Microaneurysm Turnover at the Macula Predicts Risk of Development of Clinically Significant Macular Edema in Persons With Mild Nonproliferative Diabetic Retinopathy

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OBJECTIVE—To examine the relationship between microaneurysm (MA) turnover using automated analysis of fundus photographs (RetmarkerDR, Critical Health SA) and development of clinically significant macular edema (CSME) in nonproliferative diabetic retinopathy (NPDR).

RESEARCH DESIGN AND METHODS—A prospective, monocenter, observational study was designed to follow eyes/patients with type 2 diabetes and NPDR (Early Treatment Diabetic Retinopathy Study levels 20 and 35) with no prior laser treatment for 2 years or until development of CSME. A total of 410 patients, one eye per patient, fulfilled the inclusion/exclusion criteria and were included in the study. Ophthalmologic examinations including best corrected visual acuity, fundus photography, and optical coherence tomography were performed at baseline, 6 months, and at the last study visit (24 months or before laser treatment).

RESULTS—A total of 348 eyes/patients performed the 24-month visit or developed CSME. Of these 348 eyes/patients, 26 developed CSME. HbA1c levels at baseline and MA turnover (i.e., the sum of the MA formation and disappearance rates) computed during the first 6 months of follow-up were found to be independently predictive factors for development of CSME. MA turnover was $11.2 \pm 1.2$ in the 26 eyes/patients that developed CSME and $5.0 \pm 2.1$ in the remaining 322 ($P < 0.001$). Higher MA turnover values correlated with earlier development of CSME. MA turnover predictive values for CSME development were, for the positive predictive value, 20% and for the negative predictive value, 96%.

CONCLUSIONS—MA turnover calculated with the RetmarkerDR predicts development of CSME in eyes with NPDR. Low MA turnover values identify well the eyes that are less likely to develop CSME in a 2-year period.

Diabetic retinopathy (DR) is a common and serious condition. It is the leading cause of blindness among working-age adults in the United States (1). Vision loss related to eye disease among people with diabetes is an important disability that threatens independence and can lead to depression, reduced mobility, and reduced quality of life (2).

Furthermore, a recent study by Narayanan et al. (3) demonstrated that diabetes prevalence in the United States is likely to increase dramatically through 2050, given recent increases in the incidence of diagnosed diabetes, decreases in diabetes-related mortality, and expected changes in the age of the population.

The Eye Diseases Prevalence Research Group classified DR into two major outcomes: any DR, as any DR consisting of mild, moderate, or severe DR; and vision-threatening DR (VTDR), as DR likely to result in vision loss on the absence of treatment, consisting of proliferative DR, clinically significant diabetic macular edema (CSME), or both (4).

This concept is crucial and a promising way to address the issue of management of DR in order to prevent vision loss and to identify which patients will progress to VTDR (i.e., to CSME and/or proliferative DR).

It is now clear that systemic markers of diabetes such as duration of the disease, poor glycemic control, increased blood pressure, and lipid levels are relevant factors, but they do not identify DR worsening (5). It is a well-established fact that patients under good metabolic control may worsen rapidly and develop VTDR before other patients with poor metabolic control. These observations led to the identification of three different DR phenotypes of progression (6) based on the characteristics of the retinal lesions.

It is, therefore, fundamental to identify lesions, their number, and dynamics in the earlier stages of DR and correlate their occurrence to the worsening of any stage of DR to VTDR (5).

Detection of red lesions by fundus photography has for a long time been suggested as a potential marker of DR progression. In the early stages of DR, several studies have shown that even the presence of only one or two microaneurysms (MAs) (7), but primarily MA dynamics, increases the risk of disease worsening to DR level requiring laser photocoagulation (i.e., VTDR) (8,9).

MAs appear and disappear in the retina of diabetic patients over time, disappearing by closing down due to thrombosis with new ones appearing in different locations of the vascular tree. This turnover indicates a dynamic process and disease activity and has been suggested as a predictive factor for disease worsening.

Recently, our group found in a retrospective study that MA turnover obtained

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from color fundus photographs using manual earmarking is a good predictor of DR worsening to CSME (10).

We report in this study a prospective 2-year study of a cohort of type 2 diabetic patients with mild nonproliferative DR (NPDR) analyzed using automated images analysis for MA turnover performed in color fundus photographs.

