AMERICAN JOURNAL OF PHYSIOLOGY
HEART AND CIRCULATORY PHYSIOLOGY

Am J Physiol Heart Circ Physiol 320: H23–H28, 2021.
First published December 4, 2020; doi:10.1152/ajpheart.00495.2020

RESEARCH ARTICLE

Vascular Biology and Microcirculation

Plexus-specific effect of flicker-light stimulation on the retinal microvasculature assessed with optical coherence tomography angiography

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Abstract

In neural tissues, the coupling between neural activity and blood flow is a physiological key principle in blood flow regulation. We used optical coherence tomography angiography to investigate stimulus-evoked hemodynamic responses in different microvascular layers of the human retina. Twenty-two healthy subjects were included. Vessel density before and during light stimulation was measured using optical coherence tomography angiography and assessed for the superficial, intermediate, and deep capillary plexus of the retinal circulation. Volumetric blood flow was measured using a custom-built Doppler optical coherence tomography system. Our results show that flicker stimulation induced a significant increase in the vessel density of $+9.9 \pm 6.7\%$ in the superficial capillary plexus, $+6.6 \pm 1.7\%$ in the intermediate capillary plexus, and $+4.9 \pm 2.3\%$ in the deep capillary plexus. The hyperemic response of the superficial capillary plexus was significantly higher compared to the intermediate capillary plexus ($P = 0.02$) and deep capillary plexus ($P = 0.002$). Volumetric retinal blood flow increased by $+39.9 \pm 34.9\%$ in arteries and by $+29.8 \pm 16.8\%$ in veins. In conclusion, we showed a strong increase in the retinal microvascular density in response to light stimulation, with the most pronounced effect in the superficial capillary plexus. This is compatible with the hypothesis that the microvasculature exerts an important function in mediating functional hyperemia in humans.

NEW & NOTEWORTHY We present vessel density alterations in response to flicker stimulation using optical coherence tomography angiography and identified the superficial capillary plexus as the layer with the most pronounced effect. This points out the physiological importance of the microvasculature in mediating functional hyperemia and suggests a fine-tuned plexus-specific mechanism to meet cellular metabolic demands.

healthy subjects; neurovascular coupling; optical coherence tomography angiography; retinal blood flow

INTRODUCTION

Neurovascular coupling (NVC), also referred to as functional hyperemia, is a highly sophisticated mechanism that links functional activity and blood flow in neural tissues (1). First described for the brain (2), NVC allows to spatiotemporally adapt the local blood flow to changing metabolic demands caused by firing neurons (1, 3). Although the brain has been extensively used to investigate functional hyperemia (4), the currently available imaging technologies set limits in terms of spatial and temporal resolution particularly when it comes to in vivo investigation of brain microcirculation in humans (5).

As the eye and the brain share similar embryological origins with comparable structural and physiological characteristics, the retina may offer considerable advantages for the in vivo investigation of functional hyperemia. Moreover, the retina contains a dense microvascular network organized in

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Submitted 19 June 2020 / Revised 16 October 2020 / Accepted 16 November 2020

distinct layers (6, 7), which can be easily stimulated by photic stimulation (8).

The current study takes advantage of recently introduced noninvasive imaging techniques to investigate NVC of retinal vessels in different vascular layers and compares the response of the microcirculation with the response of larger retinal arteries and veins. This is of particular importance because it is currently unclear to what extent the microcirculation, in particular the capillaries and small arterioles, contributes to the hyperemic response. Moreover, given that there is evidence that under pathological conditions, such as diabetes, capillary nonperfusion is more pronounced in some retinal plexuses than in others (9), further investigation of the depth-resolved regulation of vascular tone is warranted. Here, we use optical coherence tomography angiography (OCTA) to assess flicker-induced vascular hyperemia in different vascular layers of the retina and Doppler OCT (DOCT) to measure blood flow in major retinal vessels. A characterization of this response in the smallest retinal vessels of human circulation may add to our understanding of the neurovascular response and how changes in the microvasculature relate to the blood flow increase in major retinal vessels.

MATERIAL AND METHODS

Subjects

Twenty-two healthy subjects (12 females and 10 males) were included in this study. The study protocol was approved by the Ethics Committee of the Medical University of Vienna, and the study was performed in adherence to the guidelines of the Declaration of Helsinki as well as Good Clinical Practice Guidelines. Written informed consent was obtained from all study participants.

