Monogenic models of migraine : from clinical phenotypes to pathophysiological mechanisms

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Part II:

Retinal Vasculopathy with Cerebral Leukoencephalopathy and Systemic Manifestations – a vascular monogenic migraine model

Chapter 8.

Systemic features of retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S): a monogenic late-onset small vessel disease

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Abstract

Background: Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S) is a small vessel disease caused by C-terminal truncating *TREX1* mutations. The disease is typically characterized by vascular retinopathy and focal and global brain dysfunction. Systemic manifestations have also been reported but not yet systematically investigated.

Methods: In a cross-sectional study, we compared the clinical characteristics of 33 *TREX1* mutation carriers (MC+) from three Dutch RVCL-S families with those of 37 family members without *TREX1* mutation (MC-). All participants were investigated using personal interviews, questionnaires, physical, neurological and neuropsychological examinations, blood and urine tests, and brain MRI.

Results: In MC+, vascular retinopathy and Raynaud's phenomenon were the earliest symptoms presenting from age 20 onwards. Kidney disease became manifest from around age 35, followed by liver disease, anaemia, markers of inflammation and, in some MC+, migraine and subclinical hypothyroidism, all from age 40. Cerebral deficits usually started mildly around age 50, developing white matter and intracerebral mass lesions, and becoming severe around age 60-65.

Conclusions: RVCL-S is a rare, but likely underdiagnosed, systemic small vessel disease typically starting with vascular retinopathy, followed by multiple internal organ disease, progressive brain dysfunction, and ultimately premature death.

Keywords: systemic manifestations , three-prime repair exonuclease 1, small vessel disease, microangiopathies

Introduction

Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S) is an autosomal dominant neurovascular syndrome caused by heterozygous C-terminal frameshift mutations in *TREX1* and ultimately leading to premature death.[1,2] Before the gene was identified, the disease has been known as cerebroretinal vasculopathy (CRV),[3] hereditary vascular retinopathy (HVR),[4] hereditary endotheliopathy, retinopathy, nephropathy and stroke (HERNS),[5] and hereditary systemic angiopathy (HSA).[6] The best known features of RVCL-S are progressive blindness due to vascular retinopathy, neurological manifestations of focal and diffuse brain dysfunction, and white matter and intracerebral mass lesions on neuroimaging.[2] Less well described are systemic manifestations such as liver and kidney disease, anaemia, hypertension, and Raynaud's phenomenon.[2] As these features may precede the characteristic ophthalmological and neurological features of RVCL-S, establishing a correct diagnosis can be challenging.[2] To improve early recognition, diagnosis and treatment of RVCL-S and, in particular, its systemic manifestations, we conducted a cross-sectional study, aimed at better characterizing the extracranial manifestations of RVCL-S. To this end, we carefully investigated 33 symptomatic and asymptomatic RVCL-S *TREX1* mutation carriers (MC+) and 37 non-mutation carriers (MC-) from three Dutch RVCL-S families, using a standardized protocol specifically focussing on systemic signs and symptoms of the disease.

Materials and methods

Participants and genetic screening

We invited all established MC+ and their 1st and 2nd degree family members aged ≥ 18 years from three known (but unrelated) Dutch RVCL-S families, regardless of MC status. Consenting family members with unknown MC status were screened for *TREX1* mutations using genomic DNA extracted from peripheral leucocytes, PCR, and direct Sanger sequencing as described before.[1] As RVCL-S cannot yet be treated, participants could opt to remain unaware of the genetic test results. Family members without a *TREX1* mutation were included as controls. Matching by age and gender was not possible due to the limited number of available family members. The study was approved by the Medical Ethics Committee of LUMC. All participants provided written informed consent prior to inclusion.

Study design

This was a cross-sectional study that, for logistic reasons, consisted of three standardized visits (Figure 1). In visit 1, participants were interviewed to assess disease features and blood was sampled for DNA isolation. Raynaud's phenomenon was diagnosed using the questionnaire by Miller et al.[7]

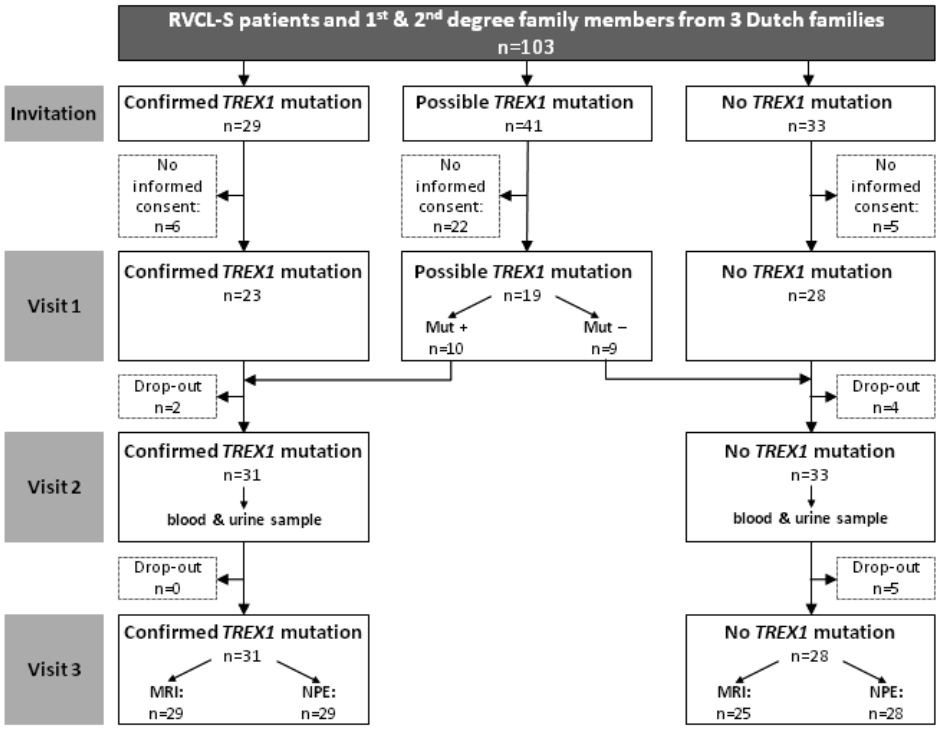

Figure 2: Flowchart depicting inclusion of participants from the three Dutch RVCL-S families into the study. Drop-outs after visit 1 (interview & questionnaires) and visit 2 (sampling and physical examination) are shown. Six participants (including two MC+) dropped out after visit 1. Five family members considered multiple visits too much of a burden and one subject was physically unable to visit the LUMC. For visit 2, the number of physical examinations, urine samples, and vena punctures for blood sampling are depicted. For visit 3, the number of brain MRIs and neuropsychological examinations (NPE) are shown. An additional five participants (all MC-) could not attend visit 3 because of lack of time. Two MC+ could not complete the neuropsychological examination because of poor vision and anarthria. Two other MC+ and three MC- withdrew from or had a relative contra-indication for brain MRI.

