The Progression of Stargardt Disease Type 4 (ProgStar-4) Study
Design and baseline characteristics (ProgStar-4 Report No. 1)

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Abstract

Background/Aims: To describe the design and baseline characteristics of patients enrolled in the multicenter, prospective natural history study of Stargardt disease type 4 (STGD4).

Methods: Fifteen eligible patients aged six years and older at baseline, harbouring disease-causing variants in the *PROM1* gene and with specified ocular lesions, were enrolled. They were examined at baseline using a standard protocol, with six monthly follow-up visits for a two-year period including best-corrected ETDRS visual acuity (VA), spectral-domain optical coherence tomography (SD-OCT), fundus autofluorescence (FAF), mesopic and scotopic microperimetry (MP). Areas of definitely decreased FAF (DDAF) and questionably decreased FAF (QDAF) were outlined and quantified on FAF images.

Results: Amongst the 15 patients (29 eyes) that were enrolled at five centers in the United States and Europe, 10 eyes (34.5%) had areas of DDAF with an average lesion area of 3.2 mm^2 ± 3.5 mm^2 (range 0.36 – 10.39 mm^2) at baseline. The mean retinal sensitivity of the posterior pole derived from mesopic MP was 8.8 ± 5.8 dB.

Conclusions: Data on disease progression in *PROM1*-related retinopathy from this study will contribute to the characterization of the natural history of disease and the exploration of the utility of several modalities to track progression and therefore to potentially be used in future interventional clinical trials.

Introduction

Prominin 1 (PROM1; also known as CD133 and AC133) [1,2] encodes a 5–transmembrane domain protein containing two large, highly glycosylated extracellular loops and a cytoplasmic tail. It was first identified as a human stem cell–specific marker, but over the last decade its crucial role during the formation and organization of disks within the outer segment (OS) of photoreceptors has been recognized [2]. Recently, a new cytoplasmic role of *PROM1* in RPE function has also been described - in regulating autophagosome maturation and trafficking [3].

Mutations in *PROM1* may show extraocular manifestations in addition to retinal dystrophy such as steroid-resistant asthma, microscopic hematuria, recurrent renal infection and renal scarring [4]. There is a heterogeneity in both the inheritance pattern and clinical phenotype, with both autosomal dominant forms of *PROM1*-related retinopathy (principally p.(R373C)) [5], and autosomal-recessive forms reported [6]. Both autosomal dominant and recessive sequence variants have been associated with a wide range of clinical phenotypes (often with a bull's-eye maculopathy appearance), including isolated macular dystrophy, cone dystrophy, cone-rod dystrophy and rod-cone dystrophy [5].

At present, there are no treatments available, however several therapeutic options for inherited retinal dystrophies are in preclinical or early clinical development phases [7,8]. The preparation for future therapeutic approaches and designing appropriate clinical trials must include an understanding of the disease itself, its variability, its progression and its correlation with visual loss [9,10]. Moreover, clinical trials that aim to slow progression and/or to restore vision require validated outcome measures to prove treatment efficacy. Such an endeavor has been undertaken for *ABCA4*-related

retinopathy in the "Natural History of the Progression of Atrophy Secondary to Stargardt Disease (ProgStar)" studies; these consist of a retrospective chart review (ProgStar-1), a prospective cohort study (ProgStar-2) and an ancillary study evaluating scotopic microperimetry (scotopic assessment of rod function in Stargardt disease SMART) [9,11]. In keeping with these studies, the "Natural History of the Progression of Atrophy Secondary to Stargardt Disease type 4 (STGD4): A Prospective Longitudinal Observational Study of Stargardt Disease type 4, *PROM1*- Related Dystrophy" (ProgStar- 4 Study) has been launched in order to determine the natural history of *PROM1*-related retinopathy. Herein we describe the study design and baseline characteristics of enrolled patients.

