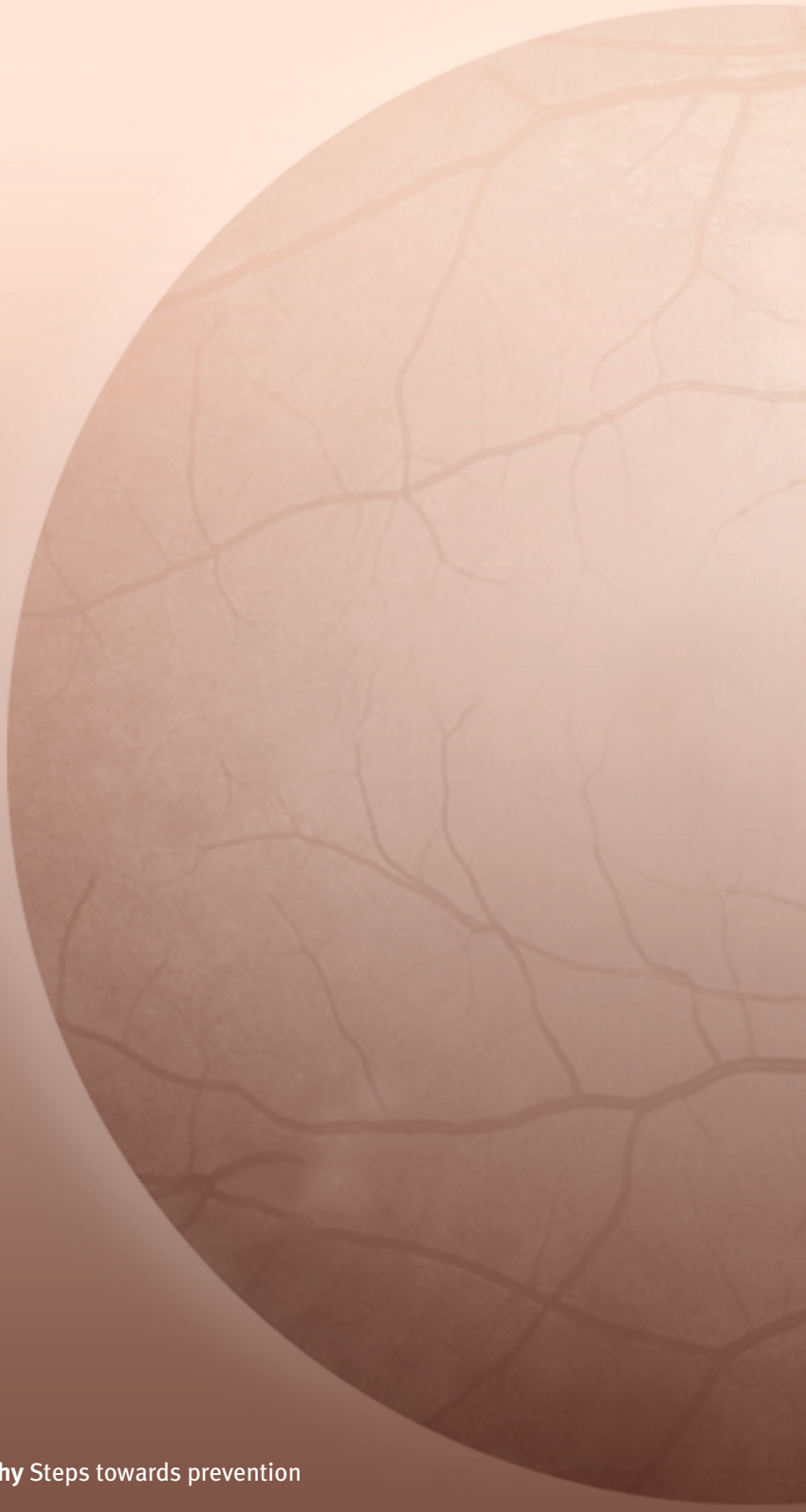

Proliferative Vitreoretinopathy Steps towards prevention