Title: Choroideremia: Molecular mechanisms and therapies

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Abstract

Choroideremia is a monogenic X-linked chorioretinal dystrophy, affecting the photoreceptors, retinal pigment epithelium and choroid, caused by mutations involving the *CHM* gene. Choroideremia is characterised by night blindness in early childhood, progressing to peripheral visual field loss and eventually leading to complete blindness from middle age. *CHM* encodes the ubiquitously expressed Rab escort protein 1 (REP1), which is responsible for prenylation of Rab proteins and is essential for intracellular trafficking of vesicles. In this

25 review, we explore the role of REP1 in the retina and newly discovered systemic
26 manifestations, and discuss the therapeutic strategies for tackling this disease, including the
27 outcomes from recent clinical trials.
28

Molecular mechanisms of choroideremia

Choroideremia (CHM; OMIM 303100) is an X-linked chorioretinal dystrophy affecting approximately 1 in 50-100,000 individuals and is characterised by the progressive degeneration of the photoreceptors, retinal pigment epithelium (RPE) and choroid. Typically, male patients present initially with night blindness in childhood, progressing to peripheral visual field loss and eventually leading to complete blindness later in life [1, 2] (Figure 1). Female carriers are typically asymptomatic but may experience night blindness and a unique speckling pattern can be detected on fundus autofluorescence imaging [3]. CHM is caused by mutations in the *CHM* gene, which is located on chromosome *Xq21.2* and encodes the 95 kDa ubiquitously expressed Rab escort protein 1 (REP1). To date, over 500 unique variants have been reported in the *CHM* gene (https://databases.lovd.nl/shared/genes/CHM accessed 12 January 2022) (Figure 1d). For in depth review of *CHM* genetics and clinical phenotype, refer to Mitsios et al 2018 [1].

REP1 plays an important role in post translational prenylation of Rab GTPases and is essential for intracellular trafficking of vesicles. Prenylation is the addition of one or more farnesyl or geranylgeranyl groups to the C-terminus of a protein by prenyl transferases. REP1 binds to and escorts unprenylated Rab proteins to Rab geranylgeranyl transferase (RabGGTase) to be prenylated, then transports the prenylated Rabs to target membranes (Figure 2a). In the absence of functional REP1, unprenylated Rabs accumulate in the cells [4].

The retina specific phenotype in CHM led to the discovery of REP2, which is encoded by the CHM-like (*CHML*) gene, shares 75% sequence homology to REP1 and is also ubiquitously

expressed [5]. The Rab protein, Rab27a, which is highly expressed in the RPE and choriocapillaris accumulates in CHM patient lymphoblasts and was found to be preferentially prenylated by REP1 [6]. Later studies revealed that Rab27a binds with the same affinity to REP1 and REP2, however the Rab27a-REP1 complex has a higher affinity for RabGGTaseII [7]. More recently, a hierarchy amongst Rabs was revealed, with Rab27a, Rab27b, Rab38 and Rab42 having the slowest prenylation rates [8]. Although the exact mechanisms by which under prenylation of certain Rabs leads to retinal degeneration is not fully understood, it is widely accepted that compensation of lack of REP activity by REP2 in cells other than the retina confines pathogenesis to the eye.

Is choroideremia a systemic disease?

With the ubiquitous expression of REP1, it has been postulated that CHM could have systemic manifestations. In an online survey carried out by Zhou and colleagues on 117 affected males, 53 carrier females and 20 unaffected males, a number of co-morbidities were particularly prevalent in CHM patients without functional vision, compared to patients with functional vision, in particular diabetes, hypertension and hypercholesterolemia, however this was not significant when corrected for age [9]. Zhang et al reported lymphocyte crystals and fatty acid abnormalities in plasma and red blood cells in a cohort of CHM patients and hypothesised that crystal deposition is due to dysregulation of fatty acid synthesis due to defects in vesicle trafficking as a result of REP1 mutations [10]. However, a later paper did not find any evidence of lymphocyte crystals or fatty acid alterations in CHM patients [11].

