Does Increased Blood Pressure Rather Than Aging Influence Retinal Pulse Wave Velocity?

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Purpose. It was demonstrated previously that retinal pulse wave velocity (rPWV) as a measure of retinal arterial stiffness is increased in aged anamnestically healthy volunteers compared with young healthy subjects. Using novel methodology of rPWV assessment this finding was confirmed and investigated whether it might relate to the increased blood pressure usually accompanying the aging process, rather than to the aging itself.

Methods. A total of 12 young 25.5-year-old (24.0–28.8) [median(1st quartile–3rd quartile)] and 12 senior 68.5-year-old (63.8–71.8) anamnestically healthy volunteers; and 12 senior 63.0-year-old (60.8–65.0) validated healthy volunteers and 12 young 33.0-year-old (29.5–35.0) hypertensive patients were examined. Time-dependent alterations of vessel diameter were assessed by the Dynamic Vessel Analyzer in a retinal artery of each subject. The data were filtered and processed using mathematical signal analysis and rPWVs were calculated.

Results. rPWV amounted to 1200 (990-1470) RU (relative units)/s in the hypertensive group and to 1040 (700-2230) RU/s in anamnestically healthy seniors. These differed significantly from rPWVs in young healthy group (410 [280–500] RU/s) and in validated healthy seniors (400 [320–510] RU/s). rPWV associated with age and mean arterial pressure (MAP) in the pooled cohort excluded validated healthy seniors. In a regression model these associations remain when alternately adjusted for MAP and age. When including validated healthy seniors in the pooled cohort only association with MAP remains.

Conclusions. Both aging (with not excluded cardiovascular risk factors) and mild hypertension are associated with elevated rPWV. rPWV increases to a similar extent both in young mildly hypertensive subjects and in aged anamnestically healthy persons. Healthy aging is not associated with increased rPWV. (*Invest Ophthalmol Vis Sci.* 2012;53:2119–2126) DOI:10.1167/iovs.11-8815

Human vasculature stiffens because of the combined effects of aging, high blood pressure, and other cardiovascular risk factors.[1] Changes in arterial distensibility and vascular stiffness contribute to the change in the wall/lumen ratio.[2]

Many established clinical methods exist to assess distensibility and vascular stiffness in the macrocirculation.[3] Most evidence has been created measuring pulse-wave velocity (PWV) and performing pulse wave analysis.[4–6] Aortic PWV has been identified as a blood pressure–independent cardiovascular risk factor, which appears to be of high prognostic relevance for cardiovascular events.[3] Population- and patient-based cohorts demonstrated a strong association between increased aortic PWV and age,[7] coronary artery disease,[8] myocardial infarction,[9] heart failure,[10] stroke,[11] and hypertension.[12] Pulse wave analysis allows the assessment of the augmentation index (AIx), which represents an indirect parameter of arterial stiffness.[13]

On the other hand, methods to investigate vascular stiffness of the microcirculation are scarce. Generally at present, microvascular assessment of vascular stiffness requires invasive generation of vascular material.[14–16] Using these methods, associations of arteriolar media/lumen ratio with blood pressure and cardiovascular organ damage were shown,[16,17] with the media/lumen ratio being predictive for cardiovascular events.[17]

Microvascular alterations mainly occur in small arteries[18] whose diameter range corresponds to the size of retinal arteries. Based on laser-Doppler flowmetry and perfusion imaging, Schmieder et al. recently introduced a noninvasive method claiming to assess wall/lumen ratio in these arteries.[19,20] This gives an indirect impression of microvascular stiffness.[20] We have recently introduced a noninvasive method for the measurement of retinal arterial stiffness, assessing retinal PWV (rPWV) in a cohort of young and old anamnestically healthy subjects.[21] In line with the expectation that vascular stiffness rises with age, we observed a higher rPWV in the elderly as compared with the young subjects.

In the present study we aimed to confirm these results in similar independent groups applying new improved methodology of rPWV assessment and to reappraise the results of our first study comparing the vascular aging process in retinal microcirculation in the presence and the absence of other cardiovascular risks.

We hypothesized that a healthy aging in the absence of other cardiovascular factors does not necessarily imply the stiffening of small retinal arterioles.

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Materials and Methods

Subjects

Four groups, consisting of volunteers and patients, were recruited at the Department of Nephrology, Department of Ophthalmology and Department of Prevention and Sports Medicine at Technische Universität München and were enrolled in this cross-sectional clinical study. Group I: 12 young 25.5-year-old (24.0–28.8) [median(1st quartile–3rd quartile)] anamnestically healthy volunteers; group II: 12 young 33.0-year-old (29.5–35.0) mildly hypertensive normalbuminuric patients; group III: 12 senior 68.5-year-old (63.8–71.8) anamnestically healthy volunteers; and group IV: 12 senior 63.0-year-old (60.8–65.0) medically validated healthy volunteers.

