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Retinal Vasculopathy with Cerebral Leukoencephalopathy and Systemic Manifestations (RVCL-S)

Theories on the Pathophysiology and on Finding Diagnostic Biomarkers and Novel Therapeutic Targets

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Layman's summary

Retinale vasculopathie met cerebrale leukoencefalopathie en systemische manifestaties (RVCL-S) is een zeldzame erfelijke ziekte. De ziekte wordt gekarakteriseerd door een aantal specifieke symptomen die aangegeven worden door de naam van de aandoening. Retinale vasculopathie laat zien dat er sprake is van ziekte van de kleine bloedvaten (=vasculopathie) in het netvlies van het oog (=retina). Klachten hiervan merken de patiënten vaak als eerst. Uiteindelijk leidt de ziekte aan de kleine bloedvaten in de retina vaak tot het ontstaan van slechtziendheid of zelfs blindheid. Naast retinale vasculopathie is er ook nog sprake van cerebrale leukoencefalopathie. Dit betekend dat er schade is aan de witte stof (=leukoencefalopathie) van de hersenen (=cerebrum). Klachten van de hersenen, zoals vergeetachtigheid, krachtverlies in één of meer ledematen, epilepsie of migraine treden ook op. Als laatste is er bij RVCL-S ook sprake van systemische manifestaties. Dat betekend dat naast de hersenen en de ogen ook andere organen betrokken zijn bij de ziekte. Bekende systemische ziekteverschijnselen zijn onder andere lever- en nierziekte, verminderde functie van de schildklier, verhoogde bloeddruk, bloedarmoede en het fenomeen van Raynaud. De leeftijd waarop de eerste symptomen van de ziekte zichtbaar worden verschilt per patiënt, maar vindt meestal plaats tussen 35 en 50 jaar. Ondanks deze duidelijke lijst aan symptomen is diagnose van RVCL-S toch lastig gebleken. Dit komt doordat RVCL-S zeldzaam is en daardoor niet genoeg artsen bekend zijn met de ziekte. Dit zorgt ervoor dat RVCL-S regelmatig aangezien wordt voor andere ziektes.

RVCL-S wordt veroorzaakt door een foutje in het erfelijk materiaal, namelijk in het stukje van het erfelijk materiaal (=gen) dat we *TREX1* noemen. Dit leidt tot de productie van een verkort eiwit. Doordat het eiwit niet volledig is, kan het zijn functie niet meer goed uitvoeren. Hoe dit precies leidt tot de hierboven genoemde ziekteverschijnselen is nog niet bekend. Deze missende informatie zorgt ervoor dat het moeilijk is om een therapie te vinden voor de aandoening. RVCL-S kan tot nu toe dan ook niet worden genezen en artsen proberen alleen klachten te voorkomen of te verlichten. Het is dus van groot belang dat er meer bekend wordt over het onderliggende ziektemechanisme van RVCL-S. Het doel van dit review is dan ook om de bestaande theorieën over de onderliggende ziektemechanismen van RVCL-S op te helderen en mogelijke nieuwe markers voor diagnose of aangrijpingspunten voor een behandeling te belichten.

In de afgelopen jaren zijn er meerdere onderzoeken gedaan naar de mogelijke onderliggende ziektemechanisme. Men denkt nu dat de binnenste laag cellen van bloedvaten (= endotheel) beschadigd raakt. Dit zou leiden tot verminderde functie van deze cellen, oftewel endotheel dysfunctie. Deze endotheel dysfunctie zou zorgen voor verslechterde doorbloeding van verschillende

organen. Daarnaast speelt mogelijk ook een ontstekingsreactie een rol. Op basis van deze theorieën lijken onder andere markers die endotheelschade, bloedvatfunctie en een ontsteking aan kunnen geven een mogelijke toevoeging voor de diagnose. Daarnaast zou een toekomstige behandeling mogelijk gebaseerd kunnen worden op het ingrijpen in de endotheeldysfunctie. Echter zal er in de toekomst nog meer onderzoek gedaan moeten worden om deze theorie te bevestigen.

Abstract

Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S) is a systemic small vessel disease that affects various organs, including the brain, retina, kidneys, and liver. The disease is characterised by retinal vasculopathy, white matter lesions, focal and global brain dysfunction, and several systemic manifestations. These symptoms arise from C-terminal truncating mutations in the *three prime repair exonuclease-1* (*TREX1*) gene. These mutations lead to the insertion of a premature stop codon, inducing the production of truncated proteins. How these truncated proteins exactly cause the disease manifestations is not yet known. However, prior studies appeared to indicate endothelial dysfunction as an important mechanism. Besides endothelial dysfunction, inflammation and serotonergic system malfunction have also been proposed as potential underlying mechanisms. Hence, these processes could potentially help to bridge the gap between the truncated TREX1 proteins and the clinical manifestations of RVCL-S. Therefore, the purpose of this review is to provide an overview of the main suggested theories concerning the pathophysiology of RVCL-S, including ideas for diagnostic markers and novel therapeutic targets.

***Keywords**: RVCL-S, pathophysiology, endothelial dysfunction, inflammation, diagnostic markers, therapeutic targets*

List of abbreviations

AGS	Aicardi-Goutières syndrome
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CARASAL	Cathepsin-A-related arteriopathy with strokes and leukoencephalopathy
CARASIL	Cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy
CNS	Central nervous system
CRV	Cerebroretinal vasculopathy
CT	Computed tomography
CVR	Cerebrovascular reactivity
ER	Endoplasmic reticulum
ESR	Erythrocyte sedimentation rate
FAZ	Foveal avascular zone
FCL	Familial chilblain lupus
FDP	Fibrin degradation product
Fs	Frameshift
HERNS	Hereditary endotheliopathy with retinopathy, nephropathy, and stroke
HSA	Hereditary systemic angiopathy
HVR	Hereditary vascular retinopathy
ICAM	Intracellular adhesion molecule 1
IFN	Interferon
iPSC	Induced pluripotent stem cells
MRI	Magnetic resonance imaging
OCT-A	Optical coherence tomography angiography
OST	Oligosaccharyltransferase
RVCL	Retinal vasculopathy with cerebral leukodystrophy
RVCL-S	Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations
SLE	Systemic lupus erythematosus
SVD	Small vessel disease
TREX1	Three prime repair exonuclease-1
VCAM	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VWF	Von Willebrand factor

