

# Characteristics and Utility of Fundus Autofluorescence in Congenital Aniridia Using Scanning Laser Ophthalmoscopy

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**PURPOSE.** To investigate fundus autofluorescence (FAF) and other fundus manifestations in congenital aniridia.

**METHODS.** Fourteen patients with congenital aniridia and 14 age- and sex-matched healthy controls were examined. FAF images were obtained with an ultra-widefield scanning laser ophthalmoscope. FAF intensity was quantified in the macular fovea and in a macular ring surrounding fovea and related to an internal reference within each image. All aniridia patients underwent an ophthalmologic examination, including optical coherence tomography and slit-lamp biomicroscopy.

**RESULTS.** Mean age was  $28.4 \pm 15.0$  years in both the aniridia and control groups. Fovea could be defined by subjective assessment of FAF images in three aniridia patients (21.4%) and in all controls ( $P = 0.001$ ). Mean ratio between FAF intensity in the macular ring and fovea was  $1.01 \pm 0.15$  in aniridia versus  $1.18 \pm 0.09$  in controls ( $P = 0.034$ ). In aniridia, presence of foveal hypoplasia evaluated by biomicroscopy correlated with lack of foveal appearance by subjective analyses of FAF images ( $P = 0.031$ ) and observation of nystagmus ( $P = 0.009$ ).

**CONCLUSIONS.** Aniridia patients present a lower ratio between FAF intensity in the peripheral and central macula than do healthy individuals. Both subjective and objective analyses of FAF images are useful tools in evaluation of foveal hypoplasia in aniridia.

**Keywords:** aniridia, fundus autofluorescence, macular hypoplasia, optical coherence tomography, widefield imaging

Aniridia is a rare congenital condition that involves the cornea, anterior chamber angle, iris, lens, retina, and optic nerve and is often named a panocular disease. In Norway, the prevalence is 1:76,000.<sup>1</sup> Aniridia usually occurs without obvious systemic involvement but may be part of syndromes.<sup>2,3</sup> Iris and foveal hypoplasia are hallmarks present from birth and commonly result in photophobia, low vision, and nystagmus. Glaucoma, cataract, and aniridia-associated keratopathy develop in many patients.<sup>3,4</sup> Aniridia is inherited in an autosomal dominant fashion with very high penetrance. Approximately two thirds of cases are familial, and one third sporadic.<sup>5</sup> A mutation in the *PAX6* gene is responsible for development of the disease in most cases.<sup>6</sup>

Autofluorescence of the ocular fundus is mainly emitted from lipofuscin in the retinal pigment epithelium (RPE).<sup>7</sup> Lipofuscin accumulates in RPE cells during phagocytosis of the photoreceptor outer segment. Fundus autofluorescence (FAF)

imaging is a useful noninvasive modality, as in assessment of achromatopsia<sup>8</sup> and prediction of visual prognosis in Stargardt disease.<sup>9</sup> The technique also proved to be helpful in combination with ultra-widefield fundus imaging.<sup>10</sup>

The FAF pattern of isolated and aniridic foveal hypoplasia has been presented in case reports,<sup>11-14</sup> but to our knowledge no systematic case-control study of FAF has been conducted in aniridia previously nor has any such study described the retinal appearance by widefield imaging in these patients. Electroretinogram recordings, however, showed alterations in the majority of aniridia subjects.<sup>15</sup> Interestingly, rod and cone function were equally affected. This suggests that not only may the central retina be affected in aniridia, evident as foveal hypoplasia,<sup>5,16</sup> but the midperipheral and peripheral part may also be affected.

The *Pax6* gene is essential in the embryonic formation of the eye, including the retina.<sup>17</sup> As *PAX6* is responsible for



development of aniridia in most cases,<sup>18</sup> we hypothesize that the FAF pattern in aniridia patients differs from that of healthy individuals. The primary aim of this study was to explore these differences and to investigate possible correlations between FAF patterns and presence of foveal hypoplasia considered by ophthalmoscopy and optical coherence tomography (OCT). It is hoped that this will support clinicians in assessing visual function and prognosis in aniridia. Furthermore, FAF may help understand the disease because of its utility in evaluation of several types of conditions and processes in the retina.

Understanding of retinal development and function in aniridia may also have implications for other eye disorders as aniridia originates from fundamental disturbances in eye formation. Moreover, insight into the aniridia phenotype could be important in monitoring treatment effects as gene therapy may be an option in the future.<sup>19</sup>

## MATERIALS AND METHODS

This prospective study included 14 aniridia patients, nine of which were women, and 14 were age- and sex-matched healthy controls. All participants had similar ethnicity. Patients were selected from a group of 35 aniridia patients who were recruited through the patient organization Aniridia Norway. Inclusion required that the diagnosis of congenital aniridia had been reported and that it was confirmed through clinical examination by the presence of typical iris hypoplasia. Associated ocular conditions could support the diagnosis if needed. Only individuals  $\geq 9$  years of age were included in order to achieve acceptable OCT images of macula.

All 35 aniridia patients in the original group underwent a general ophthalmologic examination, including ophthalmoscopy and OCT, accompanied by classification of aniridia-associated keratopathy, cataract, and foveal hypoplasia.<sup>16,20</sup> Fourteen of the participants were selected in order to obtain the best-quality fundus photographs. The selection was based on lowest degree of keratopathy and cataract. To be included, the stage of keratopathy had to be  $\leq 2$  in both eyes, and total cataract score had to be  $\leq 6$  in at least one eye. Patients with pseudophakia or aphakia were allowed to take part, but not if significant secondary cataract and/or capsular phimosis were present. Genetic analysis of participants with aniridia was performed as described by Pedersen et al.<sup>16</sup>

The study was approved by the Norwegian Regional Committees for Medical and Health Research Ethics (Application 2014/382). After receiving oral and written information about the study, all participants signed a written informed consent, as did parents of participants younger than 16 years. The study was conducted in accordance with the tenets of the Declaration of Helsinki.