Experimental Paradigm

A screening visit was performed which included the assessment of systemic hemodynamic parameters and a full ophthalmic examination.

The inclusion criteria were as follows: men and women over 18 yr of age, nonsmokers, insignificant medical history and ophthalmic examination, and ametropia <6 diopters. Exclusion criteria comprised untreated arterial hypertension, history or family history of epilepsy, best corrected visual acuity <0.5 Snellen, alcoholism, or substance abuse. Pupils were dilated by one drop of 0.5% tropicamide. After a 20-min resting period, OCTA and DOCT measurements were performed. Intraocular pressure (IOP) and systemic hemodynamics were taken before and after the measurements.

Optical Coherence Tomography Angiography

A commercially available spectral domain OCT device with OCTA module (Spectralis OCT, Heidelberg Engineering, Heidelberg, Germany) was used to perform the OCTA measurements. A square wave flicker-light pattern was applied using a 12-light-emitting diode ring placed around the front lens of the OCT device using a flicker frequency of 7 Hz, a brightness of ~450 lx, and a modulation depth of 100%. Macula-centered, high-resolution (512 B-scans, 512 A-scans/B-scan) 10° × 10° OCTA scans were performed at baseline and during flicker stimulation, respectively. A total of four different OCTA slabs were generated per timepoint and subject using the standard slab settings of the built-in Heidelberg OCTA software (v 1.10.2.0). These settings closely adhere to the plexus architecture suggested by Campbell et al. (6). The raw enface scans (Figs. 1*A* and 3) of the superficial capillary plexus (SCP), intermediate capillary plexus (ICP), deep capillary plexus (DCP), and full retina (FR) were exported using MATLAB (v 2018 b, MathWorks, MA). To account for varying scan centration at the different timepoints, recordings were registered by matching geometric features on SCP/FR images (Fig. 1*B*) and the transformation matrix was applied to the corresponding ICP and DCP images. A fovea-centered annulus with an inner diameter of 1 mm and outer diameter of 2.5 mm was created (Fig. 1*C*). On SCP/FR scans, large vessels were isolated using a thresholding method (intensity >130) (10). This step selectively removed arterioles and venules preserving the capillary portion of the SCP (Fig. 1*E*). Projection artifacts from superficial vessels were removed from ICP and DCP by applying the large vessel mask from the previous step to the ICP and DCP scans. Finally, a "Frangi filter," which is a Hessian filter vessel enhancement algorithm (11), was applied to optimize capillary visibility (Fig. 1*F*). Each image was binarized by mean values to obtain its corresponding vessel density values (Fig. 1*G*).

Dual-Beam Bidirectional Doppler Fourier Domain OCT

Flicker-induced hyperemia in major retinal vessels was measured using a prototype dual-beam bidirectional Doppler Fourier domain OCT (DOCT) device, which has been previously described (12). To analyze blood flow changes during flicker, one temporal arteriovenous (AV) pair around 1 to 2 disk diameters from the optic disk was selected and DOCT data before and during flicker stimulation were acquired at this position.

Statistical Analysis

All statistical analyses were done using IBM SPSS Statistics (v 26, IBM, Armonk, NY). Outcome parameters were checked for normality using the Shapiro–Wilk test. Vessel density and ocular hemodynamics are expressed as absolute baseline and flicker values, respectively, and as percentage change over baseline. Two-tailed paired *t* tests were used to assess the differences between measurements obtained before and during flicker stimulations. All results are presented as means ± standard deviation. A $P < 0.05$ was considered as significant.

RESULTS

Twenty-two healthy subjects were included, mean age was 27 ± 4 yr, and 12 were female, aged between 23 and 39 yr (Table 1). Blood pressure and IOP were in the normal range.