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Chapter 1: Introduction

Retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S) is a progressive small vessel disease (SVD) that affects highly vascularised organs, such as the brain, retina, liver, and kidneys (1). SVD involves pathological processes that damage the small cerebral blood vessels, including arterioles, venules, and capillaries (2). RVCL-S is a monogenic disease caused by a mutation in the *three prime repair exonuclease-1* (*TREX1*) gene and becomes evident from 35 to 50 years of age (1,3). In recent decades, RVCL-S was better known as cerebroretinal vasculopathy (CRV), hereditary systemic angiopathy (HSA), hereditary vascular retinopathy (HVR), hereditary endotheliopathy with retinopathy, nephropathy and stroke (HERNS), and retinal vasculopathy with cerebral leukodystrophy (RVCL) (1). Ultimately, due to the expanding clinical picture with related systemic manifestations, it was renamed to the current name; RVCL-S. To date, less than 30 families with RVCL-S have been identified worldwide (1). Remarkably, a relatively large proportion of these families are from the Netherlands, namely 3 families (1,4). As RVCL-S is a commonly missed diagnosis due to its rarity and a lack of awareness, it is suspected that the actual number of families is much higher. Besides misdiagnoses, no curative treatment has been established for RVCL-S. Consequently, most patients die relatively young, at a mean age of 53.1 years (1,3,4). The missing curative treatment is mainly caused by a lack of knowledge on the pathophysiology of RVCL-S. Therefore, this review aims to elucidate existing theories on the pathophysiological mechanisms contributing toward RVCL-S and highlight findings on novel diagnostic biomarkers and therapeutic targets. This will also reveal which information is missing, i.e. which studies still need to be conducted or which models should be developed in the future to fully map the pathophysiology of RVCL-S.

Chapter 2: RVCL-S genotype and phenotype

2.1 *TREX1* gene

RVCL-S is caused by a mutation in the *TREX1* gene, located on chromosome 3p21.31 (5,6). The disease is inherited autosomal dominantly, meaning that offspring have a 50% chance of getting the disease. *TREX1* encodes for the major 3'-5' DNA exonuclease DNase III (1,7). Under biological circumstances, TREX1 protein is localised in cellular cytoplasm and bound to the endoplasmic reticulum (ER) with its C-terminus. Here, it binds to the ER-associated SET complex, which is important in DNA repair. One of the functions of TREX1 proteins is that in case of oxidative stress, TREX1 proteins and SET colocalise together from the cytoplasm to the nucleus, where the TREX1 proteins start to remove nucleotides from the 3' end of single-stranded DNA (8,9). Consequently, cell death is induced in the cells with DNA damage (8).

Besides RVCL-S, several other diseases are known to involve a mutation in the *TREX1* gene, including Aicardi-Goutières syndrome (AGS), systemic lupus erythematosus (SLE) and familial chilblain lupus (FCL) (10–13). The consequences of a *TREX1* mutation depend on the type of mutation and the location on the gene. The *TREX1* gene is constructed from three exonuclease sequences, Exo I, II, and III, and a hydrophobic C-terminus associated with ER localisation (Figure 1) (1,11). Studies including AGS, SLE, and FCL patients have indicated that these diseases are associated with different types of mutations (14–17). Mutations in or near the exonuclease domains are known to alter DNase activity (11). In contrast, RVCL-S is associated with a C-terminal truncating mutation, the most common being V235 frameshift (fs) (Figure 1) (1,18). This type of mutation results in the insertion of a premature stop codon. In turn, this leads to the production of truncated TREX1 proteins. These proteins are unable to attach to the ER, causing TREX1 to be mislocalised in both the cytoplasm and nucleus (11,19). Interestingly, the DNase activity of the truncated TREX1 proteins is preserved (7,11,14). How the truncated proteins are hypothesised to cause the disease symptoms associated with RVCL-S will be addressed in section 3.2.

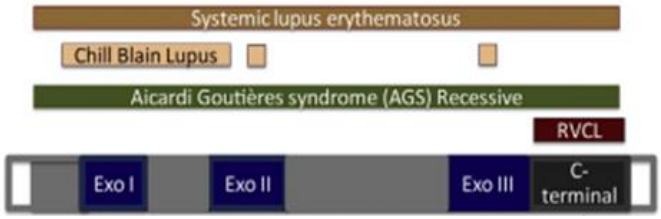

Figure 1. Diseases caused by a mutation in the TREX1 gene. *The figure illustrates in which regions of the TREX1 gene the mutations are located that cause the various diseases. In particular, RVCL-S originates in the C-terminus. Adapted from Gulati et al., 2018 (14)*

2.2 Clinical manifestations

Retinal vasculopathy is the most commonly found symptom in RVCL-S and often the first sign (18). Retinal vasculopathy causes progressive loss of visual acuity and defects in the visual field (1,8). In the early phase, it can be recognised in fundo by telangiectasia, microaneurysms, and cotton wool spots when fluorescein angiography is used. At a later stage, perifoveal capillary obliterations and neovascularisation can also be observed (Figure 2) (1,3,8). Eventually, retinal vasculopathy will lead to progressive blindness (1).

As the name further implies, RVCL-S also comprises systemic manifestations. For instance, kidney- and liver disease, anaemia, hypertension, Raynaud's phenomenon, and hypothyroidism are all common signs found in RVCL-S patients (Figure 2) (20,21).

Besides these systemic manifestations, focal and global brain dysfunction often develops, manifesting as migraines, epilepsy, cognitive decline, and loss of strength in extremities. This usually takes place between the ages of 40 and 50 (1). Around the same age, cerebral leukoencephalopathy becomes prevalent. This can be seen as supratentorial lesions, involving ring-enhancing-, punctiform-, and linear lesions on a brain magnetic resonance imaging (MRI) scan. These lesions often change in size and number over time. When lesions become enlarged and surrounded by oedema, neighbouring brain tissue can be compressed, also known as the mass effect (Figure 2) (22). Also, focal calcifications can be observed on a computed tomography (CT) scan (23).

RVCL-S is a progressive disease, implying that symptoms get worse over the years. However, it does vary between patients how the disease manifests and how rapidly it deteriorates. Ultimately, most patients die of sepsis or pneumonia (3,24).