2. PATIENTS AND METHODS

Study design & eligibility criteria

The ProgStar-4 study is a longitudinal cohort study consisting of five standardized study visits (one baseline and four follow-up visits every six months for 24 months). The time window for each visit was limited to ± 5 weeks. These include a clinical examination with refraction and best-corrected visual acuity testing, psychophysical examination by mesopic and scotopic microperimetry, and retinal imaging by fundus autofluorescence (FAF) and spectral-domain optical coherence tomography (SD-OCT).

Inclusion criteria were the following: (1) At least one well-demarcated area of atrophy in the designated study eye. The lesion size should not exceed the area to be tracked in the SD-OCT mode (20 x 20 degrees); (2) disease-causing variant(s) in the *PROM1* gene; (3) the primary study eye must have clear ocular media and adequate pupillary dilation to permit good quality FAF and SD-OCT imaging in the opinion of the investigator; (4) be

able to cooperate in performing the examinations; (5) willingness to undergo ocular examinations once every 6 months for up to 24 months; (6) minimum age of six years at baseline visit; and (7) both eyes could be included if inclusion criteria were fulfilled for both eyes.

Exclusion criteria were the following: (1) Ocular disease, such as choroidal neovascularization, glaucoma and diabetic retinopathy, in either eye that may confound assessment of the retina morphologically and functionally; (2) intraocular surgery in the primary study eye within 90 days prior to baseline visit; (3) current or previous participation in an interventional study to treat STGD4 such as gene therapy or stem cell therapy. Current participation in a drug trial or previous participation in a drug trial within six months before enrollment. The use of oral supplements of vitamins and minerals were permitted; (4) the site Principal Investigator may declare any patient at their site ineligible to participate in the study for a sound medical reason prior to the patient's enrollment into the study; (5) any systemic disease with a limited survival prognosis (e.g. cancer, severe/unstable cardiovascular disease); (6) any condition that would make adherence to the examination interfere with the patient attending their regular follow-up visits schedule of once every 6 months for up to 24 months difficult or unlikely, e.g. personality disorder, use of major tranquilizers such as Haldol or Phenothiazine, chronic alcoholism, Alzheimer's Disease or drug abuse; (7) evidence of significant uncontrolled concomitant diseases such as cardiovascular, neurological, pulmonary, renal, hepatic, endocrine or gastro-intestinal disorders.
However, there were no restrictions for visual acuity in order to be eligible for the ProgStar-4 study.

The primary objective was to assess the yearly rate of progression of STGD4 using the growth of atrophic lesions as measured by FAF imaging. The secondary objectives were (1) to assess the yearly rate of progression of STGD4 using SD-OCT to measure the rates of retinal thinning and loss of photoreceptors; (2) to assess the yearly rate of loss of retinal sensitivity as measured by mesopic and scotopic MP; (3) to assess the yearly rate of best-corrected visual acuity changes; (4) to correlate the presence and progression of morphological abnormalities in FAF and SD-OCT with visual function as measured by MP and visual acuity (VA); and (5) to perform exploratory analysis of factors associated with progression, such as e.g. the patient's smoking history.

Ethics

The study was conducted according to the ICH GCP Guidelines, the applicable regulatory requirements, the current Declaration of Helsinki and in compliance with HIPAA. Ethics committee approval was granted by the Institutional Review Board, Johns Hopkins University School of Medicine, Baltimore, U.S.A., and the local ethics committees of all participating sites. The study has been registered at www.clinicaltrials.gov (Identifier NCT02410122). All patients gave written informed consent prior to enrollment in the study.

Structural and functional retinal data

The detailed standardized protocols for FAF, SD-OCT, and mesopic and scotopic microperimetry are provided in the supplemental material. Key points are outlined in the following sections.

