POWER AND SAMPLE SIZE CONSIDERATIONS IN A PRE-MATURELY TERMINATED RANDOMIZED, DOUBLE-MASK, PLACEBO-CONTROLLED, DOSE-RESPONSE, PHASE 2 STUDY OF THE SAFETY AND EFFICACY OF THYMOSIN BETA 4 FOR THE TREATMENT OF PERSISTENT CORNEAL EPITHELIAL DEFECTS RESULTING FROM EPITHELIAL DEBRIDEMENT DURING DIABETIC VITRECTOMY SURGERY: IMPLICATIONS FOR FUTURE STUDIES

by

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of tables</td>
<td>3</td>
</tr>
<tr>
<td>List of figures</td>
<td>4</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>5</td>
</tr>
<tr>
<td>Abstract</td>
<td>6</td>
</tr>
<tr>
<td>I. Introduction</td>
<td>7</td>
</tr>
<tr>
<td>II. Rationale for a study</td>
<td>14</td>
</tr>
<tr>
<td>III. Study design and objectives</td>
<td>14</td>
</tr>
<tr>
<td>IV. Execution of the Thymosin Study</td>
<td>18</td>
</tr>
<tr>
<td>V. Statistical methods</td>
<td>44</td>
</tr>
<tr>
<td>VI. Results</td>
<td>47</td>
</tr>
<tr>
<td>VII. Discussion</td>
<td>57</td>
</tr>
<tr>
<td>VIII. Conclusions</td>
<td>72</td>
</tr>
<tr>
<td>References</td>
<td>74</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1: Schedule of observations and procedures 14
Table 2: Baseline demographics of randomized patients 46
Table 3: Adverse events reported 50
Table 4: Screening glycosylated hemaglobin levels versus time to healing 51
Table 5: Percent healing of epithelial defect from baseline to 7 and 14 days 55
Table 6: Sample sizes required for the study based on estimated rate of occurrence of AE 59
Table 7: Sample sizes required based on desired percent difference to be detected in the healing of the epithelium assuming 80% power at the 5% significance level (two-sided). 60
LIST OF FIGURES

Figure 1: Diagram of dose-escalation scheme 22

Figure 2: Kaplan-Meier Analysis (post-hoc) demonstrating the duration of days to complete epithelial healing in each group 53

Figure 3: Glycosylated hemaglobin level versus time to wound healing 65
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Persistent corneal epithelial defects (PED) are a challenging clinical condition to treat. Recently, Thymosin Beta 4 (Tβ4) has been under investigation as a novel compound for the treatment of PED. We performed a prospective, randomized clinical trial to evaluate the safety and efficacy of Tβ4 in the treatment of PED in diabetics who underwent corneal epithelial debridement during vitrectomy surgery. Although the trial was closed prematurely due to slow enrollment, the data that was collected was analyzed. In addition, study design considerations with specific regard to possible future studies are discussed.
I. Introduction

a. Persistent epithelial defects

i. Background

The corneal epithelium is composed of non-keratinized, stratified squamous epithelial cells, and it is essential to the normal functioning of the eye. In addition to providing a transparent and optically regular conduit for visual stimuli to pass to the retina and then to the brain, the epithelium provides one of the first lines of biodefense for the eye: the epithelial cells are connected by hemidesmosomes and gap junctions that prevent infectious agents from penetrating into the eye under normal circumstances. However, the maintenance of a healthy functioning corneal epithelium is dependent on having a healthy normal corneal nerve network.¹

In the event that there is a breach in the integrity of the corneal epithelium, a wound healing response occurs, and most epithelial defects heal within 2 to 3 days. This wound healing response relies on a highly organized chain of events that unfold in three mechanistic stages: migration, proliferation, and differentiation. The remaining epithelial cells migrate to the denuded area, proliferate, and differentiate: restoring the normal multi-layered epithelial cytoarchitecture. Initial migration and proliferation depend on both the interaction of the cells with the underlying substrate, as well as cell–cell adhesions.

A timely re-establishment of the epithelial barrier is of utmost importance. Reformation of the epithelial barrier not only protects the cornea from infectious agents, but also prevents epithelial fragility that leads to recurrent erosions. The wound repair
process is intricately linked to a complex inflammatory response that must be properly regulated to ensure healing and optimal visual outcome.

In certain conditions, however, the epithelial defect may be slow to heal, or it may not heal at all. These wounds are known as persistent corneal epithelial defects (PED), and they can be defined as a loss of the integrity of the corneal surface, caused by injury or disease, which does not heal within the normal timeframe and persists for weeks or even months.

Persistent epithelial defects of the cornea are uncommon, but in addition to the immediate adverse effect on vision, PED can have serious consequences for the health of the eye including infection, scarring, melting, and even perforation. Several etiologies for PED include dry eyes, limbal stem cell deficiency, diabetic keratopathy, and neurotrophic keratopathy following corneal transplant surgery or herpetic infections.

ii. Current treatment options

In the event that a certain identifiable etiology can be established for a PED, then specific treatments can sometimes be instituted such as limbal stem cell transplantation for limbal stem cell deficiency. However, in general, traditional therapy of PED consists of aggressive lubrication with preservative-free artificial tears and ointments, the use of bandage soft contact lenses, pressure patching, punctal plugging, and temporary eyelid closure through the surgical placement of tarsorrhaphies.

Amniotic membrane grafting is a newer therapeutic modality that has also been suggested for use in accelerating the corneal epithelial wound healing process in PED. In addition to acting as a temporary bandage on the ocular surface, it is believed that
amniotic membrane contains growth factors and other substances that are critical to the proliferation and migration of the corneal epithelium. Recently, the use of autologous serum eyedrops has been reported to be efficacious in treating persistent epithelial defects.\textsuperscript{7-13} Unfortunately, none of these therapies has been demonstrated to be superior to another, and often times, none is successful at healing the PED. Although limbal stem cell transplantation, tarsorrhapies, and amniotic membrane grafts can all be successful, all require surgical intervention. Furthermore, the use of autologous serum is often troublesome because there is no standardized protocol that exists for processing whole blood to be made into autologous serum eyedrops, and therefore, few clinical laboratories and clinics are equipped to prepare this product. Although Liu and colleagues have made a recommendation as to an optimized protocol for the processing of whole blood for autologous serum, this protocol has not been validated.\textsuperscript{14,15} In addition, at this time, regulatory restrictions also limit the widespread ability to produce autologous serum for therapeutic use.\textsuperscript{16}

\textit{iii. In the setting of diabetic vitrectomies}

In eyes of diabetic patients, alterations in corneal innervation occur. Both stromal and sub-basal nerves appear abnormal, with patients with proliferative diabetic retinopathy demonstrating more pronounced nerve alterations.\textsuperscript{17} In addition, the nerve fiber layer density in the retina of these patients is correlated with basal epithelial cell density and is also correlated with changes in corneal innervation.\textsuperscript{18} Furthermore, tear film abnormalities, squamous metaplasia, and goblet cell loss associated with diabetes
can contribute to ocular surface dysfunction and epithelial pathology in diabetics.\textsuperscript{19} These changes all put the diabetic cornea at risk for developing non-healing epithelial defects.

Other histological abnormalities inherent in the diabetic corneal epithelial cytoarchitecture may also substantially impede normal healing: diabetic corneas typically have thicker epithelial basement membranes (BM), decreased hemidesmosomes expression, and loss of homeostatic Laminin-5 (LM-5). A consequence of these BM aberrations is poor epithelial BM adhesion to the stroma. For example, during vitrectomy in diabetic patients, when the corneal epithelium is removed, it tends to separate as a single intact sheet with the entire thickened BM adhering to the epithelium. In contrast, during debridement of a normal epithelium, the BM typically remains attached to the stroma.

In PED after epithelial debridement during diabetic vitrectomy surgery, the delayed epithelial healing may result in sub-optimal outcomes. The mainstay of treatment for PED is to optimize the health of the ocular surface in an effort to promote epithelial healing. Unfortunately, these corneas often respond neither to standard treatment for PED, nor to any of the other treatments described above.

In eyes which undergo epithelial debridement during diabetic vitrectomy surgery, approximately 54\% of corneas actually develop PED.\textsuperscript{20} With an estimated 250,000 vitrectomies done annually in the United States,\textsuperscript{21} an estimated 25-53\% of vitrectomies being for diabetics,\textsuperscript{22,23} and an epithelial debridement rate of 17.4\%,\textsuperscript{24} an estimated 10,875 to 23,055 corneas are at risk for scarring and other serious complications in this particular setting that can ultimately limit visual outcome.
b. Thymosin beta 4

i. Background

Thymosin beta 4 (Tβ4) is a synthetically produced copy of a naturally-occurring 43-amino acid peptide. The natural peptide is found in high concentrations in the majority of tissue types, with the highest concentrations in blood platelets and white blood cells, and it is also found extracellularly in blood plasma and in wound fluid. It is one of a family of at least 16 highly conserved peptides, collectively called the beta (β) - thymosins, which are present in high concentrations in almost every cell type.