### Ophthalmoscopy and Classification of Disease in Aniridia Group

Eye refraction was determined subjectively as the correction giving best visual acuity for each eye separately. The refractive error was defined in diopters (D) by summing the spherical refractive power and half of the cylindrical power (spherical equivalent). Accordingly, five groups were defined: emmetropia (refractive error  $> -0.5$  D and  $< 0.5$  D), low-grade hyperopia ( $\geq 0.5$  D and  $< 6$  D), high-grade hyperopia ( $\geq 6$  D), low-grade myopia ( $> -6$  D and  $\leq -0.5$  D), and high-grade myopia ( $\leq -6$  D). Visual acuity (logMAR) was measured with a digital high-contrast chart at 6 m (TestChart 2000; Thomson Software Solutions, London, UK). Test distance was reduced

to 3 or 1 m if a reliable measurement was unattainable at 6 m.<sup>16</sup>

The examiner considered presence of nystagmus (yes or no) while the patient was resting and kept eyes in primary gaze position. Each subject was further examined in a slit-lamp biomicroscope. Aniridia-associated keratopathy was staged according to a previously published classification, which included four stages (0–3).<sup>20</sup> Iris hypoplasia was classified as total (aplasia) if no iris tissue was visible by slit-lamp examination or partial if some tissue could be observed. Cataract was staged in agreement with the Lens Opacities Classification System III.<sup>21</sup>

Ophthalmoscopy of the posterior segment was carried out with a 90-D funduscopy lens. Foveal hypoplasia was defined as absence of foveal appearance and/or presence of retinal vessels traversing the expected foveal area and was considered either present or not present. Foveal appearance referred to the typical light reflex and/or darker spot seen by ophthalmoscopy of central macula. Optic nerve hypoplasia was evaluated based on the distance from the temporal optic nerve head border to the foveal center. If the distance was  $>3.5$  times longer than the horizontal optic nerve head diameter, optic nerve hypoplasia was considered present. This definition was a modification built on a study by Barr et al.<sup>22</sup> If no definite foveal center could be determined, then the presence of optic nerve hypoplasia was stated based on appearance of an obvious small optic nerve head or a typical double ring sign around it.

### OCT Imaging and Grading of Foveal Hypoplasia in Aniridia Group

Spectral-domain OCT images of the macula were acquired (Heidelberg Spectralis OCT2; Heidelberg Engineering GmbH, Heidelberg, Germany). Volume scans covering a  $20^\circ \times 20^\circ$  or  $30^\circ \times 10^\circ$  area centered at the expected foveal center were obtained. Each volume scan consisted of 49 B-scans. Between 5 and 20 frames were averaged in each B-scan to reduce speckle noise in the image and compensate for eye motion (TruTrack; Heidelberg Engineering GmbH). If volume scans could not be achieved because of severe nystagmus, horizontal line scans with a  $30^\circ$  length were chosen. In these cases, the scan was moved manually across the macula, using the temporal edge of the optic nerve head as a reference point. Multiple scans were captured in the region of the expected foveal location,<sup>23</sup> and signs of foveal specialization were considered. The ratio between each participant's axial length measurement (IOL Master; Carl Zeiss Meditec AG, Jena, Germany) and the OCT default axial length (24 mm) were used to correct the lateral image scale for individual retinal magnification. This ratio was multiplied with the nominal OCT scan length. Horizontal line scans were divided into retinal layers, and foveal hypoplasia was graded as described by Pedersen et al.<sup>16</sup>

### Acquisition and Analysis of Scanning Laser Ophthalmoscopy Images

Ultra-widefield fundus images were obtained using a scanning laser ophthalmoscope (Optomap Panoramic 200Tx; Optos PLC, Dunfermline, Fife, Scotland), which allows nonmydriatic pictures of up to  $200^\circ$  of the retina. Photos were also taken of the central fundus using the central pole function in the instrument. FAF images were captured with the 532-nm laser (green-light FAF), and composite color images were acquired with a combination of the 532- and 633-nm laser. Several images were obtained in order to avoid influence from nystagmus, eyelids, and ocular media opacities. All images

were analyzed by one experienced examiner under similar conditions.

FAF images were analyzed using ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). In aniridia patients, the image with the best quality was chosen for analysis, and hence only one eye was considered. In control subjects, one image from both eyes was analyzed and an average level calculated.

Brightness in the pictures was recorded as mean gray level (GL), which represented an indirect measure of FAF intensity. Increasing GL corresponded to elevated brightness in the picture. In order to reduce sources of error in the analysis, an internal reference (IRE) was calculated in each image. The IRE was defined as mean GL in 1000 pixels of the central one third of the thickest retinal vein from the optic disk margin and distally (Fig. 1A).