The following inclusion criteria were set: no history of systemic or ocular diseases (except systemic hypertension for group II); no systemic or topical medication including contraceptive pills at the time of the examination and no history of chronic medication; no history of smoking, drug or alcohol abuse; no cardiovascular events; visual acuity 0.8 or better; ammetropia with spherical equivalent within −3.5 and 3.5 Diopter (dpt); astigmatism 1 dpt or less; intraocular pressure less than 20 mm Hg measured with Goldmann applanation tonometry; no pathologic findings in slit-lamp examination.

Senior volunteers of the group IV were validated more thoroughly. Additional inclusion criteria for this group were: body mass index (BMI): 19–28 kg/m^2; blood pressure (BP): BP systole $<$ 135 mm Hg; BP diastole $<$ 90 mm Hg; blood glucose $<$ 110 mg/dL; low density lipoprotein (LDL) cholesterol $<$ 190 mg /dL; high density lipoprotein (HDL) cholesterol $>$ 35 mg/dL.

Informed consent was obtained from all the subjects. The study protocol was reviewed by the ethics committee of Klinikum rechts der Isar (Technische Universität München). All procedures adhered to the tenets of the Declaration of Helsinki.

Blood Pressure and Other Medical Parameters

Systolic blood pressure (BP) and diastolic BP (mm Hg) were assessed in the sitting position using a multicuff blood pressure monitor (Omron Intellisence M7 BPM; Omron, Mannheim, Germany). In each subject BP was assessed three consecutive times and mean values of systolic and diastolic BP were calculated from the second and the third measurement according to the European Society of Hypertension guidelines.[22] Mean arterial pressures (MAPs) were calculated from these values.[23] The urinary albumin-to-creatinine ratio (UACR; mg/g) was determined in morning spot urine in group II. All the hypertensive subjects of group II were normoalbuminuric, with normoalbuminuria defined as UACR $<$ 17 mg/g.[24] The following medical parameters were additionally assessed in group IV: BMI (kg/m^2); HDL, LDL, and total cholesterol (mg/dL), triglycerides (mg/dL), and fasting glucose (mg/dL) from blood samples.

Measurements with the Dynamic Vessel Analyzer

By 20 minutes after pupil dilation with topical tropicamide drops (Mydriaticum Stulln; Pharma Stulln, Stulln, Germany) continuous simultaneous measurements of retinal vessel diameters of arterial and venous segments were performed in a randomly chosen eye of a subject for 1 minute (Dynamic Vessel Analyzer [DVA]; IMEDOS Systems, Jena, Germany). Vessel segments of approximately 1 mm in length located in the upper temporal quadrant 1–3 optic disc diameters away from the optic disc edge were assessed.

Properties of DVA and its measurement principles have been previously described in detail.[25–27] The device allows noninvasive online assessment of vessel diameter, depending on times and locations along the vessel. For this purpose, the DVA consists of a retinal camera (450 FF; Carl Zeiss, Jena, Germany); a digital camera for electronic online imaging; and a personal computer for system control, analysis, and recording of the obtained data. Additionally, each measurement is recorded on videotape, which provides a possibility for off-line reassessment of the data. In cases of insufficient data points in the original assessment, additional measurements of retinal arterial reaction were taken off-line from videotape recordings using DVA.

Data Acquisition

In result of a DVA assessment of a measured vessel segment a data matrix is obtained. It consists of retinal vessel diameter values changing simultaneously temporally in columns with a temporal resolution of 25 measurements/second and spatially in rows with an equidistant resolution of one measurement/10 RU.[26] Each column of such a table represents vessel diameter changes over time in a vessel cross-section with a known distance to other measured cross-sections. Such a data matrix of an investigated vessel segment was extracted from a DVA program and was analyzed thereafter.

Templates with corresponding macros in a spreadsheet (MS Excel 2000) were created to filter, process, and analyze the numerical data from DVA. In order to filter and analyze the measured samples fast Fourier transform[28] (FFT) was used, an algorithm to compute the discrete Fourier transform and its inverse. Service routines based on FFT were created in a commercial software program (MATLAB 7.0; The MathWorks, Natick, MA) for automated data processing.

Assessment of the Heart Rate

Simultaneously assessed summarized temporal retinal arterial and venous diameter changes in measured vessel segments over 1024 temporal data points (∼41 seconds) were analyzed using FFT to obtain the heart rate (HR). Power spectra of venous and arterial pulsations showed a pronounced characteristic peak of the same frequency within the frequency range 0.6–1.5 Hz. This peak frequency is related to HR and was used for further evaluations.

