Author’s response to reviews

Title: Retinal blood flow is increased in type 1 diabetes mellitus patients with advanced stages of retinopathy

Authors:
Hoang-Ton Nguyen (ht.nguyen@vumc.nl)
Eelco van Duinkerken (e.vanduinkerken@vumc.nl)
Frank. Verbraak (f.d.verbraak@amc.uva.nl)
Bettine Polak (bcp.polak@ziggo.nl)
Peter Ringens (p.ringens@mumc.nl)
Michaela Diamant (m.diamant@vumc.nl)
Annette Moll (a.moll@vumc.nl)

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Author’s response to reviews:

Reply to the comments of Reviewer 1

We thank the Reviewer for her time spent reviewing our manuscript and for the thoughtful comments. Below you can find our response to the issues raised. Text in red, italic and underlined represent the revised text.

Comment 1:

Some acronyms are not clearly explained: ICD, MRI, VU -- please add the corresponding labels.

Answer 1:

We have added the requested labels to the manuscript.
Comment 2:

The analysis performed for the panretinal laser photocoagulation included both T1DM and T2DM subjects. Based on the lack of significance observed for the BF measurements before and after the procedure and that mean results were generated with all diabetic subjects with different type of diabetes in the same dataset, I do recommend to create two subgroups and run the analysis (basically calculate the mean separated) again. It is clear the small amount of eyes in the group is not good enough for a decent statistical analysis but subtle changes may be revealed. I noticed in about 4 eyes the BF slightly increased after surgery while for 3 decreased. For example, the line/trend plotted in Figure 3 for the mean BF result should be generated for the T1 and T2 groups. I will also recommend to label/color the lines in Figure 3 based on type of diabetes.

Answer 2:

We thank the Reviewer for these thoughtful comments and agree that changes in the analysis approach and display of results provide more insight into the trends in the 2 groups of diabetes patients. We have run a paired t-test for both patient groups separately. In the type 1 diabetes (T1DM) group (4 eyes analyzed) we saw that blood flow before laser was 211.93 Arbitrary Units (AU), whereas after laser this was 220.74 AU (P = 0.28). In the type 2 diabetes (T2DM) group (4 eyes analyzed) we essentially saw a similar pattern. The mean flow before laser was 222.37 AU and after laser 230.61 AU (P = 0.303). We have changed Figure 3, and now separately show both groups, which are color coded. We have added the requested analyses as follows:

Results section, revised manuscript (page 14):

When analyzing the T1DM and T2DM patients separately, we saw similar results in both groups. In the T1DM group, blood flow before panretinal photocoagulation was 211.9 AU, whereas after photocoagulation this increased to 220.7 AU (P = 0.28, paired T-test). In the T2DM group blood flow before panretinal photocoagulation was 222.4 AU and after photocoagulation increased to 230.6 AU (P = 0.303, paired T-test).

Comment 3:

Figure 1 should identify the nasal and temporal locations. The images are inlets of a complete image of the retina. The figure should be better designed for easy interpretation mainly for readers not familiar with the software or device used in the study.
Answer 3:

Figure 1 has been revised. It now consists of a fundus photograph, in which two boxes indicate the measurement locations. An example of an image of each of the two measurement locations has been displayed.

Comment 4:

Table 1 is misleading: type of diabetes should be a category to include in the demographics info.

Answer 4:

We thank the Reviewer for pointing out to us that Table 1 is not sufficiently clearly labeled. Table 1 shows the patient characteristics of the main study, which included only type 1 diabetes patients. We have clearly added this to the title of Table 1. For clarity we have also added another table, Table 2, which includes the characteristics of the group of 4 type 1 and 4 type 2 diabetes patients separately.

Comment 5:

Some diabetic patients have hypertension. This condition really affects the BF measurements and confound the results. Therefore, the authors should consider separating such patients from the non-hypertensive diabetics.

Answer 5:

We agree with the Reviewer that hypertension can be a confounding factor in our results. To take this into account, our analyses are corrected for mean systolic blood pressure. Correcting for hypertension was not possible in the main analyses as controls with hypertension were excluded from participation. As a second step, we performed a regression analysis to determine the factors associated with blood flow in only T1DM patients. Here we did enter hypertension, but it was not related to blood flow. To gain more insight into the effect of hypertension, we split the T1DM group into patient with and without hypertension. The median flow for T1DM patients with hypertension did not differ from the patients without hypertension (236.68 and 249.72 AU respectively [P=0.17]). The median flow was also similar when analyzed with separate groups of retinopathy severity (pDRP hypertensive group 250.8 AU, non-hypertensive
group 246.4 AU; npDRP hypertensive group 273.7 AU, non-hypertensive group 228.1 AU; nDRP hypertensive group 215.6 AU, non-hypertensive group 224.1 AU). As the Reviewer requested we have repeated the analysis excluding the patients with hypertension. This left us with 12 patients with proliferative retinopathy, 9 patients with non-proliferative retinopathy and 24 patients without retinopathy. The median flow for the group with proliferative retinopathy was 246.4 AU, for the group with non-proliferative diabetic retinopathy this was 228.15 AU and for the group without retinopathy 224.4 AU, none of the controls were excluded so the median flow of this group did not alter (200.9 AU). The results of the statistical analyses remained similar. There was an overall effect of group (P=0.001) and a linear trend towards increased flow with increased retinopathy severity (P=0.01) Post-hoc between group analysis, after Bonferroni correction, showed that blood flow was statistically significantly higher in the pDRP group compared with controls (P < 0.05), but not compared to the others groups. We have added these results to the Results section, and have also added a short paragraph on this in the Discussion section.

Results section, revised manuscript (pages 12 and 13):

As hypertension can significantly influence blood flow measurements we have additionally repeated the analysis in patients and controls without hypertension only. Firstly, median flow for T1DM patients with hypertension did not significantly differ from the flow in patients without hypertension (236.7 and 249.7 AU respectively [P = 0.170]). After excluding participants with hypertension, an analysis was performed with 12 patients in de pDRP group, 9 patients in the npDRP group and 24 patients in the nDRP group. The median flow for the pDRP group was 246.4 AU, for the npDRP group 228.15 AU and for the nDRP group this was 224.4 AU. As none of the controls were excluded, the median flow in this group did not change (200.9 AU). Despite the apparent loss of sample size and thus statistical power, in the presence of an increased standard deviation this exploratory analysis showed results similar to the whole group analysis. There was an overall effect of group (P=0.001) and a significant linear trend over the groups (P &lt; 0.001). After Bonferroni correction blood flow was higher in the pDRP group compared to controls (P = 0.003). There were no significant differences in blood flow between the pDRP group and the other groups.

Discussion section, revised manuscript (pages 16 and 17):

Both hypertension and vasodilatory medication, such as some antihypertensive medication, could have an effect on blood flow and thus confound our results. Therefore, we repeated our main analysis excluding all patients with hypertension (which also included those using antihypertensive medication). Despite the loss of sample size and statistical power, this analysis showed similar results. Furthermore, blood flow was not significantly different
between the groups with and without hypertension and hypertension was not related to blood flow in the regression analysis performed in the whole group. Taken together this suggests that in this group of patients hypertension did not confound our blood flow findings.

Reply to the comments of Reviewer 2

We thank the Reviewer for his/her time spent reviewing our manuscript and for the thoughtful comments. Below you can find our response to the issues raised. Text in red, italic and underlined represent the revised text.

Comment 1:

The controls provide an unfair comparison. They are "45 gender-matched healthy controls", but they are not age-matched and the authors exclude from their controls patients with Hypertension and those using statins, so patients with hyperlipidemia. Thus, they are selecting for patients without cardiovascular risk factors who are likely significantly more healthy from a CV perspective than the diabetic patients. They find this and state "Groups differed with regard to age, diabetes duration, systolic blood pressure, HbA1c and hypertension" group with proliferative DRP was significantly older than the group without DRP and controls, had higher systolic blood pressure relative to the patients without DRP and control subjects and had longer disease duration than the group without DRP (all P < 0.05)."

Answer 1:

The Reviewer is right that the control group is healthier in terms of cardiovascular/cardiometabolic profile than the patient groups. For the purpose of the main study, assessing differences in brain and cognitive parameters between type 1 diabetes (T1DM) patients and controls, it is important to include a healthy control group, which excludes hypertension or dyslipidemia. Furthermore, given the mean age of 38 years of the control group and the age range for inclusion of 18-56, in the absence of obesity, it is not very likely control participants will already be diagnosed by their general practitioners with either hypertension or dyslipidemia. Including participants with either of these conditions may raise a bias as these may well be linked to another disorder in such young age, e.g. obesity, which excludes them from being healthy. Unfortunately, we were not able to match all groups in terms of age. Although the patients without proliferative retinopathy are age-matched with the control group, the patients with proliferative retinopathy were older. This is a natural phenomenon as it takes 15-25 years to develop the proliferative stage of retinopathy, if it develops at all, and thus these patients are older than the patients in the other groups. We have
therefore chosen to match the control group to the patients without proliferative retinopathy. To limit the influence of age on the results, all analyses were corrected for age. In the Discussion section we have added the age difference to the limitations and have briefly discussed the absence of hypertension or dyslipidemia in the control group.