(cut-off score >4) and the novel international consensus criteria (except for use of photographs, which were not available).[8] A lifetime migraine diagnosis, including its subtypes, was established according to the International Classification of Headache Disorders (ICHD) criteria,[9] using a validated migraine questionnaire.[10] Lifetime depression was established with a score of ≥8 on the Hospital Anxiety and Depression Scale (HADS-D)[11] and/or a score of ≥16 on the Centre for Epidemiological Studies Depression Scale (CES-D),[12] and anxiety with a HADS-A score of ≥8.[13] Physical and cognitive complaints, medical history, medication use, lifestyle habits and socio-demographic characteristics were assessed during a semi-structured interview.

Most family members with unknown MC status declined to know their diagnosis. Finding evidence of vascular retinopathy in a person from a family with RVCL-S would almost certain confirm the diagnosis. Not disclosing the results of an ophthalmological examination was however not an option as retinopathy can potentially be treated. We therefore decided not to screen for retinopathy in family members with unknown MC status. We did advise them, though, to consult an ophthalmologist as soon as they would get visual complaints. Information on signs of retinopathy in this study thus came solely from reports of treating ophthalmologists of established MC+.

In visit 2, physical examination was performed. Blood pressure was measured on each arm, in sitting position, using the same electronic oscillometric device with a cuff around the upper arm. Hypertension was defined as: i) use of antihypertensive medication; ii) systolic blood pressure >140 mmHg; or iii) diastolic blood pressure >90 mmHg.[14] Height and weight were measured to calculate body mass index (BMI) and a structured neurological examination was performed. To assess general disability, all participants were rated according to the modified Rankin Scale (mRS) and the Barthel index of activities of daily living.[15,16] Blood and urine samples were collected in the morning, after at least eight hours of fasting (median 12 hours, range 8-17) and were transported immediately for laboratory assays according to clinical protocols. Kidney disease was defined as glomerular filtration rates (eGFR) <60 mL/min/1.73m^2 or albumin-creatinine ratio or >3 µg/µmol.

In visit 3, all participants first underwent a neuropsychological examination in the afternoon. Examinations included the Wechsler memory scale-fourth edition (WMS-IV)[17] and the Cambridge Cognitive Examination-Revised (CAMCOG-R). This assesses orientation, language, memory, attention, praxis, calculation, abstraction and perception[18] and includes the Mini-Mental State Examination (MMSE).[19] In the evening, a brain MRI was performed. Brain white matter hyper-intensities were assessed using T1-weighted and FLAIR images, and were automatically identified using a threshold of three standard deviations above mean FLAIR signal intensity.

Statistical analysis

Variables were reported as medians (interquartile range (IQR)) or percentages. For continuous variables without a normal distribution in our population Mann-Whitney U tests were applied. Categorical variables were compared with Pearson Chi Square tests, or Fisher's Exact tests when appropriate, and by calculating relative risks (RR). Corrections for multiple testing were not applied, as analyses were meant to be exploratory and hypothesis-generating. Results with $P < 0.05$ were

considered statistically significant. All statistics were performed using SPSS 23.0 (IBM Corp., Armonk, NY, USA).

Results

Genetic testing and socio-demographic characteristics of the study population

In total 103 members of three Dutch RVCL-S families were invited to participate (Figure 1). Thirty-three (32%) declined to participate, because: i) participation was considered too much of a burden (n=21); ii) they did not want to participate in any study on RVCL-S (n=6); iii) health issues precluding visiting the hospital (n=4: one MC+ due to RVCL-S-related poor vision; three unknown); and iv) living abroad (n=2). Of 11 non-participants the MC status (6 MC+; 5 MC-) and clinical details were known from previous studies. Their clinical status did not appear to be different from participants. The remaining 22 non-participants had a 50% (n=20) or 25% (n=2) *a priori* chance of carrying a *TREX1* mutation. Their clinical status was unknown, but most (17/22=77%) were younger than 40 years of age. Overall, non-participants were younger than participants (median age 34 versus 46 years; P = 0.02) and more often male (25/33=76% versus 30/70=43%; P = 0.002).

In total 37 MC- and 33 MC+ (23 previously known, 10 newly detected) participated in the study. MC+ and MC- did not differ with respect to age, gender, education level, cigarette pack years, or alcohol use, but MC+ used less caffeine than MC- (Table 1). With respect to medication use, statins were only used by MC+ and MC+ used more antihypertensive drugs. Seven MC+ (median age 57; range 52-65 years) (but none of MC-) were unfit to work, in particular because they were too slow in performing complex tasks. This impairment was unrelated to visual impairment. A summary of all symptoms of RVCL-S is provided in Figure 2.

Association with vascular retinopathy

All known 23 MC+ had signs of vascular retinopathy, of which 15 (65%; median age 57 years) had undergone (pan)retinal laser photocoagulation. In six (median age 41 years), retinopathy was mild not yet requiring treatment. Thirteen experienced visual field defects (12 after retinal laser photocoagulation). In order to remain unaware of their mutation status, 10 MC+ (median age 24 years), who were identified during the study, were not investigated by an ophthalmologist. They did not have visual complaints.

Table 4: Demographics of RVCL-S family members with (MC+) and without (MC-) a *TREX1* mutation.

	MC+ (n=33)	MC- (n=37)	*P*-value
Age (years)			
Median (IQR)	51.9 (28.6-56.0)	45.2 (38.8-61.9)	ns
≥40 years, n (%)	20 (61%)	28 (76%)	ns
Sex			
Male, n (%)	14 (42%)	16 (43%)	ns
Males ≥40 years, n (%)	6 (30%)	13 (46%)	ns
Pedigree and *TREX1* mutation			
A: p.Val235fs, n (%)	21 (64%)	33 (89%)	
B: p.Val235fs, n (%)	7 (21%)	1 (3%)	
C: p.Leu287fs, n (%)	5 (15%)	3 (8%)	
Smoking			
Present, n (%)	5 (15%)	2 (5%)	ns
Past, n (%)	11 (33%)	12 (32%)	
Never, n (%)	17 (52%)	23 (62%)	
Median pack yrs (IQR)	0 (0-2)	0 (0-6)	ns
Alcohol use			
Median (IQR) (u/wk)	2 (0-9)	3 (0-5)	ns
Caffeine use			
Median (IQR) (u/day)	5 (3-7)	7 (4-9)	***P* = 0.02**
Medication use			
Migraine prophylactics, n (%)	1 (3%)	0 (0%)	-
Antihypertensive drugs, n (%)	8 (24%)	4 (11%)	ns
Statins, n (%)	4 (12%)	0 (0%)	-
Thrombocyte aggregation inhibitors, n (%)	5 (15%)	3 (8%)	ns
Anticoagulants, n (%)	0 (0%)	1 (3%)	-
Antidepressants, n (%)	3 (9%)	1 (3%)	ns
Education level			
Median (IQR)	4 (3-5)	4 (3-6)	ns
1. Primary school, n (%)	0 (0%)	1 (3%)	
2. Secondary 1 (low-level), n (%)	4 (12%)	8 (22%)	
3. Secondary 2 (medium-level), n (%)	5 (15%)	2 (5%)	
4. Secondary 3 (high-level), n (%)	3 (9%)	1 (3%)	
5. Tertiary 1 (low-level), n (%)	14 (42%)	10 (27%)	
6. Tertiary 2 (medium-level), n (%)	5 (15%)	15 (41%)	
7. Tertiary 3 (university), n (%)	2 (6%)	0 (0%)	

IQR=interquartile range; u=units; ns=not statistically significant.