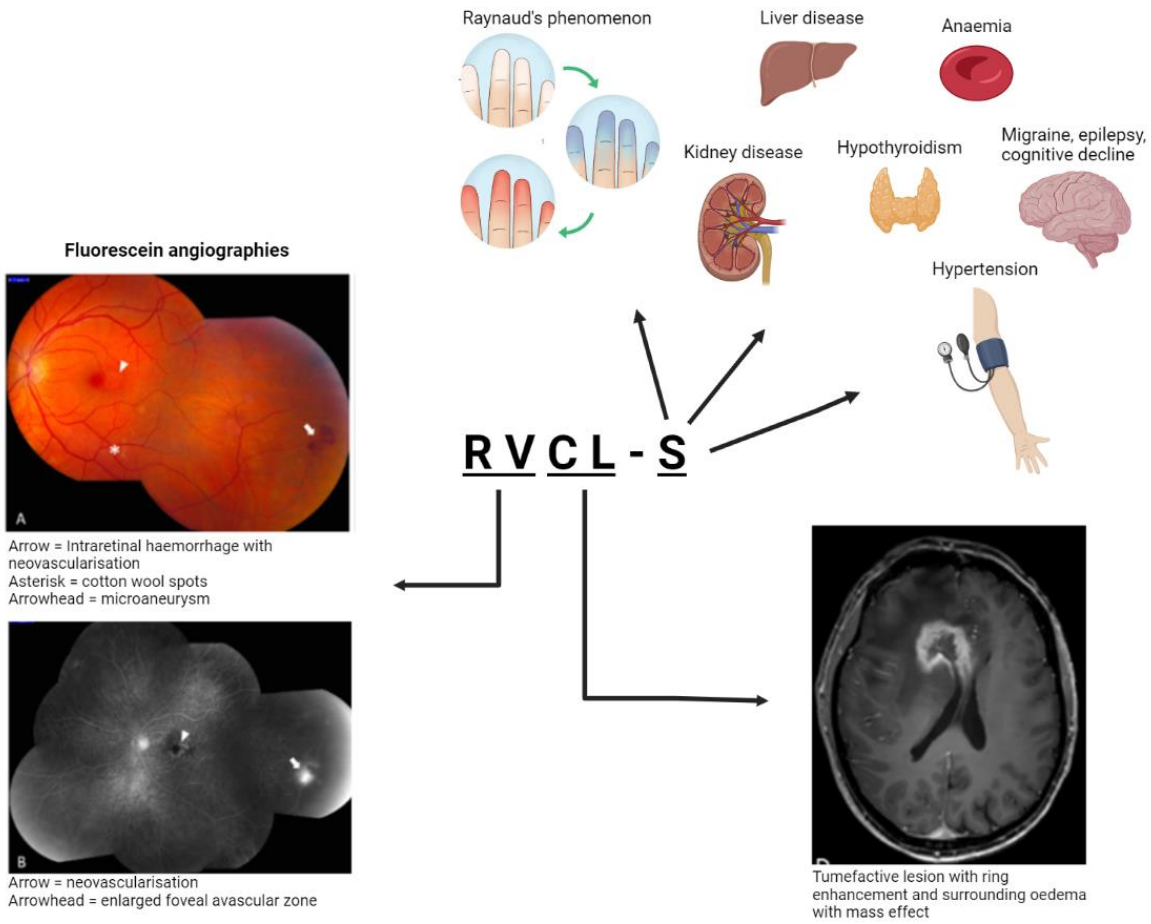

Figure 2. Overview clinical manifestations RVCL-S. *The figure depicts the various clinical manifestations associated with RVCL-S. On the left, retinal vasculopathy (RV) is visualised, involving neovascularisation, cotton wool spots, microaneurysms, and an enlarged foveal avascular zone. Furthermore, on the bottom right, an example of cerebral leukoencephalopathy (CL) is shown on an 3D-T1-weighted gadolinium-enhanced MRI brain scan; a tumefactive lesion with ring enhancement and surrounding oedema with mass effect. Lastly, the various systemic manifestations (S) associated with RVCL-S are illustrated on the upper right. Source: created in Biorender, imaging figures from (1).*

2.3 Diagnosis and treatment

2.3.1 Diagnosis

Diagnosing RVCL-S is difficult and, as a result, the disorder is often mistaken for other conditions. For example, the cerebral manifestations of RVCL-S can be mistaken for other SVDs, central nervous system (CNS) vasculitis, brain tumours or tumefactive demyelinating disease, while retinopathy striking resembles diabetic retinopathy (5,21). How these conditions can be differentiated from RVCL-S is discussed in section 4.1. To avoid misdiagnoses in the future, it is very important to create more awareness about RVCL-S.

Common clinical features are described in Table 1 (1,3). Over the years, the list of diagnostic criteria expanded to be more inclusive. For instance, in 2018, it was proposed to add gastrointestinal haemorrhage, hypothyroidism, and anaemia as well (20). However, the variability in clinical features complicates the diagnosis (21). Therefore, to be definitive of RVCL-S, molecular genetic testing can be utilised to demonstrate a pathogenic truncating mutation in the C-terminus of *TREX1* (3).

Table 1. Diagnostic criteria

Diagnostic criteria
Demonstration of a C-terminal frameshift mutation in *TREX1* to genetically confirm the diagnosis
Main features - **Vascular retinopathy** - **Features of focal and/or global brain dysfunction associated on MRI with punctate T2 hyperintense white matter lesions with nodular enhancement, and/or larger T2 hyperintense white matter mass lesions with rim-enhancement, mass effect, and surrounding oedema** - **Family history of autosomal dominant inheritance with middle-age onset of disease manifestations**
Supportive features - **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** - **Microvascular liver disease** - **Microvascular kidney disease** - **Anaemia consistent with blood loss and/or chronic disease** - **Microscopic gastrointestinal bleeding** - **Subclinical hypothyroidism**
Possibly associated features - **Raynaud's phenomenon (typically mild)** - **Migraine with or without aura** - **Hypertension**

Wilms et al., 2022 (1)

2.3.2 Current treatment

Currently, no curative treatment is available for RVCL-S. Finding such treatments is hampered by the rarity of the disease and the limited knowledge on the pathophysiology (1). Consequently, so far only the symptoms of RVCL-S are treated rather than the underlying disease mechanisms. As mentioned in section 2.2, retinal vasculopathy is one of the main clinical symptoms. This can be treated with retinal laser therapy and/or anti-vascular endothelial growth factor (VEGF) therapy (1,25). However, the use of anti-VEGF is currently not evidence-based. Besides retinal vasculopathy, other clinical symptoms also need to be treated. For example, methylprednisolone can be given to treat tumefactive white matter lesions with surrounding cerebral oedema. However, there is limited evidence for this treatment as of yet (1,5). Furthermore, migraine, hypertension, kidney disease, anaemia, hypothyroidism, and Raynaud's phenomenon are handled with standard care (1,3).

Chapter 3: Studies on the underlying mechanisms of RVCL-S

3.1 Modelling RVCL-S

3.1.1 Previously described RVCL-S models

To date, several disease models have been used to study pathological processes that might contribute to RVCL-S. Multiple mice models and cell lines have been used, all displaying some of the pathological mechanisms involved in the disease.

To start, several different mouse models have been used. Here, the three most used mice models will be discussed in detail; *TREX1*$^{-/-}$ knock-out mouse, *TREX1*-V235fs knock-in mouse with mutated human *TREX1*, and *TREX1*-V235fs knock-in mouse with mutated murine *TREX1*. As the name implies, the *TREX1*$^{-/-}$ knock-out mouse is completely lacking *TREX1*. Furthermore, in the *TREX1*-V235fs knock-in mouse with mutated human *TREX1*, the murine *TREX1* is replaced by the human *TREX1* with V235fs mutation (10,11). Lastly, the *TREX1*-V235fs knock-in mouse with murine *TREX1*. In this mouse model, not the mutated human *TREX1* exon is inserted, but a frameshift mutation is introduced into the *TREX1* gene of the mouse, the V235fs mutation (26).