**RESEARCH DESIGN AND METHODS**—The study was a prospective, observational study designed to follow eyes/patients with mild NPDR (Early Treatment Diabetic Retinopathy Study [ETDRS] grades 20 and 35) for a period of 2 years or at the time of development of a VDTR complication, CSME needing laser photocoagulation. The sample size was computed based on the results of Nunes et al. (10) for a statistical power of 90%, an $\alpha$ level of 0.05, and a dropout rate of 10% (i.e., 60% of the patients with mild NPDR and high MA turnover will develop CSME, and patients with high MA turnover represent only 20% of the patients with mild NPDR).

Four hundred ten patients, men and women, were included with the following inclusion criteria: diagnosed adult-onset type 2 diabetes, age 40–75 years, mild NPDR (grades 20 and 35 of ETDRS classification), without clinical evidence of macular edema, best corrected visual acuity (BCVA) ≥95, ETDRS letters (20/25), and refraction with a spherical equivalent less than 5D. Exclusion criteria included the presence of cataract or other eye disease that may interfere with fundus examination, glaucoma, other retinal disease, previous intraocular surgery, dilatation of the pupil <5 mm, and previous laser therapy or intravitreal injections. All patients gave written informed consent. Tenets of the Declaration of Helsinki were followed, and approval from the institutional review board was obtained (clinical trial registration number: NCT00763802).

At the baseline visit (V0), patient’s body weight, height, blood pressure, and concomitant medications were recorded. A physical examination by a diabetologist was also performed.

Laboratory analyses were performed at baseline (V0), at 6-month (V6) and at 24-month (V24) visits, or at the pretreatment visit. Laboratory analyses included glucose and HbA1c concentration, red blood cell count, white blood cell count, platelet amount, hemoglobin concentration, and packed cell volume. Metabolic control was also assessed by measuring in the plasma concentrations of HbA1c and lipid fractionation identifying total cholesterol, HDL, LDL, and triglycerides.

One eye per patient was selected as the study eye by the physician based on the inclusion/exclusion criteria. When both eyes fulfill the same criteria, one of the eyes was selected by choosing sequentially the right or the left eye.

At the three study visits, V0, V6, and V24 (or pretreatment visit), the study eyes underwent a complete eye examination, which included BCVA, as tested in the ETDRS, slit-lamp examination, intraocular pressure measurements, fundus photography, and optical coherence tomography (OCT).

**CSME**

CSME was identified on clinical examination by retinal thickening within 500 $\mu$m of the center of the fovea, the presence of exudates within 500 $\mu$m of the center of the fovea, or adjacent thickening or thickening of at least one disc area of any part within 1 disc diameter of the center of the fovea (11).

**Color fundus photography**

Color fundus photography was performed according to the ETDRS protocol. The 7-field photographs were obtained at 30° using a Zeiss FF450 camera (Carl Zeiss Meditec, Dublin, CA) for DR classification according to ETDRS grading.

The field-2 color fundus images (macula) were subjected to automated MA analysis.

The automated computer-aided diagnostic system, RetmarkerDR (Critical Health SA, Coimbra, Portugal), was used to automatically detect MA on the field-2 color fundus images. This automated computer-aided diagnostic system consists of software earmarking MAs and vascular lesions; it includes a coregistration algorithm that allows comparison within the same retinal location between different visits for the same eye. The algorithm detects the presence of MAs and red dot–like lesions. For this purpose, the images are initially converted to processing size. Next follows contrast normalization and enhancement based on principal component analysis. Then, dark objects of a given size are detected and used as candidates. For each of these candidates, features such as area, shape, intensity distribution, and gradient magnitude distribution are extracted using a region covariance descriptor. Next, a state of the art classifier, based on support vector machines, is used to classify the candidates as true or false (classifier’s training was done using a dataset of color fundus images in which graders were asked to earmark only small lesions that appeared as a round or ovoid red spot of 20–200 $\mu$m in diameter with regular borders and located within the superior and inferior arcades).

