Hypertension Research (2016) 39, 886–892

www.nature.com/hr

ORIGINAL ARTICLE

Retinal microvascular diameter, a hypertension-related trait, in ECG-gated *vs.* non-gated images analyzed by IVAN and SIVA

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The diameters of the retinal microvasculature reflect intermediate target organ damage and predict adverse health outcomes. In view of the pulsatility of the cerebral blood flow and refinement of software used for off-line analysis, we assessed the repeatability of retinal microvascular diameters in ECG-gated *vs.* non-gated images using nonmydriatic retinal photographs (Canon Cr-DGi visualization system) postprocessed by IVAN (Vasculomatic ala Nicola, version 1.1) or SIVA (Singapore I Vessel Assessment, version 3.6). Using these algorithms, we determined the central retinal arteriolar (CRAE) and venular (CRVE) equivalents and their ratio (arteriole-to-venule ratio (AVR)). The estimates of CRAE (mean, 158.5 μm), CRVE (222.5 μm) and AVR (0.71) in 10 volunteers were unaffected ($P \geqslant 0.059$) by ECG gating. We assessed intragrader repeatability by the Bland and Altman approach in 30 participants with non-gated images and 30 with ECG-gated photographs. Repeatability, which was expressed as the percentage of near maximal variability (4-s.d. range), did not improve with ECG gating. Using SIVA, CRAE and CRVE were systematically larger ($P \leqslant 0.031$), and the AVR estimates were similar ($P \geqslant 0.15$) compared with IVAN. The differences (IVAN – SIVA) averaged – 5.4 μm for CRAE, – 3.9 μm for CRVE and – 0.012 for AVR in the non-gated images and – 3.3 μm, – 6.9 μm and 0.006, respectively, in the ECG-gated photographs. In conclusion, ECG gating does not affect estimates of the retinal microvascular diameters or improve intragrader repeatability. SIVA yields slightly but significantly larger estimates of the retinal arteriolar and venular diameters. Combining historical readings analyzed by IVAN with more recent readings by SIVA is possible only for AVR and is not recommended for either CRAE or CRVE.
Hypertension Research (2016) **39,** 886–892; doi:10.1038/hr.2016.81; published online 7 July 2016

Keywords: arterioles; microcirculation; population research; repeatability; retina; venules

INTRODUCTION

Non-mydriatic retinal photography allows clinicians to measure the retinal microvessels,[1,2] which are representative of the microcirculation in the brain. This completely non-invasive technique is easily applicable in population surveys.[1–10] In cross-sectional studies,[1,2] hypertension is associated with retinal arteriolar narrowing, and in prospective studies, a lower arteriole-to-venule ratio (AVR) predicts the incidence of hypertension.[5,6] Moreover, the diameters of the retinal microvessels carry important prognostic information[7–9] as a smaller arteriolar diameter,[8,9] wider venular caliber[9] and lower AVR[7] predict cardiovascular mortality,[8] coronary heart disease[7] and lacunar stroke.[9]

The brain[11] and by extension the retina[12] are perfused at high-volume flow with a pulsatility that is maintained up to the effluent venous blood flow. In 1994, Chen *et al.*[12] reported that retinal arteriolar diameter peaked in mid-systole and venular diameter peaked in early diastole, with maximal increases in the diameters of 3.5% and 4.8%, respectively. Subsequent studies applying non-mydriatic photography[13,14] or confocal scanning laser ophthalmoscopy[15] confirmed the changes in the diameter of the retinal microvessels during the cardiac cycle. These studies had a sample size ranging from 10[12,15] to 15[13], included only healthy volunteers[12–15] and did not report on the repeatability of the microvascular diameters measured in relation to ECG gating or the software package used for off-line

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Received 25 November 2015; revised 19 April 2016; accepted 22 May 2016; published online 7 July 2016

analysis.[12–15] The prospectively defined hypotheses underlying our study were that the changes in the diameters of the retinal microvessels observed during the cardiac cycle and the refinement achieved over the past decade in the software used for the computer-aided postprocessing of retinal photographs would enhance intraobserver and interobserver repeatability. We addressed these research objectives in ECG-gated *vs.* non-gated retinal images from people enrolled in the Flemish Study on Environment, Genes and Health Outcomes (FLEMENGHO)[16] and healthy volunteers who were analyzed using the two software packages.