Tools for image acquisition and microperimetry (mesopic and scotopic)

Heidelberg Engineering® provided a custom FAF acquisition software that was formalized and deployed for exclusive use in the prospective ProgStar study [9,12]. This software can be applied on the manufacturer's commercially available confocal scanning laser ophthalmoscope (cSLO) models, such as the Heidelberg Retina Angiograph 2 (HRA2) or Heidelberg Spectralis™ and allows a laser-intensity reduction to 25%, 50% or 75% of the original laser power. The ProgStar-4 FAF acquisition protocol followed the previously published prospective ProgStar-study protocol by implementing the concept of short-wavelength reduced-illuminance autofluorescence imaging (SW-RAFI) in *ABCA4*-associated retinal dystrophies as described by Cideciyan et al [13].
Nidek® (Padova, Italy) provided the software tool "Fovea on OCT", allowing the execution of MP where the stimuli pattern is automatically centered on the anatomical fovea of a patient, after the fovea has been located using a Spectralis SD-OCT (see supplemental material) [9]. The pattern for macular sensitivity testing was performed in 68 test locations in a customized Humphrey 10-2 pattern with white Goldmann III stimuli of 200 msec duration on a white monochromatic background and a 4-2 strategy. Both aforementioned custom software tools were provided by Heidelberg Engineering® and Nidek® respectively to the participating study sites for the exclusive use in patients participating in the context of the ProgStar and ProgStar-4 studies [9].

Study organization

The overview of the organizational structure of the ProgStar-4 study is provided in figure 1. All study staff members are listed in supplemental material. Overall responsibility for the ProgStar-4 study is incumbent on the study chair. The DCC also monitored adherence to protocol and procedures, and was responsible for data analyses. It also supervises data quality apart from image quality and grading, as this

was the purpose of the reading center (Wilmer Imaging Reading Center, RC).). As the study protocols of the ProgStar-4 study significantly overlap with the previous published prospective ProgStar-2 study in *ABCA4*-related disease, clinical center staff certified for case report form completion and visual acuity measurement according to the "Early Treatment Diabetic Retinopathy Study" (ETDRS) protocols and charts used in ProgStar-2 were not required to obtain additional certification for ProgStar-4 [14]. However, a passing score (80% or higher) on a ProgStar-4 study knowledge assessment exam was required for all study coordinators. Equally, the RC grandfathered certifications for clinical center staff on the acquisition of FAF, SD-OCT images and MP. Only one site (University of Bonn, Germany) did not participate in the prospective *ABCA4*-retinopathy study and both the clinical center and clinical center staff were certified as previously described [9]. The RC had the responsibility for grading SD-OCT, FAF and MP, thereby assuring data quality in grading.

Clinical centers

Patients were recruited at five centers in the United States, United Kingdom, and Germany. A custom-built database in REDCap (http://www.project-redcap.org/cite.php) served at a central database in which all data were entered and checked for completeness and consistency by the DCC. Investigators at each clinical center identified potential study patients from their own patient populations, referral from other ophthalmologists or by self-referral. Participation was open to all interested patients and made public using an openly accessible internet webpage (http://progstar.org/progstar-home/progstar-4/).

Quality assurance and methods to minimize bias

Each site principal investigator (PI) confirmed the eligibility of patients. Data collection and procedures for all investigations were standardized and outlined in the study *Manual of Procedures* (MOP). All staff involved in performing study procedures were trained and certified prior to the start of the study. Image quality and completeness was assessed by the RC, and photographers at the centers were informed in case of poor quality or missing images. Image graders were not masked to the sequence of visits and to the patient. Images were reviewed by two RC-certified graders independently, and an adjudication process was applied in discordant cases with final determination by a RC investigator (MIA). After processing and analyzing at the RC, all data derived from grading were transferred from the RC to the DCC using the REDCap system. Case report forms were stored at each site.