The images from field-2 are coregistered (12) to indicate disease activity in the central 3,000–$\mu$m circle of the macula. Image coregistration is achieved by extracting a retinal vascular tree, which is used for landmarks during the registration process. A rigid registration estimates the translation based on fovea displacement. The rotation is estimated using a polar representation of the vascular tree. This rigid transformation is then adjusted to obtain exact pairings of selected landmarks (13).

The RetmarkerDR (Critical Health SA) therefore computes for each eye/patient the number of MA in each visit and the number of MA that appears and/or disappears from one visit to the other, allowing calculation of the number of MA appearing and/or disappearing per time interval (i.e., the MA formation rate and the MA disappearance rate, respectively). The MA turnover is computed as the sum of the MA formation and disappearance rates.

For example, a patient who has three MA on the first visit, and those same three MA remain unchanged on the second visit, will have a formation rate of zero, indicating no disease activity. In contrast, a patient can have the total of three MA on the first and second visits, but if those MA are all registered in different retinal locations, an MA formation rate of 3 and a MA disappearance rate of 3 will be calculated, indicating an MA turnover of 6 per time interval.

Previous work from our group (10,14) showed a good intergrader agreement for the total number of MAs earmarked and the MA turnover for three independent human graders. The RetmarkerDR (Critical Health SA) shows a similar intergrader agreement for the total number of MAs and the MA turnover (when compared with a human grader, intraclass correlation coefficients were 0.857 and 0.806, respectively) while showing no intergrader variability as opposed to human graders, being, therefore, a reliable tool for MA assessment.
**OCT**

OCT scans were performed using the Stratus OCT (Carl Zeiss Meditec).

To obtain a more detailed retinal thickness map in the central macular region, proprietary software for increased-resolution OCT maps was used (15). To compute the new increased-resolution OCT maps (composed by 124 equal areas), two acquisition protocols were performed in each eye, the Fast Macular acquisition protocol, acquiring six radial scans 30° apart, 6 mm long, and the Fast RNFL acquisition protocol, acquiring five circular scans within the central 6 mm in diameter area.

**Data analysis**

The following parameters were collected at baseline, computed, and compared between eyes/patients that developed CSME needing photocoagulation treatment (CSME group) and the eyes/patients that did not develop CSME (non-CSME group): age, diabetes duration, HbA1c, blood pressure, cholesterol, HDL, LDL, and triglyceride levels, macular retinal thickness (in the central 500 and 1,500 μm in diameter area), number of MA, MA formation and disappearance rates, and MA turnover.

Changes in the number of MA in field-2, including MA formation rate, disappearance rate, and turnover, were computed for the first 6-month period of follow-up, V0, and V6.

A univariate analysis was performed to test for statistically significant differences between CSME and non-CSME eyes. Due to the skewed distribution for the parameters under analysis, for the CSME and the non-CSME groups, the nonparametric Mann–Whitney test was used.

To analyze the predictiveness of the different MA parameters, a receiver operating curve (ROC) analysis was also performed. Cutoff values for CSME and non-CSME eyes were identified for the parameter that showed simultaneously the higher sensitivity and specificity (i.e., the higher ROC area). The odds ratio for the cutoff values was thereafter computed.

A multivariate analysis, considering the entire set of parameters at baseline (i.e., sex, age, diabetes duration, BCVA, diabetes treatment, systolic and diastolic blood pressure, glycos, cholesterol, HDL and LDL, triglycerides, central retinal thickness [in the 500 and 1,500 μm in diameter area], number of MA, and MA turnover [from baseline to month 6]), was also performed using a Poisson regression analysis to identify predictive factors for CSME development considering patient’s time of follow-up.

All statistical analyses were performed using the STATA software version 12.0 (StataCorp LP, College Station, Texas), and P values ≤0.05 were considered as statistically significant results.

**RESULTS**—Three hundred forty-eight eyes/patients were considered for analysis because they reached the study end point, CSME needing laser photocoagulation, or performed the last study visit (V24) (Fig. 1). Baseline characteristics for the 410 patients included are shown in Table 1 (no statistically significant differences were found between excluded and included eyes/patients except for the cholesterol and LDL levels).

Of these 348 eyes/patients, 26 were diagnosed during the 2-year period of follow-up as having CSME and treated with laser photocoagulation. The other 322 eyes/patients completed the last study visit of follow-up without developing CSME (V24). Eyes/patients characteristics by CSME and non-CSME are shown in Table 2.