The type of mouse model utilised depends mainly on the purpose of the study and the different pros and cons associated with the various models (27). For instance, a major downside of *TREX1*$^{-/-}$ knock-out mice is that they cannot distinguish between different diseases caused by *TREX1*. In other words, no distinction can be made between *TREX1* mutations in the C-terminus or exonuclease regions. In addition, the average survival of 8 to 10 weeks is short compared to other RVCL-S mouse models that can survive for over a year (10,11). In contrast to the *TREX1*$^{-/-}$ knock-out mouse, the *TREX1*-V235fs knock-in mouse model with human *TREX1* mutation can distinguish diseases caused by a mutation in the C-terminus of *TREX1* from diseases driven by mutations in the exonuclease domains. Another advantage is that the expression of the human C-terminal truncating mutation ensures that the DNase activity is maintained, which is in line with RVCL-S patients. Therefore, this model is often used to investigate the underlying mechanisms associated with the C-terminal truncating mutation related to RVCL-S (26). Lastly, the *TREX1*-V235fs knock-in mouse model with induced murine frameshift mutation has the advantage that this model is easier to make compared to the latter mouse model. In other words, inducing a frameshift mutation is easier than completely replacing an exon. Furthermore, this mouse model mimics several disease characteristics that are seen in humans. For example, the homozygous form of this mouse model shows signs of a vascular phenotype. Prior research used this feature, for instance, to investigate the link between vasculopathy and mortality (26).

Additionally, cell models have also been utilised. As an example, mouse fibroblasts were reconstituted with normal human *TREX1* and human *TREX1* with a C-terminal truncating mutation to examine the function of the *TREX1* C-terminus (10). Moreover, this study also employed lymphoblasts from RVCL-S patients that carry the V235fs mutation. This model was used to determine the role of the *TREX1* C-terminus in, among other things, immune activation. Advantages of using cell models are that it is low-cost, staining can be performed, and it allows for the investigation of specific cell types (10).

3.1.2 Studies in humans

Besides mouse- and cell models, studies have also been performed on RVCL-S patients themselves. Since the effect on humans needs to be revealed, human study samples often seem an ideal model to use. An important advantage of research in humans is the accessibility to historical datasets that can provide more information on, for instance, disease development or family history. In addition, large patient cohorts serve well to investigate the different genetic mutations appearing among patients and the variability in systemic manifestations and neuro-imaging features (18). However, research in humans also comes with disadvantages. For example, invasive procedures cannot be performed, such as extracting the brain. Additionally, human research must comply with strict medical-ethical regulations and national legal requirements. This is mainly because human health must not be compromised if possible. In animal research, strict medical-ethical regulations and national legal requirements also apply, but often more extensive research is allowed compared to humans (26).

Alongside, material from deceased RVCL-S patients is also utilised for research. Post-mortem tissue samples lend themselves well for examining patterns of gene expression with the use of staining (28). For example, dual staining was conducted on post-mortem brain tissue from RVCL-S patients to reveal which cell types express *TREX1* (19).

3.1.3 RVCL-S research in mice vs. humans

As mentioned before, mouse models are often used in research. These models can be useful in understanding disease mechanisms and testing treatments. This ensures that enough knowledge is available before trials are started on humans, minimising the risk to human health. However, there are some drawbacks to using mouse models, including the mouse models for RVCL-S. A major downside of the RVCL-S mouse models is that they do not completely replicate the human phenotype (29). For example, the mice do not show retinal vasculopathy, white matter lesions or renal disease (11,26). It is possible that these features are not expressed due to the shorter lifespan (~1.5 - 2 years) of mice compared to humans (26). However, even though the existing mouse models do not exactly replicate

the human pathological features of RVCL-S, they are still very valuable. As mentioned before, mice can be used to perform invasive procedures that cannot be performed in humans and the disease can be partly mimicked. However, care should be taken when mice studies are translated to humans, because studies often use homozygous mice in which both *TREX1* alleles are mutated. Homozygous is often chosen to increase the likelihood of capturing the disease features. However, this contrasts with the heterozygous mutation in humans that involves only one mutated *TREX1* allele. As a heterozygous mutation commonly yields a milder phenotype compared to a homozygous mutation, this difference does ensure that you have to be extra careful when translating the outcomes of these studies to humans (26).

3.1.4 Potential future models

In addition to these existing models, more need to be developed in the future. For example, designing a disease model for RVCL-S utilising induced pluripotent stem cells (iPSC). These iPSCs can be generated from somatic cells of RVCL-S patients, mimicking the specific properties of the diseased cells. In RVCL-S, endothelial dysfunction is thought to play a key role. This will be further discussed in detail in section 3.2.1. Therefore, the iPSCs could be used to differentiate into both endothelial and mural cells. These mural cells include pericytes and vascular smooth muscle cells and normally serve the function of establishing a stable vasculature. As such, an iPSC model can be used to mimic the defective interactions between endothelial- and mural cells and model the molecular mechanisms. The development of such a model could boost research into RVCL-S, but this is not available as of yet (30). In general, iPSC models are often used to model diseases *in vitro* and discover new drugs. An important advantage of an iPSC model is that the toxicity of newly devised drugs can be tested. In addition, iPSCs could potentially provide a basis for patient-specific treatment in the future. This would involve producing patient-specific cells that could replace the damaged tissue, in this case the endothelium. As the cells are patient-specific, there will be no immune rejection (31,32). Therefore, this could be a promising new model in the future for disease modelling, drug testing, and patient-specific treatment development.

3.2 Pathophysiological mechanisms

3.2.1 Endothelial dysfunction and ischaemia

Over the years, various theories have emerged on the underlying pathophysiological mechanisms of RVCL-S. The most prominent theory states that endothelial dysfunction is a primary mechanism in RVCL-S. This dysfunction would then eventually lead to ischaemia. Evidence for this theory has been presented several times in recent years. Firstly, elevated levels of several endothelial markers were

demonstrated in 3 Dutch RVCL-S families. Both Von Willebrand Factor (VWF) antigen, VWF pro-peptide, and angiopoietin-2 levels were shown to be increased. VWF is a marker for endothelial dysfunction and angiopoietin-2 for endothelial damage and activation. In addition, VWF antigen has a much longer half-life compared to VWF pro-peptide. Therefore, the ratio between VWF pro-peptide and VWF antigen can be used to indicate whether acute or chronic endothelial activation is apparent. In other words, a higher ratio, i.e. more VWF pro-peptide, indicates acute endothelial activation, whereas a lower ratio suggests chronic endothelial activation. All levels were shown to be elevated before age 40, but became more pronounced after the age of 40 (33). In addition, the ratio between VWF pro-peptide and VWF antigen was in favour of VWF antigen. Thus, these findings suggest chronic endothelial activation in RVCL-S patients, becoming more evident from the age of 40 onwards (1,33).

