Unraveling the genetic architecture of migraine: exploring the vascular components

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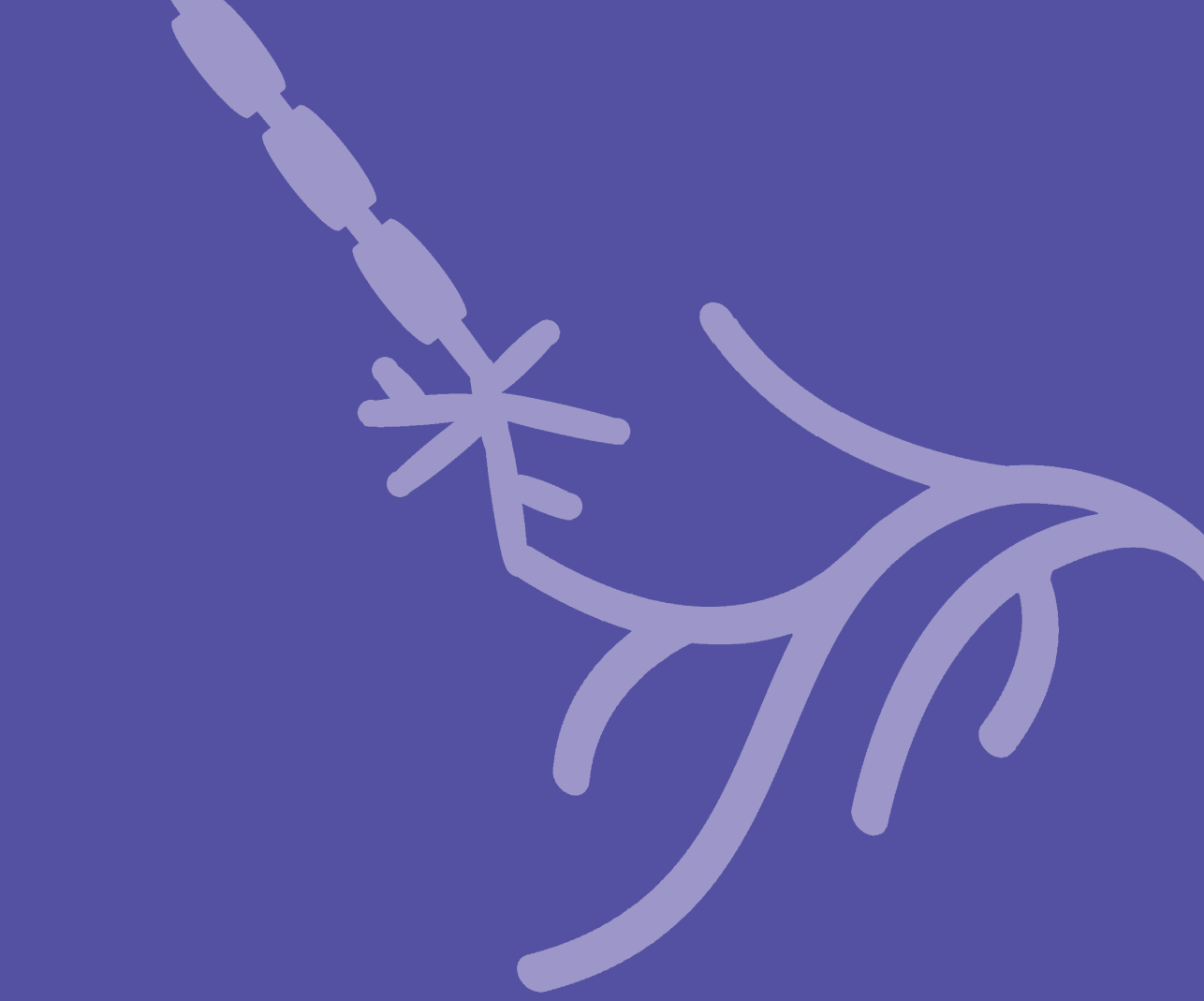

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CHAPTER 11

Retinal oxygen saturation in patients with Retinal Vasculopathy with Cerebral Leukoencephalopathy and Systemic manifestations

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Abstract

Purpose: Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S) is an autosomal dominant small vessel disease caused by mutations in *TREX1*. Early in the course of the disease, retinal vasculopathy emerges as a key feature and retinal oxygen metabolism is thought to be affected. We aimed to determine if retinal oxygen saturation differs between RVCL-S patients and controls.