METHODS

Study population

FLEMENGHO was conducted according to the principles outlined in the Declaration of Helsinki for Investigations of Human Participants. The Ethics Committee of the University of Leuven approved the study. Recruitment started in 1985 and participants underwent repeated follow-up.[16] The initial participation rate was 78.0%. Follow-up included retinal imaging without ECG gating in 1243 participants examined from January 2008 until June 2014 and retinal imaging with ECG gating in 168 participants examined from August 2014 until March 2015. We randomly selected subsets of apparently healthy participants, 30 with non-gated images and 30 with ECG-gated photographs. In addition, in accord with the sample size of previous studies,[12,15,17] we recruited 10 healthy volunteers to compare non-gated and ECG-gated images in the same subjects. All participants provided informed written consent at the time of retinal phenotyping.

Retinal photography

Participants were asked to refrain from heavy exercise, smoking and drinking alcohol or caffeine-containing beverages for at least 3 h before retinal imaging. Patients on blood pressure-lowering drugs continued their medication on the examination days. We applied a non-mydriatic approach in a dimly lit room to obtain retinal photographs, one image per eye in each participant, with the Canon Cr-DGi retinal visualization system combined with the Canon D-50 digital camera (Canon, Medical Equipment Group, Utsunomiya, Japan). We determined the central retinal arteriolar (CRAE) and venular (CRVE) equivalents, which represent the retinal arteriolar and venular diameters, respectively. We used the validated computer-assisted program IVAN (Vasculomatic ala Nicola, version 1.1, Department of Ophthalmology and Visual Science, University of Wisconsin-Madison, Madison, WI, USA)[18] or SIVA (Singapore I Vessel Assessment, version 3.6, Singapore Eye Research Institute, Singapore, Singapore) based on formulae published by Parr[19,20] and Hubbard.[21] The software returns average vessel diameters according to the revised Knudtson formula.[22] AVR was calculated by dividing the CRAE by the CRVE. We used a three-lead ECG monitoring device, along with a purpose-built microcontroller to sample the ECG signal outputted by a CardiMax FX-8322 recorder (Fukuda Denshi, Tokyo, Japan) to obtain the R wave in real time at a sampling rate of 8000 Hz and to generate a trigger pulse for acquiring the retinal image delayed by 300 ms relative to the top of the R wave.

Graders who were blinded to the identity of the study participants scored the 30 non-gated and 30 gated images to compare the performance of the two software packages in terms of intraobserver and interobserver variability. The 30 gated and 30 non-gated images were obtained from different participants; thus a test–retest design was not applicable to this part of the study. We randomized 10 healthy volunteers in a 1:1 ratio to undergo retinal imaging first with gating and then without gating or vice versa to test the difference between non-gated and ECG-gated images. The interval between the test and retest was 60 min. One reader (F-FW) who was blinded to the identity of the volunteer and the order in which the retinal pictures were taken scored all images.

Other measurements

Blood pressure was measured five times consecutively after the participants had rested for 5 min in a sitting position using a standard mercury sphygmomanometer (Riester, Jungingen, Germany). For analysis, the five readings were averaged. Mean arterial pressure was the diastolic blood pressure plus one-third of the difference between the systolic and diastolic blood pressure. Hypertension was defined as a blood pressure of at least 140 mm Hg systolic or 90 mm Hg diastolic or the use of antihypertensive drugs. Body mass index was defined as weight in kilograms divided by height in meters squared. Nurses administered a validated questionnaire to obtain information on each participant's medical history, smoking and drinking habits and intake of medications. After the participants fasted for at least 6 h, venous blood samples were drawn to measure the plasma glucose and serum cholesterol levels. Diabetes was defined as a fasting plasma glucose of at least 7.0 mmol l^{-1} or the use of antidiabetic drugs.[23]

Statistical analysis

We used the SAS software, version 9.3 (SAS Institute, Cary, NC, USA) for database management and statistical analysis. For comparison of means and proportions, we applied Student's *t*-test for paired or unpaired observations, as appropriate, and the χ^2-statistic, respectively. We assessed the agreement between paired measurements using the method described by Bland and Altman.[24] Repeatability was defined as two times the s.d. of the pairwise differences between duplicate measurements and was expressed as a percentage of the average of all first and repeat measurements. To enable comparisons with the literature, we also computed the intraclass correlation coefficient between duplicate measurements and the interclass correlation coefficient between the two observers who coded the non-gated images. Statistical significance was an α-level of <0.05 in two-sided tests.