Tβ4 has wound-healing and anti-inflammatory properties. It is thought to exert its therapeutic effect through promotion of keratinocyte and endothelial cell migration, increased collagen deposition, and stimulation of angiogenesis. Tβ4 binds to actin, blocks actin polymerization, and is the major actin-sequestering molecule in eukaryotic cells. It is released from human blood platelets and is selectively cross-linked by factor XIIIa, a tissue transglutaminase, to various molecules, including actin, collagen, fibrin, and fibrinogen.

ii. Pharmacokinetics

Given that the healing rate of diabetic corneas is slower than that of normal patients, and Tβ4 expression is recognized as a potent stimulator of epithelial cell migration, the relative concentrations of Tβ4 expression in diabetic corneas have been compared with that of normal corneas. In this study, Tβ4 expression was found to be significantly less in diabetic corneas compared with normal corneas, suggesting that the use of Tβ4 may accelerate the wound-healing process in this model.
Furthermore, given that LM-5 is a major component of corneal BM and has a crucial role in corneal epithelial cell adhesion, the effect of Tβ4 on human conjunctival and corneal epithelial cells was evaluated. Gene expression analysis revealed that Tβ4 treatment stimulates epithelial cell migration and promotes LM-5 expression in both human conjunctival (HC0597) and corneal epithelial cells (HCEC) treated with Tβ4: LM-5 production increased by more than 2-fold compared with untreated cells. These findings were confirmed by reverse transcriptase polymerase chain reaction. In light of these recent findings correlating decreased LM-5 levels with diabetic keratopathy, the findings that Tβ4 increases LM-5 production in corneal epithelial cells suggest that Tβ4 may promote a superior epithelial healing in diabetic corneas. These data suggest that it may be useful to test the ability of Tβ4 to promote and/or enhance corneal wound healing in the clinical setting.

iii. pre-clinical studies

A study of Tβ4 in a rat full-thickness wound model showed that treatment with Tβ4 increased collagen deposition and angiogenesis, and it stimulated keratinocyte migration and re-epithelialization. This study strengthens the thought that Tβ4 may be a potent wound-healing agent with multiple activities that may be useful clinically.²⁶

An in vivo mouse study examined the effect of Tβ4 treatment on corneal wound healing and inflammation in vivo following alkali injury of the eye. Mouse corneas treated topically with 5 μg of Tβ4 twice daily after alkali injury demonstrated accelerated re-epithelialization at all time points, along with decreased PMN infiltration at 7 days post-injury compared with phosphate buffered saline-treated controls.²⁹
In addition, 3 genotoxicity studies have been performed where Tβ4 was found to be non-mutagenic in the Ames mutation, mouse lymphoma mutagenesis, and the mouse micronucleus cytogenetic assay. Furthermore, a 28-day repeat dose ocular toxicity study of Tβ4 administered to New Zealand white rabbits followed by a 14-day recovery period was performed. Forty-eight rabbits were divided into 4 groups based on dosage of Tβ4: 0%, 0.01%, 0.03%, and 0.1%. All rabbits were given 4 drops 4 times daily in both eyes. No clinical pathology, no macroscopic or microscopic finding, and no adverse events were observed at any of the drug doses.

iv. experimental human uses

Results from the non-clinical studies have suggested that the potential for toxicity of topically applied Tβ4 to humans is low, and that topically applied Tβ4 has the potential to enhance wound healing. As such, Phase I studies of Tβ4 in healthy volunteers was performed on the skin, and Tβ4 was found to be well-tolerated and presented an acceptable safety profile. Currently, there are 3 Phase 2 trials underway assessing the safety and dermal wound healing properties of Tβ4 in patients with pressure ulcers, venous stasis ulcers, and the genetic disease, Epidermolysis Bullosa.

Based on the positive pre-clinical and dermal study results, Tβ4 has been used in the eye on a compassionate use basis in several cases. In these cases of long-standing neurotrophic corneal epithelial defects from herpes zoster ophthalmicus or diabetes, or both, all 4 eyes of 4 patients showed clinically significant reduction in the size of their epithelial defects. In addition, no patient reported discomfort associated with the use of the drops, and improved ocular discomfort and decreased conjunctival injection was
correlated with healing. The results of these compassionate use studies suggested that Tβ4 would be safe in humans under the conditions used for this ophthalmologic study.

II. Rationale for a study

Given that serious and blinding consequences can arise from PED that cannot be treated with standard treatment modalities, it is important that other treatment options be explored for these situations. With both animal and human data in both the skin and the eye supporting Tβ4 as being a potentially safe and efficacious novel treatment agent for PED, Tβ4 appeared to be an excellent candidate therapy for this clinical situation.

Of all of the possible underlying causes of PED, diabetic corneal wounds after vitrectomy surgery appeared to be the indication with the least number of confounding variables for which a study would have to control. Other indications such as ocular burns have significant variables such as clock hours of limbus burned (and to what extent) as well as type of chemical involved in the burn that would have various variables that would need to be addressed. As such, we were interested in designing a prospective study to assess the safety and efficacy of Tβ4 in the healing of diabetic corneal wounds that were created during diabetic vitrectomy surgery.

III. Study Design and Objectives

This was a double-masked, placebo-controlled Phase 2 proof-of-principle study using 3 concentrations of Tβ4 in diabetic male and female patients who elected to have corneal wound healing treatment using Tβ4 following epithelial debridement at the time of vitrectomy. There was to be 3 groups of patients, enrolled sequentially, with each
group having 12 patients. Patients within each group were to be randomized to Tβ4 or placebo in a 3:1 ratio. A diagram of the study design is shown in Figure 1, and the schedule of observations and procedures is shown in Table 1. The study drug (Tβ4 or placebo) was formulated as eye drops and applied topically.

Three concentrations (dose levels) of Tβ4 drops were to be used: 0.01%, 0.03%, and 0.1%. In Group 1, those randomized to Tβ4 were to receive the lowest dose (0.01%). Safety data was to be reviewed after all patients in Group 1 had received treatment for 14 days. If the safety results were acceptable, Group 2 was to be enrolled, with Tβ4 applied at the middle dose (0.03%). A second review of the safety data was to be performed after all patients in Group 2 had received treatment for 14 days. If the safety results were acceptable, Group 3 was to be enrolled, with Tβ4 applied at the highest dose (0.1%). The reviews of the safety data were to be performed using masked data (data was to be summarized for the overall group only and was not to be separated by treatment groups).
Figure 1: Diagram of dose-escalation scheme. Specifics for study termination are described below in Stopping rules.
a. Primary Objective (safety)

The primary objective of this study (hereafter referred to as the Thymosin Study) was to evaluate the safety and tolerability of Tβ4 ophthalmic solution, administered by drops to the affected eye (2 drops four times daily for 14 days), in diabetic patients with corneal wounds resulting from epithelial debridement for improved intra-operative fundus visualization during vitrectomy.

b. Secondary Objectives (efficacy)

The secondary objective of the Thymosin Study was to evaluate the wound-healing effectiveness of Tβ4 ophthalmic solution, administered by drops to the affected eye (2 drops four times daily for 14 days), in diabetic patients with corneal wounds resulting from epithelial debridement for improved intra-operative fundus visualization during vitrectomy.

c. Exploratory Objectives

The exploratory objectives of the Thymosin Study were to evaluate:

- Central corneal thickness of both eyes, as measured by ultrasound pachymetry
- Inflammation (cells and flare), as assessed clinically by slit lamp examination (SLE) and graded by Nussenblatt scale
- Plasma Tβ4 levels
- Presence of antibodies to Tβ4 in the serum
IV. Execution of the Thymosin Study

a. patient population

i. inclusion criteria

Patients were enrolled in this study only if they met all of the following criteria:

• Male or female, aged 18 years and older.
• Type 1 or Type 2 diabetes mellitus.
• Glycosylated hemoglobin (HbA₁C) level of 7% – 12%.
• Scheduled for vitrectomy.
• In the opinion of the Investigator had at least a 50% likelihood of requiring corneal epithelial debridement during vitrectomy. Lensectomy concomitant with corneal epithelial debridement was permitted.
• Signed written informed consent by patient or legal guardian.

ii. exclusion criteria

Patients were enrolled in this study only if they met none of the following criteria:

• Current herpetic eye disease or history thereof in the past 3 years.
• Evidence of any keratitis.
• Sjögren syndrome.
• Corneal scarring, opacity, or dystrophy.
• History of intra-ocular or anterior segment surgery within 3 months of baseline examination, or anticipation of such surgery during the study. (Retinal or glaucoma (intraocular) laser surgery is acceptable.)
• Abnormalities judged by the Investigator as clinically significant on screening
laboratory tests. If abnormal laboratory results were present, but were deemed to be of a chronic nature that would not in the judgment of the Investigator and Medical Monitor interfere with participation in the study, then the patient could be enrolled. Patients having any one of the following laboratory values were excluded from enrollment:

- Hemoglobin – Less than 9 gm/dL and 8.5 gm/dL, male and female, respectively.
- Hematocrit – Less than 27% and 25%, male and female, respectively.
- White blood cells – less than $3.8 \times 10^3$/mm$^3$ or greater than $11.0 \times 10^3$/mm$^3$.
- Blood Urea Nitrogen – Greater than 35 mg/dL
- Blood glucose – Greater than 250 mg/dL
- Alkaline phosphatase – Greater than 150 U/L

- History of neurological, cardiovascular, respiratory, hepatic, or renal disease which, in the opinion of the Investigator, was likely to interfere with study participation.
- History of malignancy.
- History of human immunodeficiency virus (HIV) or acquired immune deficiency syndrome (AIDS).
- Pregnancy or lactation. (A serum pregnancy test was performed at screening for female patients of childbearing potential).
- An ability to become pregnant due to the onset of menarche without completion of menopause (defined as 1 year or longer without a menstrual period), the
absence of surgical sterilization (e.g., bilateral tubal ligation or hysterectomy), the presence of a male partner lacking documented sterility (e.g., vasectomy), and/or the absence of adequate contraception during sexual activity. (Only combined hormonal methods of contraception or sexual abstinence were acceptable methods of contraception in this study).

- Allergy to fluoroquinolone antibiotics.
- Use of systemic steroidal therapy, antibiotics, immunotherapy, cytotoxic chemotherapy, or any investigational drug except for Avastin® (Genentech, San Francisco, CA) within 30 days of study entry.
- Known hypersensitivity to the study drug or any of its ingredients.
- Previous participation in this study.
- In the Investigator’s opinion, any other condition that could preclude the patient’s full participation in and completion of the study, including unreliability.
- Any complication during the surgical procedure itself, which excluded the patient from continued participation in this study.

**iii. enrollment**

If the patient satisfied both inclusion and exclusion criteria, then the patient was consented for enrollment in the study. During standard vitrectomy surgery, if corneal epithelial debridement was necessary, it was carried out by first using an 8 mm corneal trephine to mark the area for debridement, followed by standard debridement using a scalpel blade. The defect needed to measure 8 mm +/- 0.5 mm (average of the greatest three linear diameters) to confirm eligibility for randomization after surgery.
b. Randomization scheme

Once the decision was made to perform epithelial debridement because of problems with visualization during the vitrectomy surgery, an 8.0 mm corneal trephine was used to mark out the area of the cornea to be scraped. Randomization eligibility was confirmed, following surgery, by the presence of an epithelial debridement wound having a diameter of 8 mm +/- 0.5 mm (average of the greatest three linear diameters) as measured by calipers within one hour following surgery.

Given confirmation of randomization eligibility, the Investigator was to notify the study coordinator who called the randomization center at PPD (the contract research organization), which used an interactive voice response system (IVRS) to randomize the patients using a block randomization scheme. The IVRS assigned the subject randomization number, randomization record, and kit number as well as sent a notification. Each unique randomization number was digits systemized as XXX-Y-ZZ wherein the first three digits identified the site (starting at 400), the next single digit identified the group (1, 2, or 3), and the final two digits consecutively identified the patient according to the central randomization scheme. Randomized patients were assigned to Tβ4 or placebo in a 3:1 ratio.

