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Review Article

Use of the retinal vessel analyzer in ocular blood flow research

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ABSTRACT.

The present article describes a standard instrument for the continuous online determination of retinal vessel diameters, the commercially available retinal vessel analyzer. This report is intended to provide informed guidelines for measuring ocular blood flow with this system. The report describes the principles underlying the method and the instruments currently available, and discusses clinical protocol and the specific parameters measured by the system. Unresolved questions and the possible limitations of the technique are also discussed.

Key words: ocular blood flow – physiology – reproducibility – retina – retinal vessel analyzer – standardization

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Introduction

The present article describes a standard method for the continuous online determination of retinal vessel diameters using the commercially available retinal vessel analyzer (Imedos GmbH, Jena, Germany; http://www.imedos.de). As described in detail below, this instrument is available in two different versions: the retinal vessel analyzer (RVA) is designed for vessel analysis for scientific purposes, whereas the dynamic vessel analyzer (DVA) is intended for clinical use. The latter includes a capacity to provide visual stimulation while measurements are being taken.

Basically, two different aspects of retinal vessel analysis underscore its

importance. In particular, retinal vessel diameter is a major determinant of retinal blood flow. Blood flow through a specific vessel is calculated as the cross-sectional area multiplied by the mean retinal blood velocity. Thus, assuming a circular cross-section, blood flow depends on the square of the radius. Hence, retinal vessel diameter also largely determines vascular resistance. By contrast, alterations in retinal vessel diameter have been linked to several vascular-related pathologies, including systemic hypertension and diabetes, in large population-based studies. However, abnormal retinal vascular regulation may also be identified through metabolic provocation, such as flicker stimulation of the retina. Thus, the ability to obtain exact measurements of retinal vessel diameter is of crucial importance to our understanding of retinal blood flow and its regulation. Herein, we describe the basic technology and the application of the RVA, including its possible limitations and unresolved issues. Because the DVA is an extended version of the RVA, all descriptions in this paper of aspects of the RVA are also valid for the DVA.

Fig. 1. The retinal vessel analyzer/dynamic vessel analyzer system.

Basic technology

Underlying physical principles

The RVA (Imedos GmbH) is a commercially available tool for the assessment of retinal vessel diameter in relation to time. In principle, the RVA (Fig. 1) assesses retinal vessel diameter by analysing the brightness profile of the vessel using video sequences obtained with a conventional fundus camera. Specifically, the technique is based on an image analysis computer system which analyses fundus images visualized by means of a fundus camera (FF450; Carl Zeiss GmbH, Jena, Germany). The illumination light of the fundus camera is reflected by the different layers of the retina and the retinal vessels and is then delivered through the observation pathway to a video camera (charge-coupled device [CCD]). For optimal alignment, the image of the fundus can be observed by the operator on a computer display. The optical information reaching the video camera is then analysed by a computer system to which the camera is connected and simultaneously recorded by a high-quality video recorder (Fig. 2). This allows recorded data to be re-evaluated later if necessary.

Illumination light from the fundus camera enters the eye via the subject's pupil and is reflected by the different layers of the retina. The basic principle of the RVA is based on the fact that the erythrocytes within the retinal vessels absorb light at a maximum wavelength of 400–620 nm, whereas the surrounding tissue mostly reflects it. The differences between the brightness profile of the erythrocyte column within the vessel compared with that of the surrounding tissue are then used for further analysis. To achieve an optimum contrast for vessel visualization, a green filter is inserted into the illumination pathway of the fundus camera. Thus, in its strictest sense, the RVA measures the width of the red blood cell column within the selected vessel(s). Although a homogeneous distribution of erythrocytes within the vessel would, theoretically, result in a homogeneous absorption brightness profile of the red blood cell column, in reality the interpretation of the brightness map is hampered by the possible occurrence of several disturbances, such as shadowing structures or reflections on the vessel surface, which complicate the analysis. To overcome this problem, the RVA uses an adaptive algorithm based on variation in brightness, which compensates for reflections and other disturbances that occur during measurement. Furthermore, continuous assessment of the vessels allows the RVA computer to follow a selected vessel without intervention from the operator and the instrument can, in turn, automatically correct for slight movements. Additionally, based on the brightness profile, the RVA software continuously analyses the image quality provided by the video camera according to image contrast. If the image quality is inadequate dur-