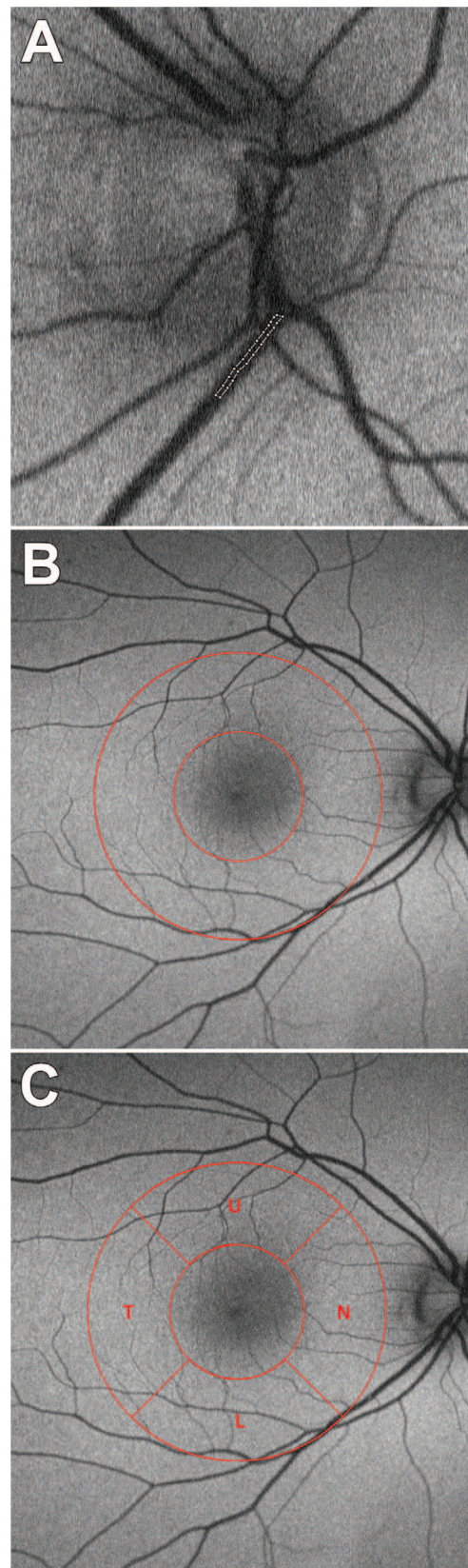
The horizontal distance (foveal distance [FD]) between the temporal edge of the optic nerve head and the foveal center was used to define the radius of two concentric circles around the foveal center (Fig. 1B). The central circle had a radius of  $0.36 \times \text{FD}$  pixels and the peripheral circle  $0.80 \times \text{FD}$  pixels. If the foveal center could not be identified in the FAF picture, its position was estimated based on analysis of the OCT scan. Mean GL was measured within the central circle (fovea) and in four segments (upper, lower, nasal, and temporal) of the ring (macular ring) created between the central and peripheral circle (Fig. 1C). Shadows from vitreous floaters, cataract, or keratopathy were outlined manually in the software and excluded from the measurement.

A ratio was calculated between mean GL in fovea and the IRE and between mean GL in each of the four macular ring segments and the IRE. The ratio between mean GL in the whole macular ring and the IRE was also determined. Finally, mean GL in the macular ring was divided on mean GL in the fovea in order to assess the ratio between brightness in these two areas.

The FAF pictures were classified (yes or no) based on whether foveal location could be determined by subjective assessment, defined by ability to localize a hypofluorescent spot in the expected foveal area (Fig. 2). Peripheral FAF was graded subjectively as either hypoautofluorescent, normal, or hyperautofluorescent. Hyperautofluorescence was considered present if the peripheral FAF was judged as equal to or greater than FAF of the central fundus. Correspondingly, normal peripheral FAF indicated FAF regarded as weaker than that of the central fundus, however clearly identifiable. Hypoautofluorescence referred to peripheral FAF that was difficult to identify. Equivalently, fundus color images were graded according to a three-step scale (dark, normal, or light fundus color).

### Statistical Analyses

Statistical analyses were performed with software (SPSS version 23.0; IBM Corporation, Armonk, NY, USA). Means, standard deviations, as well as numbers and ratios were used to describe results of the ophthalmologic examination. Univariable conditional logistic regression models and McNemar's test were chosen to compare results from cases and matched controls. Correlation analyses of parameters in the aniridia group were performed with univariable logistic regression and linear regression models and Spearman's rank correlation. Results are presented as odds ratio (OR), mean differences with 95% confidence interval (CI), correlation coefficients ( $r_s$ ), and  $P$  values.  $P$  values  $\leq 0.05$  were considered statistically significant.



**FIGURE 1.** (A) Outlining of an IRE comprising 1000 pixels of the central one third of the thickest retinal vein from the optic disk margin and distally. (B) Two concentric circles around the foveal center defining a central macular circle (fovea) and a peripheral macular ring (area between the central and peripheral circle). (C) Division of the macular ring into four sectors: upper (U), lower (L), nasal (N), and temporal (T).

