response episode to compare would denote that the vessel in volunteer 1 dilated more or less than that in volunteer 2. Which flicker episode should therefore be chosen for comparison? Smokers in the study by Heitmar et al.[1] demonstrated significant differences from the control group in the first flicker episode. Such variation may not be the case for other diseases. Using this approach on another group of healthy volunteers may demonstrate different results, despite their similar pathophysiologic status.

Finally, the reference to a single temporal time point and a single flicker episode included in the parameter ΔD is too uncertain from the standpoint of the RVA measurement technique,[2,5] since a qualitative assessment of each single flicker episode is not always possible in all subjects (e.g., elderly persons, subjects with bad fixation, or those who blink during the assessment), which represent a serious limitation of the method presented by Heitmar et al.[1]

Therefore, our main criticism of Heitmar et al. relates to each of the two suggested indices of the SDRA method. Their supposed index APR describing vascular elasticity is arbitrary. It does not convey any information regarding vascular elasticity, and its values are not reported in the paper. Their other index, ΔD, does not add any new information for an individual subject, since its definition is arbitrary. Regarding this second index, Heitmar et al.[1] discuss two additional points that supersede the standard vessel dilation assessment:

1. Their method supports the necessity of assessing the maximum dilation at the time point of its occurrence rather than at the fixed time point of flicker cessation. This aspect has been described and discussed elsewhere.[3,6]
2. It shows a possible higher rate of dilation in the first flicker episode in the group of healthy subjects, compared with subsequent episodes, and in the first flicker episode in smokers. In a healthy population, this effect has been described in other publications.[2,3] Since dilation depends on the baseline state, which is not always the same in all the flicker episodes, the value of this finding is debatable.

We would like to point out that it is worthwhile to interpret vessel diameter changes and their dynamics, which include superimposed systemic and local effects caused by cardiac pulsations, breathing rhythms, blood pressure waves and vasomotion, among others,[2,4,7] and result in a different measured diameter at any given time point. Heitmar et al.[1] acknowledge this fact and integrate it into their calculations by using BDF, which we think may be an important parameter to consider for the assessment of vascular health in the future.

Konstantin E. Kotliar[1,2]
Ines M. Lanzl[1]

Departments of [1]Ophthalmology and [2]Nephrology, Munich University of Technology, Munich, Germany.
E-mail: kotliar@lrz.tum.de

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Author Response: Can Vascular Function Be Assessed by the Interpretation of Retinal Vascular Diameter Changes?

We thank Kotliar and Lanzl for their interesting thoughts, which we believe contribute to the development of the best scientific analysis of ocular blood vessels in response to disease. This topic is rapidly developing and has clear indications for other diseases, such as diabetes. Nevertheless, we would like to take the opportunity to respond to the points raised by our colleagues.

In their opening paragraph, they suggest that our characterization of vessel elasticity is flawed by the nature of the assessment. They contend (1) that vessel elasticity cannot be characterized by a single assessment of reactive vessel dilation in response to flicker light and a determination of the magnitude of spontaneous vascular vasomotions. We are puzzled by this point, as a major premise of our new method is that a single assessment alone is insufficient because of the baseline diameter fluctuation (BDF) of the vessel. We analyzed flicker response three times to ensure a greater confidence in our data. In the final paragraph of the Results section, we present data that show no difference ($P = 0.343$) in APR taken at three time points.[1] In addition, (2) they suggest that we do not explain the reason for calling APR a measure of elasticity and that the results of the APR are not disclosed anywhere in the paper. We call APR a measure of elasticity because it accounts for the increase in vessel diameter, which then returns to baseline. This we showed with the relationship between maximum vasomotion under resting conditions and under stimulatory (e.g., flicker) conditions. In their final sentence, they say that the results of the APR are not disclosed in our paper. The data are indeed presented at end of the Results section.

In their second paragraph, our colleagues question the index that we have named ΔD, which represents the degree of vessel dilation that occurs between the time point of flicker initiation and maximum vascular dilation. They suggest that the problem inherent in this new parameter is its arbitrary limitation to a 1-second diameter assessment before flicker initiation as its main reference point. First, we would like to further explain why we believe a 1-second mean diameter is a suitable measure. The human heart beats at intervals of roughly one second, which is also visible in most RVA recordings (Fig. 1), and therefore an average of the 25 diameter recordings before flicker initiation, which are sampled in 1 second, appears to be a suitable means of reflecting the vessel size directly before the stimulation cycle. We explained that there is a limit to how much any vessel can dilate (in the Methods section on SDRA and in the Results section, paragraph five[1]). In addition, our colleagues state that reduced vessel dilation during the second and third flicker episodes in our healthy patients differed from