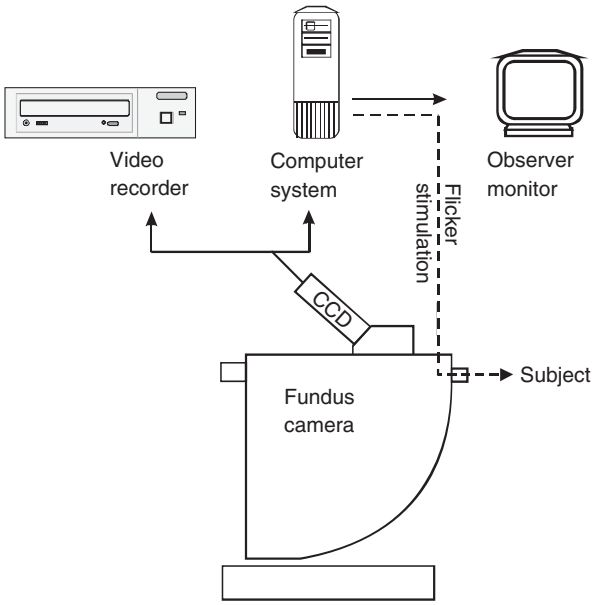

Fig. 2. Schematic view of the retinal vessel analyzer/dynamic vessel analyzer system. CCD, charge-coupled device.

ing the measurement as it occurs, such as during blinks, it is automatically removed from the analysis.

Devices available

The RVA was originally developed as an instrument for the assessment of retinal vessel diameters for scientific purposes. As well as measuring diameter, it also allows additional signals (i.e. blood pressure, pulse rate) to be recorded on separate input channels. The DVA was primarily developed for clinical use. This instrument includes all the applications of retinal vessel analysis provided by the RVA, along with an additional visual provocation system (flicker light stimulation), which will be described in detail later in this paper.

Clinical protocol

Preparation of the subject

Adequate dilation of the patient's pupil prior to measurement is crucial. Generally, a muscarinic antagonist (i.e. tropicamide) is recommended for pupil dilation. If alpha receptor agonists (such as phenylephrine hydrochloride) are necessary to obtain sufficient pupil dilation, the possibility that this drug group may influence retinal vessel diameters should be noted. A period of rest should be scheduled before the measuring process begins in order to achieve stable haemodynamic conditions. Stable haemodynamic conditions can be verified by making repeated measurements of systemic blood pressure and pulse rate. The study protocol should acknowledge that vasoactive drugs, such as caffeine and nicotine, may influence vessel measurements in the eye.

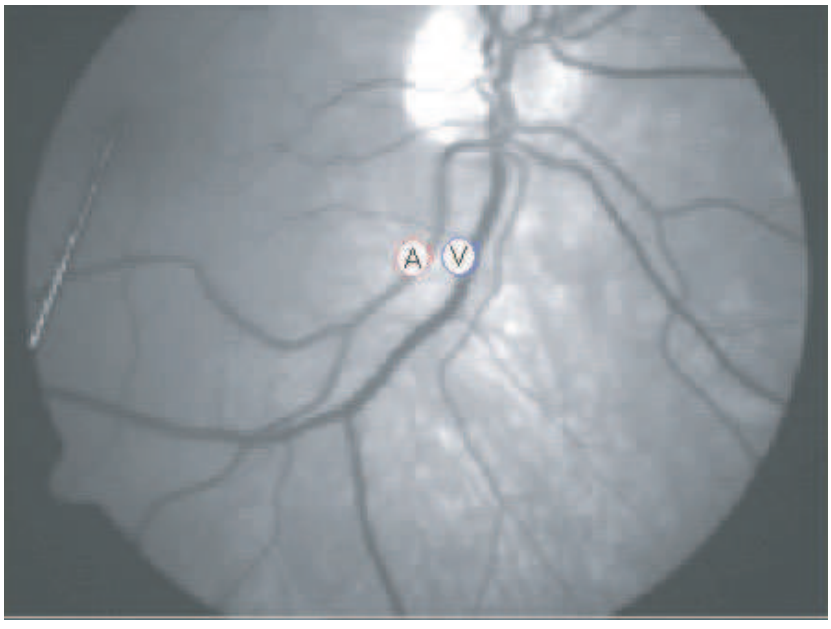

Fig. 3. Ocular fundus as visualized on the retinal vessel analyzer observer monitor. Retinal arteries (A) and veins (V) can be selected for measurement.