Association with internal organ disease

Internal organ disease was investigated at three levels: i) subjective complaints (Table 2; 33 MC+ and 37 MC-); ii) objective signs at physical examination (Table 3; 32 MC+ and 32 MC-); and iii) laboratory tests (Figure 3 and Table 4; 31 MC+ and 33 MC-).

Kidney disease was found in 36% of MC+ (Table 2). Cystitis, pyelonephritis, nephrolithiasis, and haematuria occurred equally frequent in MC+ and MC-. Urine albumin concentrations (median (IQR) 30 (10-96) mg/mL) and microalbumin/creatinine ratios (median (IQR) 6.1 (1.5-13.4) µg/µmol) were increased in MC+ (Table 4). Albuminuria was not explained by concurrent hypertension (Figure 1S). Haematuria was not routinely assessed, but absent in 24-hour urine of nine MC+.

Table 2: Complaints or self-reported diagnoses in members with (MC+) and without (MC-) a *TREX1* mutation of RVCL-S families

	MC+ (n=33)	MC- (n=37)	Statistical analysis χ^2	Statistical analysis Risk Ratio (95% CI)
Kidneys and urinary tract symptoms				
Kidney disease, n (%)	12 (36%)	1 (3%)	***P* < 0.001**	13.5 (1.8-98.0)
Frequent cystitis, n (%)	4 (12%)	6 (16%)	Ns	
Pyelonephritis, n (%)	2 (6%)	2 (5%)	Ns	
Kidney stones, n (%)	3 (9%)	4 (11%)	Ns	
Haematuria, n (%)	3 (9%)	4 (11%)	Ns	
Urine urge incontinence, n (%)	5 (16%)	4 (11%)	Ns	
Urine stress incontinence, n (%)	1 (3%)	5 (14%)	Ns	
Hepatic and gastrointestinal symptoms				
Liver disease, n (%)	9 (27%)	2 (5%)	***P* = 0.01**	5.0 (1.2-21.7)
Diarrhoea, n (%)	6 (18%)	2 (5%)	Ns	
Constipation, n (%)	12 (35%)	15 (41%)	Ns	
Faecal blood, n (%)	5 (15%)	4 (11%)	Ns	
Cholelithiasis, n (%)	3 (9%)	2 (5%)	Ns	
Jaundice, n (%)	5 (15%)	1 (3%)	Ns	
Cardiovascular symptoms				
Orthostatic hypotension, n (%)	15 (47%)	22 (60%)	Ns	
Fatigue, n (%)	15 (46%)	10 (27%)	Ns	
Anaemia, n (%)	11 (33%)	7 (19%)	Ns	
Chest pains with exercise, n (%)	2 (6%)	3 (8%)	Ns	
Exercise-induced dyspnoea, n (%)	9 (28%)	4 (11%)	Ns	
Palpitations, n (%)	16 (49%)	13 (35%)	Ns	
Swollen ankles, n (%)	16 (49%)	4 (11%)	***P* < 0.001**	4.5 (1.7-12.1)
Varicose veins, n (%)	11 (33%)	11 (30%)	Ns	
Intermittent claudication, n (%)	3 (9%)	1 (3%)	Ns	
Nocturia, n (%)	8 (24%)	6 (16%)	Ns	
Musculoskeletal symptoms				
Joint pains, n (%)	8 (24%)	12 (32%)	Ns	
Joint inflammation, n (%)	0 (0%)	3 (8%)	Ns	
Raynaud's phenomenon [a], n (%)	14 (42%)	7 (19%)	***P* = 0.03**	2.2 (1.0-4.9)
Migraine [b], n (%)	9 (27%)	14 (38%)	Ns	

ns=not statistically significant; [a]According to the international consensus criteria from 2014 [8]; [b]According to the International Classification of Headache Disorders (ICHD), third edition [9].

Liver disease, usually asymptomatic, was found in 27% of MC+ (Table 2). Five MC+ had a history of jaundice, four at a young age with jaundice simultaneously in relatives in three (i.e. highly suggestive of a shared infectious cause), and one in association with cholelithiasis. Gamma-glutamyl transferase (γ-GT), alkaline phosphatase (ALP) and aspartate aminotransferase (ASAT) were increased in MC+ (Table 4). Most MC+ with elevated liver enzymes share a same pattern of ALP and γGT 2-3x ULN (upper limit of normal) and normal to minimally elevated aminotransferase (1-1,5x ULN) and normal bilirubin levels. None had primary liver disease. One MC+ had cryptogenic liver cirrhosis (all other causes of cirrhosis were excluded) and one had hepatic steatosis. Previous liver ultrasound examination did not show abnormalities in six other MC+ with increased γ-GT levels.

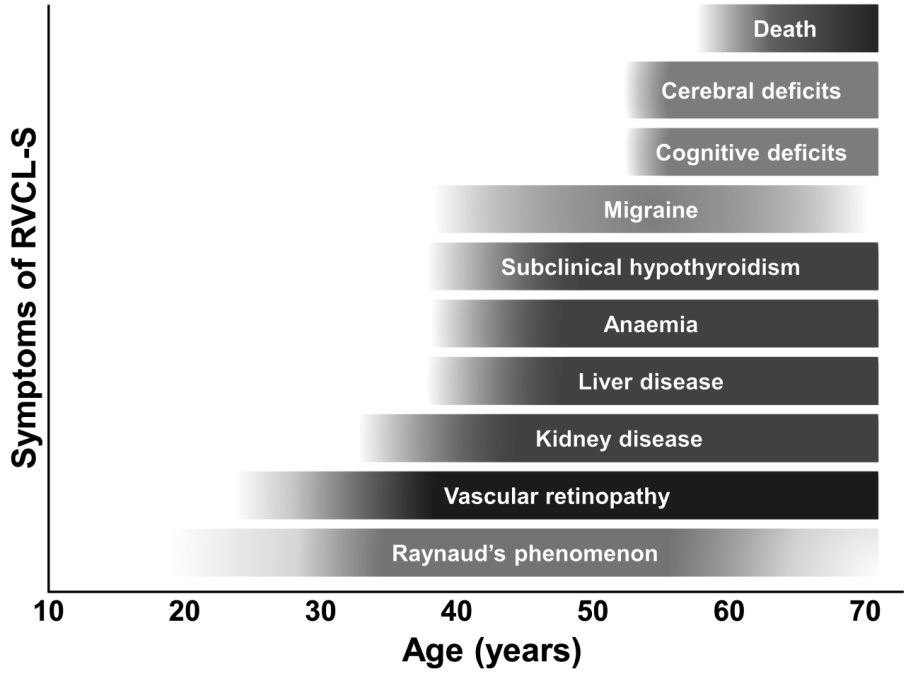

Figure 2: Clinical course of RVCL-S derived from the cross-sectional investigation of RVCL-S patients (aged 18-65 years). Vascular retinopathy and Raynaud's phenomenon were the earliest symptoms presenting from age 20 onwards. Kidney disease became manifest from around age 35, followed by liver disease, anaemia, and, in some MC+, migraine and subclinical hypothyroidism, all from age 40. Cerebral and cognitive deficits usually started mildly around age 50, developing white matter and intracerebral mass lesions, and becoming severe and ultimately lethal, around age 60-65.