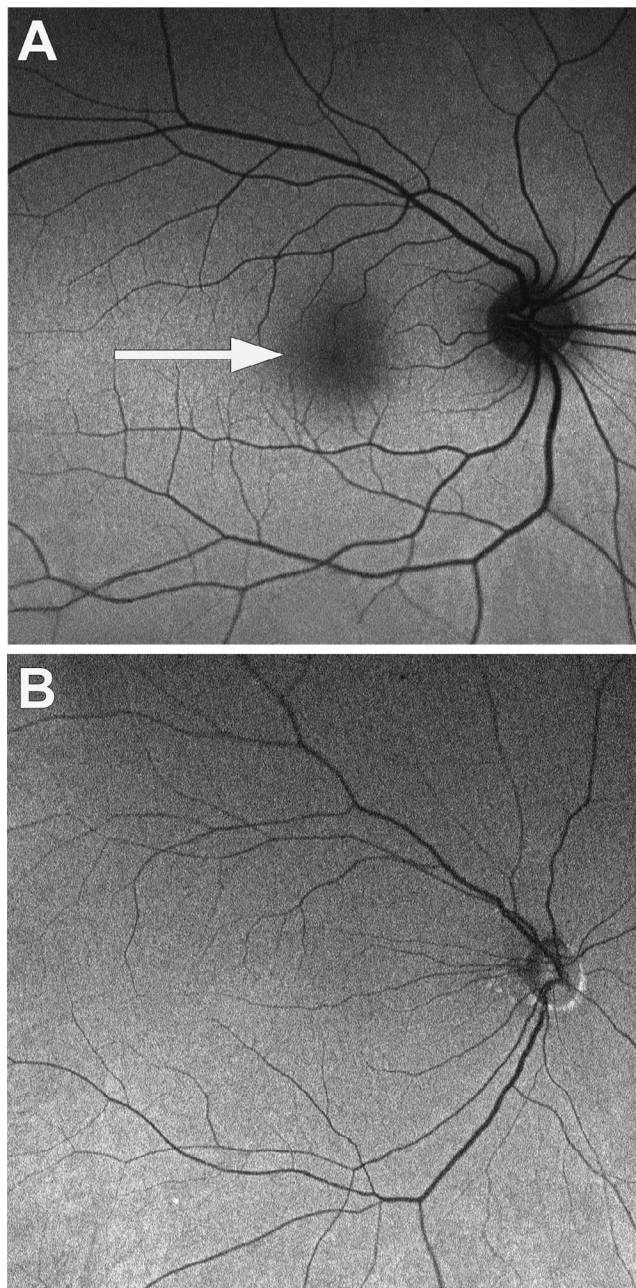


FIGURE 2. FAF images of aniridia patients showing (A) a foveal hypoautofluorescent spot (arrow) and (B) no defined fovea.

## RESULTS

Mean age in the aniridia group was  $28.4 \pm 15.0$  (range, 11–64) years and  $28.4 \pm 15.0$  (range, 9–64) years in the control group. Overview of clinical findings and grading of OCT images in the aniridia group is presented in Table 1. Supplementary Figure S1 shows OCT images from five aniridia patients with foveal hypoplasia grade 0 to 4. Genetic analysis was available in 12 of the 14 aniridia patients. *PAX6* mutation was detected in eight of these. Four patients were negative for disease-causing mutations in *PAX6* and the *PITX2* and *FOXC1* genes. *PAX6* mutation was not detected in two of the three subjects without foveal hypoplasia by ophthalmoscopy. These two had hypoplasia grade 0 and grade 1, respectively, assessed by OCT scans. In the third patient without foveal hypoplasia by ophthalmos-

TABLE 1. Results From Clinical Examination and Staging of OCT Images in Aniridia Patients ( $N = 14$ )

Parameter	Result
Visual acuity, logMAR, mean $\pm$ SD	$0.69 \pm 0.29$
Refraction, $n$ (%)	
Emmetropia	1 (7.1)
Low-grade hyperopia	4 (28.6)
High-grade hyperopia	2 (14.2)
Low-grade myopia	4 (29)
High-grade myopia	3 (21.4)
Nystagmus, $n$ (%)	9 (64)
Stage aniridia-associated keratopathy	
Mean stage $\pm$ SD	$1.14 \pm 0.77$
Stage 0, $n$ (%)	3 (21.4)
Stage 1, $n$ (%)	6 (42.9)
Stage 2, $n$ (%)	5 (35.7)
Iris aplasia, $n$ (%)	10 (71.4)
Lens status, $n$ (%)	
Phakia	10 (71.4)
Pseudophakia	3 (35.7)
Aphakia	1 (7.1)
Stage of cataract (LOCS III, mean $\pm$ SD)	
Nuclear	$1.20 \pm 0.42$
Cortical	$2.30 \pm 0.95$
Posterior subcapsular	$1.20 \pm 0.42$
Papillary hypoplasia, $n$ (%)	4 (29)
Macular hypoplasia (ophthalmoscopy), $n$ (%)	11 (79)
Macular hypoplasia (OCT)	
Mean stage $\pm$ SD	$2.43 \pm 1.34$
Stage 0, $n$ (%)	2 (14.2)
Stage 1	1 (7.1)
Stage 2	3 (21.4)
Stage 3	5 (35.7)
Stage 4	3 (21.4)

LOCS III, Lens Opacities Classification System III.

copy, genetic analysis was not available. This patient had hypoplasia grade 0 on OCT.

## Comparison of FAF Images in Aniridia and Control Group

Comparison of results in the aniridia and control group is presented in Table 2. Mean ratio between GL in the fovea and the IRE was  $3.52 \pm 1.17$  in the aniridia group and  $3.09 \pm 0.70$  in the control group (OR = 1.48, 95% CI = 0.67–3.27,  $P = 0.334$ ). A similar ratio was determined between mean GL in each of the four sectors of the macular ring and the IRE. The ratio was lower in aniridia patients for all four sectors (nasal, lower, temporal, and upper), but the results were not statistically significant (Table 2). Finally, the mean ratio between GL in the whole macular ring and fovea was  $1.01 \pm 0.15$  in aniridia and  $1.18 \pm 0.09$  in controls (OR = 0.001, 95% CI =  $5.42E-07$ –0.57,  $P = 0.034$ ). Accordingly, aniridia patients showed lower difference in autofluorescence intensity between peripheral and central macula, with a ratio close to 1.

Foveal location could be determined by subjective assessment of autofluorescence pictures in three aniridia patients (21.4%) and in all control subjects ( $P = 0.001$ ; McNemar's test). Hyperautofluorescence of the peripheral fundus (Fig. 3A) was established in six aniridia patients (42.9%) and in one control (7.1%) (OR = 6.00, 95% CI = 0.72–49.8,  $P = 0.097$ ). All other participants in both groups showed normal peripheral autofluorescence (Fig. 3B). The FAF was generally increased in the whole peripheral fundus of all six patients staged with

TABLE 2. Comparison of Results From Image Analyses in Aniridia and Control Group

Parameter	Aniridia	Control	OR*	95% CI	P Value*
Autofluorescence ratio, mean $\pm$ SD†					
Fovea	3.52 $\pm$ 1.17	3.09 $\pm$ 0.70	1.48	0.67-3.27	0.334
Macular segment‡					
Nasal	3.58 $\pm$ 1.06	3.80 $\pm$ 0.88	0.85	0.44-1.64	0.620
Lower	3.51 $\pm$ 1.19	3.87 $\pm$ 1.03	0.80	0.44-1.46	0.472
Temporal	3.74 $\pm$ 1.23	3.85 $\pm$ 0.96	0.93	0.53-1.66	0.813
Upper	3.30 $\pm$ 1.07	3.15 $\pm$ 0.65	1.15	0.55-2.40	0.702
Macular ring	3.53 $\pm$ 1.09	3.67 $\pm$ 0.87	0.90	0.47-1.72	0.757
Macular ring/fovea	1.01 $\pm$ 0.15	1.18 $\pm$ 0.09	0.001	5.42E-07-0.57	0.034
Fovea defined in FAF images, N (%)	3 (21.4)	14 (100)	N/A	N/A	0.001§
Peripheral retinal hyperautofluorescence, N (%)	6 (42.9)	1 (7.1)	6	0.72-49.8	0.097
Light fundus color, N (%)	12 (85.7)	1 (7.1)	N/A	N/A	0.001§

N/A, not applicable because of perfect fit of the outcome.