Evaluation procedure

The illumination annulus of the fundus camera must be focused on the cornea and should be concentric with the dilated pupil in order to obtain a uniformly illuminated fundus image without unwanted reflections. Furthermore, the brightness of the fundus camera light must be adjusted until the contrast between the vessels and the surrounding tissue is optimal. This can be ensured by inspecting the online fundus picture on the monitor, which should display a fundus image with optimal contrast and brightness (Fig. 3). Additionally, the RVA's software provides an analogue scale at the bottom right corner of the monitor that gives a measure of the average fundus brightness. To obtain optimal readings, the needle in this scale should remain within a narrow green range during vessel measurements.

The patient's fixation should be adjusted using a fixation target so that the site of interest lies in the middle of the fundus picture. For optimal measurement, it is recommended that superior or inferior temporal retinal vessels should be measured approximately 1–2 disc diameters from the optic nerve head. Sites where two vessels are very close to each other should not be used for vessel measurements because the system cannot distinguish between such vessels. Because of the limited resolution of the video camera and the optical system, it is generally not recommended that vessels with a cross-sectional diameter of $< 90\ \mu m$ are

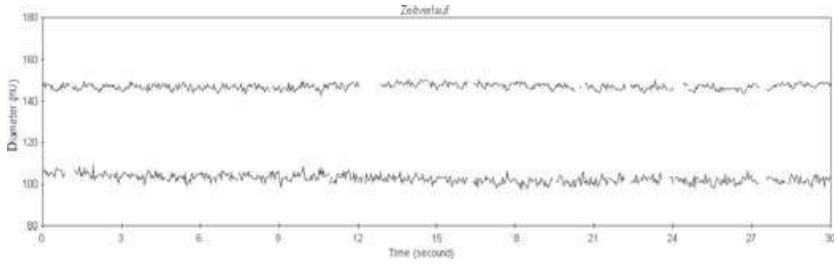

Fig. 4. Typical reading from the retinal vessel analyzer software. Retinal vessel diameters are shown as a function of time. The upper graph depicts the diameter of a retinal vein, the lower graph the diameter of a retinal artery.

measured (Seifert & Vilser 2002). A region of interest can be defined by the operator and marked by a rectangle on the screen of the observation monitor. Within a rectangular area around this target, the user selects segments of both retinal arterioles and venules by marking them axially with a straight line. The length of the selected segment along the vessels should be between 0.5 and 1 diameter of the optic nerve head to achieve representative mean vessel diameter measurements. The instrument will then start the measurement automatically and vessel diameters are continuously calculated along the selected lengths of vessels. Figure 4 shows a typical setting. Repeated measurements of the same vessel segments in the same subject across studies can be obtained using the 'repetition' feature of the instrument. The computer system will then automatically select the same segment of the vessel for the next analysis.

Potential stimuli applicable during measurement

Flicker stimulation

There is compelling evidence that visual stimulation with flickering light increases retinal vessel diameter, retinal blood flow and optic nerve head blood flow in humans (Riva et al. 2005). Therefore, stimulation with flicker light has been used as physiological provocation to investigate the regulation of vascular tone. The physiological background and effects of flicker light on the ocular circulation have been reviewed in detail elsewhere (Riva et al. 2005).

The DVA system is used for the investigation of flicker-induced changes in retinal vessel diameter. It uses the same principle for diameter measurements as the RVA system, but has an integrated flicker stimulator. This flicker system is based on an optoelectronic shutter device inserted into the fundus camera, which interrupts the illumination light over the entire 30-degree visual field of the retinal camera. This produces a rectangular-wave flicker with a bright : dark contrast ratio of $\geq 25 : 1$.