A history of **anaemia** was frequently reported by MC+ (33%), but also by MC- (19%; *P* = 0.17) (Table 2). Laboratory results clearly showed lower haemoglobin and haematocrit levels and slightly higher mean corpuscular volumes (MCV) in MC+ (Table 4). One MC+ used iron supplements, another darbepoetin (for severe kidney dysfunction), and one MC+ just had finished treatment with iron supplements. Colonoscopy results were available for four MC+; all had signs of intestinal angiodysplasia as a possible source of **(gastro)intestinal bleeding**. One patient underwent video capsule endoscopy, revealing numerous dot-sized bleeding foci throughout the small intestine. Of the nine subjects who reported incidental faecal blood loss, one MC+ was anaemic (haemoglobin 6.2), but she had noticed (bright red) blood loss only once.

Of the assessed **(cardio)vascular** complaints only swollen ankles were more prevalent in MC+ (Table 2). On physical examination there were no differences in presence of varicose veins, ankle oedema or

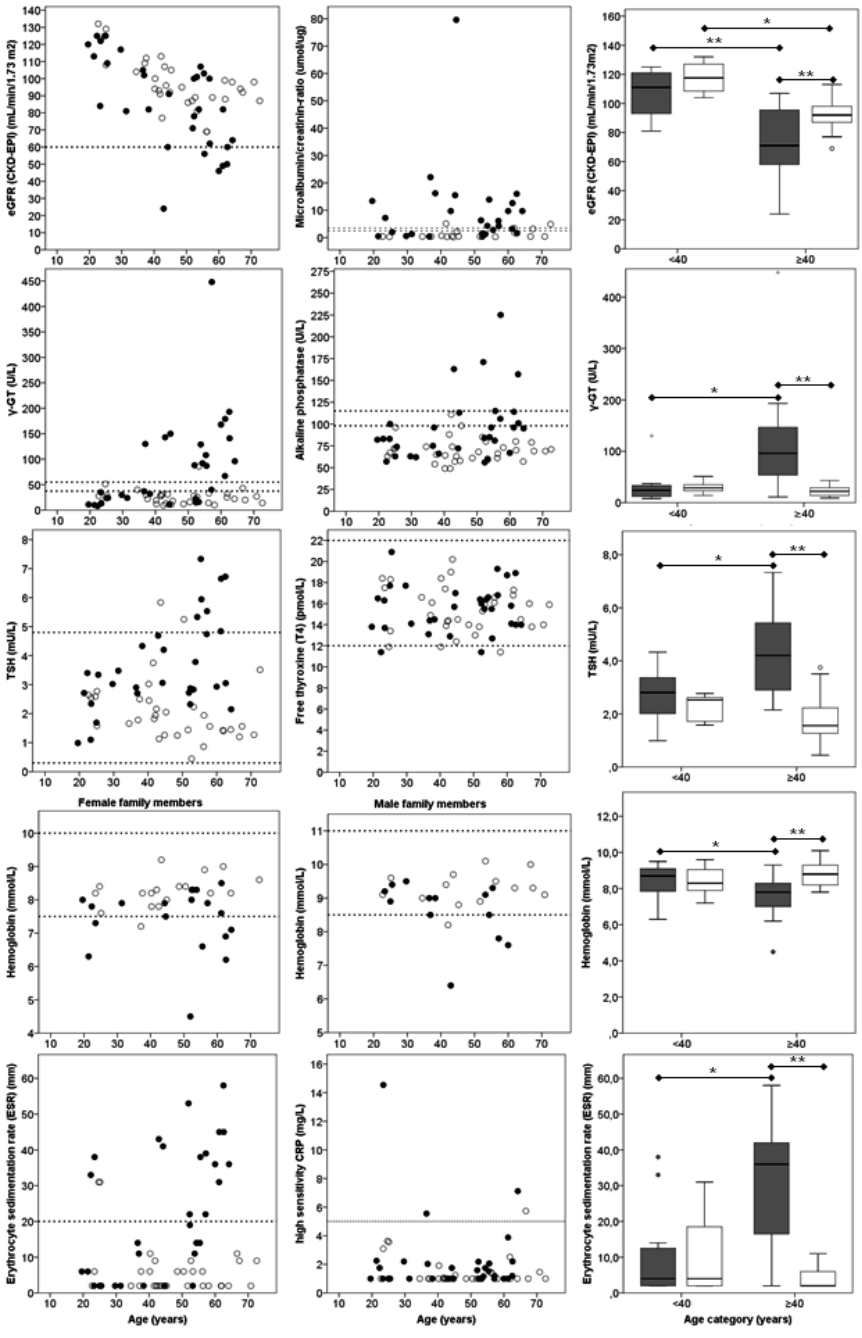

Figure 3: Laboratory parameters in blood. Parameters were investigated to assess kidney function (estimated glomerular filtration rate (eGFR) and microalbumin-creatinin-ratio), liver function (gamma-glutamyl transpeptidase (γ-GT) and alkaline phosphatase), thyroid function (thyroid stimulating hormone (TSH) and free thyroxine (T4)), blood count (haemoglobin), and inflammation (erythrocyte-sedimentation rate (ESR) and high sensitivity c-reactive protein (CRP)). One blood glucose assay and one homocysteine assay failed, both in MC+. Symbols: *Black circles and grey boxes*: MC+; *white circles and boxes*: MC-; *box plot whiskers*: medians, lower and higher quartiles, lower and higher extreme values; **P* < 0.05; ***P* < 0.001.

livedo reticularis (Table 3). Prevalence of hypertension was high in both MC+ (39%) and MC- (35%). Median systolic and diastolic blood pressures measured in visit 2 (with standardized conditions) were virtually the same in MC+ and MC- (Table 3). Only the eldest MC+ reported coronary artery disease. There were no reports of (hypertensive) cardiomyopathy in MC+, which was reported previously in RVCL-S.[6,20,21]

A novel finding in RVCL-S were increased thyroid stimulating hormone (TSH) levels with normal free thyroxine (fT4) levels, indicating **subclinical hypothyroidism** (Table 4). One MC+ had been treated for presumed Graves' disease, but had normal TSH and fT4 levels while using levothyroxine.