Data Extraction for rPWV Measurements

From the whole temporal arteriolar diameter assessments lasting for 1 minute, data matrices of $2^8 = 256$ temporal data points (≈10.2 seconds) and all the spatial data points within the measured arteriolar segment were extracted for each subject for further analysis (Fig. 1, top panel). These data matrices represented information on simultaneous vessel diameter changes in all the measured cross-sections within the assessed arterial segment over 10.2 seconds.

The temporal size of the analyzed data matrix was a compromise between the necessity of continuous analyzed data sequences and the reduction of the influence of HR variability.[21] Temporal data sequences (columns of the data matrix) with no more than 0.5 seconds of discontinuity and less than 10% of whole data missing were analyzed. Discontinuities of less than 0.5 seconds were filled up using a commercial software program (developed in MATLAB). Missed temporal points were linearly interpolated between the existing measured points.

A column of the data matrix corresponded to temporal vessel diameter changes in a reference cross-section. Another data column at another cross-section was chosen within a narrow region of the analyzed vessel segment for further paired evaluation with the first one (Fig. 1, top panel). The distance between the cross-sections was calculated as a product of 10 RU (a spatial raster of DVA) and the distance in columns between the measured cross-sections. Both continuous analyzed samples from the temporal vessel diameter assessments in two arterial cross-sections were normalized to absolute vessel diameter of the sample, and prefiltered in two steps using a high pass frequency filter and an outlier filter. The high-pass filter on the base of FFT filtered all the frequencies lower than 0.5 Hz, so that large and wide humps like those in Figure 1 (top right panel) were smoothed. The outlier filter then eliminated all the elements that were beyond the scopes of 1st quartile − 1.5 × IQR and 3rd quartiles + 1.5