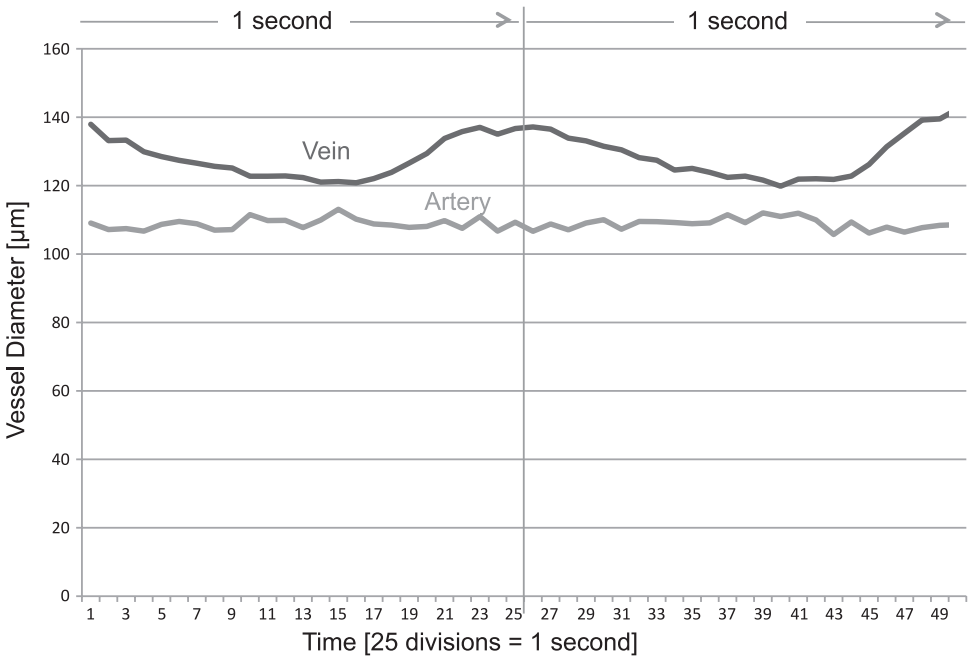

FIGURE 1. A trace of a 2-second baseline diameter recording of a retinal arteriole and venule in a healthy 40-year-old individual, obtained with the retinal vessel analyzer (IMEDOS Systems UG, Jena, Germany).

those in the smoking sample for ΔD. We want to emphasize that we showed correlations only between the 1-second mean baseline diameter and the ΔD in healthy subjects and did not compare or show the correlations for the smoking sample. Our comparison between groups is solely for the parameters shown in Tables 4, 5, and 6 of our paper. In the figure showing two individuals assessed by Kotliar and Lanzl, they present two arterial plots to justify the view that individuals differ significantly in flicker pattern during each cycle. While these data are interesting, we do not share their view, because the data for only 21 nonsmokers in our Table 4 in the original paper show that the individual parameters (BDF, DA [diameter amplitude], and BFG) for each of three flicker cycles are no different in arteries and veins. To further underline this, we present, with considerably greater power, new supporting data from 78 healthy individuals (Table 1), unequivocally demonstrating that there is no change in response to three cycles of flicker.

In paragraph three, Kotliar and Lanzl suggest a possible source of error in our calculations. First, there seems to be a basic misunderstanding: All parameters (BDF, MD [maximum dilation], MC [maximum constriction], DA, and time responses) were not set in any relation or based on or calculated to ΔD, contrary to their statement. BDF is the relative baseline diameter fluctuation, MD is the relative maximum dilation due to flicker, and so on and has been calculated from the raw data individually for each flicker episode.

Paragraph four has several issues. Kotliar and Lanzl further question the relevance of using three stimulation cycles, highlighting that, in their measurements, healthy individuals respond differently in each cycle and also that sometimes not all cycles are recorded, and so the average response should be taken. Correctly, this tripling of the number of analyzed parameters can reduce the level of statistical significance. This may be correct, but our analysis of the data that we presented in Table 4 (and elsewhere) is according to repeated-measures analysis of variance (Friedman's two-way method), which adjusts for individual variances and so is a particularly powerful method.[2] Second, our colleagues cite the paper by Kvernmo et al.[3] to explain their different hypothesis on retinal vasomotion; however, although informative in a general setting, we cannot see the relevance of this paper to our study, since Kvernmo et al. used a laser Doppler technique to examine the cutaneous blood flow of capillaries, arterioles, venules, and dermal vascular plexi, rather than single vessel motion. Third, Kotliar and Lanzl state that, in their example, the two individuals would show significant dilatory results if our analysis paradigm were used and raise the question of which flicker should be used for analysis. In addition, they question whether the difference detected between smokers and nonsmokers is unique and manifests in a similar way in other diseases, and they mention that healthy volunteers may demonstrate differing results despite their similar pathophysiologic status. In regard to the question of which flicker should be used, we stress that, if a repeated-measures protocol is used, all measures should be taken into account and that, if a data recording is incomplete, it should be excluded from further analysis. The advantage of a repeated procedure lies not only in increasing confidence but also in enabling a reaction pattern and examining any "exhaustive" effect. Any measurement taken from two individuals will have differences; these do not generally relate to disease but are part of a "normal range" within a chosen sample. For this reason, we measured a large number of people, to examine the range and dependencies of our proposed marker.