There were no signs of rheumatic disease (e.g. joint pains or inflammation), autonomic dysfunction (e.g. orthostatic hypotension, urine incontinence or erectile dysfunction), or avascular necrosis of the femur head.[6] With regard to common vascular risk factors: median BMI (P = 0.31) and cholesterol levels (P = 0.91) did not differ between MC+ and MC- (Table 3), with only four MC+ taking statins. Fasting glucose and HbA1c values were normal in all MC+, ruling out diabetes mellitus. Several markers of inflammation and coagulation (erythrocyte sedimentation rates (ESR), fibrinogen, D-dimer concentrations, and prothrombin time (PT)) were increased in MC+, other coagulation markers were not abnormal in MC+.

Correlating internal organ disease with age

By plotting laboratory parameters against age we constructed a pseudo-longitudinal course of disease (Figure 2 and 3; Table 4). Kidney disease (increased urine albumin and microalbumin-creatinine ratios) were abnormal already before age 40, while all other internal organ disease developed from age 40 onwards (Figure 2 and 3).

Association with Raynaud's phenomenon

The prevalence of Raynaud's phenomenon was increased among MC+ according to Miller's criteria (RR (95% CI)=1.90 (1.15-3.13)) and recent international consensus criteria (RR (95% CI)=2.24 (1.03-4.88)) (Table 2).[7,8] Onset was mostly before age 20 (10/20=50% MC+ and 7/12=58% MC- according to Miller's criteria; 7/14=50% MC+ and 5/7=71% MC- according to the international consensus criteria). Symptoms were mostly mild and there were no ischemic injuries.

Table 3: Results of general disability rating scales and physical examinations in RVCL-S family members with (MC+) and without (MC-) a *TREX1* mutation.

	MC+	MC-	*P*-value
Modified Rankin Scale (mRS), n (%)	(n=32)	(n=32)	***P* = 0.002**
0	11 (34%)	27 (82%)	
1	10 (31%)	5 (15%)	
2	8 (25%)	1 (3%)	
3	0 (0%)	0 (0%)	
4	2 (6%)	0 (0%)	
5	1 (3%)	0 (0%)	
Barthel index, n (%)			ns
20	24 (75%)	30 (91%)	
19	5 (16%)	3 (9%)	
4	1 (3%)*	0 (0%)	
3	1 (3%)*	0 (0%)	
2	1 (3%)	0 (0%)	
Body mass index, measured	(n=29)	(n=33)	
Median (IQR) (kg/m^2)	22.8 (21.5-26.3)	24.9 (21.4-26.9)	ns
Hypertension	(n=29)	(n=33)	
Present, n (%)	13 (39%)	13 (35%)	ns
Antihypertensive drugs, n (%)	8 (24%)	4 (11%)	
Median systolic (IQR)/ diastolic (IQR) blood pressure (mmHg), visit 1	132 (120-154)/ 85 (74-98)	128 (121-146)/ 84 (80-94)	
Median systolic (IQR)/ diastolic (IQR) blood pressure (mmHg), visit 2	129 (111-144)/ 84 (72-94)	128 (117-139)/ 86 (81-95)	
Varicose veins	(n=29)	(n=33)	
Present , n (%)	23 (72%)	23 (70%)	ns
Mild, n	14	18	ns
Moderate, n	5	0	
Severe (corona phlebectatica), n	4	5	
Ankle oedema	(n=29)	(n=33)	
Present, n (%)	6 (19%)	3 (9%)	ns
Livedo reticularis	(n=29)	(n=33)	
Present, n (%)	7 (22%)	8 (24%)	ns

IQR=interquartile range; ns=not statistically significant; *Two subjects were also diagnosed with Multiple System Atrophy, unrelated to RVCL-S.

Neurological and cognitive features

MC+ more often had subjective memory loss and focal neurological deficits. Notable neurological deficits were only found in MC+ older than 50 years.

Lifetime prevalence of migraine was similar in MC+ (27%) and MC- (38%); *P* = 0.35; RR (95% CI)=0.72 (0.36-1.42)). Aura symptoms were reported by 6/9 (67%) of MC+ and 12/14 (86%) of MC- with migraine. There was a trend for migraine starting at a later age in MC+ (median (IQR)=40 (15-43) years) versus MC- (17 (8-20) years; *P* = 0.07), which reached significance for migraine with aura (median (IQR)=40 (30-47) years in MC+ versus MC- (median (IQR)=20 (19-22) years; *P* = 0.01).

Table 4: Parameters in blood and urine in RVCL-S family members with (MC+) and without (MC-) a *TREX1* mutation.

