The Distribution of Leakage on Fluorescein Angiography in Diabetic Macular Edema: A New Approach to Its Etiology

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Purpose. To determine the distribution of leakage on fluorescein angiography (FA) and explore the clinically protective role of astrocytes against damage to the inner blood retinal barrier (iBRB) in diabetic macular edema (DME).

Methods. A consecutive case series of 87 eyes of 87 patients with DME was included. We measured the leakage area in each field of the Early Treatment Diabetic Retinopathy Study (ETDRS) grid on late-phase FA images. The normative thickness of the nerve fiber layer (NFL), in which the astrocytes are confined, was derived from a previous work using spectral-domain optical coherence tomography. We explored the difference in leakage areas in every two fields. Moreover, we investigated the correlation between the mean of the leakage area and the mean of thickness of the NFL in each ETDRS field.

Results. The leakage areas in the nasal, inferior, superior, and temporal fields were 2.34 mm^2, 2.84 mm^2, 3.03 mm^2, and 3.96 mm^2. The difference in leakage area between each two fields was significant in all cases ($P < 0.05$) except between the inferior and superior fields ($P = 0.65$). The temporal field was the only field that showed leakage in all 87 cases. The correlation between the leakage area and the thickness of the NFL in the ETDRS fields was negative and highly significant: $r = -0.96$ (95% confidence interval −0.99 to −0.02).

Conclusion. The distribution of leakage correlates inversely and statistically significantly with the thickness of the NFL, suggesting astrocytes in the NFL play a pivotal role in preventing damage to the iBRB and subsequent evolution of microaneurysms in DME. Moreover, fluid extravasation due to damage to the iBRB is expressed earlier in the temporal than in the other three fields.

Keywords: diabetic macular edema, fluorescein angiography, astrocytes, nerve fiber layer, vascular leakage

Microvascular lesions tend to develop in specific regions of the fundus in diabetic retinopathy (DR). For instance, the most common sites for neovascularization in proliferative DR are the optic disc and the superior temporal quadrant.[1] By contrast, a diffuse nonperfusion area is usually first recognized in the midperiphery of the retina.[2,3] However, no special distribution of leakage has yet been reported in diabetic macular edema (DME).

DME is still the leading cause of visual loss in diabetes.[4,5] Interest has therefore increasingly been directed to understanding its cause and the underlying vascular and neural interactions. These interactions affect the whole architecture of the retina through different pathways resulting in various and overlapping mechanisms of pathogenesis. For example, a vascular-neural effect was detected when cysts in the outer retina were found to have damaged the underlying photoreceptors in the central macula.[6] A vascular-vascular effect also has been proposed following the finding that the pressure of macular cysts in the inner nuclear layer (INL) on the adjacent retinal capillaries results in focal nonperfusion in the central macula (Haj Najeeb B, et al. *IOVS* 2013;54:ARVO E-Abstract 2364). Furthermore, astrocytes have been recently shown to play a key role in the function of the inner blood retinal barrier (iBRB) in healthy people and patients with diabetes. Astrocytes are essential in the process of angiogenesis in the retina during the embryological and neonatal periods, and for restoring the integrity of the capillary wall against the pathologic consequences of hyperglycemia.[7,8]

It is well established that the nerve fiber layer (NFL) is not equally distributed across the macula. Its thickness in the superior and inferior fields is similar, but thicker than in the temporal field. It reaches its maximum thickness in the nasal field.[9,10,11] Furthermore, astrocytes reside exclusively in the NFL and their spatial density is associated with NFL thickness.[12–14] Hence, the thickness of the NFL in the macula can be used as a good indicator for the distribution of astrocytes.