Furthermore, splitting of basement membranes, fibrinoid necrosis, and narrowing and occlusion of the vascular lumen were observed. In general, these manifestations are not necessarily caused by an endothelial problem. However, in RVCL-S that seems most likely based on the current knowledge. In turn, endothelial dysfunction tends to have important consequences for the vascular system. For instance, it was found that the vascular relaxant response to acetylcholine was reduced in RVCL-S knock-in mice with an induced frameshift mutation in murine *TREX1*. As acetylcholine is known to be an endothelium-dependent vasodilator, this appears indicative of an endothelial defect (26). Similarly, in RVCL-S patients the flow-mediated dilation was demonstrated to be impaired, which is also a measure of endothelium-dependent vasodilation. This suggests that endothelial dysfunction is indeed present in RVCL-S patients. However, this latter study was limited by the relatively small sample size (n = 10) (7). Nevertheless, a subsequent study that investigated cerebrovascular reactivity (CVR) in 21 RVCL-S patients, 23 unaffected relatives, and 31 unrelated controls supported the results of the latter study. CVR is a measure of the capacity of cerebral blood vessels to dilate in response to a vasoactive stimulus. It was observed that the CVR was reduced in both grey and white matter in RVCL-S patients compared to relatives and controls. However, this was different in patients under 40 years, in whom CVR was decreased in white matter solely (34). As CVR is considered a marker for cerebrovascular endothelial function, these findings seem to imply that the endothelial function of the cerebral vessels is reduced in RVCL-S patients.

Consequences of the hypothesised endothelial dysfunction also appear to be noticeable in both retinal- and neuro-imaging (1). For instance, it was found that thickened hyalinised arterial walls are present in the retina, which can occur in case of endothelial dysfunction (3). Moreover, in a case report of a 31-year-old man with RVCL-S, retinal peripheral capillary nonperfusion and capillary leakage were observed. Treatment with anti-VEGF reduced the capillary leakage. This seems to indicate that the

capillary leakage is associated with an endothelial defect. Furthermore, both features were revealed to correspond to the cotton wool spots. This seems to demonstrate that the cotton wool spots, that are frequently seen on fluorescein angiographies of RVCL-S patients (Figure 2), could be associated with endothelial dysfunction. Furthermore, the peripheral capillary nonperfusion appears to match the idea that ischaemia occurs in RVCL-S patients (25). However, these latter findings are not quite indicative, because it is only a single case. Therefore, a larger cohort will need to be studied in the future to determine whether this is prevalent in RVCL-S patients.

Apart from imaging of the retina, neuro-imaging has also provided some interesting observations. For instance, a study including 14 RVCL-S patients and 26 matched healthy controls revealed that the white matter oxygen-extraction fraction was elevated in RVCL-S patients and progressively increased with disease duration. The oxygen-extraction fraction is often used as a hemodynamic measure of ischaemia. Therefore, this finding suggests chronic ischaemia as a potential underlying mechanism in RVCL-S. Ischaemia can have several causes, including an endothelial defect. Therefore, the endothelial dysfunction may be the cause of the appearance of ischaemia in RVCL-S patients (29). In addition, it was also demonstrated that white matter lesions diminish in size over time, ultimately leading to periventricular ischaemic defects. These periventricular ischaemic defects were represented as necrotic periventricular white matter areas (22). In addition, a role for ischaemia in RVCL-S was also suggested in several case presentations (35,36). For example, confluent foci of ischaemia were demonstrated in a frontal lobe lesion (35). Taken together, these neuroimaging studies seem to point to ischaemia as possible consequence of the endothelial dysfunction. In turn, this ischaemia may underly the neurological deterioration seen in RVCL-S patients (22).

All in all, several pieces of evidence have been provided that seem to support the theory that endothelial dysfunction would partake in the pathological mechanism of RVCL-S (Figure 3). Among others, elevation of endothelial markers, splitting of basement membranes, fibrinoid necrosis, narrowing and occlusion of the vessel lumen, and worsening of the CVR point to endothelial dysfunction. Furthermore, the findings of peripheral nonperfusion and vascular leakage in the retina, and increased white matter oxygen-extraction fraction and necrotic white matter in the brain indicate that ischaemia ultimately occurs in RVCL-S. In other words, many findings point in the same direction, making this theory plausible.