		MC+ total[1] (n=31)	MC+ <40 yrs[2] (n=12)	MC+ ≥40 yrs[3] (n=19)	MC- total[1] (n=33)	MC- <40 yrs[2] (n=8)	MC- ≥40 yrs[3] (n=25)	Statistical comparisons		
								Total	<40 yrs	≥40 yrs
		Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	*P*-value[1]	*P*-value[2]	*P*-value[3]
Blood count	Hb (mmol/L)	7.9 (7.3-8.9)	8.7 (7.8-9.2)	7.8 (6.9-8.3)	8.6 (8.2-9.3)	8.3 (7.8-9.1)	8.8 (8.2-9.3)	***P* = 0.004**	ns	***P* < 0.001**
	Ht (L/L)	0.39 (0.37-0.43)	0.42 (0.38-0.45)	0.39 (0.35-0.42)	0.41 (0.40-0.45)	0.41 (0.38-0.44)	0.42 (0.40-0.45)	***P* = 0.02**	ns	***P* = 0.003**
	MCV (fL)	91 (89-94)	90 (88-92)	93 (90-95)	90 (87-92)	90 (87-91)	90 (88-92)	***P* = 0.04**	ns	***P* = 0.008**
	Thrombocytes (x10^9/L)	212 (181-241)	204 (166-241)	215 (181-242)	223 (202-272)	220 (194-316)	226 (202-255)	ns	ns	ns
	Leukocytes (x10^9/L)	5.0 (4.4-5.8)	5.6 (4.5-7.7)	4.9 (4.2-5.6)	5.3 (4.3-6.3)	5.9 (4.2-6.6)	5.2 (4.2-6.0)	ns	ns	ns
	Eosinophils	0.14 (0.09-0.24)	0.14 (0.05-0.29)	0.14 (0.10-0.24)	0.09 (0.06-0.14)	0.08 (0.05-0.14)	0.11 (0.06-0.15)	***P* = 0.008**	ns	***P* = 0.02**
	Basophils	0.02 (0.01-0.03)	0.02 (0.01-0.03)	0.02 (0.01-0.03)	0.02 (0.01-0.02)	0.01 (0.01-0.02)	0.02 (0.01-0.02)	ns	ns	ns
	Neutrophils	2.77 (2.30-3.63)	2.82 (2.29-4.24)	2.77 (2.49-3.52)	2.89 (2.25-3.77)	3.39 (2.12-4.53)	2.83 (2.25-3.75)	ns	ns	ns
	Lymphocytes	1.44 (1.11-1.70)	1.68 (1.35-2.54)	1.25 (0.90-1.65)	1.60 (1.33-1.93)	1.84 (1.45-2.04)	1.48 (1.32-1.87)	ns	ns	***P* = 0.01**
	Monocytes	0.42 (0.34-0.49)	0.45 (0.34-0.68)	0.42 (0.33-0.45)	0.38 (0.31-0.45)	0.38 (0.32-0.50)	0.38 (0.28-0.45)	ns	ns	ns
Kidney function	eGFR (mL/min/1.73m^2)	84 (62-107)	111 (89-122)	71 (56-100)	95 (89-108)	118 (108-128)	92 (87-98)	ns	ns	***P* = 0.01**
	albumin (urine) (mg/L)	30 (10-96)	13 (5-41)	43 (14-106)	4 (3-7)	4 (3-6)	4 (3-8)	***P* < 0.001**	***P* = 0.02**	***P* < 0.001**
	ACR (urine) (µg/µmol)	6.1 (1.5-13.4)	2.0 (0.6-14.8)	6.2 (2.5-12.9)	0.4 (0.4-2.2)	0.4 (0.3-0.4)	0.6 (0.4-3.2)	***P* < 0.001**	***P* = 0.006**	***P* < 0.001**
Liver function	Gamma-GT (U/L)	40 (22-130)	24 (12-34)	96 (40-150)	25 (14-31)	29 (23-38)	22 (14-30)	***P* = 0.004**	ns	***P* < 0.001**
	ALP (U/L)	84 (67-106)	75 (63-83)	96 (81-115)	69 (61-77)	70 (64-74)	69 (60-80)	***P* = 0.001**	ns	***P* < 0.001**
	ASAT (U/L)	26 (22-32)	25 (20-29)	28 (22-32)	22 (19-26)	20 (19-26)	22 (19-26)	***P* = 0.002**	ns	***P* = 0.004**
	ALAT (U/L)	20 (17-30)	20 (14-37)	21 (18-28)	20 (17-26)	18 (16-23)	22 (16-28)	ns	ns	ns
	Bilirubine (µmol/L)	7 (6-11)	8 (5-20)	7 (6-10)	8 (7-10)	9 (4-16)	8 (8-10)	ns	ns	ns
Thyroid gland function	TSH (mU/L)	3.1 (2.7-4.7)	2.8 (1.9-3.4)	4.2 (2.9-5.5)	1.8 (1.4-2.6)	2.5 (1.7-2.6)	1.6 (1.3-2.3)	***P* < 0.001**	ns	***P* < 0.001**
	fT4 (pmol/L)	16 (14-17)	14 (14-17)	16 (14-17)	15 (14-17)	16 (14-18)	15 (14-17)	ns	ns	ns
Cholesterol markers	Cholesterol (mmol/L)	5.3 (4.4-6.5)	4.5 (4.0-4.8)	6.0 (5.2-6.6)	5.3 (4.7-6.0)	4.8 (4.2-4.9)	5.5 (5.0-6.1)	ns	ns	ns
	LDL (mmol/L)	2.8 (2.2-3.9)	2.4 (2.1-2.6)	3.7 (2.8-4.3)	3.3 (2.5-3.7)	2.6 (2.1-3.1)	3.4 (3.0-4.0)	ns	ns	ns
	HDL (mmol/L)	1.7 (1.5-2.1)	1.7 (1.5-1.8)	1.9 (1.6-2.2)	1.6 (1.3-2.0)	1.6 (1.3-2.2)	1.7 (1.3-1.9)	ns	ns	ns
	HDL/LDL ratio	2.8 (2.5-3.3)	2.6 (2.4-3.0)	3.0 (2.6-3.8)	3.3 (2.7-3.9)	3.0 (2.0-3.3)	3.4 (2.8-4.1)	ns	ns	ns
	Triglycerides (mmol/L)	0.9 (0.6-1.0)	0.8 (0.7-1.0)	0.9 (0.6-1.1)	1.0 (0.7-1.3)	0.6 (0.5-1.0)	1.1 (0.8-1.4)	ns	ns	ns
Inflammation and coagulation	ESR (mm)	19 (2-38)	4 (2-13)	36 (14-43)	2.0 (2.0-7.5)	4 (2-25)	2 (2-8)	***P* < 0.001**	ns	***P* < 0.001**
	hsCRP (mg/L)	1.2 (1.0-2.2)	1.4 (1.0-2.2)	1.2 (1.0-2.1)	1.0 (1.0-1.4)	1.0 (1.0-3.4)	1.0 (1.0-1.3)	ns	ns	***P* = 0.03**
	Fibrinogen (g/L)	3.6 (2.9-4.1)	3.0 (2.7-3.5)	4.0 (3.6-4.1)	3.0 (2.6-3.4)	3.1 (2.6-3.9)	3.0 (2.6-3.4)	***P* = 0.001**	ns	***P* < 0.001**
	D-dimer (ng/mL)	438 (286-573)	271 (226-414)	483 (426-600)	283 (220-354)	301 (220-394)	276 (220-344)	***P* < 0.001**	ns	***P* < 0.001**
	Homocysteine (µmol/L)	11.8 (7.8-16.4)	7.7 (6.3-9.6)	13.9 (11.3-17.5)	8.7 (7.4-12.7)	7.5 (6.0-15.0)	9.6 (7.8-12.7)	ns	ns	***P* = 0.002**
	PT (s)	14.3 (13.6-14.8)	14.5 (14.0-15.0)	14.1 (13.5-14.8)	13.9 (13.4-14.3)	13.8(13.3-14.9)	13.9 (13.4-14.3)	***P* = 0.04**	ns	ns
	APTT (s)	29.0 (28.0-30.6)	29.0 (28.0-30.3)	29.0 (27.4-30.8)	29.1 (27.6-30.7)	30.0 (28.2-30.9)	29.0 (27.2-30.2)	ns	ns	ns

Statistical comparisons (Mann-Whitney U test) are shown between [1]MC+ and MC- total groups; [2]MC+ and MC- aged <40 years; [3]MC+ and MC- aged ≥40 years. Yrs=years; IQR=interquartile range; ns=not statistically significant; Hb=haemoglobin; Ht=haematocrit; MCV=mean corpuscular volume; eGFR=estimated glomerular filtration rate (CKD-EPI formula); ACR= albumin-creatinine ratio; gamma-GT=gamma-glutamyl transferase; ALP=alkaline phosphatase; ASAT=aspartate aminotransferase; ALAT=alanine aminotransferase; TSH=thyroid stimulating hormone; fT4=free thyroxine 4; LDL=low density lipoprotein; HDL=high density lipoprotein; ESR=erythrocyte sedimentation rate; hsCRP=high sensitivity C-reactive protein; PT=prothrombin time; APTT=activated partial thromboplastin time.

Table 5: Results of brain magnetic resonance (MR) imaging and neuropsychological examination.