Different measurement protocols regarding the frequency of flicker stimuli, time–course and wavelength of the flicker light have been used by various research groups. In the DVA the flicker is generated by interrupting the illumination light to produce the flicker effect. Therefore, the flicker light is of the same wavelength as the illumination light. The flicker frequency is 12.5 Hz, which is in the range of the maximal excitation flicker frequency for retinal vessels in humans (Polak et al. 2002).

We recommend a modified protocol as described previously by Nagel et al. (2004). Before starting the flicker stimulation, a baseline recording for a minimum time of about 100 seconds should be performed. Valid readings cannot be obtained without ensuring that the sampling period is not influenced by any disturbances or stimulations. After the baseline recording, flicker for a minimum of 20 seconds should be applied, followed by about 80 seconds of steady illumination. Polak et al. (2002) demonstrated that, after cessation of the flicker stimulus, arteriolar diameters decrease below baseline, reaching a minimum diameter approximately 10–40 seconds after cessation of the stimulus. The exact time–course and the reason of this decrease are unclear. However, to avoid any overlapping effects, steady illumination for a minimum of about 80 seconds is recommended between different periods of flicker stimulation.

Systemic hyperoxia

Increasing pO_2 by means of inhalation of 100% oxygen induces a pronounced vasoconstrictor effect in retinal vessels (Riva et al. 1983). This vasoconstrictor effect is fully established after 6 mins and remains stable over a period of $\geq$ 30 mins (Kiss et al. 2002). Additionally, it has been reported that the degree of vasoconstriction during inhalation of 100% oxygen is the same in all quadrants of the fundus (Jean-Louis et al. 2005). This technique for studying retinal vascular reactivity has primarily been applied in patients with diabetes. The vasoconstrictor response has been found to lessen with increasing stage of the disease and to improve after panretinal photocoagulation (Grunwald et al. 1984). The observation that, in pathological conditions, mediators, including nitric oxide and vascular endothelium growth factor (VEGF), among others, are dynamically regulated has increased our understanding of the mechanism underlying the vasoconstrictor response to hyperoxia (Pe'er et al. 1996; Izumi et al. 2008). Whether other, hitherto unknown, mechanisms may contribute to these processes remains to be investigated.

Most study protocols used a reservoir bag and a one-way valve for oxygen delivery in order to avoid re-breathing. This may, however, result in hyperventilation, leading to a reduction in pCO_2 (Becker et al. 1996). More recently, a technique to maintain isocapnia during oxygen breathing was introduced (Gilmore et al. 2004). Whenever systemic hyperoxia is used as a stimulus, pO_2 and pCO_2 should be monitored either in the exhalate or in arterial or arterialized blood.

Specific parameters

Vascular bed(s) studied

The RVA/DVA system allows for the investigation of diameters of retinal arteries and veins. Because of the resolution of the instrument, it is generally recommended that vessels with a diameter of < 90 μm should not be measured (Seifert & Vilser 2002).

Measured parameters

The main outcome variable of the RVA is the width measurement of the selected vessel(s), expressed in units of measurement (UM). In a normal Gullstrand eye, 1 UM is equivalent to 1 μm. For the stimulation with flicker light, the outcome is defined as the percent change from baseline.

Calibration

Preparation of the device

Reproducibility in healthy subjects

The reproducibility and sensitivity of the RVA in healthy subjects have been described previously (Polak et al. 2000). The short-time coefficient of variation (CV) of the RVA was assessed in a group of nine healthy volunteers. The CVs for measurements taken 12 mins apart have been reported at 1.3% and 2.6% for retinal veins and retinal arteries, respectively. The CVs for the day-to-day variability of the instrument were 4.4% for retinal veins and 5.2% for retinal arteries in the same group of volunteers (Polak et al. 2000).

In another study, short-term reproducibility was tested by means of repeated measurements of veins in 12 normal subjects (Seifert & Vilser 2002). Retinal veins in 12 healthy volunteers were assessed continuously for 5 mins and the measurements were repeated at the same vessel location after 2 hours. This resulted in a short-term CV of 1.5% for retinal veins. The same study assessed longterm reproducibility in 11 healthy subjects. Measurements were performed at baseline and after 1 month. The reported CV was 2.8% for retinal veins. Two further studies assessing long- and short-term reproducibility reported comparable results (Pache et al. 2002; Nagel et al. 2006).