To explain the clinical relevance of astrocytes for the iBRB and consequently verify a corresponding specific glial–vascular effect, we measured the distribution of leakage on fluorescein angiography (FA) in the four fields of the Early Treatment Diabetic Retinopathy Study (ETDRS) grid and explored the

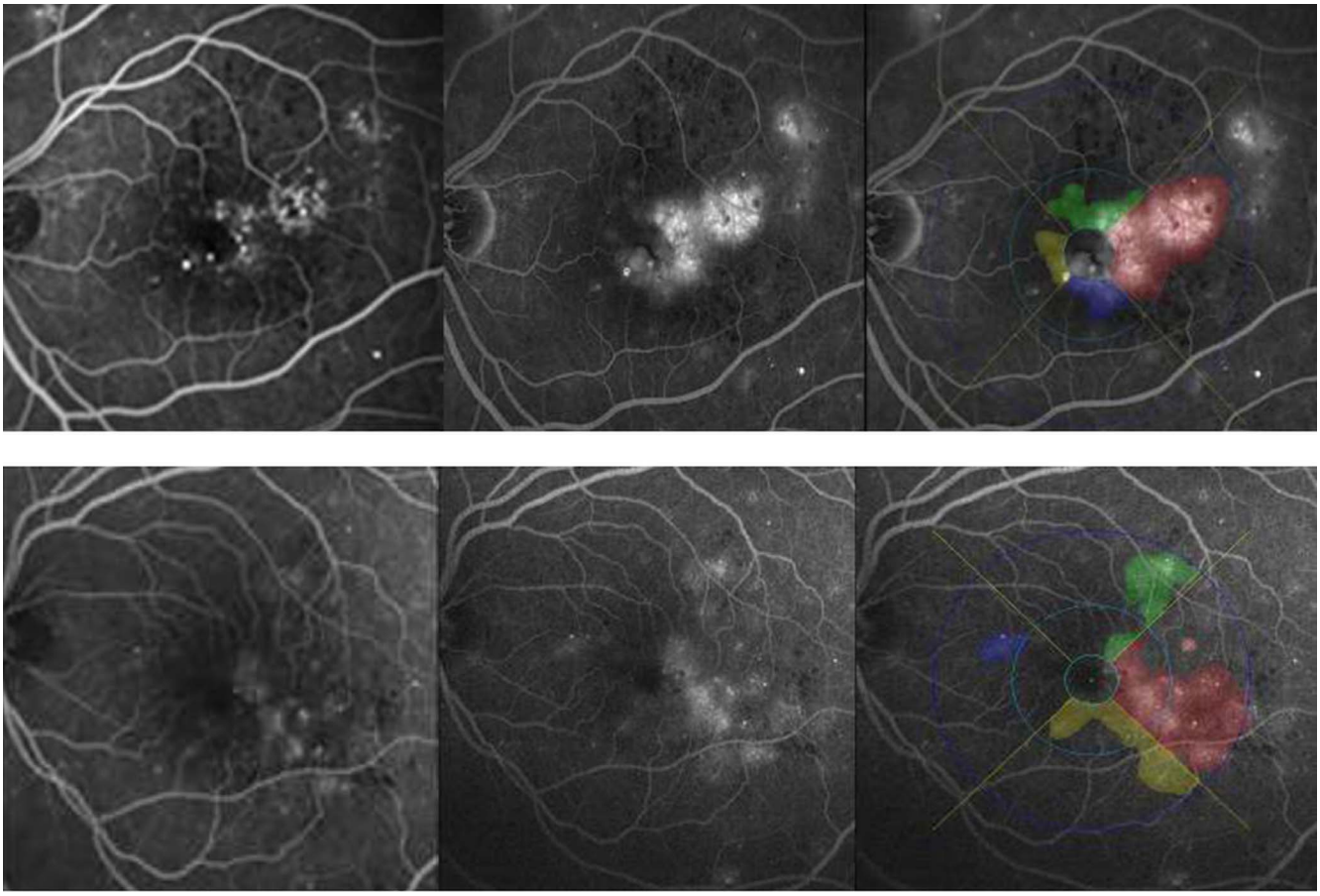

FIGURE. The FA of two eyes in the early and late phases before and after demarking the leakage using VRC custom software. Note the predominance of the microaneurysms in the temporal field on the early-phase images, which are seen afterward in the late-phase images as the main source of leakage.

correlation between the area of leakage and the thickness of the NFL, in which the astrocytes are confined.