Fifteen eyes/patients progressed to more advanced ETDRS levels. Fourteen eyes progressed to moderate NPDR (11 with 43A and 3 with 43B), and 1 eye progressed to moderate proliferative DR (65B).

The average number of MA was 6.2 ± 5.4 in the 26 eyes/patients that developed CSME and 3.3 ± 3.7 in the remaining 322 eyes/patients (P < 0.001).

The MA turnover was 11.2 ± 11.2 in the 26 eyes/patients that developed CSME and 5.0 ± 5.2 in the remaining 322 eyes/patients (P < 0.001).

The MA turnover shows a higher predictiveness for CSME than the remaining MA parameters (the ROC area was for the MA turnover, 0.695; for the number of MA, 0.625).

**Figure 1**—CONSORT flow chart.
Table 1—Baseline characteristics of the patients in the study

|                          | Dropout patients (n = 62) | Patients included in the analysis (n = 348) | P value |
|--------------------------|---------------------------|--------------------------------------------|---------|
|                          | Mean ± SD                 | Median (IQR)                                |         |
|                          |                           |                                            |         |
| Males/females¹           | 41 (65.1%)/22 (34.9%)     | 219 (62.9%)/129 (37.1%)                    | NS      |
| Patients taking insulin¹ | 14 (22.6%)                | 103 (29.6%)                                | NS      |
| Age (years)              | 62.8 ± 8.3                | 63.5 (38.0–69.0)                           |         |
| Duration of diabetes (years) | 9.3 ± 5.1              | 10.0 (5.0–14.0)                            |         |
| HbA1c (%)                | 8.0 ± 1.7                 | 7.9 (6.5–9.0)                              |         |
| Cholesterol (mg/dL)      | 209.2 ± 53.8              | 208.5 (173.0–228.0)                        |         |
| HDL (mg/dL)              | 52.4 ± 13.1               | 51.5 (43.0–59.0)                           |         |
| LDL (mg/dL)              | 137.9 ± 43.9              | 132.0 (108.0–156.0)                        |         |
| Triglycerides (mg/dL)    | 151.4 ± 77.3              | 132.0 (104.0–192.0)                        |         |
| Systolic blood pressure (mmHg) | 152.8 ± 24.8          | 152.0 (135.0–170.0)                        |         |
| Diastolic blood pressure (mmHg) | 76.2 ± 9.4              | 75.0 (72.0–82.0)                           |         |
| BCVA (letters)           | 98.4 ± 2.3                | 100.0 (95.0–100.0)                         |         |
| Retinal thickness in the central 500 μm (μm) | 178.4 ± 25.4            | 174.0 (158–197.3)                          |         |
| Retinal thickness in the central 1,500 μm (μm) | 238.9 ± 24.2            | 237.9 (221–297)                            |         |
| Number of MA             | 4.1 ± 5.2                 | 2.0 (1.0–6.0)                              |         |

¹Frequency (%). IQR, interquartile range, first and third quartiles; NS, P > 0.05. P value for statistically significant differences between dropout and included patients.

of MA, 0.676; for the MA formation rate, 0.658; and for the MA disappearance rate, 0.656).

For an MA turnover cutoff value of ≥9, a sensitivity of 57.7% and a specificity of 81.2% was achieved (i.e., 79.4% of the eyes are correctly classified).

Table 2—Patient characteristics at baseline comparing the two patient groups, the ones that did not develop CSME and the ones that developed CSME