Discussion section, revised manuscript (pages 17 and 18):

Control participants were excluded when having hypertension or dyslipidemia. This was chosen as participants included were of a young age (mean age 38 years) with an upper limit of 56 years, and in such young people hypertension and dyslipidemia are often part of the metabolic syndrome or another disorder, which excludes participants from being healthy. Lastly, patients with pDRP are older compared with the other groups. This is a natural phenomenon as it takes 15-25 or more years to reach the proliferative stage of DRP, if it develops at all. Given this age difference, we decided to carefully match in terms of age the control group to the groups without pDRP. To limit the effect of age on the results, it was treated as a confounding factor in all analyses.

Comment 2:

The study corrects for blood sugar levels and ensure it is between 4 and 15 mmol/l, and then reports there was no difference in the acute sugar values. Since the HbA1c values are different, likely the sugars were different. But they likely alter this value in their intervention.

Answer 2:

The Reviewer raises an interesting point here. It is good to mention first, that we did not do an intervention in terms of a clamp procedure to keep the blood glucose values stable. During the testing days they were checked before the start of each measurement and when the patients felt the need to do so, and acted upon if necessary. The HbA1c values are different, but only between the control group and the T1DM groups. Within the patient groups they were similar (7.8, 7.9 and 8.1%) and not statistically different from each other. It is not very likely that, with these subtle differences in HbA1c, the blood glucose levels would have been different during non-testing days. Furthermore, the upper limit of the blood glucose range is relatively high and it can be assumed that patients, in their daily lives, intervene at lower levels than 15 mmol/l. Lastly, similar glucose levels during testing are important as a study showed changes in retinal blood flow due to elevated blood glucose levels [1].
Comment 3:

Do the authors evaluate for any medications that could cause vasodilation, such as niacin?

Answer 3:

We thank the Reviewer for this input. We did not particularly exclude the use of vasodilatory medication. Vasodilatory medication will be predominantly used in the presence of hypertension. Therefore, in the revised manuscript we have added an analysis of blood flow in patients with and without hypertension, and also a group analysis without patients with hypertension. The mean flow for T1DM patients with hypertension did not differ from the patients without hypertension (236.68 and 249.72 Arbitrary Units [AU] respectively [P=0.17]). The median flow was also similar when analyzed with separate groups of retinopathy severity (pDRP hypertensive group 250.8 AU, non-hypertensive group 246.4 AU; npDRP hypertensive group 273.7 AU, non-hypertensive group 228.1 AU; nDRP hypertensive group 215.6 AU, non-hypertensive group 224.1 AU). As the Reviewer requested we have repeated the analysis excluding the patients with hypertension. This left us with 12 patients with proliferative retinopathy, 9 patients with non-proliferative retinopathy and 24 patients without proliferative retinopathy. The median flow for the group with proliferative retinopathy was 246.4 AU, for the group with non-proliferative diabetic retinopathy this was 228.15 AU and for the group without retinopathy 224.4 AU. As none of the controls were excluded, the median flow in this group did not alter (200.9 AU). The results of the statistical analyses remained similar. There was an overall effect of group (P=0.001) and a linear trend towards increased flow with increased retinopathy severity (P=0.01) Post-hoc between group analysis, after Bonferroni correction, showed that blood flow was statistically significantly higher in the pDRP group compared with controls (P < 0.003), but not compared to the others groups (all P<0.05). We have added these results to the Results section, and have also added a short paragraph on this in the Discussion section.

Results section, revised manuscript (pages 12 and 13):

As hypertension can significantly influence blood flow measurements we have additionally repeated the analysis in patients and controls without hypertension only. Firstly, median flow for T1DM patients with hypertension did not significantly differ from the flow in patients without hypertension (236.7 and 249.7 AU respectively [P = 0.170]). After excluding participants with hypertension, an analysis was performed with 12 patients in de pDRP group, 9 patients in the npDRP group and 24 patients in the nDRP group. The median flow for the pDRP group was 246.4 AU, for the npDRP group 228.15 AU and for the nDRP group this was 224.4 AU. As none of the controls were excluded, the median flow in this group did not
change (200.9 AU). Despite the apparent loss of sample size and thus statistical power, in the presence of an increased standard deviation this exploratory analysis showed results similar to the whole group analysis. There was an overall effect of group (P=0.001) and a significant linear trend over the groups (P < 0.001). After Bonferroni correction blood flow was higher in the pDRP group compared to controls (P = 0.003). There were no significant differences in blood flow between the pDRP group and the other groups.