Any patients who experienced blindness or any potentially blinding complications as a result of the vitrectomy were not to be randomized, and thus they would not receive the study drug (Tβ4 or placebo).
c. Adverse events

An adverse event (AE) is any untoward medical occurrence in a subject or clinical investigation subject administered a pharmaceutical product and that does not necessarily have a causal relationship with this treatment. An AE can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of an investigational product whether or not related to the investigational product.

A serious adverse event (SAE) is any untoward medical occurrence that at any dose:

a. Results in death

b. Is life-threatening

The term "life-threatening" in the definition of "serious" refers to an event in which the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.

c. Requires the subject’s hospitalization or prolongation of existing hospitalization

In general, hospitalization signifies that the subject has been detained (usually involving at least an overnight stay) at the hospital or emergency ward for observation and/or treatment that would not have been appropriate in the physician’s office or in an outpatient setting. Complications that occur during hospitalization are AEs. If a complication prolongs hospitalization or fulfils any other serious criteria, the event is serious. In this study, if there was any doubt as to whether “hospitalization” occurred or was necessary, the AE was to be considered serious. Hospitalization for elective treatment of a pre-existing
condition that did not worsen from baseline is not considered an AE.

d. Results in persistent or significant disability/incapacity

The term disability means a substantial disruption of a person’s ability to conduct normal life functions. This is not intended to include experiences of relatively minor medical significance such as uncomplicated headache, nausea, vomiting, diarrhea, influenza and accidental trauma, which may interfere or prevent everyday life functions, but do not constitute a substantial disruption.

e. Causes a congenital anomaly/birth defect

Abnormal laboratory findings (e.g., clinical chemistry, hematology, urinalysis) or other abnormal assessments (e.g., ECGs and vital signs) that are judged by the Investigator as clinically significant were to be recorded as AEs or SAEs if they met the definition of an AE or SAE. Clinically significant abnormal laboratory findings or other abnormal assessments that were detected during the study or were present at baseline and significantly worsen following the start of the study were to be reported as AEs and SAEs. However, clinically significant abnormal laboratory findings or other abnormal assessments that were associated with the disease being studied, unless judged by the Investigator as more severe than expected for the subject’s condition, or that were present or detected at the start of the study and did not worsen, were not to be recorded as AEs or SAEs.
d. Schedule of observations and procedures

A schedule of procedures and assessments to take place during the study is shown in Table 1 (below) and outlined below.

Table 1: Schedule of observations and procedures

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<td>Blood samples for antibodies to TB4</td>
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<td>Intra-ocular pressure (via Tono-Pen)</td>
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**Screening (Study Day -30 to -3, Visit 1)**

Screening (Visit 1) could occur from 30 to 3 days prior to the Day of Vitrectomy. At screening, patients who had signed an informed consent form (or had it signed by their legal representative) underwent the following procedures and evaluations:

- Assignment of a unique screening number
- Evaluation for inclusion and exclusion criteria
• Documentation of medical history, concurrent symptoms, and conditions
• Documentation of concomitant medications
• A complete ophthalmologic examination including SLE and fundus examination
• Clinical chemistry, hematology, and coagulation tests, and urinalysis

All patient data were recorded in the Source Documents and/or the CRF, as appropriate.

**Baseline (Study Day -2 to -1, Visit 2)**

Baseline (Visit 2) evaluations could occur 1 or 2 days prior to Day of Vitrectomy.

Baseline values for all parameters were derived from the most recent observation recorded prior to study drug application. (The first application of study drug occurred within 3 hours following completion of the Vitrectomy procedure on Study Day 0). For example, if an assessment was performed at Screening but not Visit 1 or Visit 2, then the Screening observation serves as Baseline for the purpose of comparison with final outcomes. At Baseline, patients who underwent the following evaluations at Screening did not need to repeat them at Baseline provided that Screening and Baseline evaluations were not more than 1 week apart:

• Serum pregnancy test for women capable of becoming pregnant
• Clinical chemistry, hematology, and coagulation tests, and urinalysis

At Baseline, all patients underwent the following clinical procedures:

• Documentation of demographic and baseline characteristics
• Height and weight measurements
• Physical examination and vital signs (vital signs were to be taken after the patient has been in either supine or sitting position for no less than 5 minutes)
• Electrocardiogram (ECG)
• Documentation of current physical signs and symptoms for comparison to any future adverse events (AE’s)
• Documentation of concomitant medications
• Blood collection for antibodies to Tβ4, and blood collection for Tβ4 plasma concentrations

At Baseline, all patients underwent the following ophthalmologic assessments and procedures:
• Target eye identification
• Central corneal thickness measurements in both the operative (i.e., target) and the non-operative eye by pachymetry
• Best-corrected visual acuity by ETDRS Chart
• Intraocular pressure measurement, via Tono-Pen
• A complete ophthalmologic examination, including SLE and fundus examination
• Study staff instructed the patient or caregiver in the use of the protective patch and study drug, including removal and replacement of the protective patch and application of study drug.

All patient data were to be recorded in the Source Documents and/or the CRF, as appropriate.

Day of Vitrectomy (Visit 3, Study Day 0)
At Visit 3 (Study Day 0), the previously screened patient underwent scheduled vitrectomy in the target eye. Lensectomy concomitant with corneal epithelial
debridement was permitted. The following stipulations applied:

- Vitrectomy was to be performed according to the Standard of Care practice for each site
- Standard of Care procedures were on file at each site
- The original medical records for each patient, including the surgical report and operative note, were to be available for review by the Sponsor or their designated representative
- The surgical report and operative note contained detailed information of the Vitrectomy including the date, start and end time of the operative procedure, respective patient findings, the post-operative treatment regimen, and adverse events

All information regarding the vitrectomy procedure and the patient’s condition were transcribed onto the CRF.

**Pre-Operative Procedures And Evaluations**

All patients underwent all pre-operative procedures per the Site’s Standard of Care on the Day of Vitrectomy (Visit 3).

**Randomization**

Patients who experienced blindness or any potentially blinding complications as a result of the vitrectomy/surgical procedure were not randomized and thus did not receive the study drug. In addition, patients who did not satisfy randomization criteria were withdrawn from the study and were to be followed-up per the Standard-of-Care
procedures of each site.

**Post-Operative Procedures and Evaluations for Randomized Patients**

Patients who were randomized underwent the following post-operative procedures prior to initial study drug administration:

- Documentation of concomitant medications
- Documentation of adverse events (any ophthalmologic finding which was abnormal was recorded as an AE)
- Examination of application area for any abnormalities
- A complete ophthalmologic examination including SLE
- Assessment of inflammation (cells and flare) by SLE graded by Nussenblatt scale
- Documentation of wound appearance by photography in conjunction with SLE and fluorescein eye drops
- Wound surface area was documented by digital photography
- Time to rescue medication, if applicable.

All patient data was recorded in the Source Documents and/or the CRF, as appropriate.

**Study Drug Administration in Randomized Patients**

The appropriate procedures for study drug administration were discussed with each patient:

- The study drug was to be administered at least 30 minutes following any concomitant medication.
- The first application of study drug and any dressings were to be given by the
Investigator or Sub-Investigator following all post-operative evaluations, but no later than 3 hours after surgery.

- The first dose could be given in the operating room, recovery room, or clinic at the Investigator’s discretion.
- After the first administration of the study drug, the target eye was to be covered with a protective patch designated by the Investigator per Standard of Care.
- The second, third and fourth doses were to occur at approximately four hour-intervals for Day 0 only. For all other days, the administration of the 2 drops was to occur at breakfast, lunch, dinner, and bedtime.
- The study staff could repeat instructions to the patient or caregiver regarding the use of the protective patch and study drug, including removal and replacement of the protective patch and application of study drug (such instructions first given at Baseline).
- The study staff could repeat instructions for the application of Refresh Plus® lubricant eye drops, and Standard of Care procedures to follow at home.
- The patient and/or his/her caregiver were provided with the study drug kit (containing 16 squeeze bottles).
- The patient was scheduled for his/her next visit.

All patient data were recorded in the Source Documents and/or the CRF, as appropriate.

**Visit 4 (Study Day 1)**

At Visit 4 (Study Day 1), the study staff performed the following ophthalmologic procedures:
• Removal of the protective patch
• Examination of wound for signs of healing
• Examination of application area for any abnormalities
• Intra-ocular pressure measurement, via Tono-Pen
• A complete ophthalmologic examination including SLE and fundus examination
• Assessment of inflammation (cells and flare) by SLE graded by Nussenblatt scale
• Documentation of wound appearance by photography in conjunction with SLE and fluorescein eye drops
• Wound surface area was documented by digital photography

In addition, the following data were recorded:

• Concomitant medications
• Adverse events (AEs)
• Time to use of bandage contact lens, if applicable
• Time to rescue medication, if applicable
• Dosing and Standard of Care adherence

Furthermore:

• Study staff were to review study drug application procedures and usage of protective patch.
• The staff member collected used/opened squeeze bottles of study drug returned by the patient.
• The patient will be scheduled for his/her next visit.

All patient data were recorded in the Source Documents and/or the CRF, as appropriate.
Visits 5, 6, 7, 8, and 9 (Study Days 2, 3, 4, 5, and 6)

The procedures conducted daily on Visits 5 through 9 (Study Days 2 through 6) included:

- Review of instructions for study drug application and Standard of Care procedures, as necessary
- Review of dosing and Standard of Care adherence.

The following ophthalmologic assessments were performed as directed by the protocol and the Standard of Care procedures for each site:

- Examination of wound for signs of healing
- Examination of application area for any abnormalities
- A complete ophthalmologic examination including SLE
- Assessment of inflammation (cells and flare) by SLE graded by Nussenblatt scale
- Documentation of wound appearance by photography in conjunction with SLE and fluorescein eye drops
- Wound surface area was documented by digital photography

In addition, the following data was recorded:

- Concomitant medications
- Adverse events (AEs)
- Time to use of bandage contact lens, if applicable
- Time to rescue medication, if applicable

Furthermore:

- The staff member collected used/opened squeeze bottles of study drug returned by the patient
- The patient was scheduled for his/her next visit.
All patient data were recorded in the Source Documents and/or the CRF, as appropriate.