Methods: Retinal oxygen saturation was assessed in 17 eyes from 9 RVCL-S patients and 19 eyes from 15 controls by using a non-invasive technology (Oxymap T1 retinal oximeter). Mean arterial and venular oxygen saturation and arteriovenous difference per eye were measured, as well as vessel calibre.

Results: No differences in arterial oxygen saturation were found between RVCL-S patients and controls (median [IQR]: 94.5% [92.7-98.9] vs 93.4% [87.2-98.3], p-value = 0.12). However, the venular oxygen saturation of RVCL-S patients was considerably higher than in control patients, median of 71.5% [IQR: 55.0-77.2] and 55.5% [IQR: 51.2-59.9] respectively (p-value < 0.001). In line with these findings, the arteriovenous difference was decreased in RVCL-S patients (median [IQR]: 22.6% [18.5-38.3] vs 38.5% [34.1-39.9], p-value < 0.001). Finally, RVCL-S patients exhibited a reduced calibre in both arterial and venular vessels.

Conclusion: Retinal venular oxygen saturation is higher in individuals with RVCL-S compared to controls, likely due to decreased oxygen consumption resulting from retinal atrophy. Retinal oximetry serves as a non-invasive and innovative tool for biomarker identification, aiding in the detection and prediction of complications associated with retinal vasculopathy in RVCL-S.

Introduction

Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S) is an autosomal dominant small vessel disease caused by truncating mutations located in the C-terminus of *TREX1*.[1,2] *TREX1* encodes a 3'-exonuclease that removes nucleotides from the 3'ends of DNA molecules. How these mutation lead to RVCL-S is unknown. Patients with RVCL-S mainly present with progressive visual impairment due to vascular retinopathy. Additionally, the disease is characterized by brain white matter lesions, intracerebral tumefactive lesions, migraine and internal organ dysfunction, such as kidney and liver disease.[2] Symptoms advance rapidly towards middle age, resulting in premature death.

Although RVCL-S can cause a broad spectrum of symptoms, visual impairment is often the presenting symptom.[2] As such, ophthalmologists are generally the first to see these patients. Identifying patients with RVCL-S is crucial, as retinopathy and systemic manifestations often necessitate (aggressive) treatment. Retinal vasculopathy exhibits similarities to hypertensive and diabetic retinopathy, possibly contributing to the underdiagnosis of RVCL-S. Early signs of retinopathy in RVCL-S include cotton wool spots, micro-aneurysms and small haemorrhages and can occur prior to onset of symptoms, such as visual loss.[2] Ischemia of the central and peripheral retina and neovascularisation occur in later disease stages.[2] Treatment of retinal vasculopathy in RVCL-S patients is comparable to treatment of diabetic retinopathy, consisting mainly of anti-vascular endothelial growth factor (anti-VEGF) injections for cystic macular oedema and laser photocoagulation for ischemia of the peripheral retina.[2]

No biomarkers are currently available to predict onset and progression of retinal vasculopathy or to monitor treatment response. Therefore, patients have to be strictly followed by an ophthalmologist to evaluate the need for (additional) treatment, sometimes for decades, before signs of retinopathy occur. As fundoscopy alone is not sufficient, invasive procedures such as fundoscopic angiography (FAG) have to be performed to follow disease progression.[2] Identification of predictive biomarkers would enable physicians to effectively screen and timely treat retinal vasculopathy and thereby prevent possible detrimental complications of retinal ischemia, including neovascular glaucoma.

Disturbances in oxygen saturation levels have demonstrated their potential as a biomarker in a wide range of diseases, including diabetic retinopathy, but also central nervous system diseases, such as Alzheimer's disease.[3,4] Diabetic retinopathy shows

similarities to retinal vasculopathy in RVCL-S and thus, retinal oximetry may also be a promising tool to predict and monitor RVCL-S progression.