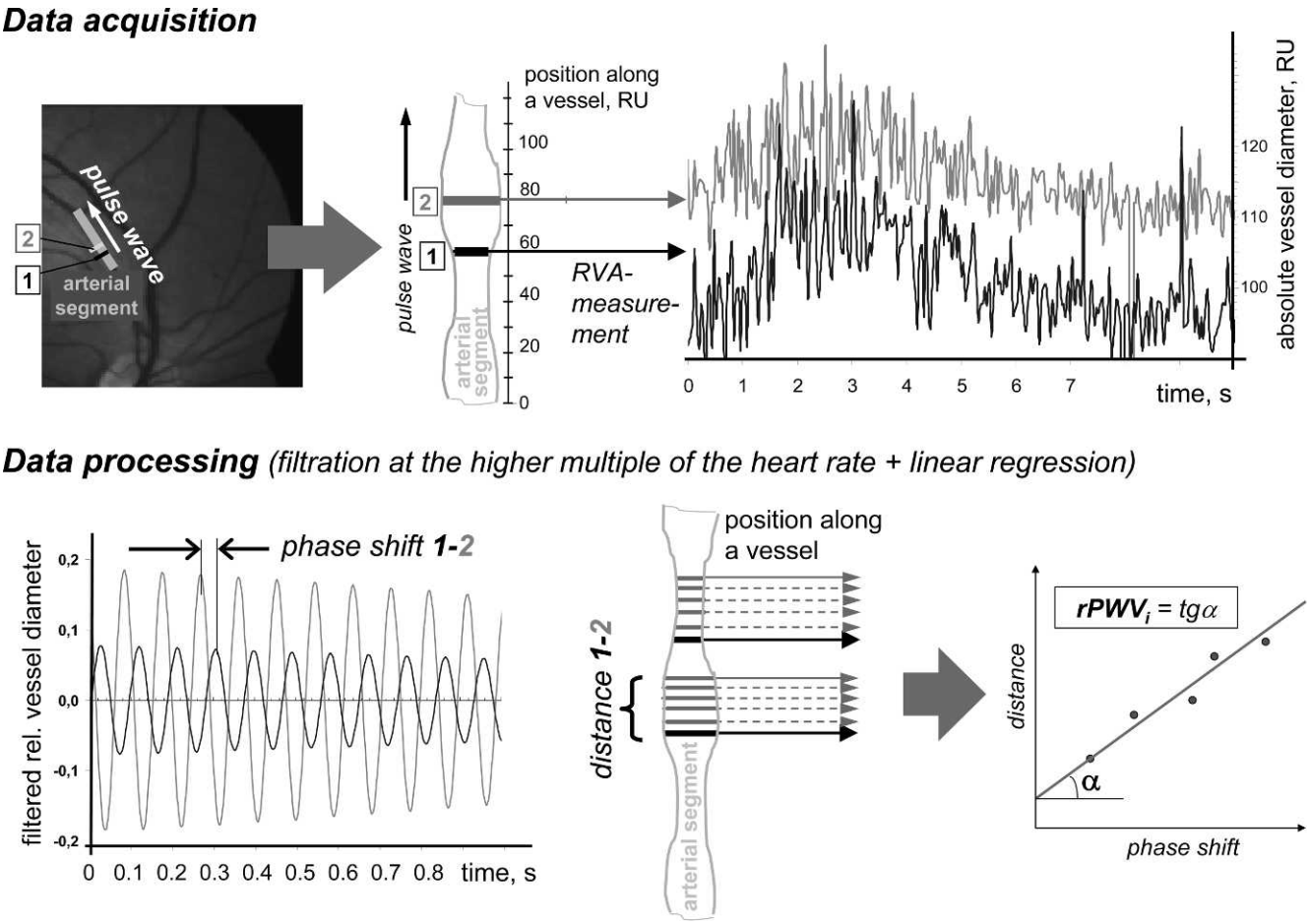

Figure 1. Methodology of rPWV assessment. *Top panel*: Dynamic vessel analyzer (DVA) simultaneously records temporal vessel diameter changes in all the points within a chosen arteriolar segment 10 relative units (RUs) apart from each other (1 RU corresponds to 1 μm in the Gullstrand's eye). Temporal arteriolar diameter changes are assessed for 10 seconds at two different places along the arteriolar segment (*black* and *gray lines*, respectively). *Bottom panel*: Calculation of rPWV: extracted time courses are spectrally filtered at the higher multiples of the heart rate and phase shift between these curves are determined. Temporal shifts are plotted against corresponding distances between the evaluated cross-sections for a narrow and a wide place along the vessel segment. rPWV is calculated as a coefficient of the ensuing linear slope. rPWV for each individual is calculated as a mean value in a narrow and a wide area along the vessel segment.

× IQR, with IQR representing the interquartile range.[29] The eliminated elements were linearly interpolated.

Calculation of the Amplitude of Vessel Diameter Changes

For each filtered and interpolated data sample the amplitude of vessel diameter change was then calculated. The algorithm searched all the local minima and maxima of the sample distant not closer than the heart period from each other. Differences between neighboring minima and maxima of the data sample were calculated and the median of these values was returned. The scattering of the amplitude was calculated as a separate parameter as the IQR of differences between neighboring minima and maxima of the data sample. Because of how their assessments were calculated, both parameters were reported as a percentage of the mean absolute vessel diameter of the initial data sequence. For each subject, median values over all assessed samples were reported for both parameters.

Calculation of rPWV

The PWV in the macrocirculation measured at the HR represents a so-called apparent wave velocity, which includes the effect of reflections in the arterial tree and deviates from the true PWV not being an appropriate measure of vessel compliance.[30] We applied these considerations for the retinal macrocirculation. Since for high HR harmonics the apparent wave velocity approaches the true PWV, both samples were filtered at the 10th harmonic (multiple) of the HR. This was the highest harmonic below the Nyquist frequency[31] of temporal resolution of DVA in the measured cohort.

We used FFT filtration applying the FFT function (from MATLAB): for a filtered data sample x of $N = 256$ elements its discrete Fourier transform X was calculated as follows:

$$X(k) = \sum_{n=1}^{N} x(n)\exp\left(\frac{-i2\pi(k-1)(n-1)}{N}\right), 1 \le k \le N$$

After the spectral filtration only the element of X' corresponded to the 10th harmonic of the HR that remained. Other elements of X were set to 0, resulting in the new sample-vector X'. Using inverse FFT transformation (IFFT-function from MATLAB) we calculated filtered sample-vector $\mathbf{x}^*$ as follows:

$$x^*(n) = \frac{1}{N}\sum_{k=1}^{N} X'(k)\exp\left(\frac{i2\pi(k-1)(n-1)}{N}\right), 1 \le n \le N$$

Applying this mathematical procedure for both samples and correcting each sample for its mean we obtained filtered vessel diameter courses as shown in Figure 1 (bottom left). The temporal shift between the ensuing curves was determined for each pair of analyzed samples. For this purpose the cross-correlation function of the pair of filtered samples $x^*(n)$ and $y^*(n)$ was calculated as:

$$crosscorr(k) = \frac{\sum_{j=1}^{N} x^*(j) \cdot y^*(j+k)}{\sqrt{\sum_{j=1}^{N}[x^*(j)]^2 \cdot \sum_{j=1}^{N}[y^*(j+k)]^2}}, -N+1 \le k \le N-1$$

The temporal shift was determined as the maximum of the function crosscorr.[21,32] rPWV was calculated in relative units per second (RU/s) as a ratio of the distance between the cross-sections and this temporal shift. The measurement unit of rPWV corresponds to μm/s in International System of Units (SI), if Gullstrand's eye model is considered.