**Visit 10 (Study Day 7)**

At Visit 10 (Study Day 7), study staff performed the following ophthalmologic procedures:

- Examination of wound for signs of healing
- Examination of application area for any abnormalities
- Intraocular pressure measurement, via Tono-Pen
- A complete ophthalmologic examination including SLE and fundus examination
- Assessment of inflammation (cells and flare) by SLE graded by Nussenblatt scale
- Central corneal thickness, as measured by ultrasound pachymetry. Central corneal thickness was assessed in both the operative (*i.e.*, target) and the non-operative eye
- Documentation of wound appearance by photography in conjunction with SLE and fluorescein eye drops
- Wound surface area was documented by digital photography

The following clinical laboratory procedures were performed:

- Clinical chemistry, hematology, and coagulation tests
- Urinalysis

In addition, the following data was recorded:

- Concomitant medications
- Adverse events (AEs)
- Time to use of bandage contact lens, if applicable
• Time to rescue medication, if applicable
• Dosing and Standard of Care adherence.
• The investigator reviewed instructions for study drug application and Standard-of-Care procedures with the patient, as necessary.

Furthermore:
• The staff member collected used/opened squeeze bottles of study drug returned by the patient.
• The patient was scheduled for his/her next visit per protocol schedule, dependent upon healing outcomes on Visit 10 (Study Day 7). If the patient experienced little or no wound closure by Visit 10 (Study Day 7), then the patient was managed according to the options outlined below:

"Little or no wound closure" was defined as a decrease in wound surface area of approximately 25% or less. Patients who had achieved little or no wound closure by Study Day 7 had two options, per discretion of the Investigator:

1) Patients could have a bandage contact lens applied, be withdrawn from the study, and discontinue study drug. Such patients would undergo an Early-Termination Visit (i.e., End-of-Treatment Visit procedures). The patient would return for a Follow-Up Visit two (2) weeks later and undergo all Follow-Up procedures. The patient would be followed for safety (i.e., unscheduled visits) until complete re-epithelialization occurred, if applicable

2) Patients could have a bandage contact lens applied, continue to receive study
drug, and be followed per protocol for safety at each subsequent evaluation through Study Day 28. Patients would be followed for safety until complete re-epithelialization occurred.

Patients who had a bandage contact lens applied for indications other than lack of wound closure were to be withdrawn from the study. All patient data were recorded in the Source Documents and/or the CRF, as appropriate.

Visits 11, 12, and 13 (Study Days 9, 11, and 13)
Study Visits 11, 12, and 13 occurred every other day. The procedures for patients who had not achieved complete wound closure have been described above. The procedures for patients who had been withdrawn from the study and discontinued study drug, for patients with little or no wound closure or incomplete wound closure, and for patients with complete wound healing are described below.

Patients Withdrawn from the Study with Study Drug Discontinuation
Patients withdrawn from the study with study drug discontinuation were to undergo an Early-Termination Visit (i.e., End-of-Treatment Visit procedures). At the discretion of the Investigator, the patient could be followed for safety (e.g., unscheduled visits). The patients were to return for a Follow-Up Visit two (2) weeks later and undergo all Follow-Up procedures. The patient would be followed for safety (i.e., unscheduled visits) until complete re-epithelialization occurs, if applicable.
Patients with Little or No Wound Closure or Incomplete Wound Closure

Patients defined at Visit 10 (Study Day 7) as either having had little or no wound closure or incomplete wound closure were to be followed for safety and efficacy if a contact bandage lens was not applied. As such, the procedures for patients with little or no wound closure or incomplete wound closure during Visits 11, 12, and 13 (Study Days 9, 11, and 13) included:

- Ophthalmologic assessments (per site Standard of Care)
- Examination of wound for signs of healing
- Examination of application area for any abnormalities (Application Site Abnormalities)
- A complete ophthalmologic examination including SLE
- Assessment of inflammation (cells and flare) by SLE graded by Nussenblatt scale
- Documentation of wound appearance by photography in conjunction with SLE and fluorescein eye drops
- Wound surface area documented by digital photography

In addition, the following data was recorded:

- Concomitant medications
- Adverse events (AEs)
- Time to use of bandage contact lens, if applicable
- Time to rescue medication, if applicable
- Dosing and Standard of Care adherence
• Review instructions for study drug application and Standard-of-Care procedures, as necessary.

All patient data was recorded in the Source Documents and/or the CRF, as appropriate. The staff member collected open squeeze bottles of study drug returned by the patient. On Visit 13 (Study Day 13), the patient was scheduled for the End-of-Treatment Visit (Visit 14, Study Day 14) and instructed to return all used and unused study drug bottles to the investigative site on Visit 14 (Study Day 14) in the pharmacy cooler bag containing an ice pack.

**Patients with Complete Wound Closure (Complete re-epithelialization)**

The procedures for patients with complete wound healing prior the End of Treatment (Study Day 14) Visit continued to receive study drug and were followed for safety. Complete wound healing (*i.e.*, epithelial closure) is defined as the absence of fluorescein-positive areas. As such, the procedures for patients with complete wound healing during Visits 11, 12, and 13 (Study Days 9, 11, and 13) included:

The following ophthalmologic assessments were performed:

• Examination of application area for any abnormalities (Application Site Abnormalities)

• A complete ophthalmologic examination including SLE

• Assessment of inflammation (cells and flare) by SLE graded by Nussenblatt scale

In addition, the following data were recorded:

• Concomitant medications
• Adverse events (AEs)

• Time to rescue medication, if applicable

• Review instructions for study drug application and Standard-of-Care procedures, as necessary.

• On Visit 13 (Study Day 13), the patient was scheduled for the End-of-Treatment Visit (Visit 14, Study Day 14) and instructed to return all used and unused study drug squeeze bottles to the investigative site on Visit 14 (Study Day 14) in the pharmacy cooler bag containing an ice pack.

All patient data were recorded in the Source Documents and/or the CRF, as appropriate.

End-of-Treatment Visit 14 (Study Day 14) [Early-Termination Visit]

At the End-of-Treatment Visit (Visit 14, Study Day 14), a member of the study staff applied one last dose of study drug for pharmacokinetic purposes (evaluation of blood samples for plasma Tß4 levels). The following pharmacokinetic procedure were performed:

• a blood sample was drawn immediately prior to study drug administration

• 2 drops of study drug were administered to the target eye

• blood samples were drawn 20, 40, and 60 minutes after study drug administration.

In addition, all patients attending the End-of-Treatment Visit will underwent the following ophthalmologic assessments per the site’s Standard of Care for each procedure:

• Examination of wound for signs of healing

• Examination of application area for any abnormalities (Application Site Abnormalities)
• Central corneal thickness, as measured by ultrasound pachymetry. Central corneal thickness was assessed in both the operative (i.e., target) and the non-operative eye.

• Best-corrected visual acuity by ETDRS Chart

• Intraocular pressure measurement, via Tono-Pen

• A complete ophthalmologic examination including SLE

• Fundus examination

• Assessment of inflammation (cells and flare) by SLE graded by Nussenblatt scale

• Documentation of wound appearance by photography in conjunction with SLE and fluorescein eye drops

• Wound surface area was documented by digital photography

In addition, the following data were recorded:

• Physical examination and vital signs (vital signs are to be taken after the patient has been in either supine or sitting position for no less than 5 minutes)

• ECG

• Clinical chemistry, hematology, and coagulation tests

• Urinalysis

• Concomitant medications

• Adverse events (AEs)

• Time to use of bandage contact lens, if applicable

• Time to rescue medication, if applicable

• Dosing and Standard of Care adherence.

All patient data were recorded in the Source Documents and/or the CRF, as appropriate.
The staff member collected all used and unused study drug bottles in the patient's possession, as necessary. (The patient was to return all unused study drug bottles to the investigative site in the pharmacy cooler bag containing an ice pack.) If the patient terminated or was withdrawn from the study prior to Study Day 14, he/she underwent the End of Treatment assessments as described in this Section. All patients, including those who were terminated or withdrawn early, were scheduled for the Follow-Up Visit (Visit 15, Study Day 28).

**Follow-Up Visit 15 (Study Day 28)**

All randomized patients were to return for the Follow-Up Visit (Study Day 28). The following ophthalmologic assessments were performed per the site’s Standard of Care for each patient:

- Examination of wound for signs of healing
- Examination of application area for any abnormalities (Application Site Abnormalities)
- Best-corrected visual acuity by ETDRS Chart
- Central corneal thickness, as measured by ultrasound pachymetry. Central corneal thickness was assessed in both the operative (*i.e.*, target) and the non-operative eye
- A complete ophthalmologic examination including SLE
- Assessment of inflammation (cells and flare) by SLE graded by Nussenblatt scale
- Documentation of wound appearance by photography in conjunction with SLE and fluorescein eye drops
• Wound surface area was documented by digital photography

In addition, the following procedures were performed:

• Blood collection for antibodies to Tβ4

• A serum pregnancy tests for patients who may be able to become pregnant

• The staff member collected all used and unused study drug bottles in the patient's possession, as necessary. (The patient was to return all unused study drug squeeze bottles to the investigative site in the pharmacy cooler bag containing an ice pack).

In addition, the following data were recorded:

• Concomitant medications

• Adverse events (AEs)

• Time to rescue medication, if applicable

• Dosing and Standard of Care adherence.

When patients completed the Follow-Up visit, they were considered to have completed the study. However, patients with unresolved serious or study-related AEs were to be followed until the AE resolved or was considered stable, even if this extended beyond the time of the Follow-Up visit. Patients whose corneas had not completely re-epithelialized by the end of the study were to continue to be followed for safety until complete re-epithelialization occurred, even if this extended beyond the time of the Follow-Up visit (i.e., unscheduled visits).

d. Exit Rules

All patients had the right to withdraw from the study at any time, for any reason, without
prejudice. *Withdrawn patients were not to be replaced.* Patients were not required to state their reasons for withdrawing consent. If a patient was withdrawn from participation in the study at any time at his/her request, at the investigator’s discretion, or at the sponsor or CRO’s request, the reason(s) for withdrawal was to be documented as thoroughly as possible in the source documents and CRFs. If there was more than one reason for withdrawal, one reason will be shown in the CRF as the primary reason and the others were to be shown as secondary reasons. Reasons for which a patient would be withdrawn from the study included:

- Corneal epithelial debridement not performed
- Failure to satisfy any one of the eligibility requirements (inclusion or exclusion)
- Use of a bandage contact lens because of epithelial sloughing or haze development at wound base
- Use of a rescue medication
- Discretion of Investigator
- Study termination for clinical or administrative reasons.