Retinal oximetry is a non-invasive tool to measure the retinal arterial and venular oxygen saturation. Retinal vascular dysregulation can be part of disease pathophysiology, but also be a consequence of disease. By measuring the retinal arterial and venular oxygen saturation separately, retinal oximetry can provide information on the underlying mechanisms that cause abnormalities in retinal oxygenation. Thus, retinal oximetry might provide new insights into pathophysiology of diseases and might ultimately lead to the identification of new treatment targets.

This study aimed to evaluate whether retinal oxygenation is abnormal in RVCL-S patients by comparing oxygen saturation of the retinal vessels between RVCL-S patients and controls with the goal to elucidate pathophysiological mechanisms involved in small vessel diseases and to identify possible non-invasive biomarkers.

Method

Study population

The Leiden University Medical Centre (LUMC) is a tertiary referral centre for RVCL-S. Confirmed *TREX1* mutation carriers who visited the ophthalmological outpatient clinic between July 2012 and August 2020 were eligible for this cross-sectional study. Controls were selected at random from an ophthalmological cohort at the LUMC. Controls were excluded if they had metabolic, neurological or cerebrovascular diseases. In controls, only healthy eyes were included without abnormalities affecting retinal oxygenation, thus excluding eyes that were affected by present or past retinal or optic nerve diseases, for example choroidal melanoma, papilledema or glaucoma, as this could affect retinal oxygenation. Participants were above the age of 18. The study was approved by the LUMC medical ethics committee and conducted in accordance with the criteria of the Declaration of Helsinki.

Ophthalmological examination

All participants underwent standard ophthalmologic examination by an ophthalmologist consisting of measuring best-corrected visual acuity, slit lamp examination and fundoscopy. Moreover, intraocular pressure (IOP) was measured using Goldmann applanation tonometry after instillation of topical oxybuprocaine monofree 0.4% and fluorescein dye. Prior to performing retinal oximetry, pupils were dilated with tropicamide monofree 0.5% and phenylephrine monofree 5.0%.

Retinal oximetry

The Oxymap T1 retinal oximeter (Oxymap, Reykjavic, Iceland) was used to assess oxygen saturation in retinal vessels. The technique's reliability and reproducibility have been previously established.[5,6] The Oxymap T1 consists of two high resolution digital cameras that are attached to a Topcon TRC-50DX fundus camera (Topcon Corporation, Tokyo, Japan). Images were simultaneously acquired in a 50° field of view at wavelengths of 570 nm (oxygen insensitive) and 600 nm (oxygen sensitive) centred on the optic nerve head.

After combining the images, the oxygen saturation and vessel diameter was automatically calculated by specialised software (Oxymap Analyzer software version