Macrovasculature as Assessed with DOCT

As expected, a significant increase in diameters of major retinal vessels was observed during flicker stimulation. Diameters in retinal arteries increased by 3.7 ± 3.1% from 118.0 ± 17.6 to 122.3 ± 17.6 μm ($P < 0.001$) and in veins by 4.5 ± 3.0% from 147.9 ± 21.5 to 154.4 ± 22.2 μm ($P < 0.001$, Fig. 2). Similarly, a significant increase in retinal blood flow

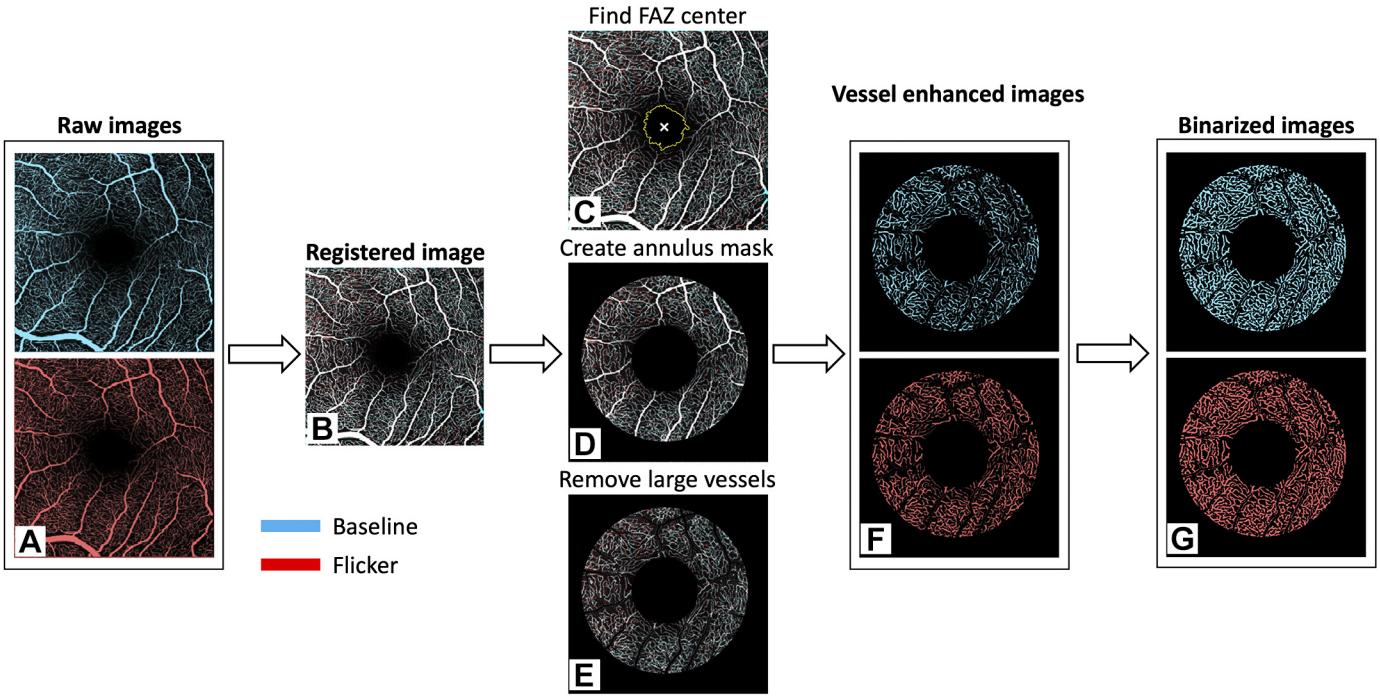

Figure. 1. Framework of OCTA image postprocessing. *A*: raw images extracted from the OCTA machine. *B*: overlaid image from baseline and flicker recordings. *C*: the FAZ was roughly delineated automatically, and the center was computed (white cross). *D*: an annulus centered at FAZ center with inner diameter of 1 mm and outer diameter 2.5 mm was generated as a mask to the 3 × 3 mm image. *E*: large vessels were isolated and excluded. *F*: vessel enhancement algorithm (Frangi filter) was applied to image *E*, and then, it was binarized to achieve image *G*. Blue: baseline; red: flicker. FAZ, foveal avascular zone; OCTA, optical coherence tomography angiography.

was observed in arteries as well as in veins during flicker. Retinal arterial blood flow as measured with DOCT increased from 7.1 ± 3.7 to 9.4 ± 4.5 μL/min (+39.9 ± 34.9%, $P < 0.001$) and blood flow in retinal veins from 10.2 ± 4.1 to 13.2 ± 5.5 μL/min (+29.8 ± 16.8%, $P < 0.001$, Fig. 2).