	MC+ total[1)]	MC+ <40 yrs[2)]	MC+ ≥40 yrs[3)]	MC- total[1)]	MC- <40 yrs[2)]	MC- ≥40 yrs[3)]	Statistical comparisons		
							Total	<40 yrs	≥40 yrs
	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	*P*-value[1)]	*P*-value[2)]	*P*-value[3)]
Brain MR imaging									
	(n=29)	(n=11)	(n=18)	(n=25)	(n=6)	(n=19)			
WMH volume†, mL	0.97 (0.23-3.93)	0.19 (0.08-0.34)	2.66 (0.99-7.67)	0.48 (0.27-1.52)	0.34 (0.24-0.41)	0.82 (0.27-2.20)	ns	Ns	***P* = 0.004**
				Neuropsychological examination					
	(n=30)	(n=12)	(n=18)	(n=28)	(n=7)	(n=21)			
MMSE	29.0 (29.0-30.0)	29.5 (29.0-30.0)	29.0 (28.0-30.0)	30.0 (29.0-30.0)	29.0 (29.0-30.0)	30.0 (29.0-30.0)	ns	ns	ns
	(n=29)	(n=12)	(n=17)	(n=28)	(n=7)	(n=21)			
CAMCOG-R	93.0 (89.0-96.0)	95.0 (89.0-96.8)	93.0 (88.0-96.0)	93.5 (89.0-97.8)	89.0 (85.0-94.0)	95.0 (90.0-98.5)	ns	ns	ns
	(n=29)	(n=12)	(n=17)	(n=28)	(n=7)	(n=21)			
WMS	116.0 (106.0-137.5)	115.0 (106.0-128.3)	118.0 (106.5-141.5)	122.0 (109.5-138.8)	108.0 (96.0-122.0)	126.0 (116.0-140.0)	ns	ns	ns
	(n=31)	(n=12)	(n=19)	(n=30)	(n=8)	(n=22)			
HADS-D ≥8 and/or CES-D ≥16, n (%)	7 (21%)	1 (8%)	6 (30%)	2 (5%)	1 (11%)	1 (4%)	ns	ns	***P* = 0.04**
HADS-A ≥8, n (%)	7 (21%)	2 (15%)	5 (25%)	3 (8%)	1 (11%)	2 (7%)	ns	ns	ns

Statistical comparisons are shown between [1)]MC+ and MC- total groups; [2)]MC+ and MC- aged <40 years; [3)]MC+ and MC- aged ≥40 years. Yrs=years; IQR= interquartile range; ns=not statistically significant; † Brain white matter hyperintensities (WMH) volume: assessed using tools of the FSL software package (Functional MRI of the Brain [FMRIB] Software Library, www.fmrib.ox.ac.uk/fsl) [35]. T1-weighted (TR=9.8 msec, TE=4.6 msec, flip angle of 8°, voxelsize of 0.86 x 0.85 mm, 130 slices with a thickness of 1.2 mm) and FLAIR (TR=4.8 sec, TE=291 msec, flip angle of 90°, voxel size of 1 x 1 mm, 310 slices with a thickness of 1.1 mm) images were brain extracted using the brain extraction tool and co-registered using FLIRT (FMRIB's linear image registration tool). FLAIR images were registered to the MNI152 standard space using FLIRT. White matter hyperintensities in a conservative MNI white matter mask were automatically identified using a threshold of three standard deviations above mean FLAIR signal intensity. MMSE: Mini-Mental State Examination (maximum score 30 points); CAMCOG-R: Cambridge Cognitive Examination-Revised (maximum score 107 points); WMS: Wechsler Memory Scale (fourth edition); HADS-A/D: Hospital Anxiety and Depression Scale- Anxiety/Depression; CES-D: Centre for Epidemiological Studies Depression Scale.

Except for a higher prevalence of depressive symptoms in MC+ ≥40 years, neuropsychological tests did not reveal clear differences between MC+ and MC- (Table 5). We found no major cognitive impairment in MC+ (median (IQR) CAMCOG-R score of 93.0 (89.0-96.0)) in MC+ versus 93.5 (9.0-97.8) in MC-). Only the eldest MC+ fulfilled criteria for dementia.

The volume of white matter hyperintensities on brain MRI was increased in MC+ ≥ 40 years (Table 5). While several MC+ had suffered from intracerebral mass lesions in the past, new 'pseudotumours' were not detected.

Table 6: Current diagnostic criteria for RVCL-S.[2]

Major Diagnostic Criteria
1. Vascular retinopathy (which in the early phases is associated with retinal hemorrhages, intraretinal microvascular abnormalities, and/or cotton wool spots)
2. Features of focal and/or global brain dysfunction associated on MRI with (i) punctate T2 hyperintense white matter lesions with nodular enhancement; and/or (ii) larger T2 hyperintense white matter mass lesions with rim-enhancement, mass effect, and surrounding edema
3. Family history of autosomal dominant inheritance with middle-age onset of disease manifestations [a)]
4. Demonstration of a C-terminal frameshift mutation in *TREX1* to genetically confirm the diagnosis
Supportive features
1. On CT focal white matter calcifications and/or on MRI non-enhancing punctate T2 hyperintense white matter lesions at an age that non-specific age-related white matter hyperintensities are infrequent
2. Microvascular liver disease (nodular regenerative hyperplasia)
3. Microvascular kidney disease (arterio- or arteriolonephrosclerosis, glomerulosclerosis)
Possibly associated features
1. Anemia consistent with blood loss and/or chronic disease
2. Microscopic gastrointestinal bleeding
3. Hypertension
4. Migraine with or without aura
5. Raynaud's phenomenon (typically mild)

[a)] *De novo* mutations may be possible although none have been reported to date.

Table 7: Proposed new diagnostic criteria for RVCL-S.

Major Diagnostic Criteria
1. Vascular retinopathy (which in the early phases is associated with retinal hemorrhages, intraretinal microvascular abnormalities, and/or cotton wool spots)
2. Features of focal and/or global brain dysfunction associated on MRI with (i) punctate T2 hyperintense white matter lesions with nodular enhancement; and/or (ii) larger T2 hyperintense white matter mass lesions with rim-enhancement, mass effect, and surrounding edema
3. Family history of autosomal dominant inheritance with middle-age onset of disease manifestations [a)]
4. Demonstration of a C-terminal frameshift mutation in *TREX1* to genetically confirm the diagnosis
Supportive features
1. On CT focal white matter calcifications and/or on MRI non-enhancing punctate T2 hyperintense white matter lesions at an age that non-specific age-related white matter hyperintensities are infrequent
2. Microvascular liver disease (nodular regenerative hyperplasia)
3. Microvascular kidney disease (arterio- or arteriolonephrosclerosis, glomerulosclerosis)
4. Anemia consistent with blood loss and/or chronic disease*
5. Microscopic gastrointestinal bleeding*
6. Subclinical hypothyroidism[#]
Possibly associated features
1. Raynaud's phenomenon (typically mild)
2. Migraine with or without aura (typically relatively late onset)
3. Hypertension

[a)] *De novo* mutations may be possible although none have been reported to date.