\* Calculated from univariable conditional logistic regression models.

† Ratio between light intensity in retina and IRE (retinal vein).

‡ One fourth of the macular ring around fovea.

§ Performed with McNemar's test due to perfect distinction between cases and controls.

peripheral hyperautofluorescence. However, peripheral hyperautofluorescent spots were observed in one of these patients (Fig. 3A).

Fundus color was considered as light (Fig. 3C, 3D) in 12 aniridia patients, but in only one control ( $P = 0.001$ ; McNemar's test). The remaining participants (one in aniridia and 11 in control group) had normal fundus color.

### Factors Associated With Foveal Hypoplasia in Aniridia

Presence of foveal hypoplasia by ophthalmoscopy (yes or no) in the aniridia group was correlated with results from digital analyses of FAF images and from the ophthalmologic examination, including subjective assessment of FAF images. Data are presented graphically in Figure 4A and 4B.

The mean ratio between GL in fovea and the IRE was  $3.07 \pm 1.01$  (95% CI = 0.57-5.57) in patients with normal-appearing macula by ophthalmoscopy and  $3.65 \pm 1.24$  (95% CI = 2.82-4.48) in those with foveal hypoplasia (Fig. 4A). This numerical difference indicates that hypoplasia is associated with a more

hyperfluorescent fovea. The mean ratio between GL in the macular ring and IRE was  $3.48 \pm 1.15$  (95% CI = 2.71-4.25) in foveal hypoplasia and  $3.73 \pm 1.01$  (95% CI = 1.21-6.24) in normal-appearing macula, suggesting darker peripheral macula in hypoplasia. Eventually, the mean ratio between GL in the macular ring and fovea was compared in the two groups. In hypoplasia, the ratio was  $0.96 \pm 0.06$  (95% CI = 0.91-1.00) and in those without hypoplasia was  $1.23 \pm 0.19$  (95% CI = 0.75-1.71). This implies that foveal hypoplasia by ophthalmoscopy is connected to a lower difference between autofluorescence intensity in peripheral and central macula, with a ratio close to 1.

Foveal location could be determined by subjective analyses of FAF images in 1 (9.1%) of the 11 patients with foveal hypoplasia by ophthalmoscopy and in two (66.7%) of the three subjects with normal macular appearance (Fig. 4B;  $P = 0.031$ ). Hence, foveal hypoplasia was associated with the lower possibility of detecting fovea on FAF pictures. In addition, the sensitivity of subjective FAF analysis in assessment of foveal hypoplasia was 90.9% (10/11) and the specificity 66.7% (2/3).

Macular pictures were analyzed subjectively in the three patients without foveal hypoplasia by ophthalmoscopy. In one of these subjects, it was not possible to identify a clear hypofluorescent foveal spot on the FAF image. Instead, a sparse, patchy hypofluorescence was seen in the foveal area. In the two other patients, the diameter of the hypofluorescent spot was judged as smaller than that in matched controls. Moreover, the diameter of the foveal reflex on color images was considered to be smaller in these two patients than in control subjects.

Nystagmus was present in 81.8% of patients with foveal hypoplasia but not in any of those with normal-appearing macula (Fig. 4B;  $P = 0.009$ ). Four aniridia subjects (36.4%) with hypoplasia by ophthalmoscopy also had optic nerve hypoplasia, but there was none in those with normal macula ( $P = 0.217$ ). All three patients without foveal hypoplasia by ophthalmoscopy had aniridia-associated keratopathy stage 0. Patients with hypoplasia had either keratopathy stage 1 or 2 (mean 1.45). One of the patients without hypoplasia was pseudophakic. The other two had lowest degree (stage 1) of both nuclear, cortical, and posterior subcapsular cataract. In the hypoplasia group, the mean stage of nuclear cataract was 1.25; of cortical cataract, 2.625; and of posterior subcapsular, 1.25.

The mean ratio between GL in the macular ring and the fovea was calculated in patients with same grade of foveal hypoplasia on OCT (Fig. 4C). The ratio was  $1.25 \pm 0.27$  in