Reasons for which a patient could be withdrawn from the study included:

- Serious or severe adverse event(s)
- Violation of the protocol or deviation from the treatment plan specified in the protocol (*e.g.*, incorrect administration of study drug, failure to attend study visits)
- Use of a bandage contact lens because of little or no wound closure at Study Day 7 (Visit 10)

All randomized patients who were withdrawn prematurely and who received at least 1
dose of study drug were to undergo all End-of-Treatment and Follow-Up assessments. If any patient was to withdraw his or her consent and stopped using the study drug, the patient was to undergo an immediate Early Termination Visit to be considered an End-of-Treatment Visit in terms of procedures to be performed and the data to be captured. The patient was to be requested to return for a Follow-Up Visit on Day 28. If a patient was withdrawn because of an AE, the patient was to be followed until the AE resolved or the patient was clinically stable. If a patient was withdrawn because of the use of a bandage contact lens or any rescue medication, the patient was to have an Early-Termination Visit (i.e., End-of-Treatment Visit procedures as described in Section 8.9). At the discretion of the Investigator, the patient could be followed for safety (e.g., unscheduled visits). The patient was to return for a Follow-Up Visit two (2) weeks later and undergo all Follow-Up procedures. The patient was to be followed for safety (i.e., unscheduled visits) until complete re-epithelialization occurred, if applicable.

e. Stopping Rules

Safety data was to be reviewed after all patients in Group 1 had received treatment for 14 days and again after all patients in Group 2 had received treatment for 14 days. A review of safety data could also have been performed at any time during the study. Medical decisions relevant to the study were made by the Sponsor, in consultation with the Primary Investigator, the Scientific Coordinating Investigator, and the Medical Monitor. Reviews of the safety data were performed using masked data (data was to be summarized for the overall group only and was not to be separated by treatment groups).

Under the following circumstances, enrollment was to be stopped immediately, but
patients already enrolled could continue to receive study drug.

- Occurrence of 3 or more adverse events (AEs) all assessed as serious and possibly, probably, or definitely related to study drug, within the same system organ class (SOC).
- Occurrence of 2 or more nonfatal, non-life-threatening SAEs assessed as possibly, probably, or definitely related to study drug.

Safety data would be evaluated. Based on this evaluation, the decision would be made either to re-open enrollment or to enroll no more patients.

Under the following circumstances, enrollment was to be stopped immediately, and patients already enrolled were to stop receiving study drug:

- Occurrence of AEs, all assessed as serious and possibly, probably, or definitely related to study drug and within the same SOC, in more than half of the patients currently enrolled in the group currently receiving the highest dose.
- Occurrence of 1 or more fatal or life-threatening SAE assessed as possibly, probably, or definitely related to study drug.

Safety data was to be evaluated. Based on this evaluation, the decision would be made either to re-open enrollment and resume treatment of enrolled patients or to terminate the study.

Other conditions that could have warranted termination included, but were not limited to:

- Any circumstances that presented an unexpected, significant, or unacceptable risk to patients enrolled in the study, such as infection and sloughing of the corneal epithelium.
• Decision by the Sponsor to suspend or discontinue development of Tβ4

• Failure to enroll an adequate number of patients.

For safety monitoring purposes, patients who were randomized at the time of early termination and had received study drug were to complete an Early Termination visit. Under these circumstances, all procedures described for the Follow-Up visit were to be performed at the Early Termination visit for those patients terminating early from the study, regardless of termination reason.

V. Statistical Methods

a. Sample size calculations (based on primary objective)

i. Which adverse events were sought

A number of adverse events can occur with the application of any topical ophthalmic medication. Disastrous events such as corneal melting and perforation can occur, but are considered to be extremely rare (less than 1 in 1,000 chance). Corneal infections can also occur in this setting, but occur at a rate of less than 1 in 100. The most common adverse event that can occur in this setting would be corneal epithelial toxicity from the topically applied medication. This is particularly relevant for a medication that is being used for epithelial healing. As such, we chose to use the enlarging of the epithelial defect being treated as a proxy for local toxicity as the primary AE of interest in the Thymosin study.

ii. Incidences of such adverse events

From a sample size calculation standpoint, the rate of enlarging of the epithelial defect temporally associated with the use of Tβ4 (thus being a proxy for local toxicity) was
initially used for calculations. In the investigator’s opinion, it was estimated that
enlargement of non-healing epithelial defects in diabetic eyes which have just undergone
epithelial debridement during vitrectomy surgery due to toxicity from topical medications
occurs in approximately 25% of eyes.

**iii. Required sample size to detect these rates**

Thus, having 9 eyes in each treatment dose group would allow us to expect to see 2 eyes
(22.2%) in which local toxicity was seen as a result of the study drug. Any more cases of
toxicity seen beyond 2 could suggest a safety issue. As such, if 3 (33.3% of eyes) such
treatment related AE’s were seen at any of the treatment doses, then the study would be
halted to evaluate for safety.

**iv. Power of the final sample for detecting the adverse events**

No formal power calculations were used for determining the sample sizes in this study
beyond the suppositions above.

**b. Analysis methods**

Descriptive summaries by treatment group were provided for safety and efficacy
parameters. For categorical variables, summaries included the number and percentage in
each category. For continuous variables, summaries included descriptive statistics such as
mean, median, standard deviation, minimum, and maximum.
In general, there were no imputations for missing data. Baseline values for all parameters
were derived from the most recent observation recorded before study drug application.
For example, if an assessment was performed at Visit 2 but not at Visit 3, then the screening observation served as the baseline value for the purpose of comparison with final outcomes.

**i. Safety analysis**

The intent-to-treat population was analyzed for drug safety. The number of AE’s and SAE’s were analyzed.

**ii. Efficacy analysis**

Summary statistics for observed values of wound surface area and for epithelial healing from baseline were computed by visit, and corresponding mean time-response curves for epithelial healing from baseline were drawn for each treatment group. For epithelial healing, 2 regression models were fitted, one with epithelial healing as a categorical variable (healed versus not healed) and the other with epithelial healing as a continuous variable (percentage healed). Where possible, all statistical models were adjusted for the effects of study center, which was included in the statistical model as a categorical class variable.

No interim analyses for efficacy were performed. For the final analyses, a subset of the original analyses focusing primarily on safety measures and secondarily on efficacy measures were performed.

Two post hoc investigations were conducted: an additional Kaplan-Meier analysis based on the investigator’s measurement of the wound and a simple linear regression of time to healing versus screening glycosylated hemoglobin level.
iii. Exploratory endpoint analysis

For categorical variables, summaries included the number and percentage in each category. For continuous variables, summaries included descriptive statistics such as mean, median, standard deviation, minimum, and maximum.

VI. Results

Due to slow patient enrollment and financial reasons, the study was closed after the first 12 patients were enrolled in the lowest treatment dose. The results presented here represent only these 12 eyes that were randomized (out of 25 enrolled) in the lowest treatment dose of Tβ4. Baseline demographics are listed in Table 2.

Table 2: Baseline demographics of randomized patients.

<table>
<thead>
<tr>
<th></th>
<th>Tβ4 0.01% (n = 9)</th>
<th>Placebo (n =3)</th>
<th>Total (N = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>9</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>51.4 (15.00)</td>
<td>43.3 (3.06)</td>
<td>49.4 (13.37)</td>
</tr>
<tr>
<td>Median</td>
<td>49.0</td>
<td>44.0</td>
<td>45.0</td>
</tr>
<tr>
<td>Minimum, maximum</td>
<td>28, 77</td>
<td>40, 46</td>
<td>28, 77</td>
</tr>
<tr>
<td>Sex, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>5 (55.6)</td>
<td>1 (33.3)</td>
<td>6 (50.0)</td>
</tr>
<tr>
<td>Female</td>
<td>4 (44.4)</td>
<td>2 (66.7)</td>
<td>6 (50.0)</td>
</tr>
<tr>
<td>Race, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>African-American (not Hispanic or Latino)</td>
<td>9 (100.0)</td>
<td>3 (100.0)</td>
<td>12 (100.0)</td>
</tr>
<tr>
<td>Iris color, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brown</td>
<td>9 (100.0)</td>
<td>3 (100.0)</td>
<td>12 (100.0)</td>
</tr>
<tr>
<td>Type of diabetes, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 1</td>
<td>2 (22.2)</td>
<td>1 (33.3)</td>
<td>3 (25.0)</td>
</tr>
<tr>
<td>Type 2</td>
<td>7 (77.8)</td>
<td>2 (66.7)</td>
<td>9 (75.0)</td>
</tr>
<tr>
<td>Target eye, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>4 (44.4)</td>
<td>0</td>
<td>4 (33.3)</td>
</tr>
<tr>
<td>Right</td>
<td>5 (55.6)</td>
<td>3 (100.0)</td>
<td>8 (66.7)</td>
</tr>
</tbody>
</table>
Measurements of Treatment Compliance

Treatment compliance was calculated as the number of doses taken divided by the number of prescribed doses taken, multiplied by 100. Mean (SD) treatment compliance was 95.2% (6.56%) for all patients, 96.4% (5.42%) for patients receiving Tβ4, and 91.5% (9.58%) for patients receiving placebo. These figures were based on analyzing the returned single-use medication vials.

a. Safety data

No patients died and no SAEs occurred in this study. One patient in the Tβ4 treatment group was discontinued from treatment because of a moderate AE of increased IOP that was not believed to be related to the study treatment. Although AEs were reported for a greater proportion of patients receiving Tβ4 than for those receiving placebo, no AEs were considered probably or definitely related to study treatment. Notable differences between treatment groups were a greater percentage of patients in the Tβ4 treatment group who experienced AEs of eye pain, increased IOP, and headache compared with patients in the placebo treatment group. A lower percentage of patients in the Tβ4 treatment group experienced AEs of anterior chamber cell, anterior chamber flare, corneal disorder, corneal edema, and corneal erosion compared with patients in the placebo treatment group (Table 3).

For the ophthalmological examination, no notable differences with respect to treatment groups were observed: no notable changes from baseline to Day 14 were
observed in the eyelid, conjunctiva, cornea, lens, vitreous, or fundus examination results for target eyes. Postsurgical increases from baseline in mean anterior chamber cell and anterior chamber flare were observed for patients in both treatment groups; however, by Day 28, values for both were decreased from baseline.