RESULTS

Characteristics of the participants

Table 1 lists the characteristics of the FLEMENGHO participants, 30 with non-gated images and 30 with ECG-gated retinal photographs, and of the 10 healthy volunteers who underwent both non-gated and ECG-gated imaging. In these three groups, the age ranged from 21.0 to 79.8 years, from 18.9 to 86.1 years and from 24.1 to 54.5 years, respectively. Participants who underwent ECG-gated imaging tended to have higher systolic (131.4 *vs.* 123.4 mm Hg; $P=0.080$) and diastolic (83.0 *vs.* 79.0 mm Hg; $P=0.15$) blood pressures with no difference in the prevalence of hypertension (30.0% *vs.* 23.3%; $P=0.56$) compared with those with non-gated imaging. Otherwise, there were no differences between these two groups in any of the continuous and categorical variables listed in Table 1 ($P\geqslant 0.31$).

Repeatability of the measurements derived from the non-gated photographs

In the 30 participants with non-gated photographs (Table 2), the differences (repeat − first) between two photographs read by the same observer (F-FW) were 0.75 μm ($P=0.18$) for CRAE, 0.39 μm for CRVE ($P=0.40$) and 0.002 units ($P=0.52$) for AVR, as measured by IVAN, and − 0.26 μm ($P=0.56$), − 0.48 μm ($P=0.36$) and 0.001 units ($P=0.70$), respectively, as measured by SIVA. Expressed as a percentage of the near maximal variability (the 4-s.d. interval), the repeatability for each of these measurements was 11.0, 6.4 and 9.3% for IVAN and 9.1, 6.5 and 8.3% for SIVA, respectively, with higher values indicating worse repeatability. The intraobserver correlation coefficients were 0.98, 0.99 and 0.98 and 0.98, 0.99 and 0.98, respectively.

With regard to interobserver repeatability, the mean differences between two observers (F-FW − Z-YZ) were -1.4 ± 5.3 μm (95% confidence interval (CI), − 3.4 to 0.53 μm; $P=0.14$), -0.79 ± 2.9 μm (CI, − 1.9 to 0.30 μm; $P=0.15$) and -0.004 ± 0.029 units (CI, − 0.015 to 0.007 units; $P=0.49$) for CRAE, CRVE and AVR, respectively, as estimated by IVAN. The corresponding differences for the SIVA measurements were -0.50 ± 4.6 μm (CI, − 2.2 to 1.2 μm; $P=0.56$), -0.22 ± 7.9 μm (CI, − 3.2 to 2.7 μm; $P=0.88$), and -0.002 ± 0.03

Table 1 Characteristics of participants

Characteristic	*Participants with non-gated imaging*	*Participants with ECG-gated imaging*	*Volunteers*
Number (%) with characteristics	30	30	10
Women (%)	16 (53.3)	16 (53.3)	7 (70)
Current smoker (%)	7 (23.3)	4 (13.3)	0
Drinking alcohol (%)	23 (76.7)	23 (76.7)	4 (40)
Diabetes mellitus (%)	0 (0)	1 (3.3)	0
Hypertension (%)	7 (23.3)	9 (30.0)	0
Treated hypertension (%)	2 (6.7)	3 (10.0)	0
Mean (± s.d.) of characteristics			
Age (years)	49.1 ±16.0	52.8 ± 17.0	34.3 ± 9.1
Body mass index (kg m^{-2})	26.3 ± 4.5	27.4 ± 5.7	22.5 ± 4.2
Systolic pressure (mm Hg)	123.4 ± 16.3	131.4 ± 18.1	112.0 ± 11.5
Diastolic pressure (mm Hg)	79.0 ± 10.1	83.0 ± 11.2	72.6 ± 7.5
Heart rate (beats min^{-1})	65.7 ± 9.7	65.1 ± 9.1	71.0 ± 10.6
Total cholesterol (mmol l^{-1})	5.06 ± 1.05	4.98 ± 1.10	—
Plasma glucose (mmol l^{-1})	4.62 ± 0.50	4.60 ± 0.51	—

Diabetes was a fasting plasma glucose ⩾7.0 mmol l^{-1} or use of antidiabetic drugs. Hypertension was a blood pressure of ⩾140 mm Hg systolic or ⩾90 mm Hg diastolic or use of antihypertensive drugs.