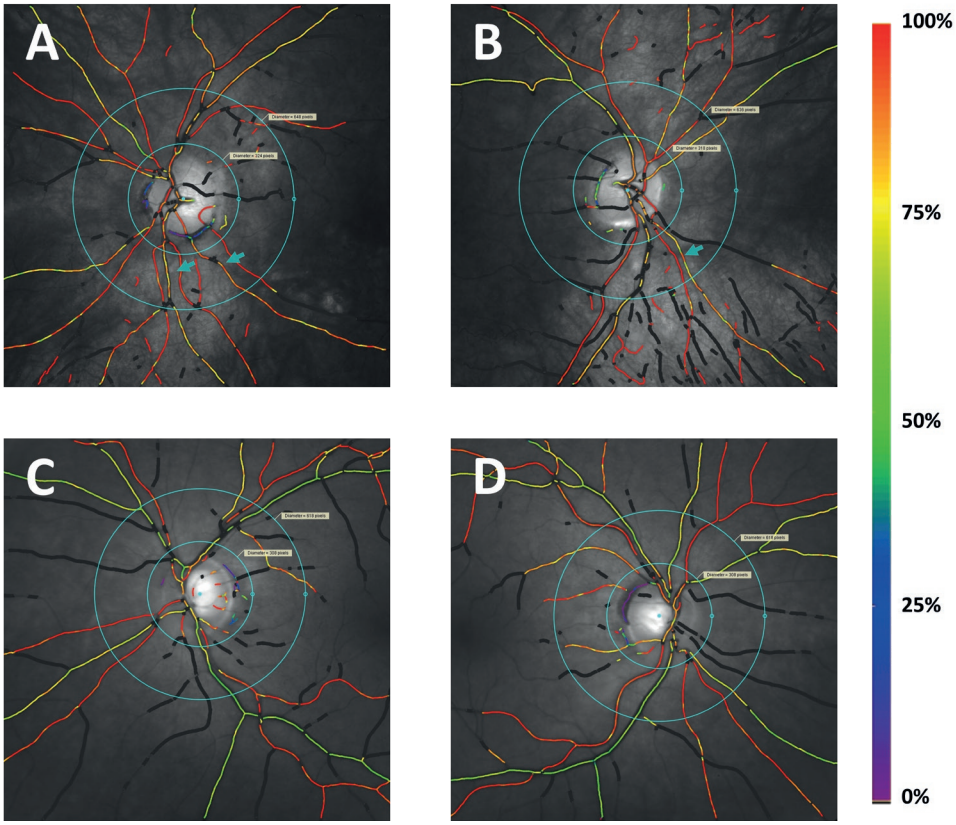

Figure 1. Pseudo colour fundus map of RVCL-S patient and control.

The image is focused around the optic disc. Retinal oximetry images of the left (A) and right (B) eye of a RVCL-S patient and the left (C) and right (D) eye of a control individual are shown. The vessel segments located in the area between the two circles (blue), which have a diameter of 1.5 and 3.0 times the optic disc diameter, were included. Colours reflect oxygen saturation as shown in the colour scale. The blue arrows indicate retinal venules of a RVCL-S patient with increased oxygen saturation.

2.2.1, revision 10927, Oxymap, Reykjavik, Iceland). Images were acquired and analysed in accordance with the protocol for acquisition and analysis of Oxymap T1 oximetry images.[7] Arterioles and venules wider than 8 pixels and longer than 50 pixels were measured (1 pixel corresponds to approx. 9 µm) in a peripapillary annulus within a grid of 1.5 to 3.0 disc diameters from the optic disc centre (fig 1).[7] If needed and available, fundoscopic angiography (FAG) images were used to discern retinal arterioles from venules. An ophthalmologist made the final decision concerning vessel allocation. Images with an overall image quality <5.0, as reported by the Oxymap Analyzer software, were excluded from analysis. Calibration parameters and the vessel detection algorithm are stated in table S1.

To correct for differences in vessel diameter in the retinal circulation, mean arterial saturation and venular saturation per eye were calculated by weighing with the vessel diameter as shown in fig S1. The arteriovenous difference (AV-difference) was calculated by using the mean weighted arterial and venular saturation. Additionally, the mean width of selected arterial and venular vessel segments was reported.

Statistical analysis

Demographics were described as median and interquartile range (IQR) or counts and percentages. They were compared using Mann-Whitney U tests for continuous variables and Fischer's Exact Test for categorical variables. To correct for inter-eye correlations within participants, retinal oximetry parameters were compared between groups using generalized estimating equation (GEE) with an exchangeable correlation structure. All analyses were performed using R version 4.2.1. and RStudio version 2022.02.3+492. P-values of ≤0.05 were considered statistically significant.

Results

Patient characteristics

Retinal oximetry was performed on 20 eyes from 10 RVCL-S patients and 30 eyes from 15 controls. Three eyes from two RVCL-S patients were excluded due to overall image quality <5.0. In the control group 11 eyes from 11 control patients were excluded due to the presence of choroidal melanoma (n = 9) or a peripapillary choroidal naevus (n = 2). As such, 19 eyes, without any (previous) abnormalities, were included in the control cohort. The final study population thus consisted of 17 eyes from 9 RVCL-S patients and 19 eyes from 15 controls. No differences regarding age, sex and IOP were present (table 1). Panretinal laser photocoagulation therapy was performed in 52.9% (9/17) of the eyes of RVCL-S patients.

Table 1. Characteristics study population.