Visual acuity data were available for the target eyes of 6 patients. Among 4 patients who received Tβ4, changes from baseline to Day 28 in visual acuity were as follows: 1 patient showed a 1-line decrease in visual acuity, 1 patient showed no change in visual acuity, 1 patient showed a 1-line increase in visual acuity, and 1 patient showed a 4-line increase in visual acuity. Among 2 evaluable patients who received placebo, changes from baseline to Day 28 in visual acuity were comprised of 1 patient with a 7-line decrease in visual acuity and 1 patient with a 3-line increase in visual acuity.

Increases from baseline in mean IOP for target eyes were greater for the Tβ4 treatment group than for the placebo treatment group at all time points assessed. Mean (SD) change from baseline in IOP for target eyes among patients in the Tβ4 treatment group was greatest on Day 1 (10.0 [12.12] mm Hg) and trended downward to Day 14 (4.9 [10.43] mm Hg). Among patients in the placebo treatment group, mean change from baseline in IOP for target eyes was below baseline at each assessment.

No application site infections were observed during the study. Epithelial defects were observed in all patients immediately after surgery, all of which were healed by Day 11 among patients receiving Tβ4 and by Day 4 among patients receiving placebo. At last follow-up (Day 28), a moderate AE of corneal epithelial defect was reported for 1 patient in the Tβ4 treatment group. This event resolved and was considered not related to study treatment. Inflammation ranging from 1+ to 3+ cell and 0 to 3+ flare was noted for the
majority of eyes immediately after vitrectomy, but decreased in the postoperative period. At the Day 28 follow-up visit, no inflammation was noted for 10 study eyes, and either trace or 1+ inflammation were noted for 1 patient each.

Abnormal laboratory test results were expected in this patient population. Most of the clinically significant abnormal test results for any specific patient were reported both before and after the patient began receiving study treatment and could be attributed to the patient’s known medical condition. Overall, no notable changes from baseline in laboratory assessments were observed. The glycosylated hemoglobin levels (HgA1C) versus time to healing in both groups are listed in Table 4. The mean HgA1C level of the Tβ4 group was 8.9 compared to the placebo group which was 8.0 (p=0.10).

With respect to physical examination, compared with baseline, the results of physical examination at EOT were either the same or improved for each treatment group. For vital signs, changes from baseline were minimal for the Tβ4 treatment group, but were noted for systolic and diastolic blood pressure (decrease) as well as pulse (slight increase) for the placebo treatment group. A difference between treatment groups was also noted in mean change from baseline in ECG QT interval and corrected QT interval results; however, interpretation of these findings is difficult given the small size of the patient population. Of note, 11 of the 12 patients receiving treatment had a medical history of hypertension.

Issues with drug stability arose during the study. Lot Number 1340319 was found to be out of specification at the 18-month evaluation (87.6% of label claim) and Lot Number 1340378 was found to be out of specification (83.6% of label claim) at the 9-month evaluation. The test specification for Tβ4 is 90% to 110% of label claim. Six
patients may have received subpotent drug, 4 from Lot Number 1340319 and 2 from Lot Number 1340378. One patient received and completed treatment with Tβ4 0.01% from Lot Number 1340378 that was known to be subpotent. No AEs related to study drug were reported for this patient. Since the companion placebo lots were within specifications and any out of specification test product received was subpotent, no safety implications were anticipated.

Table 3. Adverse events reported

<table>
<thead>
<tr>
<th>Adverse events</th>
<th>Tβ4 0.01% (n = 9)</th>
<th>Placebo (n = 3)</th>
<th>Total (N = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eye disorders</td>
<td>9 (100.0%)</td>
<td>3 (100.0%)</td>
<td>12 (100.0%)</td>
</tr>
<tr>
<td>Anterior chamber cell</td>
<td>6 (66.7%)</td>
<td>3 (100.0%)</td>
<td>9 (75.0%)</td>
</tr>
<tr>
<td>Anterior chamber flare</td>
<td>6 (66.7%)</td>
<td>3 (100.0%)</td>
<td>9 (75.0%)</td>
</tr>
<tr>
<td>Conjunctival haemorrhage</td>
<td>7 (77.8%)</td>
<td>2 (66.7%)</td>
<td>9 (75.0%)</td>
</tr>
<tr>
<td>Conjunctival oedema</td>
<td>6 (66.7%)</td>
<td>1 (33.3%)</td>
<td>7 (58.3%)</td>
</tr>
<tr>
<td>Punctate keratitis</td>
<td>5 (55.6%)</td>
<td>2 (66.7%)</td>
<td>7 (58.3%)</td>
</tr>
<tr>
<td>Corneal disorder</td>
<td>4 (44.4%)</td>
<td>2 (66.7%)</td>
<td>6 (50.0%)</td>
</tr>
<tr>
<td>Corneal oedema</td>
<td>4 (44.4%)</td>
<td>2 (66.7%)</td>
<td>6 (50.0%)</td>
</tr>
<tr>
<td>Eye pain</td>
<td>4 (44.4%)</td>
<td>0</td>
<td>4 (33.3%)</td>
</tr>
<tr>
<td>Corneal erosion</td>
<td>2 (22.2%)</td>
<td>1 (33.3%)</td>
<td>3 (25.0%)</td>
</tr>
<tr>
<td>Eyelid oedema</td>
<td>3 (33.3%)</td>
<td>0</td>
<td>3 (25.0%)</td>
</tr>
<tr>
<td>Anterior chamber fibrin</td>
<td>1 (11.1%)</td>
<td>1 (33.3%)</td>
<td>2 (16.7%)</td>
</tr>
<tr>
<td>Corneal staining</td>
<td>4 (44.4%)</td>
<td>2 (66.7%)</td>
<td>6 (50.0%)</td>
</tr>
<tr>
<td>Intraocular pressure increased</td>
<td>3 (33.3%)</td>
<td>0</td>
<td>3 (25.0%)</td>
</tr>
<tr>
<td>Gastrointestinal disorders</td>
<td>2 (22.2%)</td>
<td>0</td>
<td>2 (16.7%)</td>
</tr>
<tr>
<td>Nausea</td>
<td>1 (11.1%)</td>
<td>0</td>
<td>1 (8.3%)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>1 (11.1%)</td>
<td>0</td>
<td>1 (8.3%)</td>
</tr>
<tr>
<td>Nervous system disorders</td>
<td>2 (22.2%)</td>
<td>0</td>
<td>2 (16.7%)</td>
</tr>
<tr>
<td>Headache</td>
<td>2 (22.2%)</td>
<td>0</td>
<td>2 (16.7%)</td>
</tr>
<tr>
<td>Total number of TEAEs</td>
<td>74</td>
<td>21</td>
<td>95</td>
</tr>
<tr>
<td>Number of patients with at least 1 AE</td>
<td>9 (100.0%)</td>
<td>3 (100.0%)</td>
<td>12 (100.0%)</td>
</tr>
</tbody>
</table>
Table 4: Screening glycosylated hemaglobin levels versus time to healing

<table>
<thead>
<tr>
<th>Patients</th>
<th>Screening glycosylated hemoglobin level (%)</th>
<th>Time to healing (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.5</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>9.2</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>9.6</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>7.0</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>9.1</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>7.7</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>10.3</td>
<td>11</td>
</tr>
<tr>
<td>8</td>
<td>7.3</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>9.8</td>
<td>6</td>
</tr>
<tr>
<td>10 (placebo)</td>
<td>7.4</td>
<td>2</td>
</tr>
<tr>
<td>11 (placebo)</td>
<td>7.5</td>
<td>2</td>
</tr>
<tr>
<td>12 (placebo)</td>
<td>9.0</td>
<td>4</td>
</tr>
</tbody>
</table>

b. Efficacy data

The efficacy objective of this study was to evaluate the effectiveness of Tβ4 ophthalmic solution in healing corneal wounds resulting from epithelial debridement during vitrectomy. Endpoints for assessment of this objective were the incidence of healing, time to complete healing, and percent change in wound surface area.
Interpretation of efficacy results is limited by the small number of patients who received study treatment (underpowered).

\[ i. \text{Incidence of Healing} \]

With respect to efficacy assessments, no superiority of Tβ4 treatment in wound healing was observed for percent change in wound surface area at the treatment endpoint, or for area under the curve (AUC) of percent change in wound area from baseline to Day 7 and to Day 14, as compared with placebo. All patients’ wounds remained healed through the Day 14 EOT visit; however, a moderate AE of corneal epithelial defect was reported at the posttreatment follow-up visit for 1 patient in the Tβ4 treatment group. This event was considered not related to study treatment. Consequently, the incidence of wound healing at the Day 28 follow-up visit was 97.7% (11 of 12 patients) overall: 88.9% (8 of 9 patients) in the Tβ4 treatment group, and 100.0% (3 of 3 patients) in the placebo group. Due to the small number of patients in the statistical model, the logistic analysis was not able to provide estimates of the inferential statistics at the treatment endpoint (Day 14) and at each treatment day.

\[ ii. \text{Time to Complete Healing} \]

Kaplan-Meier Analysis

The time to complete healing (complete re-epithelialization) of the wound was defined in days and calculated as the date of the first study visit on which epithelial closure was achieved minus the date of vitrectomy (Visit 3). Wound-healing time was longer for patients receiving Tβ4 (all 9 patients healed within 11 days) than for patients
receiving placebo (all 3 patients healed within 4 days) based on the investigator’s assessment of the wound \((P=0.05)\). However, after unmasking of the data, it was noted that the wounds of 2 patients were marked "healed" in the CRF 1 day after the investigator’s wound measurement was zero when rounded to 1 decimal place. A post hoc Kaplan-Meier analysis based on the new definition of wound healing showed no statistically significant \((P=0.09)\) difference between treatment groups for time to complete healing. Figure 2 presents a graphic display of the post hoc Kaplan-Meier curve for time to complete healing.

Figure 2: Kaplan-Meier Analysis (post-hoc) demonstrating the duration of days to complete epithelial healing in each group. Log-rank \(P\)-value: 0.09.
iii. Percent Change in Wound Surface Area

Calculations based on the central reader and investigator assessments yielded similar results: mean percent change in wound area at any visit was never greater for patients receiving Tβ4 treatment than for patients receiving placebo treatment. Mean percent change in wound area from baseline for the Tβ4 treatment group compared with the placebo treatment group was either lower or equal on all days assessed for both the central reader and investigator assessments. The statistically significant difference between treatment groups observed on Day 1 (Visit 4) for analyses on data from both the central reader and investigator assessments, favored placebo ($P=0.01$ and $P=0.02$, respectively).