Table 2 Intraobserver repeatability

				Difference		*Repeatability*		
Software ECG gating trait	*First measurement*	*Repeat measurement*	P-*value*	*Estimate*	*95% CI*	*RC*	*%M*	*%MV*
IVAN								
Non-gated								
CRAE (μm)	152.2 ± 14.0	151.4 ± 13.3	0.18	0.75 ± 3.0	−0.37 to 1.9	6.0	4.0	11.0
CRVE (μm)	219.4 ± 19.3	219.0 ± 19.7	0.40	0.39 ± 2.5	−0.54 to 1.3	5.0	2.3	6.4
AVR (unit)	0.70 ± 0.07	0.70 ± 0.07	0.52	0.002 ± 0.013	−0.003 to 0.006	0.03	3.7	9.3
ECG-gated								
CRAE (μm)	153.4 ± 14.7	153.1 ± 15.0	0.65	0.35 ± 4.2	−1.2 to 2.8	8.4	5.5	14.1
CRVE (μm)	226.6 ± 19.3	226.2 ± 19.0	0.44	0.40 ± 2.8	−0.65 to 1.4	5.6	2.5	7.3
AVR (unit)	0.68 ± 0.04	0.68 ± 0.04	0.83	0.001 ± 0.017	−0.006 to 0.007	0.03	5.3	17.6
SIVA								
Non-gated								
CRAE (μm)	157.1 ± 13.3	157.3 ± 13.1	0.56	−0.26 ± 2.4	−1.1 to 0.63	4.8	3.0	9.1
CRVE (μm)	222.8 ± 22.1	223.3 ± 20.8	0.36	−0.48 ± 2.8	−1.5 to 0.58	5.6	2.5	6.5
AVR (unit)	0.71 ± 0.06	0.71 ± 0.06	0.70	0.001 ± 0.012	−0.004 to 0.005	0.02	2.8	8.3
ECG-gated								
CRAE (μm)	156.3 ± 15.6	156.9 ± 15.8	0.34	−0.64 ± 3.6	−2.0 to 0.71	7.2	4.6	11.5
CRVE (μm)	232.8 ± 17.5	233.8 ± 18.6	0.21	−0.95 ± 4.1	−2.5 to 0.57	8.2	3.5	11.4
AVR (unit)	0.67 ± 0.04	0.67 ± 0.04	0.96	−0.0001 ± 0.013	−0.005 to 0.005	0.03	4.5	16.2

Abbreviations: AVR, arteriole-to-venule ratio; CI, confidence interval; CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent; IVAN, Vasculomatic ala Nicola, version 1.1; RC, repeatability coefficient; SIVA, Singapore I Vessel Assessment, version 3.6.
First and second measurements and differences (first minus repeat measurement) are means ± s.d. The RC is twice the s.d. of the signed differences between duplicate measurements expressed in μm or unit or as a percentage of the mean (%M) or four times the s.d. (%MV) of the averaged duplicate measurements. Greater values indicate worse repeatability. *P*-value is the significance of the difference between duplicate measurements.

units (CI, −0.014 to 0.010 units; $P=0.73$). Expressed as a percentage of the near maximal variability, the repeatability was 19.8, 7.6 and 21.4% for CRAE, CRVE and AVR, respectively, using IVAN and 18.4, 19.4 and 25.0%, respectively, using SIVA. The corresponding inter-observer correlation coefficients were 0.92, 0.99 and 0.91 and 0.94, 0.93 and 0.87, respectively.

Repeatability of the measurements derived from the ECG-gated photographs

In the 30 participants with ECG-gated images (Table 2), the differences between the two photographs read by the same observer (F-FW) were 0.35 μm ($P=0.65$) for CRAE, 0.40 μm for CRVE ($P=0.44$) and 0.001 units ($P=0.83$) for AVR as measured by IVAN and −0.64 μm ($P=0.34$), −0.95 μm ($P=0.21$) and −0.0001 ($P=0.96$), respectively, as measured by SIVA. Expressed as a percentage of the near maximal variability, repeatability amounted to 14.1% for CRAE, 7.3% for CRVE and 17.6% for AVR as read by IVAN and to 11.5% for CRAE, 11.4% for CRVE and 16.2% for AVR as processed by SIVA. The corresponding intragrader correlation coefficients were 0.96, 0.99 and 0.92 and 0.97, 0.97 and 0.95, respectively.

Table 3 Difference in retinal microvascular traits between non-gated and ECG-gated measurements in 10 volunteers

				Difference		*Repeatability*		
Software trait	*Non-gated*	*ECG-gated*	P-*value*	*Estimate*	*95% CI*	*RC*	*%M*	*%MV*
IVAN								
CRAE (μm)	159.4±12.4	158.4±13.2	0.36	0.92±3.0	−1.2 to 3.1	6.0	3.8	11.8
CRVE (μm)	220.9±20.6	218.2±19.7	0.059	2.7±3.9	−0.13 to 5.5	7.8	3.6	9.8
AVR (unit)	0.72±0.06	0.73±0.06	0.63	−0.004±0.017	−0.016 to 0.008	0.03	4.6	12.5
SIVA								
CRAE (μm)	158.0±9.1	158.3±9.5	0.71	−0.29±2.4	−2.0 to 1.4	4.8	3.0	13.0
CRVE (μm)	226.1±13.4	224.8±11.6	0.21	1.2±2.9	−0.84 to 3.3	5.8	2.6	11.7
AVR (unit)	0.70±0.04	0.70±0.05	0.42	−0.004±0.016	−0.016 to 0.007	0.032	4.6	20.0