	RVCL-S patients (n = 9)	Controls (n = 15)	P-value
Age (median (IQR))	59.5 (46.8-65.3)	62.0 (54.0-67.2)	0.35
Sex (female, n (%))	4 (44.4)	7 (46.7)	1.00
IOP[a] (median (IQR))	16.5 (10.3-19.3)	14.0 (13.0-16.8)	0.82
Visual acuity (logMAR)	0.0 (0.0-0.1)	-0.1 (-0.2-0.0)	0.001
Retinopathy (yes, n(%))	8 (88.9)	-	-
Neurological manifestations (yes, n(%))[b]	8 (88.9)	-	-
Internal organ dysfunction (yes, n(%))[c]	6 (66.7)	-	-

[a] IOP (in mm Hg) was missing in two eyes of one *TREX1* MC and one eye of one control patient. [b] Neurological manifestations were defined as i) cognitive dysfunction, ii) focal neurological manifestations, iii) MRI lesions typical of RVCL-S. [c] Internal organ dysfunction was defined as organ dysfunction (liver, kidney and thyroid disease and anaemia) requiring treatment. MC, mutation carrier; IOP, intraocular pressure.

Retinal oximetry

No difference in arterial oxygenation was found (table 2, fig 2A). Venular oxygenation was considerably higher in RVCL-S patients than in controls with a median of 71.5% [IQR: 55.0-77.2] and 55.5% [IQR: 51.2-59.9] respectively (p-value < 0.001) (table 2, fig 2B). The AV-difference was found to be lower in RVCL-S patients (median AV-difference [IQR]: 22.6% [18.5-38.3] vs 38.5% [34.1-39.9], p < 0.001) (table 2, fig 2C). In the eyes treated without pan-retinal laser photocoagulation therapy, 3 out of 8 eyes (37.5%) still had a venular saturation of above 70%. Both controls and the patient without retinopathy had no venular saturation values exceeding 65%.

Both arterial and venular vessels were thinner in RVCL-S patients compared to controls. Median [IQR] arterial and venular diameters were 10.4 [9.8-11.2] pixels vs. 12.1 [11.2-12.8] pixels, p < 0.001 and 12.9 [11.8-15.3] pixels vs. 14.9 [14.1-16.8] pixels; p<0.001, respectively (table 2).

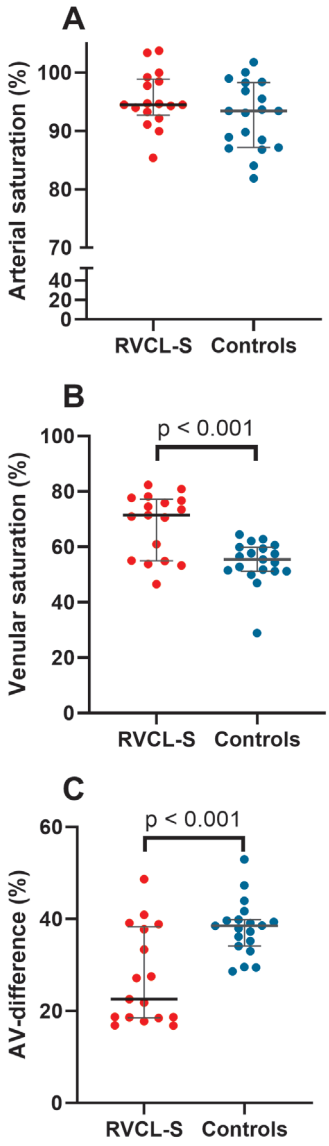

Figure 2. Retinal oxygen saturation in RVCL-S patients and controls.

Arterial oxygen saturation (A), venular oxygen saturation (B) and arteriovenous difference (AV-difference) (C) in RVCL-S patients and controls. AV-difference was calculated using the arterial and venous saturation. Thickened line displays the median and the bars the interquartile range. P-value were calculated using generalized estimating equation (GEE) analyses.

Table 2. Retinal oxygen saturation in RVCL-S patients.

	Median (IQR) RVCL-S patients (%) (n = 17)	Median (IQR) controls (%) (n = 19)	Estimate	CI 95%	P-value
ArtSat (%)	94.5 (92.7-98.9)	93.4 (87.2-98.3)	2.8	–0.7 : 6.2	0.12
VenSat (%)	71.5 (55.0-77.2)	55.5 (51.2-59.9)	14.0	6.6 : 21.5	<0.001
AV-diff (%)	22.6 (18.5-38.3)	38.5 (34.1-39.9)	-11.2	–17.7 : -4.7	<0.001
ArtDiam (pixels)[a]	10.4 (9.8-11.2)	12.1 (11.2-12.88)	-1.5	-2.3 : -0.7	<0.001
VenDiam (pixels)[a]	12.9 (11.8-15.3)	14.9 (14.1-16.8)	-2.2	-3.5 : -1.0	<0.001

[a] One-pixel width corresponds to approx. 9 µm. ArtSat, arterial saturation; VenSat, venous saturation; AV-diff, arteriovenous difference; ArtDiam, arterial diameter; VenDiam, venular diameter; MC, mutation carrier; CI, confidence interval.