METHODS

The patients were participants of a randomized, double blinded, active controlled, phase III study of the efficacy and safety of repeated doses of an intravitreal anti–vascular endothelial growth factor (VEGF) drug in patients with DME. DME diagnosis was confirmed using fundus biomicroscopy, FA, color fundus photographs, and spectral-domain optical coherence tomography (SD-OCT). The reading of this multicenter study was carried out in the Vienna Reading Center (VRC) and images used in our study are part of the VRC database. We took advantage of the data we received to test our theory in FA images from only baseline visits independent of the findings related to the anti-VEGF drug. Ethical approval was obtained from the institution's review board of each participating center for the inclusion into the multicenter trial and from the Ethics Committee of the Medical University of Vienna for the retrospective post hoc data analysis. The study was performed according to the tenets of the Declaration of Helsinki. Inclusion criteria were adults 18 years or older with type 1 or 2 diabetes mellitus and central DME. Exclusion criteria were patients with a previous retinal medical or surgical intervention, previous laser treatment in the macular region, fluorescein leakage covering the whole ETDRS grid (circle of 6-mm diameter), active proliferative DR, and macular nonperfusion. In addition, we excluded other diseases that may cause or exacerbate macular edema, such as retinal vascular occlusions, uveitis, Irvine-Gass syndrome, vitreomacular traction, and epiretinal membrane.

Study of Leakage

We retrospectively measured the distribution of leakage in the four macular regions (superior, nasal, inferior, and temporal) of 87 eyes of 87 consecutive patients with DME. The FA images had a minimum resolution of 1024×1024 pixels, but devices from different manufacturers were used by the various participating study centers to acquire the images. Leakage, which represents the hallmark of the loss of integrity of the iBRB, was seen as a hyperfluorescent area on the images. This hyperfluorescent area of iBRB breakdown enlarges over time and usually has blurred borders. We ignored any leakage emerging from disease outside the ETDRS grid. The image with the best quality where the macula was in the center after 10 minutes had elapsed was chosen for measuring the leakage. An experienced retina specialist and VRC supervisor outlined the leakage in each field manually after applying the ETDRS grid using VRC custom software. The leakage areas demarked in the four fields of the ETDRS grid were then measured separately (Fig.). All imported FA images were provided with test eye measurements as reference measurements, which allow our software program to transform the outlined leakage area from pixel into mm^2 measures. Subsequently, we calculated the

Table 1. The Mean, SD, Minimum, and Maximum of Leakage in All Four Fields of the ETDRS Grid

ETDRS Field	Mean of Leakage, mm²	SD	Minimum, mm²	Maximum, mm²	Mean of NFL Thickness, µm
Nasal	2.34	1.73	0	6.44	34.85
Inferior	2.84	2.15	0	6.56	33.43
Superior	3.03	2	0	6.79	31.78
Temporal	3.96	2.21	0.17	6.99	19.7

The table also shows the mean of the normative NFL thickness measured by SD-OCT of the inner and outer ETDRS subfield given from previous work.[9]

statistical significance of the difference between the means of the four leakage areas.

Study of NFL

We noticed a distinct distortion of the NFL on reviewing the SD-OCT scans in this study. The swelling of the NFL led to an inaccurate measurement of its thickness. Moreover, it obscured any thinning as a result of neurodegeneration caused by DR.[15] Therefore, we did not measure the thickness of the NFL on our images. Instead, we imported the normative NFL thickness in each inner and outer ETDRS subfield of 60 healthy subjects from a Spectralis SD-OCT device (Heidelberg Engineering, Heidelberg, Germany) used in a previous study.[9] Then, we calculated the means of the inner and outer subfields in each field. Finally, we explored the correlation between the means of leakage area in each ETDRS field on FA and the means of NFL thickness in the same field on SD-OCT.

The statistics were carried out in the Department of Medical Statistics of Medical University of Vienna. Results are reported as means (SDs). A linear mixed model was calculated to account for correlations of measurements from the same patients. Post hoc analysis included pairwise comparisons of means. To correct for multiple testing, the reported P values were adjusted by the Tukey method. A $P \leq 0.05$ was considered statistically significant. The correlation was determined by the Pearson correlation test. Results are reported as correlation coefficients (confidence intervals [CIs]).