Specifically, for percent change in wound area from baseline to Day 7 and Day 14, no statistically significant differences between treatment groups were observed for results based on either central reader assessments or investigator assessments (Table 5).
Table 5. Percent healing of epithelial defect from baseline to 7 and 14 days.

<table>
<thead>
<tr>
<th>Patient</th>
<th>% healing at 7 days</th>
<th>% healing at 14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>2</td>
<td>84%</td>
<td>100%</td>
</tr>
<tr>
<td>3</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>4</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>5</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>6</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>7</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>8</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>9</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>10 (placebo)</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>11 (placebo)</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>12 (placebo)</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

c. Exploratory data

i. Central Corneal Thickness

Central corneal thickness was assessed at baseline, Day 7, Day 14, and Day 28. The overall mean (SD) change from baseline to Day 14 in central corneal thickness of the target eye was greater for patients in the Tß4 treatment group (40.3 [66.29] μm) than for patients in the placebo treatment group (10.7 [30.44] μm). A similar difference between
treatment groups was observed at the Day 28 follow-up visit (Tβ4: 31.6 [52.15] μm; placebo: 6.7 [42.67] μm).

ii. Inflammation

Inflammation ranging from 1+ to 3+ cells and 0 to 3+ flare was noted for the majority of eyes immediately after vitrectomy, but decreased in the postoperative period. At the Day 28 follow-up visit, no inflammation (cell and flare) was noted for 10 target eyes (83.3%), and either trace or 1+ inflammation (cell and flare) was noted for 1 patient (8.3%) each. No readily apparent difference between treatment groups was noted. Other application site abnormalities were noted for 4 (33.3%) to 8 patients (66.7%) on any particular study day and for 5 patients (41.7%) on Day 28.

VII. Discussion

Phase 2 studies are the first controlled clinical studies of a drug, and the primary objectives of these studies are to evaluate both the efficacy of a drug based on defined clinical endpoints, as well as to determine the dosing ranges, the common short-term side effects, and the risks associated with the drug. With regard to dosing ranges specifically, phase 2 studies aim to identify the minimum effective and maximum tolerable doses of the therapeutic agent under study. Clinical studies designed to evaluate dosing are referred to as phase 2A studies whereas studies designed to determine the effectiveness of a drug are phase 2B studies.31

Under the assumption of linear pharmacokinetics of the study drug, dose-ranging phase 2 studies usually include at least 3 doses. Since these dose-ranging studies are
usually being conducted at a stage where the efficacy of the study drug has not yet been established, it is also important to include a placebo control to provide an estimate of the absolute efficacy for each dose, in addition to the dose-response relationship.\textsuperscript{31}

Although the 3-6 patients per dose level seen in dose-ranging, conventional toxicity studies is feasible, these small sample sizes are only sufficient to exclude high toxicity rates.\textsuperscript{32} In the case of our study, this was both a phase 2A (dosing) and phase 2B (efficacy) study. Given our primary interest in safety, and specifically surface toxicity, we designed the study such that we would halt it if more than 25% of the eyes developed what appeared to be surface toxicity. As such, if 3 or more of the 9 eyes (33%) at each of the treatment doses in the non-placebo arm of the study developed toxicity, the study would be halted. However, we did not set this sample size to detect other, rarer complications such as infection or corneal melting. In addition, we did not calculate the sample size with specific consideration for efficacy. Finally, our calculations were based on the raw number of events only, hoping to detect a higher than expected number of events without specific consideration for statistical significance.

a. Comparision of a priori and post-hoc power and sample size considerations

In retrospect, as a phase 2A and 2B study, despite our calculations for sample size with regard to toxicity, we should have also considered calculating the sample size such that there would be adequate power to determine efficacy.

i. New power and sample size calculations

1. broadening the range of possible AE’s
The current dosing of the study was that of a traditional phase II study to evaluate safety, here in the form of ocular surface toxicity. Given our estimated high rate of this AE in topical medications (25%), a relatively small sample size was used. However, if we were to have wanted to explore the other less common possible side effects of the study medication, then this would have resulted in one of the larger phase II studies that may have involved several hundred patients, or even possibly resulted in an expanded Phase II study that involved several thousand patients.\textsuperscript{31}

For example, if we were to have looked at infection as a possible AE, then assuming a rate of infection of 1%, we would have needed at least 100 patients in each dose level of the study arms to see a higher than expected rate of infection. For a rare complication such as corneal perforation which occurs in 1 in 500 eyes, we would have needed 500 eyes in the study group at each dosing level. (Table 6) This would have required 2,000 eyes to complete enrollment in this study. Given that our previous annual estimate of cases of persistent epithelial defects in this setting was estimated at 10,875 to 23,055 corneas nationwide, a trial (though multicenter) needing to recruit 2,000 patients would be impractical. Thus, it would have been impractical to run the trial.

While the argument could then be made to broaden the indications for persistent epithelial defects to include not just those who are post-diabetic vitrectomy surgery, this would have created more statistical issues with different variables for which to account.

In addition, phase II studies are typically highly controlled and use highly defined (narrow) patient groups so that extraneous variation is kept to a minimum.\textsuperscript{33}
Table 6. Sample sizes required for the study based on estimated rate of occurrence of AE.

<table>
<thead>
<tr>
<th>Estimated rate of occurrence of AE</th>
<th>Sample size required to detect a higher than expected number of events</th>
<th>Total sample size required including 3 dosing levels and placebo group</th>
</tr>
</thead>
<tbody>
<tr>
<td>25%</td>
<td>9</td>
<td>36</td>
</tr>
<tr>
<td>1%</td>
<td>100</td>
<td>400</td>
</tr>
<tr>
<td>0.2%</td>
<td>500</td>
<td>2,000</td>
</tr>
</tbody>
</table>

2. efficacy as the primary outcome

If efficacy were to have been used as another component of determining the sample size to have adequate power, and if we would have measured efficacy as the percentage of healing of the epithelial defect by day 14, then the sample size would have needed to be larger than what we had, but still smaller than if we choose to look at rarer AE’s. If we assume a standard deviation of 46% around the endpoint of complete epithelial healing at 2 weeks, then in a dose-escalation study with 3 levels, we would need 14 eyes per treatment arm, and therefore 64 total eyes (3 dosages plus placebo) to provide 80% power at the 5% significance level (two-sided) to detect a 50% difference in the healing of the epithelium from baseline to day 14. As the percent difference in the healing that is to be detected decreases, then the sample size increases. (Table 7)
Table 7. Sample sizes required based on desired percent difference to be detected in the healing of the epithelium assuming 80% power at the 5% significance level (two-sided).

<table>
<thead>
<tr>
<th>Percent difference to be detected</th>
<th>Sample size per arm</th>
<th>Total sample size required</th>
</tr>
</thead>
<tbody>
<tr>
<td>50%</td>
<td>14</td>
<td>64</td>
</tr>
<tr>
<td>45%</td>
<td>17</td>
<td>76</td>
</tr>
<tr>
<td>40%</td>
<td>21</td>
<td>84</td>
</tr>
<tr>
<td>35%</td>
<td>28</td>
<td>112</td>
</tr>
<tr>
<td>30%</td>
<td>37</td>
<td>148</td>
</tr>
<tr>
<td>25%</td>
<td>54</td>
<td>216</td>
</tr>
<tr>
<td>20%</td>
<td>84</td>
<td>336</td>
</tr>
</tbody>
</table>

**ii. New sample size**

If we considered efficacy in our sample size calculations, then assuming the parameters outlined in the above section, we would have needed to recruit a total of 64 subjects for this study, based on the above calculations to detect a difference of 50% in the healing of the epithelium. If we would have considered giving a conservative allowance of 15% potential loss to follow up (which we did not do in our original protocol), then we would need 17 eyes in each arm and 76 eyes total. This figure would have powered the study for both safety and efficacy at the above described parameters.
b. Challenges of designing a multicenter study

Because of the limitation in the capacity of a single site, as well as a desire to increase patient enrollment and therefore shorten the duration of the study, most clinical trials are designed as multicenter studies. However, there are some difficulties that can be associated with a multicenter trial. Particularly in sponsored multicentered studies, if enrollment is slow, the sponsor may decide to close slower-enrolling sites, increase enrollment for the more aggressively recruiting sites, open new site. Each of these actions can introduce bias to the study. In addition, the sponsor may decide to ship unused study medication from closed sites to other sites, which can increase the chance of mixing up the randomization schedules, introducing yet another possible source of bias.\textsuperscript{31}

Furthermore, the United States Food and Drug Administration (FDA) requires that treatment-by-center interaction be carefully evaluated, and no overall conclusion can be made across study sites if any interaction is present. As such, it is generally more desirable to have fewer study sites than the number of patients within each site, allowing patients to be compared within each site, as well.\textsuperscript{31}

Different centers may also have different standards for clinical evaluation and laboratory tests.\textsuperscript{31} Another challenge of executing a multicentered study is ensuring that the manual of operations is adhered to at each center. Variations in procedures and techniques, however small, can adversely affect the collection and interpretation of data, which can ultimately induce bias and affect the final results. In our study, we started with 6 centers, and later opened 6 more centers with the hope of recruiting a total of 36 patients. Thus, with an average planned recruitment of 3 patients per site, this number
violates the desirability of having fewer study sites than the number of patients within each site. By this rule, we should have only opened 5 or 6 sites. Opening the second round of 6 sites not only violated this rule, but also introduced the bias of opening new sites while the trial was open. In our study, we ended up only having 2 sites enroll patients (7 at one site, and 5 at the other). Other sites recruited patients, but they were not randomized.

i. Differences in photographic quality

In our study, the photographic quality was standardized by providing the same camera to all sites. Each investigator and coordinator was trained on how to use this camera, as well as the exact protocol to follow to take the pictures. Despite this attempt at standardization, there was still some variation in the distance from the eye that a photograph was taken, as well as the amount of fluorescein dye that was used prior to photography. Both of these variations made the collection of data, specifically the image analysis, more difficult than it should have been, and it also introduced bias.

ii. Differences in population enrolled (ethnic)

Our final enrollment composition of the patients was skewed much more dramatically towards African-Americans than would have been predicted based on epidemiology. It is estimated that African-American diabetics comprise only 19.1% (4.9 million of 25.6 million people) of all diabetics greater than 20 years of age in the United States, but in our study, all of the randomized patients were African-American. While this was not intentional, it was a result of the two centers that enrolled all of the patients.
being comprised of a predominantly African-American population. Although there is not enough data to suggest that ethnicity may have an effect on rates of corneal epithelial healing, this makes our results less generalizable to the general population.

iii. Collection of data

Collection of data requires compliance of the study coordinators at each center to collect and send the data to the reading centers. In our case, the photographs were sent to our central reading site for standardized measurement of the corneal epithelial defect for each image, as is recommended for multicentered trials. However, because of variations in following the photographic protocol at the different centers, measurement of the defects was more challenging than anticipated: some of the photos demonstrated less fluorescein dye in the eye than is necessary to allow for the central reader to visualize the epithelial defect easily.

c. Challenges to having appropriate external validity

As described above, for the most part, our sample of patients reflected the general population in that there was a wide range of diabetic pathology, including varying HgA1C levels. However, due to difficulty with enrollment at many sites, all of the patients were enrolled though two sites which had a high African-American population. Ultimately, all of the patients enrolled in the study were African-American, and as such, unfortunately, the racial demographics did not reflect the general population.

d. Effect of early termination of study on interpretation of data
Early termination of this trial by the sponsor (done for financial reasons), severely affected our ability to interpret what data we collected.