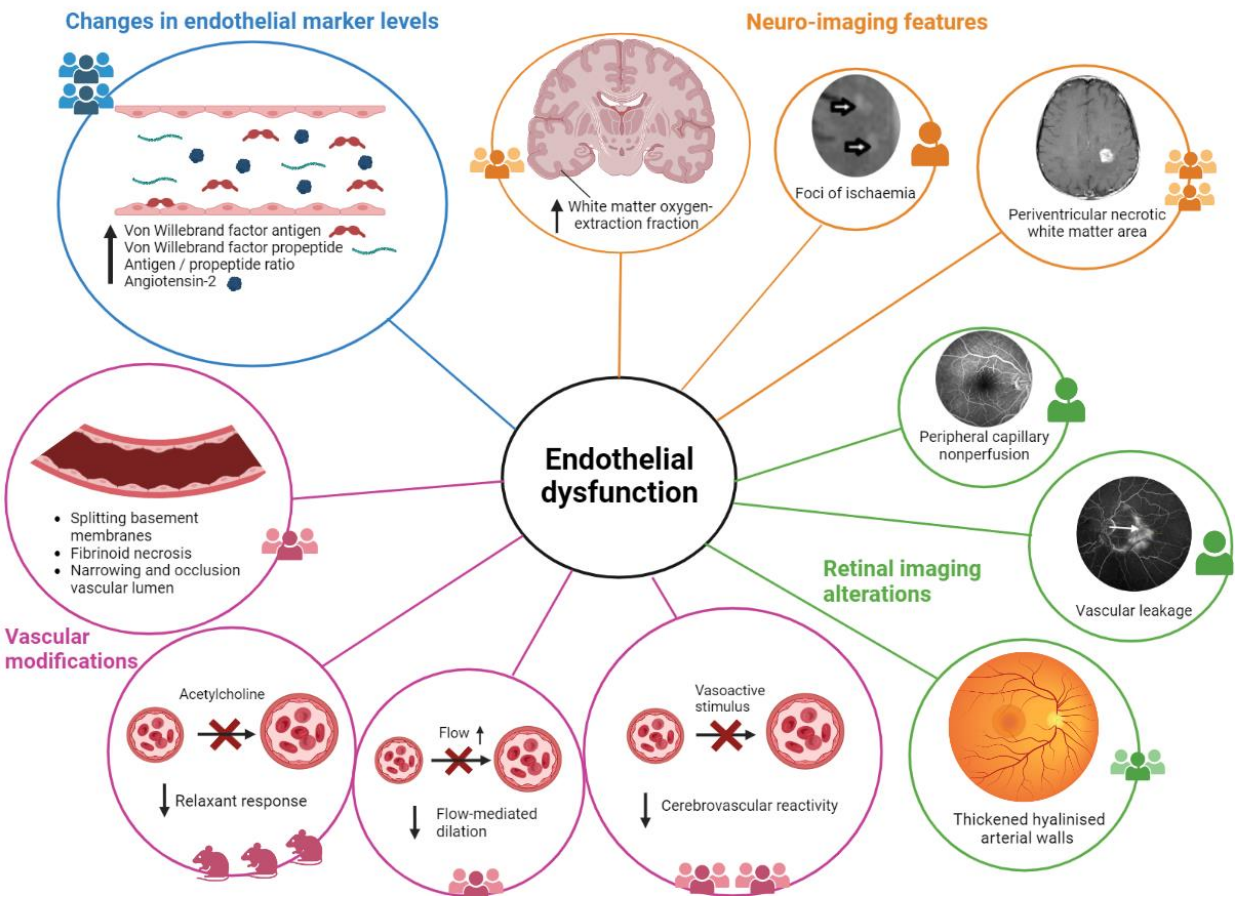

Figure 3. Overview findings pointing to endothelial dysfunction as underlying pathophysiological mechanism*. The different findings are divided into four categories, indicated by a different colour. The size of the circles represents how strongly the findings contribute as evidence for the theory. This depends on the size of the study population, the reliability of the study, and the certainty of it being caused by endothelial dysfunction. The people and mice figures show whether it was tested in humans or mice, and the number of figures indicate the size of the study population. One figure indicates n = 1, three figures indicate 5 < n < 20, and six figures reflect n > 20. Source: created in Biorender*

3.2.2 Alternative theories

3.2.2.1 Inflammation

Apart from the endothelial dysfunction theory, additional hypotheses exist on the pathophysiological mechanisms of RVCL-S. For example, it is hypothesised that inflammation plays a significant role. Besides a role for the C-terminus of *TREX1* in ER localisation, it is also implicated in the regulation of the oligosaccharyltransferase (OST) complex. The OST complex is embedded in the membrane of the ER and is involved in transferring glycans to asparagine residues within nascent polypeptides that are located in the cytoplasm of the ER. Under physiological conditions, TREX1 protein binds to the OST complex with its C-terminus, leading to normal functioning of the complex. However, the C-terminal

truncating mutations in RVCL-S are associated with dysregulation of the OST complex. Consequently, this is thought to lead to reduced glycosylation of the cytoplasmic proteins, leaving the protein peptides exposed. As a result, the protein peptides can be recognised by the immune system and seen as foreign, initiating an immune response (Figure 4) (29). Indeed, in both human lymphoblasts with V235fs mutation and *TREX1*-V235fs knock-in mice with inserted human *TREX1* mutation, it has been observed that this OST complex dysregulation leads to immune activation and autoantibody production (10,11). Supporting this, elevated levels of several inflammatory markers were seen in RVCL-S patients, involving fibrin degradation product (FDP), erythrocyte sedimentation rate (ESR), D-dimer, C-reactive protein, and interleukin-6 (21). Therefore, these studies seem to support the theory that inflammation is implicated in the pathophysiology of RVCL-S.

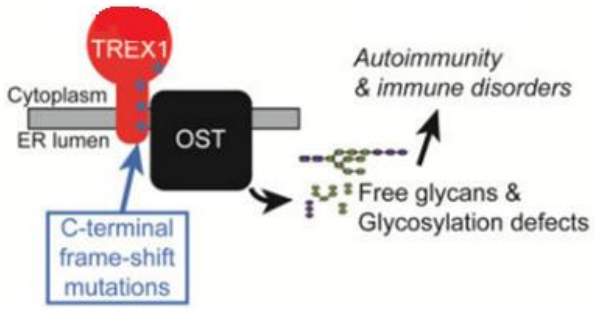

Figure 4. Overview effect TREX1 C-terminal truncating mutation on OST complex. *The figure demonstrates that the C-terminal truncating mutations in TREX1 cause dysregulation of the OST complex. This leads to auto-immunity and activation of the immune system via an error in the glycosylation. TREX1 = three prime repair exonuclease-1*; *OST = oligosaccharyltransferase. Source: modified from Sakai et al., 2017 (11)*

In addition to these studies, various other findings suggest an association between *TREX1* and neuroinflammation. For instance, *TREX1*-expressing microglia and macrophages were found in human brain tissue from RVCL-S patients. *TREX1* was thought to assist the microglia and macrophages in phagocytosis and clearance of necrotic debris. In addition, an increase in *TREX1*-expressing microglia was demonstrated in areas of ischaemia in brain tissue of RVCL-S patients. Therefore, these findings appear to indicate that *TREX1*-expressing microglia are involved in vascular homeostasis and response to ischaemia in the brain (19).

Moreover, as mentioned before in section 2.3.2, methylprednisolone can potentially be used to treat tumefactive white matter lesions with surrounding cerebral oedema (1). The mechanism of this treatment is based on the underlying idea that neuroinflammation is implicated in RVCL-S. Methylprednisolone is a corticosteroid that is frequently used as an immunosuppressant. It can bind

to intracellular glucocorticoid receptors, whereafter the promotor sites of pro-inflammatory gene products are blocked and the expression of anti-inflammatory gene products is promoted (37). In contrast to methylprednisolone, treatment with an interferon-α (IFN-α) inhibitor seems not convenient to use. Previous research in 30 RVCL-S patients has shown that the IFN-α levels were not elevated. This appears to indicate that the hypothesised inflammation does not involve the IFN-α pathway (38).

Together, these aforementioned findings make the idea that inflammation is implicated in the pathophysiology of RVCL-S seem reasonable. Future research should show whether inflammation indeed has a significant role in RVCL-S, if this inflammation then coexists with the endothelial dysfunction, and whether these would then be independent or dependent from one another. In addition, it will be necessary to examine carefully which inflammatory pathways are involved to select the right treatment.

3.2.2.2 Serotonergic system malfunction

An alternative theory that has been proposed in the past is a malfunctioning of the serotonergic system. In 1998, a study was performed including 12 HVR patients, 10 relatives, and 19 controls. In these patients and relatives, reduced concentrations of serotonin were found in plasma and platelets compared to controls. Serotonin is known to function as a vasoconstrictor. However, this finding does not seem applicable anymore for the following reasons. Firstly, the presence of HVR was not genetically confirmed. In other words, a C-terminal truncating mutation in *TREX1* was not proved. Secondly, the HVR participants were selected based on the presence of angioscopic and/or fundoscopic abnormalities, retinal haemorrhages, and areas of capillary non-perfusion. This does not fully cover the current diagnostic criteria of RVCL-S discussed in section 2.3.1. Lastly, the results of this study seem biased by the difference in age in the various study groups. The HVR patients were on average 47 years old, whereas the relatives and controls had a mean age of 36 years old. Given that age is a possible confounder of serotonin levels, the variability in age between the groups could very well have skewed the outcome (39). Therefore, the results of this study do not seem useful for supporting the serotonergic system malfunction theory.