Microvasculature as Assessed with OCTA

As shown in Fig. 3, stimulation with flicker light induced an increase in vascular density in all layers. A significant increase in FR vessel density from 39.7 ± 1.7 to 41.2 ± 1.6% (+3.8 ± 2.7%, $P < 0.001$) was observed. When looking at the different layers separately, vessel density increased from 23.0 ± 2.3 to 25.2 ± 2.4% (+9.9 ± 6.7%, $P < 0.001$) in the SCP, from 34.8 ± 1.4 to 37.0 ± 1.4% (+6.6 ± 1.7%, $P < 0.001$) in the ICP, and from 34.6 ± 2.1 to 36.2 ± 2.2% (+4.9 ± 2.3%, $P < 0.001$) in the DCP, respectively. Thus, the increase in retinal vascular density was significantly higher in the SCP compared to the ICP (P = 0.02) and DCP (P = .002). Also, when comparing the amount of increase in vessel density during stimulation with flicker light in the ICP and DCP, a significantly higher effect was found in the ICP ($P < 0.001$). A significant correlation was found between SCP and ICP at baseline ($P < 0.001$, r = 0.697) and during flicker stimulation (P = 0.002, r = 0.625).

Table 1. *Baseline characteristics of the study population*

Age, yr	27 ± 4
SBP, mmHg	117 ± 13
DBP, mmHg	72 ± 7
MAP, mmHg	89 ± 9
HR, beats/min	66 ± 9
IOP, mmHg	13 ± 2
OPP, mmHg	47 ± 6

Values are means ± SD. DBP, diastolic blood pressure; HR, heart rate; IOP, intraocular pressure; MAP, mean arterial pressure; OPP, ocular perfusion pressure; SBP, systolic blood pressure.

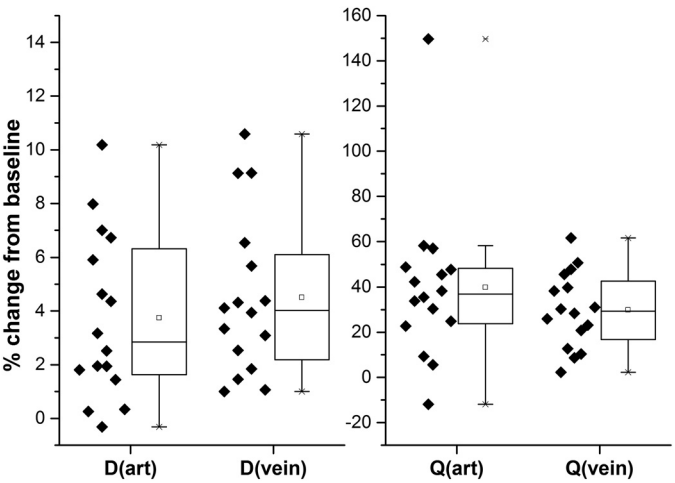

Figure. 2. Relative change from baseline during stimulation with flicker light in diameters in retinal arteries (D_{art}) and veins (D_{vein}) and in blood flow in retinal arteries (Q_{art}) and veins (Q_{vein}). Data are presented as individual values (*left*) and as boxplots. *significant vs. baseline.

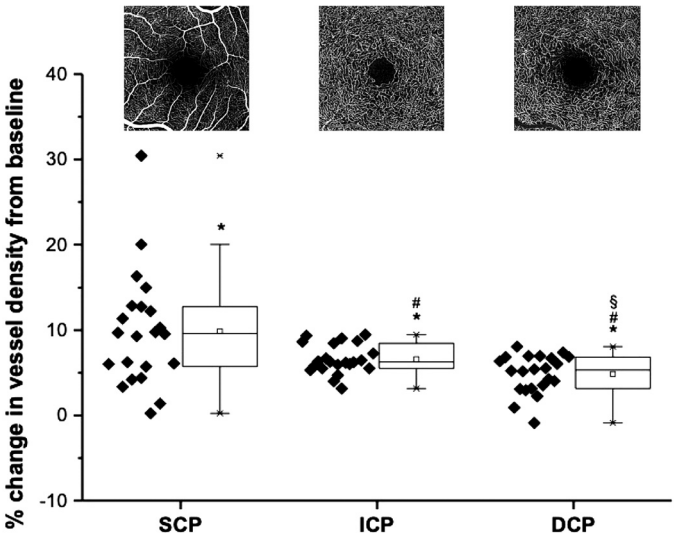

Figure. 3. Relative change from baseline during stimulation with flicker light retinal vessel density in the three assessed layers (SCP, ICP, and DCP). Data are presented as individual values (*left*) and as boxplots. *significant vs. baseline, #significant vs. SCP, §significant vs. ICP. DCP, deep capillary plexus; ICP, intermediate capillary plexus; SCP, superficial capillary plexus.