Abbreviations: AVR, arteriole-to-venule ratio; CI, confidence interval; CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent; IVAN, Vasculomatic ala Nicola, version 1.1; RC, repeatability coefficient; SIVA, Singapore I Vessel Assessment, version 3.6.
Non-gated and ECG-gated measurements and differences (non-gated minus ECG-gated measurement) are means±s.d. The RC is twice the s.d. of the signed differences between non-gated and ECG-gated measurements expressed in μm or unit or as a percentage of the mean (%M) or four times the s.d. (%MV) of the averaged non-gated and ECG-gated measurements. *P*-value is the significance of the difference between repeated measurements.

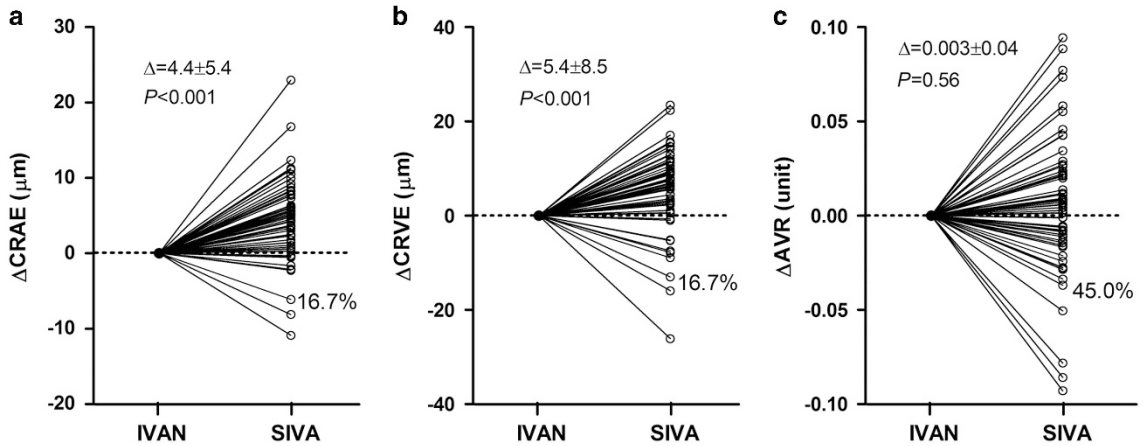

Figure 1 Differences between the 60 individual participants in the estimates of retinal microvascular diameters in images that were postprocessed by the same observer (F-FW) using IVAN and SIVA. The differences (Δ) and corresponding significance (*P*) are provided for the central retinal arteriolar equivalent (CRAE) in panel (**a**), for the central retinal venular equivalent (CRVE) in panel (**b**) and for the arteriole-to-venule ratio (AVR) in panel (**c**). The percentage of values refer to the proportion of participants with smaller CRAE (**a**), CRVE (**b**) or AVR (**c**) using SIVA compared with IVAN.

Differences between the non-gated and ECG-gated measurements
Among the 10 volunteers who underwent both non-gated and ECG-gated retinal imaging (Table 3), the two approaches yielded similar point estimates of the mean CRAE, CRVE and AVR, with differences (non-gated − ECG-gated) averaging 0.92 μm for CRAE ($P=0.36$), 2.7 μm for CRVE ($P=0.059$) and −0.004 units for AVR ($P=0.63$) using IVAN and −0.29 μm ($P=0.71$), 1.2 μm ($P=0.21$) and −0.004 units ($P=0.42$), respectively, using SIVA. Expressed as a percentage of the near maximal variability, repeatability amounted to 11.8% for CRAE, 9.8% for CRVE and 12.5% for AVR using IVAN and to 13, 11.7 and 20%, respectively, using SIVA (Table 3).

Furthermore, with adjustments applied to sex, age, systolic blood pressure, smoking and antihypertensive drug treatment, we compared CRAE between the two groups of FLEMENGHO participants who underwent retinal imaging without and with ECG gating. In these non-paired comparisons, the differences in CRAE (non-gated − ECG-gated) averaged 4.9 μm ($P=0.11$) using IVAN and 2.3 μm ($P=0.50$) using SIVA.