Results

We measured the distribution of leakage in the four ETDRS fields in 87 eyes with DME. The mean of leakage in the nasal, inferior, superior, and temporal areas was 2.34 mm² (1.73), 2.84 mm² (2.15), 3.03 mm² (2), and 3.96 mm² (2.21) (Table 1). Moreover, the pairwise comparisons in leakage among the four ETDRS fields showed a statistically significant difference ($P \leq 0.05$), except in the mean between the superior and inferior fields ($P = 0.65$) (Table 2). Interestingly, the temporal field was the only field in which the leakage was present in all 87 eyes.

The means of normative NFL thickness in the nasal, inferior, superior, and temporal fields were 34.85 µm, 33.43 µm, 31.78 µm, and 19.7 µm (Table 1). The correlation coefficient between the means of the leakage areas and the means of NFL thickness was negative and highly significant $r = -0.96$ (95% CI −0.99 to −0.02).

Table 2. The Estimates of Differences With Adjusted P Values of Pairwise Comparisons of the Leakage Areas, and the Correlation Coefficient With the CI

	Mean Difference, mm²	P Value
Nasal-inferior	−0.5	0.0068
Superior-inferior	0.19	0.6541
Temporal-inferior	1.12	<0.001
Superior-nasal	0.69	<0.001
Temporal-nasal	1.62	<0.001
Temporal-superior	0.93	<0.001

The correlation coefficient $r = -0.96$, 95% CI −0.99 to −0.02.

Discussion

We evaluated the distribution of fluorescein leakage in the central macula in 87 DME eyes of 87 patients. The means of the leakage areas showed a consistent increase starting from the nasal area toward the temporal area; that is, the nasal field had the smallest mean of leakage area, then the inferior and superior fields, followed by the temporal field, which had the largest mean of leakage area (Table 1). This characteristic leakage distribution correlated significantly and inversely with the thickness of the NFL from a normative database: $r = -0.96$ (95% CI −0.99 to −0.02). The NFL, in contrast, had a maximum thickness in the nasal field, followed by the inferior and superior fields, followed by the temporal field, where it was thinnest. As astrocytes are found only in the NFL and their density is associated with the thickness of the NFL,[12–14] this correlation can be extended to the density of astrocytes in each field.

Of note, only the difference in mean areas between the inferior and superior fields was not statistically significant (Table 1), which could be explained by their similar thicknesses in healthy people and the symmetrical and progressive thinning caused by diabetes.[1,15] In the other pairwise comparisons, the P value was, however, statistically significant because of the distinctive difference in the thickness of NFL.

Interestingly, microaneurysms (MAs), the number and turnover rate of which have been considered an important biomarker for the progression to DME,[16,17] have previously been shown to have the same spatial distribution as the distribution of leakage seen in our study.[18] Thus, the leakage of fluids seen in DR comes essentially from strongly leaking MAs (Fig.). In addition, MAs in the superficial retina (i.e., the NFL, which in the astrocytes are confined across the retina) have smaller diameters and occur less frequently than those that occupy the INL, which is devoid of astrocytes.[19] Thus, the characteristic horizontal distribution of leakage across the macula caused by MAs seen in our study, as well as the distinctive vertical discrepancy in number and size of the MAs along the inner retinal layers are both contrariwise proportionate with the distribution of astrocytes. Therefore, our results led us to hypothesize that astrocytes play a paramount role in maintaining the integrity of the iBRB in the NFL against the effects of hyperglycemia, including the early pathologic vascular changes and formation of MAs.

Many researchers have found that astrocytes are very important in supporting the iBRB. This support is demonstrated physically by enveloping the capillaries comprising the neurovascular unit as well as forming and preserving the tight vascular junctions,[8,20–22] and chemically by secreting a variety of agents that regulate the intercellular communication to keep

the iBRB intact.[21,23,24] Moreover, under hyperglycemia, a pronounced and progressive loss of the NFL precedes the pathologic microvascular changes.[15,25] The subsequent dysfunction of astrocytes can facilitate an increased expression of inflammatory cytokines, oxidative stress, and VEGF, which affect the proliferation, adhesion, and migration of the astrocytes and provoke the extravasations of fluids from the retinal capillaries culminating in DME.[26–28] These notable functions of the astrocytes in the physiologic and pathologic processes confirm their unique role in protecting the iBRB and explain our finding that the area of leakage and the density of astrocytes in the NFL correlated statistically significantly and inversely in each ETDRS grid.