Discussion section, revised manuscript (pages 16 and 17):

Both hypertension and vasodilatory medication, such as some antihypertensive medication, could have an effect on blood flow and thus confound our results. Therefore, we repeated our main analysis excluding all patients with hypertension (which also included those using antihypertensive medication). Despite the loss of sample size and statistical power, this analysis showed similar results. Furthermore, blood flow was not significantly different between the groups with and without hypertension and hypertension was not related to blood flow in the regression analysis performed in the whole group. Taken together this suggests that in this group of patients hypertension did not confound our blood flow findings.

Comment 4:

It would be helpful to evaluate the large vessels along with the choroidal/choriocapillaris circulation.

Answer 4:

Michelson et al (1995) has described the principles of scanning laser Doppler flowmetry of the retina. The Heidelberg Retinal Flowmeter employs measurement of Doppler broadening, which is limited at 2000 Hz. Measurements in major vessels produce a Doppler broadening over 2000 Hz, and therefore measuring major vessels are not possible [2]. Furthermore, because of the retinal pigment epithelium, laser light of the Heidelberg Retinal flowmeter is absorbed. Therefore the choroidal vasculature cannot is not measured. Pandav et al. (2008), using a pig model, showed the measurement of the Heidelberg Retinal flowmeter to only be in the retina [3]. We have added this as follows to the Discussion section:
Discussion section, revised manuscript (page 18):

It would have been interesting to also measure the large vessels as well as the choroidal/choriocapillaris circulation to capture a more complete picture of retinal blood flow. Unfortunately, due to technical limitations of the Heidelberg Retinal Flowmeter and using the AFFPIA software, it is not capable of capturing flow in these parts of the retina [31,32].

Comment 5:

"Large vessels and photocoagulation scars were manually excluded, leaving only capillaries to be analyzed." We know that there is capillary drop-out and peripheral nonperfusion with DM. Since we are not assessing the entire eye but only a small region, could there not be an increase in this region and a decrease adjacent to this? Doesn't this call the entire analysis into question?

Answer 5:

The reviewer raises a very interesting point as indeed areas of capillary drop-out and peripheral non-perfusion are present, particularly in advanced stages of diabetic retinopathy such as proliferative diabetic retinopathy. We did not measure peripheral blood flow, therefore these non-perfusion areas could not be assessed. Given the distribution of blood flow findings in the proliferative diabetic retinopathy group (Figure 2), as compared to the other groups, capillary drop-out was modest in the measured areas as we did not measure extremely low retinal blood flow values. For this study, we attempted to measure blood flow in a uniform location. Because of the anatomy of the optic disc, these areas were chosen. A limitation of our measurements is that the data cannot be extrapolated to the entire retina. This has been mentioned in the discussion section.

Discussion section, revised manuscript (page 16):

Capillary drop-out and peripheral areas of non-perfusion are present in proliferative diabetic retinopathy (REF). As we did not find extremely low blood flow values in the pDRP group, it can be assumed that capillary drop-out was modest in the measured areas. The peripheral retina could not be measured using our technique and therefore, and because our measurements were limited to the peripapillary area, we are not able to extrapolate our findings to the entire retina.
Comment 6:

With PRP, the authors evaluate a heterogeneous group of 8 patients with both type 1 and 2 DM and find about the same with 2 going up slightly, 2 going down slightly, and 4 staying around the same, so no overall difference. However, they have highly variable follow-up, ranging from 2 to 12 months. This would be better to standardize this time.

Answer 6:

We agree with the Reviewer that the range of follow-up time is large, and ideally should have been smaller. This larger range is a result of a number of participants not showing up for the check-up after laser photocoagulation. As an addition we have added the follow-up time as a covariate to the analysis and found essentially similar results (no statistical difference in blood flow \( P = 0.404 \)). We have added the results of this analysis to the Results section and have added the follow-up range as a limitation to the Discussion section.

Results section, revised manuscript (page 14):

Figure 3 shows retinal blood flow before and after panretinal photocoagulation. Blood flow did not significantly change after panretinal photocoagulation, compared to before panretinal photocoagulation (\( P > 0.05 \), paired T-test) When adding follow-up time as a covariate, change over time remained statistically non-significant (\( P = 0.404 \)).

Discussion section, revised manuscript (page 17):

We did not find any significant change in blood flow before and after panretinal photocoagulation, although the substudy was limited by a small sample size and variable time to follow up (2 - 12 months). This variable follow-up time was due to a number of participants not showing up for the routine check-up after laser photocoagulation, and adjusting for this did not change the results.

Reference List