Differences between the off-line readings using IVAN and SIVA
Although there was interindividual variability (Figure 1), the estimates of CRAE and CRVE were systematically larger with SIVA compared with IVAN (Table 4), with no differences in AVR, regardless of whether ECG gating was applied. The differences (IVAN − SIVA) averaged −5.4 μm ($P<0.001$) for CRAE, −3.9 μm ($P=0.031$) for CRVE and −0.012 units ($P=0.15$) for AVR in the non-gated images and −3.3 μm ($P<0.001$), −6.9 μm ($P<0.001$) and 0.006 units ($P=0.20$), respectively, in ECG-gated photographs.

DISCUSSION

The retinal microcirculation is a pivotal trait in clinical and population research, because narrowing of the retinal microvessels reflects target organ damage and predicts adverse health outcomes. Our current study addresses two questions, which to the best of our knowledge, have both previously been addressed. First, in view of the pulsatility of the retinal blood flow, does ECG gating improve the precision of retinal microvascular diameter measurements? Second, are estimates of retinal microvascular diameters consistent if retinal photographs are postprocessed with newer (SIVA) software compared with older (IVAN) software? Regarding the first question, our key finding was that ECG gating had no influence on estimates of the retinal microvascular diameters and did not improve intraobserver repeatability, irrespective of the software used. For the second question, we showed that in the presence of large intraindividual variability, on

Table 4 Difference in retinal microvascular traits according to the software used for off-line analysis in two sets of 30 FLEMENGHO participants

				Difference		*Repeatability*		
ECG-gating trait	*IVAN*	*SIVA*	P-*value*	*Estimate*	*95% CI*	*RC*	*%M*	*%MV*
Non-gated (n = 30)								
CRAE (μm)	151.8 ± 13.6	157.2 ± 13.2	<0.001	−5.4 ± 6.7	−7.9 to −2.9	13.4	8.7	26.0
CRVE (μm)	219.2 ± 19.5	223.0 ± 21.4	0.031	−3.9 ± 9.4	−7.4 to −0.38	18.8	8.5	23.6
AVR (unit)	0.70 ± 0.07	0.71 ± 0.06	0.15	−0.012 ± 0.045	−0.029 to 0.005	0.09	12.8	34.6
ECG-gated (n = 30)								
CRAE (μm)	153.3 ± 14.7	156.6 ± 15.6	<0.001	−3.3 ± 3.4	−4.6 to −2.0	6.8	4.4	11.3
CRVE (μm)	226.4 ± 19.1	233.3 ± 17.9	<0.001	−6.9 ± 7.5	−9.7 to −4.1	15.0	6.5	20.7
AVR (unit)	0.68 ± 0.04	0.67 ± 0.04	0.20	0.006 ± 0.027	−0.004 to 0.016	0.05	7.5	31.2

Abbreviations: AVR, arteriole-to-venule ratio; CI, confidence interval; CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent; FLEMENGHO, Flemish Study on Environment, Genes and Health Outcomes; IVAN, Vasculomatic ala Nicola, version 1.1; RC, repeatability coefficient; SIVA, Singapore I Vessel Assessment, version 3.6.
The analyses included 30 FLEMENGHO participants with non-gated retinal images and 30 with ECG-gated images. IVAN and SIVA measurements and differences (IVAN − SIVA) are means ± s.d. The RC is twice the s.d. of the signed differences between IVAN and SIVA measurements expressed in μm or unit or as a percentage of the mean (%M) or four times the s.d. (%MV) of the averaged IVAN and SIVA measurements. *P*-value is the significance of the difference between IVAN and SIVA measurements.

average, SIVA produced significantly larger estimates of the retinal microvascular diameters compared with IVAN. Among the 16.7% of subjects with smaller CRAE or smaller CRVE using SIVA compared with IVAN, only two had both smaller CRAE and CRVE. The smaller diameters obtained with IVAN compared with SIVA are clinically relevant. Indeed, as estimated in the same study population[1] and in accord with most published papers, as reviewed in Liu *et al.*,[1] CRAE decreases by ~3 μm and CRVE by ~4 μm per 10-year increment in age. CRAE narrows by ~4 μm for a 10-mm Hg increase in mean arterial pressure. These estimates must be compared with the 3–5 μm lower CRAE and the 4–7 μm lower CRVE, when IVAN was used for postprocessing instead of SIVA (Table 4).