Grading of atrophic lesions on fundus autofluorescence

Previously established grading protocols were applied for grading of atrophic lesions on FAF images using a semi-automated software tool (Heidelberg Engineering® RegionFinder) [9,12,15]. Areas of decreased autofluorescence (DAF) were quantified in three distinct types with the level of darkness being used to define an area of DAF qualitatively being "definite "or "questionable". Reference points were the optic nerve head (ONH) for "100% level of darkness", while the retinal background autofluorescence seen in the periphery in less affected retinal areas, served as the reference for normal autofluorescence. Areas with level of darkness close to 100% (at least 90%) in reference to the ONH or blood vessels were defined as "definitely decreased autofluorescence" (DDAF). Such lesions were well-demarked by nature of contrast differences with surrounding areas, though sometimes ill-organized (see figure

2). Areas with darkness levels ranging between 50% and 90% darkness were defined as "questionably decreased" AF (QDAF).

Further grading included contiguity of DDAF lesions (unifocal/multifocal) and qualitative grading parameters: presence/absence of an edge of increased autofluorescence and presence/absence of fleck-like lesions. The background autofluorescence was graded as homogeneous or heterogeneous as previously described [9]. The autofluorescence of the foveal center (when regarded as a point) was determined as normal, DDAF, QDAF or increased autofluorescence.

Grading of microperimetric assessments

Mesopic microperimetry was performed under dim room light conditions, and scotopic microperimetry under completely dark conditions after at least 30 minutes dark adaption. The sensitivity in each of the 68 (mesopic microperimetry) and 40 (scotopic microperimetry) retinal locations was determined by iteratively adjusting the light intensity until the dimmest visible stimulus was found. A scale of 0 dB to 20 dB served to determine the sensitivity for each test location. The term "deep scotoma" was defined for test locations with 0 dB (i.e., retinal locations where only the brightest stimulus was detected or no stimulus at all was detected), and the term "relative scotoma" for test locations with more than 0 dB but less than 12 dB [16]. Mean sensitivity across all tested locations, and the number of absolute and relative scotomas were calculated.

Fixation results were obtained during microperimetric macular sensitivity testing (dynamic testing) by tracking the patient's retina and generating of a scatter plot of all fixation locations [17]. Fixation stability was quantified as a continuous variable, the

bivariate contour ellipse area (BCEA): global BCEA for one, two, and three standard deviations was calculated using the following equation:

$$BCEA = 2k\pi\sigma_H\sigma_V(1 - \rho^2)^{1/2}.$$

σ_H and σ_V are the standard deviations of horizontal and vertical eye movements, ρ is the Pearson product-moment correlation coefficient of fixation positions in the horizontal and the vertical meridian, k is a constant dependent on the chosen probability area which is given by the equation:

$$P = 1 - e^{-k}$$

P is the probability area and e is the base of the natural logarithm. P is the chosen probability for the SD that the BCEA is based on and the equation is solved for k [16-18]:

$$k = -\ln(1 - P)$$

Spectral-domain optical coherence tomography

Patients were tracked with 20° x 20° cube comprising SD-OCT scans. Single B-scans were semi-automatically graded using the Heidelberg Spectralis V version 6.3.4. The following layers were segmented and analyzed: retinal nerve fiber layer (RNFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), external limiting membrane (ELM), inner segments/outer segments (IS/OS), retinal pigment epithelium (RPE), Bruch's membrane (BM) and choriocapillaris (CC); choroidal stroma was assessed by analysis of enhanced depth imaging (EDI). Segmentation errors were manually corrected.[19] Results from SD-OCT grading will be reported separately; for the purpose of this

manuscript, values of the central subfield derived from clinical report forms (CRF) are provided.

Clinical and demographic factors

Data sets include demographic information, presence of mutations in *PROM1*, and best-corrected visual acuity assessed according to the "Early Treatment Diabetic Retinopathy Study" (ETDRS) charts and protocols.[14] Data from biomicroscopy of the anterior segment including the status of the lens (presence of lens opacities or cataracts), as graded according to the Age-related Eye Disease Study (AREDS) cataract grading scheme [20], and from dilated fundus examination were also recorded. Smoking history and concomitant diseases were recorded, with emphasis on those related to ciliopathies; these were assessed using a custom-built questionnaire that specifically included questions regarding hearing problems, use of hearing aids, recurrent sinusitis, and kidney disorders.