Nevertheless, another study was conducted in *TREX1*-V235fs knock-in mice with induced frameshift mutation that investigated the maximal contraction in response to serotonin. This study mentioned that the maximum contraction was lower in *TREX1*-V235fs knock-in mice, which seems to support the serotonin hypothesis. However, the maximum contraction was lower, but no significant reduction could be demonstrated compared to control mice (26). In addition, unfortunately, no other

vasoconstrictors were tested. This would reveal whether the reduced maximal contraction depended purely on serotonin or whether the vasoconstriction capacity was impaired regardless of which vasoconstrictor was used. Thus, the results of this study are not indicative enough to serve as evidence for a malfunctioning of the serotonergic system. Overall, this serotonin system malfunction theory does not seem plausible with the current knowledge.

Chapter 4: Ideas for future diagnosis and treatment

4.1 Differential diagnosis

As mentioned earlier, RVCL-S is frequently misdiagnosed. Hence, the differential diagnoses of RVCL-S are listed below in Table 2. The list is not exhaustive, but highlights the most important ones. In particular, the main focus of the list is on the neurology and retinopathy. The major contrasts with RVCL-S are highlighted in bold. To start, RVCL-S can be confused with other monogenic SVDs, such as cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), cathepsin-A-related arteriopathy with strokes and leukoencephalopathy (CARASAL), and cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL). A genetic test can be performed to determine which disorder is present. However, a distinction can already be made based on common clinical manifestations and neuroimaging features. An important example is the occurrence of retinal vasculopathy in RVCL-S, which is not present in the other three monogenic SVDs (3,40). In addition, in the case of CADASIL, microbleeds and enlarged perivascular spaces can be recognised on MRI scans that are not prominent in RVCL-S. On the contrary, CARASIL makes a greater contrast with RVCL-S in clinical features. For instance, spasticity in the lower extremities, gait disturbances, and depression are common in CARASIL whereas these are not observed in RVCL-S. Next, in the case of CARASAL, haemorrhagic strokes often occur, in contrast to the ischaemic strokes in RVCL-S (40).

Besides these SVDs, RVCL-S has been confused with other diseases in the past. For example, the MRI scans of tumefactive demyelinating disease and CNS lymphoma can resemble that of RVCL-S. However, CNS lymphoma can distinguish itself by the location of the lesions, affecting the corpus callosum and basal ganglia (41,42). Tumefactive demyelinating disease should be distinguished based on clinical manifestations, as this disease only affects the brain (42–44). Hence, characteristic RVCL-S symptoms such as retinal vasculopathy and liver- and kidney disease do not occur. Subsequently, diabetic retinopathy can also be confused with RVCL-S as it involves vascular retinopathy and vision loss. However, the deterioration in vision with eventual vision loss is preceded by damage in the occipital cortex, which is not apparent in RVCL-S (45). Lastly, CNS vasculitis characterises itself by defects in the spinal cord that are not seen in RVCL-S. Taken together, a clear distinction can be made between RVCL-S and other differential diagnoses based on genetic background, clinical manifestations, and neuroimaging features (46).

Table 2. Differential diagnosis RVCL-S

	Genetics	Affected organs	Main clinical features	Neuroimaging features
RVCL-S	C-terminal truncating mutation in *TREX1*	Brain Eyes Liver Kidneys	Vascular retinopathy Focal and/or global brain dysfunction Microvascular liver disease Microvascular kidney disease	White matter lesions with nodular enhancement White matter lesions with rim-enhancement, oedema, and mass effect
CADASIL	Missense mutation in *NOTCH3*	Brain	Migraine with aura Cognitive decline Gait and mood disorders Ischaemic stroke	Symmetrical white matter hyperintensities Lacunes **Microbleeds** **Enlarged perivascular spaces**
CARASIL	Frameshift mutation in *HTRA1*	Brain	Cognitive decline **Spasticity in low extremities** Vascular dementia **Gait disturbance** **Depression**	Symmetrical white matter hyperintensities **Lacunes**
CARASAL	Point mutation in *CTSA*	Brain	**Haemorrhagic strokes** Ischaemic stroke Cognitive decline Hypertension Dementia Migraine **Movement disorder**	Leukoencephalopathy involving the brainstem and subcortical white matter
Tumefactive demyelinating disease		Brain	Headache Cognitive decline Aphasia Apraxia Seizures	Large lesions (>2cm) with mass effect, perilesional oedema, and/or ring enhancement
Primary CNS lymphoma	Point mutation in *MYD88*, CD79B, *BCL6, MYC, BCL2, SPIB, TNFAIP3,* or *CIITA*	Brain Eyes Ears	Cognitive impairment Personality changes **Hemiparesis** Aphasia **Hearing loss** Vision problems Headache Seizures	T1 supratentorial and periventricular lesions with contrast-enhancement (mainly affect **corpus callosum or basal ganglia**)
Diabetic retinopathy	TBD	Brain Eyes	Vascular retinopathy **Microaneurysms in retina** Blurry vision Spots or dark strings floating in vision Vision loss Cognitive decline	**Reduced grey matter volume in occipital cortex**
CNS vasculitis	TBD	Brain Spinal cord	Headache Cognitive decline Seizures **Movement disorder** Brainstem, hemispheric or **spinal deficits**	T2 hyperintense unilateral and multifocal lesions (**both grey and white matter**)

Abbreviations: CADASIL = cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; CARASAL = cathepsin-A-related arteriopathy with strokes and leukoencephalopathy; CARASIL = cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy; CNS = central nervous system; RVCL-S = retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations; TBD = to be determined

4.2 Potential novel biomarkers

Prior studies have provided a basis for possible future biomarkers for RVCL-S. As discussed in section 3.2.1, elevated levels of VWF antigen, VWF pro-peptide, and angiopoietin-2 have been found in RVCL-S patients. These endothelial markers could potentially be used as biomarkers of disease activity. In fact, these endothelial markers have already been detected in RVCL-S patients younger than 40 years. Therefore, these markers could potentially indicate early onset of the disease (33). However, it is not yet known whether these endothelial markers could also reflect clinical progression. So, this will have to be investigated in the future.

Additionally, adhesion molecules might also be used as a biomarker, such as intracellular adhesion molecule 1 (ICAM-1) or vascular cell adhesion molecule 1 (VCAM-1). Both are involved in endothelial cell activation, and could potentially also indicate early endothelial activation (47). The extent to which these adhesion molecules can contribute remains to be investigated in the future.