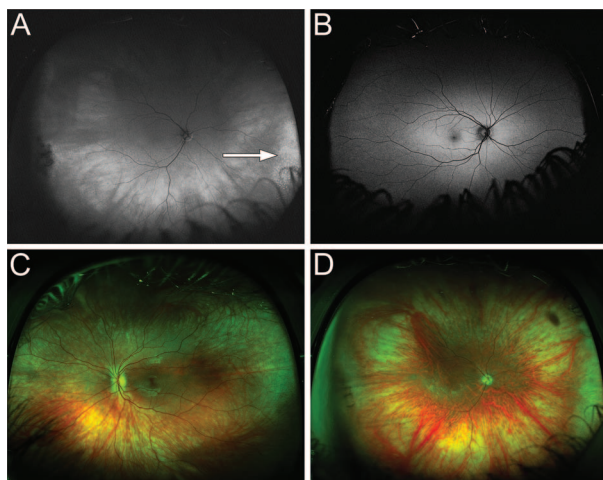
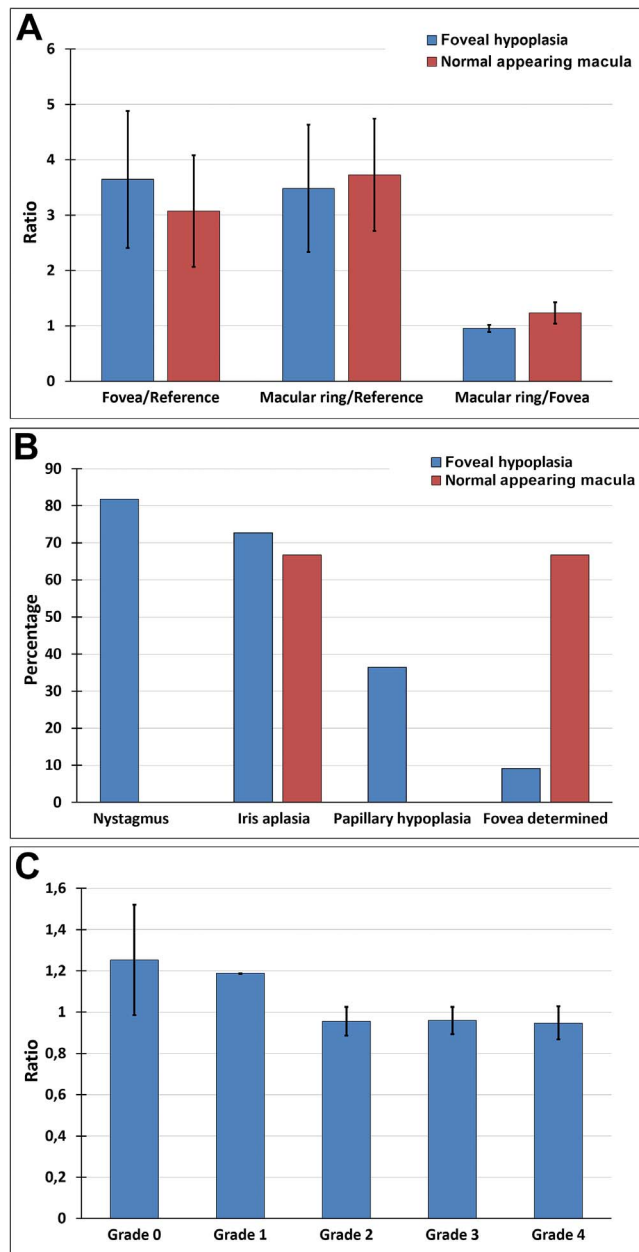


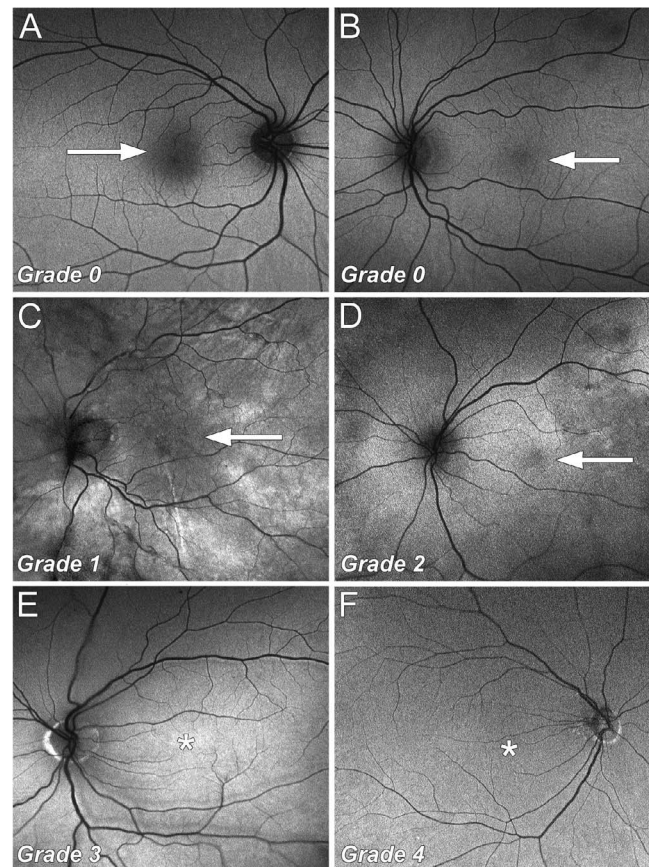
FIGURE 3. (A) FAF image of an aniridia patient demonstrating hyperautofluorescence of the peripheral fundus and peripheral hyperautofluorescent spots (white arrow). (B) FAF image from aniridia patient with normal peripheral autofluorescence. (C, D) Examples of two aniridia patients with light fundus color.



**FIGURE 4.** (A) Ratios (means with standard deviations) between FAF intensities in aniridia patients with foveal hypoplasia by ophthalmoscopy (blue bars) and normal-appearing macula (red bars). (B) Percentage of patients with foveal hypoplasia and normal macula who had nystagmus, iris aplasia, papillary hypoplasia, and fovea determined by subjective assessment of FAF pictures. (C) Ratios (means with standard deviations) between FAF intensity in peripheral and central macula in different grades of foveal hypoplasia considered by OCT. Macular ring, area of peripheral macula around fovea; reference, IRE in the picture.

patients with hypoplasia grade 0 and 1.19 in grade 1. In grade 2, 3, and 4, the mean ratio was  $0.96 \pm 0.07$ ,  $0.96 \pm 0.07$ , and  $0.95 \pm 0.08$ , respectively. Consequently, the ratio was relatively stable above 1 in grade 0 and 1 and under 1 in grade 2, 3, and 4. Linear regression showed that the ratio was significantly higher in patients with either grade 0 or 1 than in those with either grade 2, 3, or 4 ( $P = 0.001$ ).