* Moved from possibly associated to supportive features. [#] New supportive criterion

Discussion

We conducted an extensive and detailed cross-sectional study into the clinical course and ophthalmological, cerebral, and notably systemic manifestations of RVCL-S in 33 MC+ and 37 MC- from three Dutch RVCL-S families. Typically, vascular retinopathy and Raynaud's phenomenon presented from age 20 onwards, followed by kidney disease from age 35, and liver disease, anaemia likely due to gastrointestinal bleeding, migraine, and subclinical hypothyroidism, from age 40. The characteristic clinical and neuroimaging manifestations of global and focal brain dysfunction started mildly around age 50, to progressively worsen and becoming fatal over the next 10-15 years. We suggest to add anaemia, gastrointestinal bleeding, and subclinical hypothyroidism as supportive diagnostic criteria for RVCL-S (Tables 6 and 7).

The sample size of our study, although considerable in view of the low prevalence of the disease, is probably too small for an accurate detailed assessment of the full clinical spectrum of RVCL-S. Moreover, the cross-sectional design also precludes an accurate estimate of the exact disease course over four decades. On the other hand, the clinical pattern across families and individual patients appears rather consistent in the present and previous studies.[2,4,5] We therefore believe that, despite the above limitations, the clinical characteristics and tentative disease course we found in our study do seem to reflect the grand clinical picture of RVCL-S rather well. The gold standard, a detailed, prospective follow-up study over several decades in large numbers of MC+ and families, seems rather impossible.

Other than micro-vasculopathy due to RVCL-S, we did not reveal any other good explanation for internal organ disease in RVCL-S. For instance, MC+ with albuminuria did not have high blood pressure and those with liver disease did not have high alcohol intake. Gastrointestinal bleeding might explain anaemia in at least some patients,[2] warranting endoscopic evaluation with gastro- and colonoscopy and possibly targeted treatment. Large angiodysplasias might require treatment with endoscopic argon plasma coagulation. Kidney disease was mild (eGFR > 40 mL/min/1.73m^2) and therefore not considered a likely explanation for anaemia, except perhaps in one MC+ who had an eGFR of 21 mL/min/1.73m^2 and accordingly was treated with darbepoetin.

Seven MC+ in our study proved to have subclinical hypothyroidism, which is a novel finding in RVCL-S. We did not find any other good explanation in these MC+, including use of medication.[22] Screening of thyroid function has not been routinely done in RVCL-S.[24] We are aware of only one report of hypothyroidism in RVCL-S, a 44-year-old male, but the association with RVCL-S was considered

fortuitous.[23] Of interest, hypothyroidism has been reported in 14 patients with Aicardi-Goutières syndrome, which can be caused by homozygous missense mutations in *TREX1*,[25] i.e. different to the heterozygous C-terminal truncating mutations causing RVCL-S. Subclinical hypothyroidism is also considered a marker of endothelial dysfunction in diabetic retinopathy and chronic kidney disease.[26,27]

We found an increased prevalence of Raynaud's phenomenon in RVCL-S (42%), in line with previous reports.[2] Prevalence figures were higher (both in MC+ and MC-) when using Miller's criteria[4,7] than when using the recent international consensus criteria[8] and greatly exceeded the prevalence figures of around 5% usually found in the general population[28]). As a positive family history of Raynaud's phenomenon increases the odds of developing the condition nearly 17-fold,[28] additional genetic risk factors seem to play an important role as well.

Lifetime prevalence of migraine was lower (27%) than previously reported (59%).[2] This might have been due to that 13/33=39% of the newly identified MC+ in our study was younger than 40 years, the usual age at onset of migraine in RVCL-S. Many of these MC+ might thus still develop migraine later on in life. In the general population, migraine typically starts before age 25.[30] Why migraine begins so much later in RVCL-S is unknown but one could envisage that migraine in RVCL-S is a secondary phenomenon due to progressive vasculopathy.

Neurological manifestations were associated with higher white matter hyperintensity-volume on MRI and often remained mild and unnoticed. Cerebral mass lesions usually present around age 55 and were, without exception, associated with major neurological deficits.[2] Depressive symptoms were more common among MC+. There was no evidence of major cognitive impairment.

How RVCL-S *TREX1* mutations lead to multiple organ disease is unknown. Most organs affected in RVCL-S heavily rely on an intact endothelial barrier to maintain normal function. We found pharmacological and biochemical evidence for endothelial dysfunction in RVCL-S.[31,32] Patients with RVCL-S had abnormal increased circulating levels of the markers of endothelial function angiopoietin-2 and Von Willebrand Factor antigen and propeptide.[32] Altogether, this seems to suggest that RVCL-S is a systemic endotheliopathy.

The underlying mechanisms for the putative endothelial dysfunction are also unknown. Atherosclerosis does not seem to play a major role as vascular risk factors were no more common

among MC+ than in MC-. Auto-immune mechanisms have been implicated. TREX1 is a DNA exonuclease that may prevent auto-immune activation by self-DNA.[33] RVCL-S frameshift mutations result in a truncated C-terminus leaving the enzymatic activity of the N-terminal truncated TREX1 protein intact. *TREX1* escapes nonsense-mediated decay, usually seen with truncations, as it is a one-exon gene. Lack of the C-terminal part of the TREX1 protein results in dysregulation of oligosaccharyltransferase activity of the endoplasmic reticulum, which in turn leads to aberrant glycosylation and production of free glycans that may trigger the auto-immune response.[34] Inflammatory mechanisms might also be involved as we found increased levels of inflammatory markers (i.e. ESR, hsCRP, fibrinogen, D-dimer and homocysteine) in older MC+.

In summary, RVCL-S is a rare, fatal, and probably underdiagnosed systemic small vessel disease, clinically typically starting around age 20 with progressive blindness due to vascular retinopathy and Raynaud's phenomenon. From age 35-40 onwards, most patients will develop multiple internal organ disease, justifying regular screening, followed a decade later by progressive characteristic and ultimately fatal cerebral deficits. World-wide, only 16 unrelated families with confirmed RVCL-S have been described, of which three originate from and live in The Netherlands.[2] As this is a small country with only 17 million inhabitants, this striking observation does seem to suggest that many families and patients with RVCL-S remain, globally, unidentified.

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Online supplemental materials

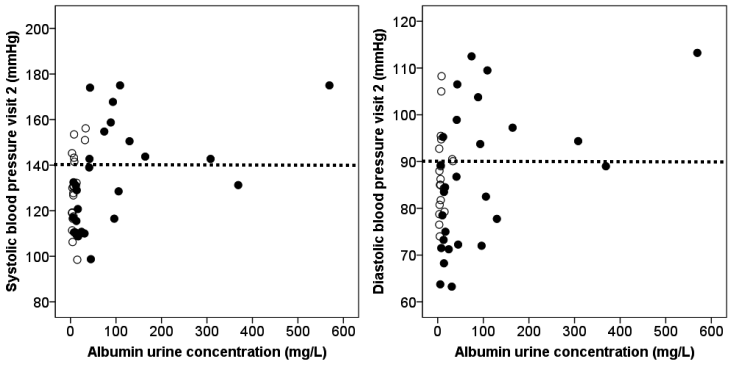

Figure 1S: Lack of association between albumin urine concentration versus systolic and diastolic blood pressure measured in visit 2. Black circles: family members with *TREX1* mutation; White circles: family members without *TREX1* mutation.