We believe astrocytes are the only glial cells that can explain the characteristic distribution of leakage that we observed. Astrocytes are located only in the NFL, where there are fewer and smaller MAs than in the INL, and they are most dense in the macula.[12,19] Müller cells, which are the other type of glial cell found in the NFL, do not show the same distinctive pattern of leakage. Rather, they distribute randomly and extend along the whole thickness of the retina and are at their lowest density in the macula.[29] We found a distribution of leakage typical for astrocytes in each of the ETDRS fields we examined. Therefore, we consider the astrocytes are the only glial cells that can clarify the distinguishing distribution of leakage.

Embryologically, this supportive role of astrocytes can be understood to a certain extent by their prime function of forming and orientating retinal vessels and keeping their walls intact.[7,30]

Loss of pericytes has been assumed to be the main mechanism in developing MAs.[27,31,32] Otherwise, histologic studies have shown a disparity between the regional density of MAs, which were found to be more frequent in the superior temporal quadrant of the fundus, and the location of pericyte ghosts, which were equally distributed throughout the retina.[1] Hence, pericytes are not the only decisive cells. Other types of cells, such as astrocytes, also have a remarkable influence in deterring the evolution of MAs, because pericytes are normally absent from the connecting points where the astrocytes envelop and protect the retinal capillaries.[8] Furthermore, the development of MAs in the retina under the hyperglycemic state is never accompanied by a similar formation of MAs in the brain, albeit both the brain and the retina have similar embryologic origins.[33] This definitive prophylactic role of the astrocytes on the capillaries of the brain could be equally effective in the retina in maintaining the integrity of the retinal capillaries and inducing the subsequent characteristic extension of leakage in each field of the ETDRS grid, as seen in our study.

The tendency of the temporal field to demonstrate extensive vascular changes in DR has been explained by assuming the existence of widespread anastomoses of capillary beds in this field.[34] Otherwise, recent peri- and parafoveal imaging studies using OCT angiography have not revealed any statistically significant increase in the vascular density of the temporal field.[35] Therefore, this phenomenon can be explained by a glial rather than a vascular mechanism: specifically, because of the lower density of the astrocytes in the NFL of the temporal field compared with the other three fields.

The temporal field on the ETDRS grid was the only field showing leakage in all 87 eyes (Table 1), which, for the first time to our knowledge, indicates that fluid extravasation due to damage to the iBRB is earlier expressed in the temporal than in the other three fields. We believe this finding will aid clinicians to focus attention on the temporal macular field when carrying out screening in DR.

At the VRC we do not have access to the demographic data for the individual patients included in the study because the study and reading protocols of the studies usually specify that all graders must be blinded to patients' data. Therefore, we were unable to compare the demographic characteristics of our patient cohort with those of the patients in the control study. Nonetheless, many studies including different ethnic groups also have shown the same distribution of the normative thickness of the NFL.[10,11] The use of FA devices from different manufacturers could be a limitation in our study. For this reason, we chose only good-quality images with a minimum resolution of 1024 × 1024 pixels. Other limitations are inclusion of both patients with type 1 and type 2 diabetes, and the lack of a parallel comparative histologic study showing the corresponding change in the spatial density of the astrocytes in the NFL in patients with DME. The wide range of the CI is due to the few (four) correlating items. Furthermore, due to a lack of access to reliable NFL thickness values from patients for the ETDRS fields, we could not make individual correlations of leakage areas and NFL thickness. Instead, we compared the mean leakage areas with the mean thickness of normal NFL, which usually shows symmetrical progressive loss of 0.25 μm every year in patients with diabetes with no to minimal DR.[15] Therefore, the highly statistically significant negative correlation we found only supports rather than proves our theory, and the use of NFL measurement from another study could be a limitation.

In conclusion, we propose that leakage in DME does not occur at random, rather astrocytes have a definite influence on its characteristic distribution due to their function in maintaining the integrity of the iBRB and preventing the evolution of vascular lesions. The temporal field tends to become involved with leakage earlier than the other three fields. Further studies on the cellular level are necessary to understand this intricate interplay between the glial and vascular components of the retina and to interpret the consequent nonuniform distribution of lesions in DR.

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