By subjective assessment of FAF images, a hypofluorescent spot was identified in the fovea of both participants with hypoplasia grade 0 on OCT (Fig. 5A, 5B). In the single subject



**FIGURE 5.** FAF images of aniridia patients with different grades of foveal hypoplasia by OCT. (A) Hypoautofluorescence in central macula (fovea; arrow) in foveal hypoplasia grade 0. (B) Hypoautofluorescent spot (arrow) present in fovea in hypoplasia grade 0, but less evident than in A. (C) Patchy hypoautofluorescent area (arrow) in the expected foveal region of patient with hypoplasia grade 1. A hyperautofluorescent streak (linear degeneration) is evident below the fovea. (D) A subtle hypoautofluorescent spot (arrow) in fovea of a patient with hypoplasia grade 2. (E) Absence of foveal hypoautofluorescence in hypoplasia grade 3. Foveal center (asterisk) estimated based on OCT scans. (F) Lack of foveal hypoautofluorescence in hypoplasia grade 4, including retinal vessel traversing the estimated foveal center (asterisk).

with hypoplasia grade 1, a patchy hypoautofluorescent area was observed in the expected foveal region, but it was not considered sufficient to determine the foveal location (Fig. 5C). A subtle hypoautofluorescent spot was noticed in the fovea of one patient with hypoplasia grade 2 (Fig. 5D). Otherwise, the foveal location was not possible to identify based on FAF images in any other participants with either hypoplasia grade 2, 3 (Fig. 5E), or 4 (Fig. 5F). The sensitivity of subjective FAF analysis in determining foveal hypoplasia by OCT (grade 1, 2, 3, or 4) was 91.7% (11/12), and the specificity was 100% (2/2).

Additionally, FAF pictures were analyzed subjectively in order to detect other differences in macula of patients with various grades of foveal hypoplasia on OCT. Variation was then observed in the appearance of the retinal vessels. The vessels in grade 0 and 1 did not manifest any definite abnormalities. In all patients with hypoplasia grade 2, 3, or 4, however, the presence of retinal vessels in the peripheral fovea was more evident than in matched controls. In three out of eight patients with either hypoplasia grade 3 or 4, the vessels also extended into or traversed the foveal center (Fig. 5F).

Among those four aniridia patients with optic nerve hypoplasia determined by ophthalmoscopy, two had foveal hypoplasia grade 3 on OCT and the other had two grade 4. Linear regression demonstrated a significantly higher frequency of optic nerve hypoplasia in patients with either foveal hypoplasia grade 3 or 4 than in those with either grade 0, 1, or 2 ( $P = 0.045$ ). This finding indicates coexistence of optic nerve hypoplasia and the most severe grades of foveal hypoplasia considered by OCT. No significant differences were found in FAF between the four patients with both optic nerve hypoplasia and foveal hypoplasia and the other 10 patients.

Finally, correlation analyses were performed between visual acuity and results from FAF and OCT analyses in the aniridia group. Better visual acuity was associated with a higher ratio between GL in the macular ring and fovea ( $r_s = -0.30$ ). However, the association was not statistically significant ( $P = 0.299$ ). On the other hand, poorer visual acuity correlated significantly with increasing grade of foveal hypoplasia on OCT ( $r_s = 0.73$ ,  $P = 0.003$ ).

## DISCUSSION

Our study showed lack of foveal hypofluorescence in most aniridia patients (78.6%) by subjective analysis of FAF pictures, while a hypofluorescent spot could be localized in the foveal area of all control subjects. This finding corresponded well with the objective method in which the ratio between autofluorescence intensity in the peripheral and central macula was higher in controls than in patients and close to one in aniridia. Results from the subjective and objective examination of FAF images were both related to the grade of foveal hypoplasia evaluated with OCT. Moreover, absence of foveal demarcation on FAF pictures was associated with presence of foveal hypoplasia by ophthalmoscopy. Ultra-widefield color images demonstrated a light fundus in most aniridia patients (85.7%) but in only one control (7.1%).

In the normal retina, macular fovea appears dark on FAF pictures. This could be attributed to absorption of excitation light by macular pigment,<sup>7</sup> which has its highest density in the central fovea.<sup>24</sup> Absorption in RPE melanin may also contribute to reduction in autofluorescence in this area.<sup>7</sup> Finally, the cellular content of lipofuscin is possibly lower in central macula.<sup>25</sup> As foveal hypoplasia is common in aniridia,<sup>5</sup> alteration in macular autofluorescence could be expected. In our study, this was evident as the ratio between FAF intensity in the peripheral and central macula was lower than in controls. Additionally, foveal hypofluorescence was observed in only three aniridia patients (21.4%) by subjective analysis of FAF images, while it was observed in all control subjects. The ratio between FAF intensity in the peripheral and central macula was close to 1 in aniridia patients. This suggests an increase in foveal FAF in aniridia to a level equal to the peripheral macular fluorescence. It is likely that increased central macular FAF (or lack of hypofluorescence) could be ascribed to a reduced amount of macular pigment, since this substance has its highest concentration in the central fovea. Theoretically, lower density of RPE melanin could be an additional explanation for the lack of foveal hypofluorescence in aniridia.

Absence of a hypofluorescent foveal spot on FAF images was associated with presence of foveal hypoplasia by ophthalmoscopy in our study. Additionally, difference in FAF pattern could be observed between patients with normal appearing macula by ophthalmoscopy and OCT, and matched, healthy controls. This included a smaller hypofluorescent foveal spot in aniridia patients. One patient with normal macula by ophthalmoscopy and hypoplasia grade 1 on OCT

displayed a sparse, patchy hypofluorescence in the foveal area. Interestingly, a hypofluorescent spot was present in one patient with foveal hypoplasia by ophthalmoscopy and hypoplasia grade 2 on OCT. FAF imaging could therefore be a useful tool in assessing foveal hypoplasia in aniridia patients and yield information that cannot be obtained by ophthalmoscopy or OCT. Scanning laser ophthalmoscopy is easy and quick to perform, even in small children. The modality is also helpful in patients with photophobia and nystagmus due to its low light level. Moreover, widefield imaging provides a valuable overview of the peripheral fundus. Thus, FAF imaging using scanning laser ophthalmoscopy could bring clinically relevant information that is accessible in a wide range of patients.