Several previous studies[12–15] reported the pulsatility of the retinal blood flow during the cardiac cycle and suggested that strategies that account for pulsatility might be relevant in the assessment of retinal microvascular diameters. Chen *et al.*[12] reported maximum increases of 3.46% in the arteriolar diameter and 4.82% in the venular diameter in mid-late systole and early diastole, respectively. Kumar *et al.*[14] observed increases in diameter averaging 3.3 μm for arterioles and 6.6 μm for venules when accounting for pulsatile motion. Without visualization of the pulsatile motion, these diameter changes were 2.1 and 4.7 μm, respectively.[14] Hao *et al.*[13] reported increases in the arteriolar and venular diameters of up to of 3.9 μm (6.3%; $P<0.001$) and 3.0 μm (3.4%; $P<0.001$), respectively. What all previous studies had in common is that 8^{12-14} to 45 (9 per second)[15] retinal images were taken at fixed intervals triggered by the ECG signal, spanning one or more cardiac cycles. The measurements were performed on a single microvascular segment that was carefully selected based on image quality. Hao *et al.*[13] also used SIVA to obtain 'summary' measurements of the vessel caliber in the left eye. Using this approach, which we applied in our current study, the diameter increases during the cardiac cycle by up to 3.8 μm (2.5%) for arterioles and up to 8.7 μm (4.1%) for venules, but the difference was not statistically significant ($P \geqslant 0.12$).

In the present study, we sampled the ECG signal in real time at 8000 Hz and acquired a single retinal photograph of the left and right eye with a 300-ms delay relative to the top of the R wave. This delay ensured that the trigger for the retinal photographs coincided with left ventricular ejection during the ST-segment of the ECG for heart rates ranging from 60 to 100 beats per minute. Thus, in view of the report by Hao *et al.*,[13] the use of summary measurements probably explains why ECG gating did not affect the estimates of the retinal microvascular diameters in the present study. Indeed, the propagation of the pulse wave from the six largest arterioles to the six largest venules takes time. Therefore, the average changes in the diameters across 'the big six estimated' from a single photograph of both eyes with a shutter time of 1/60 s might obscure the diameter changes picked up by photography and off-line analysis of selected segments, as reported in studies describing the pulsatility of the retinal microvasculature.[12–15] Furthermore, Knudston *et al.*[25] reported that the variations across photographs taken in a single individual within the same point in the cardiac cycle were too large to detect differences across the three points in the cardiac cycle. Other potential confounders obscuring the caliber changes graded by IVAN or SIVA are the pulsatile motion of the microvessels and auto-regulation of the retinal microvasculature.

A key finding was that the use of SIVA instead of IVAN for post-processing produced slightly but significantly larger estimates of CRAE and CRVE, with no difference in AVR. The implications of these observations are that it is only possible to combine historical AVR readings analyzed by IVAN with more recent readings analyzed by SIVA, but this process cannot be recommended for CRAE or CRVE. AVR is a relative measure that combines information from the arteriolar and venular side of the retinal microcirculation, is normally distributed and decreases with increasing risk, as exemplified by hypertension.[21] It has the advantage of controlling for magnification differences resulting from camera lenses or refractive error.[26] Our study adds the use of different software packages as an additional factor that can control for the use of AVR. From this perspective, one should note that AVR was the key prognostic variable in influential studies relating adverse health outcomes to the retinal microcirculation. Among the 10 358 participants in the Atherosclerosis Risk in Communities study,[27] with adjustments applied for multiple risk factors, including mean arterial pressure, plasma glucose and total and high-density lipoprotein cholesterol, the lower AVR (mean, 0.84) over 3.5 years of follow-up was associated with a higher stroke risk (110 incident cases; $P=0.03$). In a case–control study (cases/controls, 413/1198) nested within the Beaver Dam Eye Study,[8] the odds ratio of any cardiovascular death over the 10 years of follow-up in relation to generalized arteriolar narrowing, which was defined as the lowest quintile of the AVR distribution, was 1.5 (95% CI, 1.1–2.1). In the Rotterdam Study,[3] AVR was inversely associated with carotid intima-media thickness and plaque score. On the other hand, in 386 patients

with essential hypertension, Masaidi *et al.*[28] failed to reveal formally significant multivariable-adjusted correlations of carotid intima-media thickness and left ventricular mass index ($P=0.06$ for both) with AVR. According to the authors' interpretation, the lack of significance in the study might have resulted from the small sample size and the inclusion of patients with a narrow spread of demographic and clinical characteristics, most of whom were undergoing antihypertensive treatment.[28]

In our present study, the intraobserver variability in both the ECG-gated and non-gated images was within acceptable limits. The variability calculated using Bland and Altman's method ranged from 2.3% to 17.6%. Other groups reported the repeatability of retinal phenotypes as a correlation coefficient[29,30] or as a coefficient of variation.[17] However, the use of a correlation coefficient to compare repeated measurements is less accurate, as it measures the strength of a relation but not the agreement between two variables.[24] Furthermore, coefficients of variation represent a normalized measure of the dispersion of a probability distribution. When the mean value is close to zero, the coefficient of variation will approach infinity and is therefore sensitive to small changes in the mean.