DISCUSSION

Here, we show using OCTA that there is a significant increase of vessel density in the parafoveal microcirculation during stimulation with flicker light in healthy subjects. Our data also demonstrate a more pronounced flicker-induced hyperemic response in the superficial layer compared to the deeper vascular layers of the retina. The present study is the first to apply hemodynamic measurements during flicker stimulation in both the microvasculature and the larger retinal vessels.

By the means of OCTA, we observed a statistically significant increase in parafoveal vessel density of ~5%–10%. As a major advantage, OCTA allows for noninvasive and depth-resolved imaging of the microcirculation. Comparative studies between OCTA and postmortem histological samples show that this technique is capable of reliably visualizing vessels down to a size of 8 μm (13). As we were mainly interested in the precapillary and capillary vasculature in this study, larger vessels in the size of ~30 μm or more were removed during image processing. These larger vessels would contribute disproportionally to the signal and therefore reduce the performance in detecting changes in the microcirculation. Interestingly, our data show that the SCP shows a higher variance compared to the deeper plexus. The reason for this effect is not entirely clear. However, as the highest response was observed in the SCP and considering the high variability observed in the flicker-induced hyperemia in healthy subjects (14), it is reasonable to suggest that this variability mainly reflects the interindividual variability of the flicker response per se.

Our data are compatible with the current hypothesis that in the human retina the microvasculature, most importantly small vessels in the size of 30 μm or less, significantly contributes to the large blood flow response seen in the upstream vessels and is therefore in good agreement with our previous modeling of retinal blood flow during flicker stimulation (15). It is also in agreement with our previous vessel analysis data showing that the flicker-induced vasodilation is inversely proportional to the absolute vessel diameter (16). During flicker stimulation, retinal arterial blood flow in large vessels increased by ~40% and was accompanied by a vasodilatation of ~4% only. Given that the microcirculation is the major site of resistance to flow, the proportional dilatation in the microcirculation may have a much greater effect on the upstream vessels. Consequently, relatively small alterations in particular in the small resistance vessels can translate to large changes in blood flow. The same was reflected in larger veins, where blood flow increased by ~30% and diameters by 4.5%. Our data in larger retinal vessels are well compatible with previously published data (14, 17, 18) that show a vasodilation of retinal arterial and venous calibers in the range of 3%–4% together with an increase of 40%–60% in retinal blood flow.

Using OCTA, a recently published study investigated the effect of light and dark transitions as well as flicker stimulation in different layers of the human retina (19). The study shows that capillary recruitment was higher in the deeper capillary layer during photic stimulation, which is in contrast to the findings of the present study (19). However, as the study of Nesper et al. used a mixture of dark-light transitions and flicker stimulation, the data are difficult to compare with previously published studies and our current work, which studied light-adapted eyes. Previous studies have shown that light adaptation in itself is associated with a retinal vascular response (20). In addition, in the experiment of Nesper et al., measurements were done not during but after flicker stimulation. This makes it difficult to fully appreciate the results, because it has been shown that cessation of the flicker stimulation is followed by a decrease in retinal vessel diameters indicating for a transient reduction of retinal blood flow (21, 22).

Furthermore, using adaptive optics, a previous report in humans indicates that vessels of a diameter of 8 μm or less showed the greatest proportional diameter change, supporting the hypothesis that mainly the small vessels are responsible for the blood flow increase (23). This study was, however, limited to three subjects, and the field of observation was very small. Hence, it is unclear to which degree these results can be extrapolated to the entire retinal microvasculature.