A full-field electroretinogram (ffERG) according to ISCEV standards [21] was performed once at the baseline visit or the results were submitted if performed within five years of the baseline visit. Color fundus photos could be obtained at the discretion of the site-PI and sent to the RC.

Statistical analysis

VA measures were converted to LogMAR scale. Visual acuity was then divided into categories of visual impairment as proposed by the World Health Organization (WHO) [22,23]: (i) BCVA better than or equal to 20/25 (LogMAR≤ 0.1) (i.e. no visual impairment [VI]); (ii) worse than 20/25 to 20/70 (LogMAR 0.1-0.54) (i.e. mild VI); (iii)

worse than 20/70 to 20/200 (LogMAR 0.54-1.0) (i.e. moderate VI); (iv) worse than 20/200 to 20/400 (LogMAR 1.0-1.3) (i.e. severe VI); and (v) worse than 20/400 (LogMAR>1.3) (i.e. blindness) [22].

Descriptive statistics are shown for characteristics at patient and eye level; the mean (standard deviation) median and range for continuous variables and proportions for categorical variables are used. To describe the correlation between total area of DAF and number of absolute scotomas, the Spearman correlation coefficient is presented. All analyses were conducted in SAS 9.4,

Results

Demographic characteristics

A total of 29 eyes (15 patients) were enrolled in the ProgStar-4 study between December 2nd, 2014 and May 6th, 2015 at five clinical centers: six at Moorfields Eye Hospital, London, three at Retinafoundation of the Southwest, Dallas and Department of Ophthalmology, University of Bonn, respectively, two at the Department of Ophthalmology, Eberhard-Karls University Hospital Tübingen, and one at the Wilmer Eye Hospital, Johns Hopkins University, Baltimore. All had disease-causing mutations in the *PROM1* gene and were white; demographic data are summarized in table 1.

Mean BCVA at baseline was 52.6 (± 26.1 sd, range 0-91) ETDRS letter score (LogMar 0.65 ± 0.52, range -0.12 – 1.66); 15 eyes (51.7%) had no or mild visual impairment (BCVA 20/70 or better (LogMar <0.54)), six eyes (20.7%) had moderate (worse than 20/70 to 20/200 (LogMAR 0.54-1.0)), six eyes (20.7%) had severe (worse than 20/200 to 20/400 (LogMAR 1.0-1.3)) visual impairment, and two eyes (6.9%) were legally blind (worse than 20/400 (LogMAR>1.3)).

On clinical fundus examination, six eyes (20.7%) showed pallor of the optic nerve and seven eyes (24.1%) showed vascular attenuation. In 27 eyes (93.1%) RPE-atrophy was present on clinical exam, and RPE pigmentation abnormalities in 19 eyes (65.5%); fleck-like lesions were described in eight eyes (27.6%), in two eyes (6.9%) also beyond the vascular arcades. Genetic data of enrolled patients are presented in table 2.

Baseline characteristics in fundus autofluorescence, microperimetry and spectral-domain optical coherence tomography

At baseline, 29 eyes had FAF images graded. Ten eyes (34.5%) showed areas of DDAF, out of which 3 were unifocal and 7 multifocal. Mean lesion size of DDAF was 3.2 mm^2 (± 3.5 mm^2, range 0.36 – 10.39 mm^2; figure 2, table 3).

A signal of increased autofluorescence was present in 17/29 (58.6%) of these eyes. All 29 eligible eyes had QDAF lesions. When regarded as a point, the foveal center was normal in 8/29 (27.6%) eyes, had increased FAF signal in 4/29 (13.8%) eyes, 13/29 (44.8%) with QDAF and 4/29 (13.8%) with DDAF.

Results derived from both photopic and scotopic microperimetric exams (figure 3) are also summarized in table 3. There was a positive correlation between areas of total area of DAF and absolute scotoma (Spearman Correlation coefficient $\rho=0.61$, $p=0.02$).