Discussion

In this study, we demonstrated that retinal saturation is affected in RVCL-S. There was an increase in venular saturation with a decreased AV-difference, but without large differences in arterial saturation. In addition, retinal vessel attenuation was observed in RVCL-S patients.

There are several possible explanations for the elevated venular saturation in RVCL-S. These mechanisms can be divided in the following categories: 1) inadequate distribution of blood flow and oxygen, 2) increased oxygen supply due to increased total retinal blood flow, 3) decreased oxygen consumption, 4) thickening of capillary walls or 5) a combination of the above mechanisms. Poor distribution (mechanism 1) encompasses both closure of retinal capillaries and widening of other capillaries in the retina. When oxygen rich blood passes through wider shunt capillaries, it flows rapidly to the venous side, reducing the amount of oxygen delivered to the tissue per unit volume of blood decreases, causing an increase in venular oxygen saturation. Interestingly, both occluded capillaries and collateral vessels have been seen on fluorescein angiography in RVCL-S.[8] A decreased vascular reactivity has also been demonstrated in both cerebral vessels and in the forearm in RVCL-S.[9,10] Here, we showed a reduction in arteriolar and venular diameter. Together, this renders increase blood flow (mechanism 2) as an improbable explanation. Additionally, a decrease in oxygen consumption, for instance due to less metabolic activity (mechanism 3), could be considered. Thinning of retinal layers, such as the ganglion cell layer which occurs in RVCL-S,[11] which could lead to less metabolic activity, resulting in an increase in venular saturation. Moreover, mechanisms such as ischemia, inflammation and/or neurodegeneration could contribute to cell death, leading ultimately to less oxygen

consumption. Finally, thickening of capillary vessel walls (mechanism 4), can cause decreased oxygen delivery, as the increase in diffusion distance leads to more oxygen being retained in the blood. In RVCL-S, retinal arteries have been shown to have thickened hyalinized walls on histopathological examination, possibly adding to the increase in venular saturation.[8] The mechanisms considered above may also interact in diverse ways to lead to an elevation in venular saturation.

Elevated venular saturation and decreased AV-difference have previously been found in other vascular retinopathies such as diabetic retinopathy and have shown to be related to disease severity.[12,13] Retinal vasculopathy in RVCL-S shows similarities to diabetic retinopathy regarding clinical signs and treatment. Interestingly, this study indicates that retinal oxygenation is also affected in a similar way. Therefore, it could be valuable to determine whether saturation of the retina in RVCL-S also predicts disease severity in a longitudinal study. Besides retinal vasculopathy, RVCL-S is characterised by cerebral white matter disease and accompanying neurological deficits. As the central nervous system and the retina have a shared embryonic origin, it has been hypothesised that changes in the retina potentially reflect abnormalities in the central nervous system. In other diseases that affect or originate from the central nervous system, including multiple sclerosis and Alzheimer's disease, increased venular saturation and decreased AV-differences have indeed been observed.[4,14] In addition, studies indicate that retinal oximetry might serve as an early biomarker for diagnosing Alzheimer's disease.[4,15] If retinal abnormalities indeed reflect changes in the central nervous system, retinal oximetry might therefore not only serve as a biomarker for retinal vasculopathy, but also for overall disease status and disease onset of RVCL-S.