The data indicate that reproducibility is slightly higher for retinal veins than for retinal arteries. Whether this reflects a resolution size phenomenon (in comparable fundus locations, veins are larger than arteries) or differences in the absorption properties of arteries compared with veins remains to be clarified.

Reproducibility in patients

The exact reproducibility of measurement in patients has not yet been published in detail.

Main limitations

An important limitation of the instrument is that good measurement quality is strongly dependent on clear optical media. Thus, measurements in patients with opaque media caused by cataract or corneal disease are often of poor quality. Secondly, optimal readings require good fixation abilities. Although, as stated above, the RVA can adjust for slight eye movements, measurements in subjects with central vision loss or poor visual acuity will result in increased variability. Thus, it is generally recommended to consider fixation ability when planning a study in patients or subjects with impaired vision. This represents a severe problem for sample size calculations because no variability data for patients with opaque media or fixation problems have been published.

Reporting

Retinal vessel diameters should be reported in relative units of measurement (UM) as recorded by the RVA, which are equal to micrometres (μm) in the normal Gullstrand eye. For flicker stimulation, flicker response (ΔFl) is reported as:

$$\Delta Fl = 100 \times (Fl_{flicker} - Fl_{baseline})/Fl_{baseline}$$

where $Fl_{baseline}$ is the average of a steady section of the baseline recording, and $Fl_{flicker}$ is the average of a steady section of the same duration during flicker stimulation.

Unresolved open questions

Despite much effort, several aspects of retinal vessel diameter measurement remain to be clarified. Particularly, current data on the reproducibility of flicker responses are insufficient. This is mainly as a consequence of the different approaches used by the various research groups and the limited number of subjects investigated. A multicentre study would enable investigation in a large number of subjects and might generate new insight into the reliability of flicker data. This is important for the design and planning of future studies.

As stated above, in the normal Gullstrand eye, 1 UM equals 1 μm. However, this changes if the eye is non-emmetropic. Currently, there is no generally accepted procedure for correcting data obtained with the RVA in the non-emmetropic eye.

Clinical use

In recent years it has become clear that measurements of vessel calibre are important, and not only for scientific purposes. Although we have known for a long time that hypertension, diabetes and other systemic diseases are reflected in morphological changes in the retina, the introduction of new and sophisticated technology such as the RVA now allows for the exact quantification of theses changes.

Several large epidemiological studies have consistently reported the existence of a correlation between systemic disease factors and retinal vessel calibre. In particular, it has been demonstrated that increased systemic blood pressure is reflected in the generalized arterial vasoconstriction of retinal vessels (Wong & Mitchell 2007), whereas systemic inflammation or obesity lead to wider venous diameters (Liew et al. 2008). Thus, there is increasing evidence that retinal vessel diameters not only carry information about the retinal circulation *per se*, but may also reflect systemic pathologies. In particular, changes in retinal vessel diameters have been shown to predict risk for coronary heart disease, stroke and stroke mortality (Wong et al. 2001, 2002). Pooled data from the Beaver Dam Eye Study and the Blue Mountains Eye Study also showed that smaller arterial diameters and larger retinal venous diameters are associated with increased risk for stroke mortality (Wang et al. 2007). These data clearly support the suggestion that retinal vessel diameter may serve as a predictor for events in other vascular beds, such as in the heart or brain.

Additionally, it has been shown that changes in retinal vessel calibres observed during systemic pathologies may be influenced by treatment. In particular, retinal arterial and venous diameters were found to be significantly reduced in diabetes patients who underwent laser treatment com-

pared with non-treated subjects (Klein et al. 2006). This was interpreted by the authors as a sign for decreased blood flow need and better oxygenation of the tissue. Moreover, the oxygen-induced regulatory response of the retinal vasculature was observed to improve in patients with proliferative diabetic retinopathy, particularly in those who also showed regression of neovascularization (Grunwald et al. 1989). Whether this indicates that retinal vessel diameter can be used as an indicator of treatment success in such patients has yet to be clarified in large-scale, longitudinal studies.

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