Spectral-domain optical coherence tomography imaging showed a mean retinal thickness of the central subfield (1000 microns diameter) of 196 microns which is significantly below the normal mean of 283 +/-27 microns [24].

Discussion

Natural history studies such as herein for *PROM1*-related retinopathy have also been undertaken in the ProgStar study of *ABCA4*-related disease [9]. While there is a major

overlap of the study design, there exists several differences: first, the inclusion criteria were broadened for the ProgStar-4 study after a survey to identify potential study patients, and hence the definition of the atrophic lesion size of the study eye(s) was confined by the possibility to track disease progression using 20 x 20 degree SD-OCT scans rather than by a definite size threshold; secondly, there was no threshold for minimal visual acuity; thirdly, the manual of procedures, especially for the acquisition of FAF images was amended; for the purpose of the ProgStar study in *ABCA4*-related disease, the concept of short-wavelength reduced autofluorescence imaging (SW-RAFI) as proposed by Cideciyan et al was implemented [9]. This approach is based on the potential light-toxicity, especially in *ABCA4*-related disease due to accumulation of A2-dihydropyridine-ethanolamine (A2E) as one of the major components of lipofuscin and may lead to acceleration of disease progression [13]. Indeed, ABCA4-related STGD1 shows elevated levels of lipofuscin-related autofluorescence intensity,[25] and this facilitates the use of SW-RAFI leading to comparable grading results with conventional FAF imaging [12]. Because photoreceptor cell degeneration of *PROM1*-knockout mice was shown to be light-dependent based on histologic and functional examinations [26], we adopted this concept also for the ProgStar-4 study. However, we realized that a reduction of the laser power in *PROM1*-related disease may result in underexposed images and therefore the acquisition of an image with 25% LP appeared not to be optimal for the ProgStar-4 study.

The patients enrolled into the Prog-Star-4 study comprise a wide spectrum of PROM1-related maculopathy both anatomically (as determined by changes in FAF and SD-OCT) and functionally (as determined by changes in mesopic/scotopic microperimetry and ffERG). The study will permit a determination of structure-function correlations and longitudinal changes and deepen the understanding for the natural progression of

STGD4. This is the first step towards possible therapies for *PROM1*-related maculopathies: As an example, administration of Fenretinide, which lowers the level of the toxic lipofuscin, has been shown to slow down the degeneration of photoreceptor cells in *Prom1*$^{-/-}$-knockout mice [26]. Other strategies such as reduction of oxidative stress [27] to slow down progression as well as restoration of sight by using optogenetics, stem cells or retinal prosthesis offer alternatives for future therapies [7].

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Figure legends:

Figure 1: Organizational structure of the ProgStar-4 study group.

Figure 2: Fundus autofluorescence images were graded for background changes and atrophic lesions (definitely decreased autofluorescence (DDAF) and questionably decreased autofluorescence (QDAF) 1A and 1C). RegionFinder™ software tool was then used to determine each individual lesion's size (Figure 1B and D): the example shows an area of QDAF with a normal-appearing foveal center and two adjoining areas of DDAF. Example of QDAF in figure 1C: manual restrictions were applied to allow correct lesion demarcation. In this case QDAF measured 6.797 mm^2. Nonconfluent lesions were seen as in figure 2D: the subfoveal QDAF measured 7.210 mm^2 and all lesions summed up to 7.331 mm^2.

Figure 3:
Microperimetric macular threshold testing was performed under both photopic (Figure 2A, left) and scotopic (Figure 2B, right) conditions. Threshold was determined for 68 (photopic) and 40 (scotopic) retinal loci, respectively. Individual thresholds were color-coded (green = normal retina; yellow = relative scotoma; red open squares = absolute scotoma). Fixation was recorded (turquois dots) and fixation stability was quantified as a continuous variable, the bivariate contour ellipse area (BCEA).