\textit{i. Inadequate sample size to make meaningful conclusions or even draw inferences}

Given that the study was underpowered to begin with, the early termination with only one-third of the anticipated enrollment (9 patients in the lowest treatment group and 3 patients in the placebo group) resulted in this study having an inadequate sample size to make any meaningful conclusions about safety or efficacy.

\textit{ii. Lack of toxicity seen at lowest drug concentration (single dose in a dose-escalation study) may not reflect that seen in higher concentrations}

The fact that no safety issues were seen in our enrolled patients could only allow us to conclude that at the lowest drug dosing, no AE’s of ocular surface toxicity were seen. However, the lack of toxicity seen at the lowest drug concentration (the lowest level of this dose-escalation study) does not reflect what may be seen at higher concentrations of the study drug. On top of this, we were plagued by the fact that on post-hoc analysis, several patients were known to have received subpotent doses of the drug.

\textit{iii. Difficulty in determining if HgA1C level was a confounder in this small sample size}

Although higher HgA1C levels reflect poorer diabetic control and are thought to be correlated with more diabetes-associated complications, this variable was not controlled
for in our study. The mean HgA\textsubscript{1C} levels of the Tβ4 group (8.9%) and the placebo group (8.0%) were not statistically significant (p=0.1), but there definitely was a trend towards a difference. When linear regression analysis was performed of the two groups comparing HgA\textsubscript{1C} level to time to epithelial healing, there was no difference in the slopes of the regression lines (p=0.73) (Figure 2). This suggests that there is indeed a relationship: the higher the HgA\textsubscript{1C} level, the longer it will take an epithelial defect to heal. A larger sample size may have been able to tell us if this relationship actually existed, and if HgA\textsubscript{1C} is indeed a confounding variable.

Figure 3: Glycosylated hemoglobin level versus time to wound healing.

![Graph showing glycosylated hemoglobin level versus time to wound healing.]

e. Improvements in trial design

i. Design issues in dose escalation phase 2 studies

The design of our study has already been reviewed and critiqued above in relation to the ideal phase 2 study. While we could have more accurately powered this study for
safety (ocular surface toxicity) and efficacy, one other option would have been to power
the study for rarer AE’s to be even more stringent on evaluation of safety. However, the
large number of patients (400) that would need to be enrolled would have been
financially impossible for this particular study.

1. Stringency of entry criteria

The entry criteria for this study were admittedly very stringent, both in terms of
the indication (persistent epithelial defect) and in the specific subpopulation (diabetics
undergoing vitrectomy surgery). While the argument could then be made to broaden the
indications for persistent epithelial defects to include not just those who are post-diabetic
vitrectomy surgery, this would have created more statistical issues with different
variables for which to account. Phase II studies are typically highly controlled, as we had
it, and they use highly defined (narrow) patient groups so that extraneous variation is kept
to a minimum.33 Our main problem was that the entry criteria was so stringent, and the
population of available patients was so small, that it had negative effect on our ability to
recruit enough patients in a timely manner.

2. Accuracy of sample size calculations

Our mistakes in sample size calculations have been extensively discussed above:
our sample size calculations were incorrect, and the study was severely underpowered for
safety and efficacy.

3. Inability to assess safety of higher doses of drug if study terminated
Because one of the main points of dose-escalation studies to identify the minimum effective and maximum tolerable doses of the therapeutic agent being studied, if a dose-escalation study is terminated early, the safety (and efficacy) of the higher doses cannot be assessed. Because safety must be determined by rising doses, it is not possible to design a trial to test higher doses first, in the event that the study is terminated early.

\[iii. \textit{Threats to successful completion of clinical trials}\]

One of the major threats to successful completion of clinical trials is patient compliance. Patient compliance has an impact on the evaluability of patients for clinical evaluation of safety and efficacy. Regardless of the severity of illness, it is well-recognized that overdoing, underdoing, and erratic dosing commonly occur in all patient populations. For once daily dosing, there is approximately 87% compliance, with this rate dropping sharply to 39% compliance for 4 times daily dosing. Compliance relates to taking the drug at the prescribed dosing, whereas adherence refers to taking the drug at the scheduled times. Poor compliance may result in treatment failure and possible adverse drug reactions.

1. Difficult regimen for patients

The proposed administration of the medication was a threat to not only completion of the trial, but also to enrollment. Upon hearing the complex regimen for administration of the study medication, some patients were likely dissuaded from enrolling in the study. In addition, after enrollment and randomization, some patients found it very
difficult to adhere and comply to the medication regimen (although for the most part, adherence and compliance was good). In general, a simpler medication regimen (rather than 2 drops 4 times daily) would dissuade fewer patients from enrolling in clinical trials, and a simpler regimen could also result in better adherence and compliance with the medication regimen.

2. Intensive follow-up schedule for patients

In addition to the issues of adherence and compliance with the study medication, the intensive follow-up schedule, along with the intensive testing at each visit, were also threats to both enrollment and completion of the trial. Many patients were not interested in participating in the trial because of the frequent follow-up visits, as well as the time involved at each visit. While it may be inevitable that a significant amount of time is required for participation in clinical trials, better enrollment may be achieved if patients are promised that they will be expedited during their study visits. In addition, tangible incentives (such as meal vouchers, parking vouchers, etc…) can help keep the interest of some patients, but care must be taken to ensure that the amount of compensation offered to patients is not considered coercive.

3. High screen failure rate

On top of the difficulties with recruiting patients because of the difficult medication regimen and visit schedule, as designed, the present study also suffered from a very high screen failure rate of 52%. In practice, it is very difficult to predict which patients will ultimately need to have corneal epithelial debridement during diabetic
vitrectomy surgery, and as such, many patients who were enrolled in the study pre-operatively ultimately did not receive scrapings, and therefore did not complete the trial. The main reason this is a threat to successful completion of this clinical trial, as well as other trials, is the high cost of enrolling patients who ultimately do not get randomized.

In light of this, a better design for our study could have been to enroll and randomize patients on the first post-operative day after they had corneal epithelial debridement. Unfortunately, one drawback of this design would be that the extensive pre-operative tests that were performed at baseline to monitor for safety would be laborious for a patient who just had surgery. In addition, it could be difficult to make some assessments such as corneal endothelial cell count with a potentially edematous cornea right after surgery. Furthermore, measurements such as anterior chamber inflammation and corneal edema on the first day post-operatively are usually a function of having had surgery, and thus it would not be a good baseline measurement in this situation.

4. Lack of enthusiasm of investigators

Low enthusiasm of the investigators is a huge threat to completion of a clinical trial. In our trial, several investigators had found the high screen failure rate disappointing, and thus their enthusiasm for the trial decreased as time progressed. Consequently, enrollment at these centers suffered. For a clinical trial to be successful, all investigators must be fully committed and interested in the trial. Otherwise, enrollment will not be as robust as it could be. Regular meetings with investigators (could be by teleconference) may remind investigators to try to enroll patients in the trial.
5. Other reasons

There are many other threats to successful completion of a clinical trial including the presence of competing studies, competitive market forces, as well as lack of focus from the sponsor. At the time of our study, there was a prevailing high level of interest in the development of a product to help with corneal epithelial wound healing, and there was no other competing product available, even in clinical trials. Furthermore, our sponsor was indeed very vested and interested. However, as with most clinical trials, the financial incentive for any individual center is low, and success needs to be driven by the investigators. Unfortunately, in this study, as described above, there appeared to be a low level of enthusiasm and commitment by the investigators.

f. Potential future studies

Given the erroneous sample size calculations and assumptions, it is not possible for us to make a determination regarding neither the safety nor the efficacy of Tβ4 in the healing of diabetic wounds after epithelial debridement during diabetic vitrectomy surgery, except to say that at the lowest dose of the study medication, no evidence of ocular surface toxicity was seen. The enrollment scheme also resulted in a high screen failure rate, which drove up costs of the study, and it resulted in its early closure.

As a result of the trial’s early closure, there were not enough patients randomized to determine if HgA1C levels were a confounding variable in this study. Given the possibility that the longer healing times in the Tβ4 group may have been due to the higher baseline HgA1C levels, Tβ4 may still have potential as an efficacious treatment modality.
for persistent epithelial defects. If this compound still does hold promise from a scientific standpoint regarding healing of corneal epithelial defects, future studies should be designed to assess the safety and efficacy of this compound for treatment of corneal epithelial defects in a more common corneal condition, such as after phototherapeutic keratectomy. In addition, the dosing and follow-up schedules should be simplified, and fewer study centers should be employed. The evaluation of Tβ4 in this situation would allow for faster recruitment for more patients at fewer centers leading to completion of a dose-exalation study with less bias and better generalizability at a lower cost.

VIII. Conclusions

Our inability to determine the safety and efficacy of Tβ4 in healing diabetic corneal epithelial defect was due to the erroneous sample size calculations that were performed prior to the start of the trial. The lack of sample size calculations for efficacy resulted in a gross underestimation of the sample size required for this study. In addition, no considerations were made for possibly evaluating safety in the setting more uncommon AE’s. As such the study was underpowered from the outset, and no conclusions regarding the safety or efficacy of Tβ4 can be made from this study, especially in the light of the fact that the study was terminated prematurely.

The early termination of the study was driven by finances: the study had too many open sites and had slow enrollment. A better study design may have been able to help avert the failure of this study to be completed.
Given that the results of this study did not generate significant interpretable data, future studies evaluating Tβ4 should indeed be carried out. However, the mistakes made in this study should be carefully reviewed to avoid having the same outcome.
References


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