In all patients presenting foveal hypoplasia by ophthalmoscopy, macular OCT scans were classified with either hypoplasia grade 2, 3, or 4. In the three subjects without hypoplasia by ophthalmoscopy, two were categorized as grade 0 (normal configuration) and one as grade 1 on OCT. Accordingly, assessment of foveal hypoplasia by slit-lamp biomicroscopy seemed to correspond well with level of macular development considered by OCT. OCT grading was also related to the ratio between FAF intensity in the peripheral and central macula. A significantly higher ratio was detected in hypoplasia grade 0 and 1 than in grade 2, 3, and 4. Consequently, foveal hypoplasia grade 0 to 1 can be differentiated from grade 2 to 4 by objective evaluation of FAF images in aniridia patients. Importantly, grade of foveal hypoplasia on OCT correlated significantly with visual acuity. However, a significant correlation was not found between visual acuity and the ratio between FAF in peripheral and central macula. This indicates that macular OCT is better than macular FAF to predict visual acuity in aniridia.

Additional information about the grade of foveal hypoplasia may be achieved by subjective assessment of FAF pictures as increasing central involvement of vessels in the fovea seemed to be associated with more advanced grades of hypoplasia considered with OCT. Consistently, it has been argued that formation of a foveal depression probably is connected to development of an avascular zone in the fovea.<sup>26</sup> In the current classification, foveal hypoplasia grade 2 was differentiated from grade 1 by absence of a foveal depression on OCT. A foveal depression could be identified by fetal age 25 weeks<sup>27</sup> with increasing depth until postnatal age 15 months.<sup>27,28</sup> Simultaneously, the foveal cones are maturing.<sup>27-29</sup> Thus, foveal development might have been influenced at the latest from midgestation in patients with hypoplasia grade 2 and above. A relation between distribution of macular pigment and size of the foveal avascular zone and the thickness of the central fovea has also been found.<sup>30</sup> Hence, it could be hypothesized that macular pigment plays a role in foveal development. It is possible that the presence of optic nerve hypoplasia could also be a factor in the process leading to foveal hypoplasia.<sup>31</sup> All patients with optic nerve hypoplasia in our study had either foveal hypoplasia grade 3 or 4. This finding may support that optic nerve hypoplasia contributes to more severe foveal hypoplasia or that these two conditions coincide because of the genotype.

Fundus color was classified as light in 12 aniridia patients (86%) but in only one control (7%). Hence, a light or hypopigmented fundus seems to be a characteristic finding in aniridia. Few differential diagnoses exist in a patient with hypopigmented fundus. Among these are oculocutaneous or ocular albinism and Angelman syndrome.<sup>32</sup> Aniridia should therefore be considered a possible diagnosis in a patient with hypopigmented fundus. *Pax6* is involved in the differentiation of RPE in embryogenesis of vertebrates.<sup>33</sup> It is further shown that absence of *Pax6* reduces production of pigment in RPE cells. Thus, it is likely that *PAX6* mutations in aniridia influence

RPE cells and explain the presence of a hypopigmented fundus in most patients.

Aniridia subjects in our study were selected from a larger group of patients in order to reduce influence from ocular media opacities on the fundus images. Additionally, the image with the best quality was chosen for analyses, and hence only one eye was considered. This might have favored a certain phenotype. For instance, all patients with normal-appearing macula by ophthalmoscopy had the lowest degree of aniridia-associated keratopathy. However, Hingorani et al.<sup>5</sup> found foveal hypoplasia by ophthalmoscopy in 37 of 43 individuals with aniridia (86%), which is comparable with our results (79%). Despite selection of participants and the clearest picture for analyses, aniridia-associated keratopathy and cataract could still have influenced our findings. Theoretically, different refractive status and variations in IRE in matched individuals may also play a role. Mutation in *PAX6* was not detected in those two genetically tested patients without foveal hypoplasia by ophthalmoscopy. Hence, it seems more likely that these patients have another gene mutation causing aniridia. However, *PAX6* mutation was not detected in two patients with foveal hypoplasia. Additionally, in about 90% of aniridia cases, the genetic origin could be identified in the *PAX6* gene.<sup>6</sup> In our study, analysis of *PITX2* and *FOXC1* was included as well. Thus, negative mutational analysis may also be related to lower detection rate and not only to a specific ocular phenotype. More comprehensive genetic analysis is required to fully rule out *PAX6* involvement in the cases where no mutation was found.

Our results showing connection between FAF and foveal hypoplasia could be useful in further research on congenital aniridia. For instance, gene therapy has increased packing density of photoreceptor nuclei in aniridia mice.<sup>19</sup> Furthermore, such treatment increased the thickness of all retinal layers. As distribution of macular pigment is related to central foveal thickness,<sup>30</sup> FAF might be utilized to monitor effects of gene therapy in aniridia patients in the future. Interestingly, supplementation of formulas containing macular carotenoids could improve vision in healthy individuals.<sup>34</sup> Such treatment might be an option in future studies of aniridia, in which any alterations in FAF should be observed.

In conclusion, our study demonstrates lack of foveal hypofluorescence on FAF images in most aniridia patients. Correspondingly, objective image analysis confirms a lower ratio between peripheral and central macular autofluorescence in aniridia than in controls. FAF imaging could be a useful tool in clinical evaluation of aniridia patients as absence of foveal hypofluorescence correlates well with presence of foveal hypoplasia. Additionally, FAF imaging can provide information about macular morphology that is not detectable using ophthalmoscopy or OCT.

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