Temporal shifts were plotted against corresponding distances between the evaluated cross-sections for a narrow and a wide topographic point along the vessel segment. rPWV was determined as a coefficient of the ensuing linear fit, calculated with the least-squares method. rPWV for each individual was calculated as a mean of resultant rPWV values in a wide and a narrow area along the measured arterial segment.

Since the sampling rate of DVA assessment of 40 ms was insufficient for most measured temporal shifts in the present study, the cross-correlation function was interpolated near its absolute maximum with a polynomial fit (using MS Excel; Microsoft, Redlands, WA) for a more precise assessment.[21,33] The maximum of this fit was considered as the absolute maximum of the cross-correlation function, with the corresponding temporal lag being the desired temporal shift between the vessel diameter assessments.

Statistical Analysis

Statistical analysis was performed using commercial software programs (MS Excel 2000 for Windows, Microsoft; SPSS version 15.0; SPSS Inc., Chicago, IL). Because it was impossible to prove normal distribution of measurement data, nonparametric statistics were applied for the evaluation. Data were presented as median (first quartile − third quartile). Differences between the subgroups were analyzed using the Kruskal-Wallis and the Mann-Whitney tests. We used Bonferroni correction to adjust for multiple comparisons and to reveal significant differences between the subgroups.[29] This adjustment did not imply correction for multiple parameters since we aimed to test local differences for each parameter in this pilot study. Statistical tests were applied on the level of significance of $P = 0.05$ for each evaluated parameter. Nonparametric Spearman correlation was consistently used to reveal statistical associations between parameters.

Results

Blood pressure values and other biometric parameters of the evaluated cohort are presented in Table 1. Mean arterial pressure differs between the young healthy group and both the hypertensive group ($P < 0.01$) and the group of anamnestically healthy seniors ($P < 0.05$). MAPs of validated healthy seniors and hypertensive patients differed from each other as well ($P < 0.01$).

Average rPWV in groups amounted to 1200 (990-1470) RU/s in the hypertensive group and to 1040 (700-2230) RU/s in anamnestically healthy seniors (Table 1, Fig. 2). These differed significantly from rPWVs in the young healthy group: 410 (280–500) RU/s ($P < 0.01$), and in validated healthy seniors: 400 (320–510) RU/s ($P < 0.05$).

Table 1. Biometric and Retinal Parameters of the Participants of the Study

	I			II			III			IV		
	Young Anamn. Healthy ($N = 12$)			Young Hypertensive ($N = 12$)			Old Anamn. Healthy ($N = 12$)			Old Validated Healthy ($N = 12$)		
Parameter/ Group	Median	First–Third Quartile	Significance	Median	First–Third Quartile	Significance	Median	First–Third Quartile	Significance	Median	First–Third Quartile	Significance
Age (y)	25.5	24.0–28.8		33.0	29.5–35.0	* I	68.5	63.8–71.8	** I and II	63.0	60.8–65.0	** I and II
Mean arterial pressure (mm Hg)	92.5	88.9–96.6	*III	107.0	104.8–108.8	** I and IV	100.0	93.6–108.7		97.3	93.3–99.5	
Systolic blood pressure (mm Hg)	123.0	120.5–128.3		140.0	138.8–147.0	** I and IV	134.0	127.5–151.0		127.5	125.9–134.0	
Diastolic blood pressure (mm Hg)	78.5	71.8–82.3		90.0	87.8–93.3	** I and IV	81.5	78.8–87.8		80.5	77.1–84.1	
Mean arterial diameter (RU)	108.2	98.1–127.6		113.7	107.8–128.0		112.0	105.9–119.4		111.1	107.8–116.0	
rPWV (RU/s)	410	280–500	** II; * III	1200	990–1470	* III	1040	700–2230		400	320–510	** II; * III
Amplitude of vessel diameter change (%)	9.7	7.7–12.6	** III	10.0	8.6–10.9	* III	18.0	12.2–24.4		11.4	9.3–13.6	
Scattering of the amplitude (%)	0.31	0.29–0.33	* II	0.27	0.25–0.30	* IV	0.30	0.28–0.32		0.36	0.30–0.42	

Significance: $^*P < 0.05$; $^{**}P < 0.01$: Mann–Whitney test with Bonferroni correction for multiple comparisons between the subgroups, local for each parameter. 1 RU (relative unit) corresponds to 1 μm in the Gullstrand's eye.