The decreased retinal vessel calibre found in RVCL-S could be due to decreased metabolic demands of the degenerating retina. Prior research employing quantitative assessments of retinal vessel calibre has indicated that narrower retinal vessels are linked to various subclinical and clinical diseases, extending beyond ocular conditions to disorders involving other organs. For example, narrow retinal vessels are associated with lacunar infarcts,[16] predicting incident stroke,[17] and diabetes.[18] Reduced retinal nerve fibre layer thickness, as seen in RVCL-S,[11] is likewise correlated with narrower retinal vessels.[19]

Our study may be subjected to several limitations. To begin, an investigator discerned arteriolar from venular vessel segments based on anatomical characteristics, which could not be fully performed in a blinded manner. Signs of retinopathy and laser photocoagulation could not be blinded, and therefore eyes of RVCL-S patients could be distinguished from eyes from controls. This could potentially have introduced

bias. Uncertainties were, however, very rare (2.3%) and evaluated by a trained ophthalmologist. Another limitation may be the small sample size. However, as RVCL-S is a rare disease, this study already consists of a relatively large cohort. It should be mentioned that laser photocoagulation was performed in a subset of RVCL-S patients. Particularly pan-retinal laser photocoagulation leads to wide-scale atrophy in the retina, thereby reducing oxygen demand and thus potentially increasing venular saturation and decreasing the AV-difference. In practice, however, previous work in diabetic retinopathy demonstrated that only a slight increase in venular and arterial saturation was measured after pan-retinal laser photocoagulation, while the AV-difference remained unchanged.[20] Moreover, several RVCL-S patients in our study that were not treated with pan-retinal laser photocoagulation still had a venular saturation exceeding 70.0%. Finally, it should be noted that oxygen saturation values rely on calibration, and are therefore not absolute, potentially surpassing 100%. However, our method has been shown to give repeatable results, and to be sensitive to changes in oxygen saturation.[6] Several strengths of this study should also be taken into consideration. This was, to our knowledge, the first study to investigate retinal saturation and vessel calibre in patients with RVCL-S. Another strength of our study is the use of a unique study population of RVCL-S patients, a disease that serves as a monogenic model for common vascular retinopathies and small vessel diseases. Our study also provides insights into the pathophysiology of retinal disease in RVCL-S, as we demonstrated that retinal oxygenation is altered in RVCL-S. Monitoring retinopathy in RVCL-S using parameters that indicate the initial metabolic or ischemic phases of the condition, rather than the subsequent morphological alterations, would be preferable. Thus, a next step would be to evaluate the oxygen saturation status in presymptomatic patients.

To conclude, we demonstrated that there is an increase in venular saturation with a decreased AV-difference in RVCL-S. Additionally, we observed retinal vessel attenuation. Our study, also considering similar findings in other related disorders, sheds light on the possible role of retinal oximetry as an objective, non-invasive biomarker for retinal vasculopathy in RVCL-S. Retinal oximetry is a promising non-invasive tool to monitor progression of retinal vasculopathy in RVCL-S and will likely complement conventional evaluation of the retina in the future.

Author contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Irene de Boer, Wendy Winter and Mays Al-Nofal. The first draft of the manuscript was written by Irene de Boer and Wendy Winter and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Statements and Declaration