In their fifth paragraph, Kotliar and Lanzl state that a qualitative assessment of each single flicker episode is not always possible in all subjects (elderly population, subjects with bad fixation, and those with excessive blinking during the assessment), which represents a serious limitation in our novel method. We are aware that it can be difficult, in some patients, to obtain all three cycles; however, in our study we analyzed only the data sets in which all three cycles were completely recorded. Assessments of individuals with poor fixation or excessive blinking should be removed if all cycles were not recorded (especially when evaluating normative parameters, good fixation is essential), since eye movements can introduce a significant error especially in regard to diameter fluctuations attributable to vasomotion. The conclusion that fixation, eye movements, and incomplete recordings can influence the result is therefore correct but does not apply to our data set, since we included only subjects with good fixation and in whom all three cycles were completely recorded.

Table 1. Retinal Arterial and Venous Dilatory Parameters in Response to Flickering Light Stimulation

Parameter	Nonsmoker (n = 78)	Friedman ANOVA (within group)
Arterial Response		
BDF		
Flicker 1	4.04 (2.13)	0.654
Flicker 2	4.16 (2.13)	
Flicker 3	4.31 (2.59)	
MD		
Flicker 1	4.64 (2.69)	0.432
Flicker 2	4.81 (2.69)	
Flicker 3	5.04 (2.66)	
DA		
Flicker 1	7.94 (3.56)	0.581
Flicker 2	8.02 (3.26)	
Flicker 3	8.33 (3.50)	
BFR		
Flicker 1	3.75 (2.63)	0.174
Flicker 2	3.85 (2.68)	
Flicker 3	4.01 (2.78)	
Venous Response		
MD		
Flicker 1	5.80 (2.63)	0.750
Flicker 2	5.81 (2.29)	
Flicker 3	6.08 (2.45)	
MC		
Flicker 1	−1.27 (1.34)	0.697
Flicker 2	−1.28 (1.33)	
Flicker 3	−1.23 (1.31)	
DA		
Flicker 1	7.08 (3.17)	0.171
Flicker 2	7.09 (2.86)	
Flicker 3	7.30 (3.02)	

Data are expressed as the mean (SD). All relative values are expressed as the percentage of change in relation to baseline diameter.

Main Criticism

In the first point of their summary, our colleagues state that it is necessary to assess the maximum dilation at the time point of its occurrence rather than at the fixed time point of flicker cessation, as we proposed in our paper, and they refer to two publications[4,5] stating that this problem had been addressed before our publication. To clarify this matter, our publication had been submitted before the publication date of one of these studies[5]; furthermore, both referenced studies involved only 10 healthy subjects, and despite evaluating individual time responses, some dilatory parameters were averaged over three flicker cycles, or averaged diameter values of up to 4 seconds were used. We also want to stress that these papers examined pathologic changes rather than analysis validity, as there was a maximum of 11 healthy volunteers who were matched to the pathologic group, and a cross-sectional view of all ages was therefore lacking. We presented data of 78 healthy individuals with an age range of 17 to 70 years.

In point two of Kotliar and Lanzl's main criticism they state that we reported "a possible higher rate of dilation in the first flicker episode in the group of healthy subjects, compared with subsequent episodes, and in the first flicker episode in smokers." Again, we refer to Table 4 in our paper for clarification. It clearly shows a similar dilatory response at baseline and during provocation for each flicker cycle in the 21 healthy controls but not in the smokers. We included the data for all 78 healthy subjects in Table 1 herein, which are similar to those of the 21 subjects randomly chosen to match the smoking group.

Summary

A protocol with repeated stimulation cycles should be analyzed stepwise, in that each stimulation is evaluated, and a reaction pattern is identified. No two subjects will react identically, in that dilation and recovery times can vary; however, this is not reason enough to abandon a multiple stimulation cycle with fixed recovery and stimulation times. Furthermore, it enables us to examine and determine the range in which a normal subject will be placed and can then be compared to different pathophysiological states (i.e., smokers and different diseases). The purpose of our paper was to highlight the importance of evaluating these different cycles and the danger of false interpretation when averaging results. There are many different ways of evaluating dilatory responses and elasticity, but each of them must be carefully evaluated and should not be overaveraged, which can result in a loss of sensitivity and specificity.

Rebekka Heitmar[1]
Andrew Blann[2]
Robert Peter Cubbidge[1]
Gregory Lip[2]
Doina Gherghel[1]

[1]Aston University, School of Life and Health Sciences, Birmingham, United Kingdom; and the [2]University of Birmingham Centre for Cardiovascular Sciences, Department of Medicine, City Hospital, Birmingham, United Kingdom.
E-mail: r.heitmar1@aston.ac.uk

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