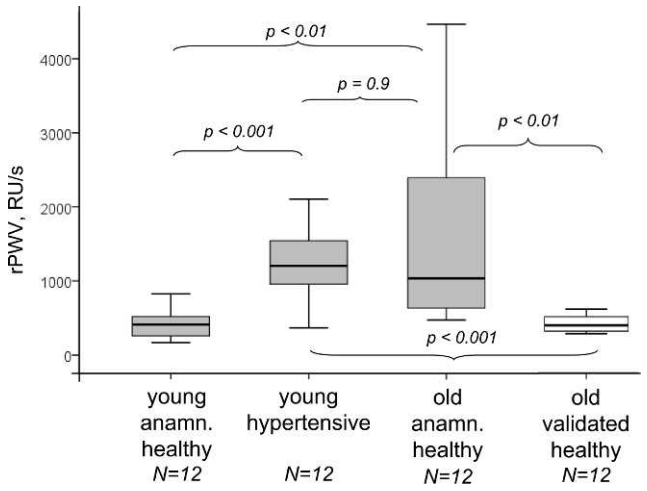

Figure 2. rPWV values in four subgroups of the study participants. Statistics: ANOVA + multiple comparisons (Mann–Whitney test with Bonferroni correction).

rPWV showed strong significant association with age and MAP in the pooled cohort excluded validated healthy seniors (Spearman correlation: $r = 0.49$ and $r = 0.63$ correspondingly, $P < 0.001$, Fig. 3, top panel). In a regression model these associations remained when alternately adjusting for MAP and age. When including validated healthy seniors in the pooled cohort only the association with MAP remained ($r = 0.61$, $P < 0.001$, Fig. 3, middle panel). Averaged absolute arteriolar diameters in the site of measurement did not differ between the groups (Table 1). In the whole pooled cohort or in the pooled cohort that excluded validated healthy seniors, vessel diameters did not show any significant association either with MAP or with age ($P > 0.5$, Fig. 3, bottom panel).

Amplitude of vessel diameter change differed significantly between old anamnestically healthy volunteers and both young groups (Table 1; $P < 0.05$, Mann–Whitney test with Bonferroni correction). There were no significant correlations between rPWV and the amplitude of vessel diameter change, either within each separate subgroup or in the whole pooled cohort ($P > 0.28$, Spearman correlation). The scattering of the amplitude of vessel diameter change differed significantly between young healthy volunteers and two further groups: young hypertensives and old validated healthy volunteers (Table 1; $P < 0.05$, Mann–Whitney test with Bonferroni correction). rPWV did not correlate with the latter parameter,

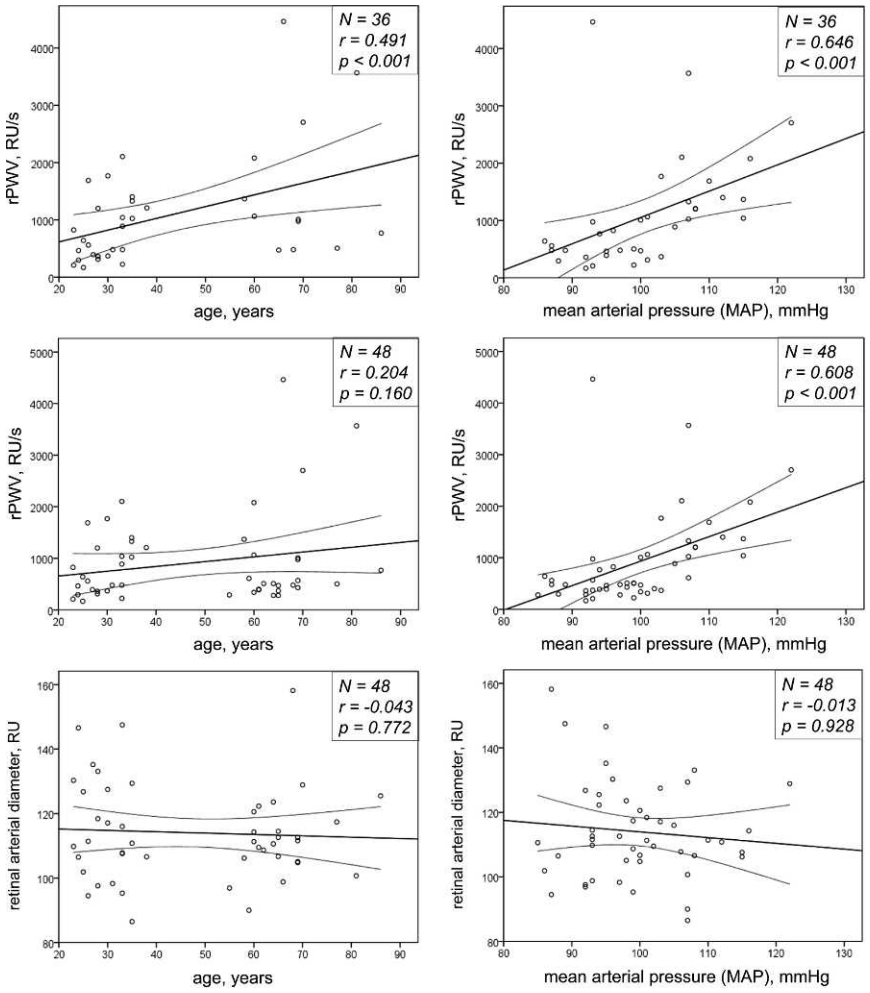

Figure 3. *Top panel*: Spearman correlation between rPWV and two clinical parameters: blood pressure and age of participants for groups I–III. *Middle panel*: Spearman correlation between rPWV and two clinical parameters: blood pressure and age of participants for groups I–IV. *Bottom panel*: Spearman correlation between mean arterial diameter and the same parameters: blood pressure and age of participants for groups I–IV. Linear slopes are shown with *black solid lines*; *gray thin lines* show 95% confidence intervals.