Animal and ex vivo studies suggest that small and precapillary arterioles play an important role in regulating blood flow and mediating functional hyperemia (24–26). Using a confocal microscopy-based technique, studies in the rat retina show a fast and pronounced flow increase in small arteries caused by photic stimulation (27). Furthermore, this study indicates a considerable difference in the hyperemic response depending on the retinal layers, among which the intermediate layer showed the most pronounced flicker-evoked vasodilatation. In contrast, deeper and superficial layers showed only minor reactions (27). Studies using OCTA to investigate rat and mice retinas also show large vascular changes in the microcirculation (28–30). In the mouse, again the intermediate and deep layers showed the most pronounced response (28, 29). This is in contrast to our results

in humans that show the most pronounced vascular responses in the SCP. Species differences as well as unknown effects of the anesthesia in the animal models may account for this difference. For example, using invasive measurement of retinal oxygen tension it has been shown that even small differences in anatomical properties result in considerably different retinal oxygen profiles (31).

Our data indicate that flicker-induced hyperemia is most pronounced in the superficial layer, which is supported by other experiments. In humans, the superficial layer consists of a dense network of precapillary arterioles, which accordingly plays a key role in retinal vascular resistance. Furthermore, anatomically speaking, the structures supplied by the SCP mainly consist of the nerve fiber layer, the ganglion cell layer, and the inner plexiform layer (6). Although the relative contribution in energy demand of different neural cells is currently unknown, our results are in keeping with the previous hypothesis that activated ganglion cells and amacrine cells of the inner retina are a major component of increased metabolic demand mediating functional hyperemia (8). This is supported by the comparable response of blood flow and retinal ganglion cell activity to flicker stimulation with regard to temporal frequency, modulation depth, and relative chromaticity (32, 33). Moreover, it has been shown that adaption to temporal contrast is, at least in part, a retinal function and is reflected by a modulation of retinal ganglion cell firing rate (34, 35). Finally, the second harmonic of the heterochromatic red-green flicker, which contains a significant contribution from the retinal ganglion cells (36), is significantly correlated to the blood flow response to flicker under different experimental conditions (37, 38).

Several limitations need to be considered when interpreting our findings. First, because of the limited scanning speed of the OCTA instruments currently available, our OCTA scanning pattern covers only the 10° of the central retina. Thus, we cannot fully exclude that the vascular responses are different in other regions of the retina. Moreover, DOCT measurements were performed in only one major temporal AV pair, whereas OCTA measurements were done macula centered. Thus, given that temporal vessels not solely supply the macular region, our DOCT data also contain information regarding blood flow of the more peripheral parts of the retina. Measurements with larger acquisition frames including wide-field OCTA instruments will be able to overcome these limitations in the near future. Finally, OCTA vascular data itself require careful interpretation. As the OCTA parameter vascular density is based on decorrelation analysis, an increase in vessel density may be caused by either capillary recruitment, an increase in retinal vessel diameter in small arterioles or an increase in blood velocity. However, the current OCTA technology is not capable of distinguishing between these options, and further studies using multimodal imaging approaches are required.

In summary, our data show that OCTA is an appropriate approach for the in vivo investigation of flicker-induced hyperemia of the retinal microcirculation in humans. Furthermore, we demonstrate that the SCP shows a more pronounced vasodilator response than the deeper layers of the retinal vasculature indicating an important role that ganglion cells play in NVC in the human retina. Our results strengthen the concept that microvascular changes play a major role in the flicker light-induced hyperemic response of the retina.

GRANTS

Financial support from the Austrian Science Foundation FWF Projects KLIF529, KLIF721, and the Singapore National Medical Research Council (NMRC/CG/C010A/2017_SERI and NMRC/OFIRG/0048/2017, and NMRC/OFLCG/004c/2018) is gratefully acknowledged.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.K., M.P., and G.G. conceived and designed research; M.K., N. H., M.P., and A.S. performed experiments; B.T., J.C., D.S., L.S., and G.G. analyzed data; M.K., D.S., L.S., and G.G. interpreted results of experiments; M.K., B.T., and D.S. prepared figures; M.K., R.M.W., D.S., and G.G. drafted manuscript; M.K., N.H., B.T., M.P., A.S., R.M.W., J.C., D.S., L.S., and G.G. edited and revised manuscript; M. K., N.H., B.T., M.P., A.S., R.M.W., J.C., D.S., L.S., and G.G. approved final version of manuscript.

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