|                          | Non-CSME (n = 322) | CSME (n = 26) | P value |
|--------------------------|-------------------|--------------|---------|
|                          | Mean ± SD         | Median (IQR) |         |
| Age (years)              | 60.8 ± 8.2        | 62.0 (55.0–67.0) |         |
| Duration of diabetes (years) | 10.0 ± 5.1         | 10.0 (6.0–14.0) |         |
| HbA1c (%)                | 7.9 ± 1.4         | 7.6 (6.9–8.8)  |         |
| Cholesterol (mg/dL)      | 195.2 ± 39.3      | 193.0 (167.0–218.0) |         |
| HDL (mg/dL)              | 50.9 ± 12.9       | 49.0 (42.0–57.0) |         |
| LDL (mg/dL)              | 126.1 ± 30.0      | 124.0 (104.0–146.0) |         |
| Triglycerides (mg/dL)    | 175.2 ± 73.3      | 162.0 (119.0–225.0) |         |
| Systolic blood pressure (mmHg) | 151.5 ± 21.0     | 151.0 (137.0–164.0) |         |
| Diastolic blood pressure (mmHg) | 75.9 ± 10.9      | 76.0 (69.0–82.0)  |         |
| BCVA (letters)           | 98.8 ± 2.1        | 100.0 (100.0–100.0) |         |
| Retinal thickness in the central 500 μm (μm) | 181.5 ± 24.8    | 179.6 (164.8–199.3) |         |
| Retinal thickness in the central 1,500 μm (μm) | 241.6 ± 21.0     | 243.4 (227.6–255.4) |         |
| Number of MA             | 3.3 ± 3.7         | 2.0 (1.0–4.0)  |         |
| MA formation rate        | 2.5 ± 3.5         | 1.0 (0.0–3.0)  |         |
| MA disappearance rate    | 2.5 ± 2.8         | 2.0 (0.0–3.0)  |         |
| MA turnover              | 5.0 ± 5.2         | 4.0 (1.0–7.0)  |         |

P value for statistically significant differences between CSME and non-CSME eyes. IQR, interquartile range, first and third quartiles; NS, P > 0.05.

Eyes with an MA turnover >9 during the initial 6-month period showed a higher risk for CSME development than eyes with a lower MA turnover (odds ratio 5.886 [95% CI 2.503–13.844]). The MA turnover predictive values for CSME development were: for the positive predictive value, 20.0%; and for the negative predictive value, 95.9%, showing that a low MA turnover value is associated with less likelihood for CSME development in a 2-year period.

Furthermore, considering only the eyes with an MA turnover ≥9, eyes that...
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developed CSME before the 24-month visit presented higher MA turnover values (26.6 ± 15.9) when compared with the eyes in which CSME was detected only at month 24 (12.8 ± 3.6) (P = 0.018), indicating again that there is a correlation between high turnover values and risk for the development of CSME for eyes with the same ETDRS retinopathy level.

Considering the central retinal thickness values at baseline, a statistically significant difference was found for the central 500 μm in diameter area (P = 0.025) but not for the central 1,500-μm area (P = 0.052).

For BCVA, no statistically significant differences were found between CSME and non-CSME eyes at baseline (P = 0.499) or in the last study visit (P = 0.593). However, there was an overall decrease in BCVA in both CSME and non-CSME (P < 0.018).

When considering the systemic parameters examined, the eyes that developed CSME during the 2-year study period had higher HbA1c level at baseline (P = 0.039). However, there was an overall decrease in BCVA in both CSME and non-CSME (P < 0.018).

Table 3—Multivariate Poisson regression analysis

| Parameter                          | IRR   | 95% CI   | P value |
|-----------------------------------|-------|----------|---------|
| Males/females                     | 0.601 | 0.239–1.511 | 0.279   |
| Age (years)                       | 1.03  | 0.976–1.098 | 0.251   |
| Duration of diabetes (years)      | 1.008 | 0.931–1.092 | 0.842   |
| Patients taking insulin           | 0.957 | 0.355–2.582 | 0.932   |
| HbA1c (%)                         | 1.400 | 1.039–1.886 | 0.027   |
| Cholesterol (mg/dL)               | 0.990 | 0.949–1.033 | 0.651   |
| HDL (mg/dL)                       | 1.002 | 0.949–1.058 | 0.934   |
| LDL (mg/dL)                       | 1.016 | 0.967–1.068 | 0.521   |
| Glycose (mg/dL)                   | 0.996 | 0.988–1.004 | 0.339   |
| Triglycerides (mg/dL)             | 0.998 | 0.993–1.004 | 0.997   |
| Systolic blood pressure (mmHg)    | 0.996 | 0.970–1.022 | 0.759   |
| Diastolic blood pressure (mmHg)   | 1.007 | 0.960–1.056 | 0.779   |
| BCVA (letters)                    | 1.010 | 0.816–1.251 | 0.924   |
| Retinal thickness in the central 500 μm (μm) | 1.012 | 0.985–1.039 | 0.398   |
| Retinal thickness in the central 1,500 μm (μm) | 1.008 | 0.971–1.046 | 0.680   |
| Number of MA                      | 1.023 | 0.908–1.151 | 0.709   |
| MA turnover                       | 1.085 | 1.014–1.160 | 0.018   |

Incidence rate ratios (IRR) and 95% CI for the IRR. P value for predictive parameters for CSME eyes.