either within each separate subgroup or in the whole cohort ($P > 0.80$, Spearman correlation).

Discussion

Using methodologic principles from the macrocirculation we introduced and applied retinal PWV (rPWV) as a noninvasive measure of microvascular stiffness in the retinal microcirculation.

We demonstrated that rPWV is increased both in young hypertensive subjects and in aged anamnestically healthy volunteers with nonexcluded cardiovascular risk factors. The exclusion of the following cardiovascular risk factors in the aged healthy volunteers—increased blood pressure, increased cholesterol level, and increased blood glucose—results in lower rPWV values, which are well comparable with those of young healthy persons.

Based on these results we can consider that increased blood pressure and related cardiovascular risk factors rather than aging itself do influence rPWV and thus the microvascular stiffness in the retinal microcirculation. A "healthy aging" in the absence of other cardiovascular risks does not necessarily seem to imply the stiffening of retinal arterioles.

In the macrocirculation PWV is strongly associated with blood pressure.[1] In our study rPWV demonstrated a strong association with blood pressure in the whole pooled cohort and in the pooled groups I, II, and III (individual groups were not large enough to show a significance). This represents a necessary condition for a parameter of microvascular stiffness, since BP is associated with eutrophic remodeling, characterized by an increase in the wall-to-lumen ratio.[17] The strong association with BP is an important feature of the new parameter of rPWV, which we were able to demonstrate in other cohorts elsewhere as well.[34]

We did not find any differences in averaged absolute arteriolar diameters in the site of measurement between the groups. The parameter does not show any significant association either with MAP or with age in the cohort. This means that although pulse wave propagation in the arterial tree seems to be influenced by arterial diameter, the diameter itself was not directly responsible for the changes in rPWV between the groups in our study. As expected, these changes are rather related to the structural and mechanical properties of retinal arterioles.

Recently we described, for the first time, the opportunity to assess rPWV noninvasively in young and old anamnestically healthy subjects.[21] A thorough analysis of the methodology presented in that study has shown the lack of its linearity, an important characteristic of a quantitative measure. We reconsider some steps of the data processing, improving the methodology reported in the present study. An ability to already detect temporal shifts between vessel segment pulsations at small distances represents the principal difference to our previous methodology. Moreover, in the new methodology numbers of measurements are evaluated at different sites of a retinal artery to access one rPWV value. The introduction of the new methodology results in physiologic rPWV values being lower than those reported previously.[21]

PWV in the microvessels was observed to be at least two to three orders of magnitude slower than that in the aorta.[35] These experimental findings were shown to be consistent with linear pulse wave transmission theory in a branching system of vessels.[36] Thus, the rPWV values reported in the present study seem to be in line with contemporary vascular physiology.

The aims of the study, to confirm our previous results[21] applying new improved methodology for rPWV and to reappraise those results comparing microvascular aging in the presence and the absence of other cardiovascular risks, determined the choice of the study groups. Group I was represented by young participants with reduced or without cardiovascular risk. Group II contained young subjects with well-defined cardiovascular risk, that is, hypertension. Group III included aged participants, most of whom possessed cardiovascular risk except the aging itself. Finally, group IV represented aged participants without cardiovascular risks other than aging.

Following the inclusion criteria of the study two groups of anamnestically healthy volunteers (groups I and III) reflected an average healthy population. We did not control thoroughly all the cardiovascular risk factors in the young healthy cohort (group I). However, BP values in this group and young age of its participants allowed us to affirm that most of these subjects would pass the appropriate medical validation. Unlike this group I, most aged anamnestically healthy volunteers of group III possessed high cardiovascular risk, which was reflected in increased BP values and their excess scattering in this group. Correspondingly, rPWV values scatter much more in group III than that in other groups. Showing a quantifiable and measurable microvascular manifestation of latent cardiovascular risk in the majority of aged representatives of the average healthy cohort is one of the strengths of our study.