Furthermore, neuroradiological features might also act as a biomarker. For example, the CVR is already reduced in the white matter of patients younger than 40 years of age. Since CVR is a marker for the dilation capacity of cerebral blood vessels, this could be used as an early biomarker to indicate vessel wall changes associated with *TREX1* mutations (34). In addition, the speed of white matter decline could also be a potential candidate for RVCL-S biomarker. In RVCL-S patients, white matter volume diminishes faster on average compared to non-mutation carriers. However, it is not known whether this differs between pre-symptomatic and symptomatic patients (1). Therefore, future studies will have to find out whether the speed of white matter decline can be used as biomarker and if it also decreases faster in pre-symptomatic patients, as it could then be employed biomarker for disease onset.

Moreover, identification of biomarkers related to the development of retinal vasculopathy would also be beneficial, as this is often the first sign of RVCL-S. To achieve this, optical coherence tomography angiography (OCT-A) is employed as a novel non-invasive method to visualise retinal layers with high resolution. Several important findings have already been made with this method that might have identified new biomarkers. For instance, the foveal avascular zone (FAZ), which is a zone in the centre of the retina that lacks vasculature, was found to be significantly larger in RVCL-S patients compared to controls. Furthermore, it was observed that the vascular density in the superficial capillary networks of the retina was considerably lower. This network involves the blood vessels from the ganglion- and nerve fibre layer (48). Together FAZ size and vascular density seem to reflect blood flow regulation.

Therefore, these markers appear to have the potential to be used as an early biomarker to detect dysfunctions in retinal blood flow regulation in RVCL-S patients. Besides, OCT-A seems to be a promising method to decipher more potential biomarkers. However, future research will have to unravel that.

Lastly, retinal layer thickness could also be considered as a future biomarker for RVCL-S. Earlier research already demonstrated retinal thinning in RVCL-S patients. For example, the thickness of the peripapillary retinal nerve fibre layer, containing the extensions of the optic nerve fibres, can potentially be used as a marker for retinal thinning (49). However, more research needs to be performed on the value of all proposed biomarkers.

4.3 Suggestions therapeutic targets

Due to the gap in knowledge on the underlying pathophysiological mechanisms, it has proved difficult to establish a curative treatment for RVCL-S. Something that we do know about RVCL-S is the associated genetic mutation. One therapy that would fit well with this is CRISPR-Cas9 gene editing. This type of gene therapy can be used to correct the C-terminal truncating mutation in the *TREX1* gene. However, gene therapy has proved to be challenging and is not available for RVCL-S as of yet (1).

Apart from gene editing, intervening in the underlying pathophysiological mechanisms could also offer a possibility of treating or curing RVCL-S in the future. For example, interfering with the hypothesised endothelial dysfunction. As mentioned in section 4.2, the endothelial markers VWF and angiopoietin-2 are proposed as possible candidates for early biomarkers of disease activity in RVCL-S. However, these markers may also potentially act as therapeutic targets (33). Previous studies have already looked at the effect of anti-VWF in thrombotic thrombocytopenic purpura and the functionality of antagonists of angiopoietin-2 in diabetes (50,51). As diabetes more resembles RVCL-S, this study will be discussed. To start, angiopoietins control vessel permeability, -remodelling, and -inflammation. In particular, angiopoietin-2 is involved in counteracting the anti-inflammatory effects of angiopoietin-1. In turn, angiopoietin-1 is an agonist ligand for the Tie2 receptor and implicated in maintaining quiescent endothelium. This seems to suggest that inhibiting angiopoietin-2 signalling or increasing the ratio between angiopoietin-1 and -2 may cause activated endothelium to revert to quiescent endothelium (50). Earlier studies demonstrated that if angiopoietin-2 signalling is inhibited, an antagonist such as angiopoietin-1 is used, another Tie2 agonist is employed, or if the ratio between angiopoietin-1 and -2 is high, this can help to prevent or delay the onset of vascular complications associated with diabetes (52–54). All in all, these findings appear to indicate that angiopoietin-2 could

be a potential therapeutic target for diseases in which endothelial dysfunction is implicated, as believed in RVCL-S. Hence, the effect of targeting angiopoietin-2, e.g. reducing the angiopoietin-2 levels and/or increasing angiopoietin-1 levels, could be tested in RVCL-S patients in the future to determine whether the onset of vascular complications is also counteracted or delayed.

Along with angiopoietins, other endothelial factors are also thought to be implicated in endothelial dysfunction, such as VEGF. As stated in sections 2.3.2 and 3.2.1, anti-VEGF has been trialled to treat retinal vasculopathy (1,25). As VEGF is known to be implicated in promoting endothelial leakage, anti-VEGF counteracts this. Nevertheless, anti-VEGF may also have a more systemic effect. In other words, VEGF may have the potential to become a therapeutic target to treat both retinal-, cerebral-, and systemic vasculopathy (25). This could be beneficial for RVCL-S patients, as all these forms of vasculopathy are believed to be involved (3). Thus, future work should test the potential usefulness of VEGF as therapeutic target in RVCL-S.

Lastly, as mentioned in 3.2.2.1, it is hypothesised that a dysfunction of the OST complex is integrated into the pathophysiological mechanisms of RVCL-S. This dysfunction would then be responsible for immune activation and autoantibody production (11). This knowledge gives the idea that targeting the OST complex, thus inhibiting OST complex dysfunction, could be a possible way of treating the inflammation in RVCL-S. This notion has already been supported by a study that deployed an OST inhibitor in both human lymphoblasts and knock-in mice with human V235fs mutation. In this study, it was observed that the immune defects were reversed (10). Nevertheless, this has not yet been trialled in RVCL-S patients. So, testing the effect of an OST inhibitor in RVCL-S patients is required to determine its effect in humans and the potential value as a therapeutic target.

Taken together, multiple possibilities have been proposed for therapeutic targets to treat RVCL-S. Future studies will have to reveal which of these could be of value for RVCL-S patients and whether perhaps a combination of these therapeutic targets would be beneficial.

Chapter 5: Conclusion

This review aimed to present the most pivotal findings related to the pathophysiological mechanisms underlying RVCL-S. Moreover, we aimed to reveal potential novel diagnostic markers and therapeutic targets. Based on prior findings, endothelial dysfunction seems to be the most plausible theory, which appears to lead to ischaemia. In addition, there have also been several studies that identified inflammation as a possible contributing mechanism. Knowledge on these underlying pathophysiological mechanisms is needed to gain insight into opportunities to improve diagnosis and to find a treatment for RVCL-S. Multiple suggestions for novel diagnostic markers and therapeutic targets are proposed based on previous studies. However, future studies will have to delve further into this to enable development of a curative treatment. Research into the pathophysiology of RVCL-S could be enhanced by the development of new disease models, such as an iPSC-derived endothelium model.

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