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References

1. Richards A, van den Maagdenberg AM, Jen JC, et al. C-terminal truncations in human 3'-5' DNA exonuclease TREX1 cause autosomal dominant retinal vasculopathy with cerebral leukodystrophy. *Nat Genet.* 2007;39:1068-1070.
2. Wilms AE, de Boer I, Terwindt GM. Retinal Vasculopathy with Cerebral Leukoencephalopathy and Systemic manifestations (RVCL-S): An update on basic science and clinical perspectives. *Cereb Circ Cogn Behav.* 2022;3:100046.
3. Guduru A, Martz TG, Waters A, Kshirsagar AV, Garg S. Oxygen Saturation of Retinal Vessels in All Stages of Diabetic Retinopathy and Correlation to Ultra-Wide Field Fluorescein Angiography. *Invest Ophthalmol Vis Sci.* 2016;57:5278-5284.
4. Stefánsson E, Olafsdottir OB, Einarsdottir AB, Eliasdottir TS, Eysteinsson T, Vehmeijer W, et al. Retinal Oximetry Discovers Novel Biomarkers in Retinal and Brain Diseases. *Invest Ophthalmol Vis Sci.* 2017;58:BIO227-BIO233.
5. Palsson O, Geirsdottir A, Hardarson SH, Olafsdottir OB, Kristjansdottir JV, Stefánsson E. Retinal oximetry images must be standardized: a methodological analysis. *Invest Ophthalmol Vis Sci.* 2012;53:1729-1733.
6. Hardarson SH, Harris A, Karlsson RA, et al. Automatic retinal oximetry. *Invest Ophthalmol Vis Sci.* 2006;47:5011-5016.
7. Hardarson S. Protocol for Acquisition and Analysis of Oxymap T1 Oximetry Images. Reykjavik, Iceland: Oxymap. 2013.
8. Stam AH, Kothari PH, Shaikh A, et al. Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations. *Brain.* 2016;139:2909-2922.
9. de Boer I, Stam AH, Buntinx L, et al. RVCL-S and CADASIL display distinct impaired vascular function. *Neurology.* 2018;91:e956-e963.
10. Hoogeveen ES, Pelzer N, de Boer I, van Buchem MA, Terwindt GM, Kruit MC. Neuroimaging Findings in Retinal Vasculopathy with Cerebral Leukoencephalopathy and Systemic Manifestations. *AJNR Am J Neuroradiol.* 2021;42:1604-1609.
11. de Boer I, Steenmeijer SR, Pelzer N, et al. Spectral Domain Optical Coherence Tomography in Retinal Vasculopathy With Cerebral Leukoencephalopathy and Systemic Manifestations: A Monogenic Small Vessel Disease. *J Neuroophthalmol.* 2022;42:e130-e136.
12. Khoobehi B, Firn K, Thompson H, Reinoso M, Beach J. Retinal Arterial and Venous Oxygen Saturation Is Altered in Diabetic Patients. *Invest Ophthalmol Vis Sci.* 2013;54:7103-7106.
13. Jorgensen CM, Hardarson SH, Bek T. The oxygen saturation in retinal vessels from diabetic patients depends on the severity and type of vision-threatening retinopathy. *Acta Ophthalmol.* 2014;92:34-39.
14. Einarsdottir AB, Olafsdottir OB, Hjaltason H, Hardarson SH. Retinal oximetry is affected in multiple sclerosis. *Acta Ophthalmol.* 2018;96:528-530.
15. Olafsdottir OB, Saevarsdottir HS, Hardarson SH, et al. Retinal oxygen metabolism in patients with mild cognitive impairment. *Alzheimers Dement (Amst).* 2018;10:340-345.
16. Lindley RI, Wang JJ, Wong MC, et al. Retinal microvasculature in acute lacunar stroke: a cross-sectional study. *Lancet Neurol.* 2009;8:628-634.
17. Wong TY, Klein R, Couper DJ, et al. Retinal microvascular abnormalities and incident stroke: the Atherosclerosis Risk in Communities Study. *Lancet.* 2001;358:1134-1140.
18. Wong TY, Klein R, Sharrett AR, et al. Retinal arteriolar narrowing and risk of diabetes mellitus in middle-aged persons. *JAMA.* 2002;287:2528-2533.
19. Zheng Y, Cheung N, Aung T, Mitchell P, He M, Wong TY. Relationship of retinal vascular caliber with retinal nerve fiber layer thickness: the singapore malay eye study. *Invest Ophthalmol Vis Sci.* 2009;50:4091-4096.
20. Jorgensen C, Bek T. Increasing oxygen saturation in larger retinal vessels after photocoagulation for diabetic retinopathy. *Invest Ophthalmol Vis Sci.* 2014;55:5365-5369.

Supplementary

$$\text{Mean weighted oxygen saturation} = \frac{Sat_1 * d_1^4 + Sat_2 * d_2^4 + Sat_3 * d_3^4 + Sat_4 * d_4^4}{d_1^4 + d_2^4 + d_3^4 + d_4^4}$$

Figure S1. Calculating the mean arteriolar or venular oxygen saturation by weighing with the fourth power of the vessel diameter.

Sat_x = saturation (in %) of each arteriolar or venular vessel segment; d_x = diameter (in pixels) of each arteriolar or venular vessel segment.

Table S1. Specifications of Oxymap Analyzer software, version 2.2.1

Calibration parameters	
a	–1.28
b	1.24
c	0.01
d	-0.14
Minimum vessel length	50 pixels
Minimum vessel width	8 pixels
Vessel detection algorithm	V2