Some technical details of rPWV assessment need to be clarified. With respect to the heart, the retinal microcirculation is located in the periphery, close to the capillary bed. Our recent results have shown that the effect of reflections in the vascular bed needs to be considered in the rPWV concept as well as in the macrocirculation.[34] Additionally, we presumed in the novel rPWV concept that the temporal shift increases with the greater distances between measuring points, because of pulse wave propagation in retinal arteries. Because of the discreteness of the spatial diameter assessment in DVA, the calculation of the linear slope between measured points was shown to be more successful than the simple assessment of the average value of several rPWV measurements.[34]

The temporal resolution of DVA is 25 measurements/s. This theoretically limits rPWV assessment down to 5000 RU/s while simultaneously increasing inaccuracy (degree of closeness of measurements of a quantity to its true value) up to 60% for rPWV = 1500 RU/s. The problem was solved by mathematical signal processing of raw data as shown previously.[21,33] This approach allows the measurement of rPWV values up to 50,000 RU/s with an inaccuracy of 10%, which is limited only to the spatial resolution of DVA.

The amplitude of retinal vessel diameter change due to PWV propagation did not directly associate with the parameter rPWV in the present study. This result was to be expected, since to our opinion the amplitude of the arterial pulsation in the retina is related to the relative stiffness of retinal vessels and upstream vessels rather than to retinal arterial stiffness alone. The difference in the amplitude of retinal vessel diameter change in young and old anamnestically healthy volunteers is well in accordance with the results of our previous study.[32] Another amplitude parameter, the scattering of the amplitude of retinal vessel diameter change, shows an additional aspect of retinal arterial pulsatility: the uniformity of arterial pulsations. The applicability of this parameter for the clinical assessment of retinal arterial pulsations needs to be investigated in the future.

The detailed check of the reproducibility of rPWV assessment represents the matter of a separate methodologic study, which is currently in preparation. Primarily, results show satisfactory results on the short-term reproducibility of the methodology, tested in 10 young healthy volunteers before and after a physiologic response to a standardized stimulus. rPWV

values were evaluated at the same arterial segment during the baselines within 30 seconds before the first and the second flicker stimulation of the standardized protocol of DVA assessment[25,37] The time interval between the measured temporal sequences amounted to 100–140 seconds. The average rPWV values at these sequences amounted to: 439 (362–516) and 399 (346–463) RU/s correspondingly ($P = 0.35$). The coefficient of variation (CV) amounted to 3.9% (0.5–16.7); the Spearman correlation coefficient amounted to 0.70 ($P < 0.05$). These results show that the current methodology allows for a reproducible assessment in the group of volunteers, but might need some technical improvement for individual assessments. In any event, the reported short-term rPWV alterations were much less than, for example, the difference of rPWV between young normotensive and young normoalbuminuric hypertensive subjects in the present study. How rPWV as a physiologic parameter changes with circadian rhythms and over a long time needs to be elucidated.

There are some limitations to the present study. The relatively small sample size does not allow for generalized conclusions related to the normotensive and hypertensive populations. Larger studies are warranted to determine clinical applicability of the new parameter and to enable arriving at general conclusions.

One potential limitation is that one arbitrary retinal arterial segment was chosen to assess the rPWV of a subject. Since the whole retinal vasculature undergoes structural pathologic alterations we assumed that rPWV values in large retinal vessels of a subject are of the same order and depend on the vessel diameter and other factors. The reliability of the assumption needs to be elucidated.

Since we assessed rPWV both in a narrow and a wide topographic point along a measured vessel segment, we were able to estimate relative differences of rPWV calculated in different locations within a subject. Although average rPWV values in narrow 580 (371-1195) RU/s and in wide 503 (362–907) RU/s topographic points were similar ($P = 0.83$), averaged individual CV of rPWV measurements in different locations amounted to 33.1% (17.4–48.3) in the whole cohort. Such a relatively large intraindividual variation in PWV measurements can be related to several factors, including different BP status of the subjects as well as different vessel diameters and vessel wall thicknesses in the sites of measurement. It may also be explained in part by temporal BP variations. The lack of simultaneous measurements of rPWV and BP in the present study did not allow us to investigate the issue in detail. This could be considered as another limitation to the study, which needs to be elucidated in future studies.

In summary, we present an improved method to assess retinal PWV. It implies the assessment of temporal delay between vessel pulsations at different topographic points along a measured vessel, which is similar to common approaches of PWV measurements in large vessels. Retinal PWV as a measure of microvascular stiffness showed a strong correlation with blood pressure in the whole pooled cohort. In the present study increased blood pressure and related cardiovascular risk factors rather than aging itself do influence rPWV and thus the microvascular stiffness in the retinal microcirculation. This allows consideration of the fact that "healthy aging" in the absence of other cardiovascular risks does not necessarily imply the stiffening of retinal arterioles. However, for general conclusions larger groups of patients and healthy volunteers need to be investigated.

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