CONCLUSIONS—This 2-year prospective, longitudinal study of patients with diabetes type 2 and mild NPDR (ETDRS levels 20 and 35 at baseline) shows that MA turnover in field-2 is a good indicator of retinopathy worsening and development of CSME needing photocoagulation.

On ophthalmoscopic examination and color fundus photography, red-dot lesions, including MA and small hemorrhages, are the initial changes detected in the diabetic retina. They may be counted, and retinal MA counting has been proposed as an appropriate marker of retinopathy worsening (16).

In a previous study by our group (10), determination of MA formation rates taking into account the exact location of new MA in successive color fundus photographs showed higher sensitivity in predicting worsening of retinopathy for a 10-year period of follow-up than the simple counting of MA at one time. Moreover, we found that MA formation rates obtained during an initial 2-year follow-up period gave more accurate information on the activity of the retinopathy and that there was much better agreement between graders when determining MA formation rates than MA counts (14).

MA turnover values in this study show a high SD relatively to the mean, indicating that MA turnover values vary widely between eyes having similar ETDRS grading level. This confirms previous observations by Sharp et al. (17) and may indicate that MA turnover is an indicator of different vascular disease activity in different eyes.

Of particular interest in this study is the observation that MA turnover values, including both the MA formation rate and MA disappearance rate, determined over a period of only 6 months predicts with a high degree of confidence the eyes that do not go on to develop CSME for at least a period of 2 years. This observation is interpreted as indicating that eyes with low MA turnover have less disease activity and therefore less risk of worsening. It should be realized that retinal thickness was only measured in the central 6,000 μm in diameter area, within the vascular arcades, corresponding to ETDRS field-2. Therefore, any retinal edema outside of this central area was not considered.

The observation that, in this group of patients with diabetes type 2, the level of metabolic control, given by HbA1c values, correlates with retinopathy worsening confirms a previous report (10). It is also of major interest that at these early retinopathy stages, other variables, such as blood pressure and blood lipid levels, do not appear to be associated with the development of CSME. However, cholesterol fractions were not measured.

It is also considered of major interest that MA turnover can be determined in diabetic retinas using only noninvasive color fundus photographs, without the need for fluorescein angiography (18). The identification of the eyes that show very slow worsening of retinopathy and low disease activity with low risk for development of vision-threatening complications, based on computer-assisted detection of MA turnover using digital color fundus images, is considered of potential relevance for management of diabetic retinal disease.
The validation of digital color fundus cameras as a tool for following DR worsening to VTRD is considered a valuable development, taking into account their cost and availability. Quality assurance of digital color fundus imaging is straightforward and will allow more efficient utilization of widespread screening and prevention programs. Automated analysis techniques offer advantages of repeatability and consistency, and, although not better than trained graders, particularly in identifying all MA, they avoid the variability inherent to human grading, which has its own subjective reference standards. It is also considered relevant that MA turnover calculated by the RetmarkerDR (Critical Health SA) is much less time consuming than MA counting by an expert grader.

The observations reported in this article are also expected to have impact on clinical trial design. Excluding from the trials eyes/patients that show little disease activity and are not expected to progress during the trial period would offer a better chance for development of VTRD in the placebo control eyes, thus creating the conditions to identify efficacy of the drug being tested.

Limitations of this study include the relatively short duration of the study (2 years) and the lack of more detailed information on the systemic parameters such as lipid stratification.

The results of this study confirm that in a prospective study of a relatively large number of patients, MA turnover values obtained from noninvasive color fundus photography based solely on field-2 images may help to identify the eyes/patients at risk for developing CSME and potential vision loss.

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M.L.R. researched data and reviewed and edited the manuscript. S.G.N. analyzed data, contributed to the discussion, and reviewed and edited the manuscript. J.G.C.-V. designed the study, researched data, wrote the manuscript, and contributed to the discussion. J.G.C.-V. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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