Compared with IVAN, SIVA generates information over and beyond the diameter of the retinal microvasculature, including fractal dimensions, branching pattern and tortuosity. Retinal vascular branching and tortuosity might be early indicators of microvascular damage. Indeed, in a population-based cross-sectional study, Cheung *et al.*[31,32] showed that the retinal arteriolar branching asymmetry ratio and arteriolar and venular tortuosity were independently correlated with the mean arterial blood pressure in multivariable linear regression analysis. In another cross-sectional study of 1159 participants with type-1 diabetes, Sasongko *et al.*[33] observed that retinopathy (odds ratio, 2.01; CI, 1.23–3.29), early kidney dysfunction (1.56; CI, 1.06–2.28) and the coexistence of both complications (1.96; CI, 1.21–3.24) were associated with higher arteriolar tortuosity.

Our current study must be interpreted within the context of some potential limitations. First, we specifically recruited healthy normotensive volunteers to compare ECG-gated and non-gated images. To our knowledge, all previous reports on the changes in the diameter of the retinal microvasculature during the cardiac cycle also included normotensive volunteers.[12–15] One might hypothesize that the changes in the retinal arteriolar diameter observed during the cardiac cycle might be proportional to the extending pressure and therefore would be larger in hypertensive patients than in normotensive people. Our current findings showed similarities in the retinal arteriolar diameters in the gated and non-gated images and therefore cannot be generalized to patients with elevated systolic blood pressure. Second, the characteristics listed in Table 1 were similar among the selected and non-selected participants from the 2008–2014 cohort, with the exception of systolic blood pressure (123.4 mm Hg *vs.* 130.4 mm Hg; $P=0.025$) and the prevalence of hypertension (23.3% *vs.* 45.1%; $P=0.018$). These variables were not different among the selected and non-selected participants from the 2014–2015 cohort. The mismatch in the 2008–2014 cohort occurred by chance, as the participants were randomly selected, but it might limit the generalizability of our findings related to the non-gated retinal images. However, our findings for intraobserver variability (Table 2) and for the differences between software packages were consistent, regardless of whether the participants were recruited among the 2008–2014 cohort (non-gated images) or the 2014–2015 cohort (ECG-gated images). Third, we only assessed interobserver repeatability in 30 participants from the population study who underwent non-gated imaging that was post-processed by both IVAN and SIVA. Because there were no differences in the retinal microvascular measurements associated with ECG gating, we did not repeat the assessment of interobserver repeatability in the 30 participants who underwent ECG-gated photography.

In population-based research, retinal microvasculature is an established key trait because of its association with hypertension[1,2,5,6] and its role as a predictor of adverse cardiovascular outcomes.[7–9] Our study adds to the current knowledge base by showing that ECG gating does not affect estimates of the retinal microvascular diameters or improve intraobserver repeatability and that SIVA yields larger and clinically relevant estimates of CRAE and CRVE with no differences in AVR compared with IVAN. The implications for research are twofold. First, despite the pulsatility of retinal blood flow,[12–15] ECG gating does not enhance the accuracy of summary measurements of the retinal microvascular diameters by IVAN and SIVA. Second, it is possible to combine historical AVR readings analyzed by IVAN with more recent readings postprocessed by SIVA, but it is not possible for CRAE or CRVE. One possible way to pool CRAE and CRVE estimates obtained with IVAN and SIVA is to use standardized units by expressing each individual reading in units of s.d. after stratification for use in IVAN or SIVA. However, the validity of this approach still requires further investigation before it can be proposed as an alternative to re-measuring all historical images using the newer software.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

The European Union (HEALTH-2011.2.4.2-2-EU-MASCARA, HEALTH-F7-305507 HOMAGE and the European Research Council Advanced Researcher Grant-2011-294713-EPLORE) and the Fonds voor Wetenschappelijk Onderzoek Vlaanderen, Ministry of the Flemish Community, Brussels, Belgium (G.0881.13 and G.088013) currently support the Studies Coordinating Centre in Leuven. We acknowledge the contribution of the nurses working at the examination centre (Linda Custers, Marie-Jeanne Jehoul, Daisy Thijs and Hanne Truyens) and the clerical staff at the Studies Coordinating Centre (Annick De Soete and Renilde Wolfs).

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