A recent paper ~~by our group~~ has provided further evidence that CHM may be a systemic condition, consistent with the ubiquitous expression of REP1 [12]. Whole metabolomic analysis of serum plasma from 25 CHM patients and 25 age- and sex- matched controls revealed a total of 85 compounds that were significantly different between patient and control groups, including sphingolipids and phospholipids. Sphingolipids are a large family of lipids, including sphingosine, ceramide and sphingosine-1-phosphate (S1P), that are metabolised via the action of many enzymes, and are involved in numerous cell processes such as cell growth, migration, adhesion, apoptosis and inflammation. Disruptions in sphingolipid metabolism has been implicated in neurodegenerative diseases, metabolic disorders, immune function and cancer [13]. S1P was significantly increased in CHM patient plasma [12]. The S1P pathway is disrupted in patients with Sjögren-Larsson syndrome (SLS), a systemic disease characterised by ichthyosis, mental retardation and spastic diplegia, caused by mutations in *ALDH3A2*, the gene encoding fatty aldehyde dehydrogenase (FALDH). FALDH is expressed in the retina, RPE and choroid and ocular defects have been found in SLS patients, including perifoveal crystalline inclusions, RPE atrophy, deficient macular pigment and retinal thinning [14]. FALDH requires prenylation for proper localisation and function, therefore ~~we proposed that~~ FALDH was proposed as a potential target for REP1 (Figure 2b). Further study is required to determine the interaction between REP1 and FALDH. The lipidomic changes observed in patient samples were also seen in the *chm*^{ru848} zebrafish model. Twelve compounds were found at differential levels between the chm and wt fish, which were also in the top 30 compounds in the human study [12].

Additionally, markers of oxidative stress were increased in the patient group and levels of antioxidants decreased [12]. Increased oxidative stress was also detected in the *chm*^{ru848}

zebrafish retina [15]. Oxidative stress has damaging affects on the cell, triggering endoplasmic reticulum stress, autophagy, DNA damage, mitochondrial dysfunction and lipid peroxidation, and has been linked to cardiovascular disease, cancer and neurodegenerative diseases [16, 17]. Systemic application of antioxidant treatments may therefore be beneficial for CHM patients.

Tryptophan metabolism was also enriched in the patient group, with significantly increased serotonin levels in CHM patient plasma [12]. ~~Preliminary data also indicates increased serotonin levels were also in the *chm*^{ru848} zebrafish (unpublished data).~~ Serotonin regulates sleep, behaviour and mood and increased serotonin levels may cause anxiety, muscle tremors, rapid heartbeat and high blood pressure [18]. As well as regulating brain function, serotonin regulates many biological processes including cardiovascular, endocrine, gastrointestinal and reproductive function and serotonin receptors are widely expressed throughout the body [19]. Serotonin is also produced in the photoreceptors as a precursor to melatonin [20]. One particularly interesting pathway where serotonin and REP1 overlap is serotonylation, a posttranslational modification where serotonin is added to GTPases. Serotonylation of Rab3a and Rab27a, a known target of REP1, regulates insulin secretion in pancreatic β-cells [21] (Figure 2c).

Haemoglobin synthesis and porphyrin metabolism pathways were also enriched, suggesting reduced liver cytochrome P450 (CYP450) activity, with lower levels of methylxanthines and steroids detected in patients [12]. CYP450 enzymes are essential for metabolism of many compounds, including cholesterol, steroids, methylxanthines, fatty acids and drugs [22] (Figure 2d). Diabetes has also been linked to a significant reduction in activity of the hepatic

CYP450 enzyme, CYP450 3A4 [23]. No differences in long chain fatty acids were observed in the CHM patients however branched and dicarboxylic fatty acids were reduced, indicative of impaired lipid oxidation in CHM patients. Further studies are required to determine the significance of these altered metabolites and investigate REP1 target enzymes.

In addition, the *Chm^{Flox}, Tyr-Cre+* mouse, a conditional knockout of the *Rep1* gene in pigmented RPE cells, showed signs of premature aging, with an accumulation of lipofuscin, uneven basal infoldings and extracellular basal deposits within 6 months, compared to age matched littermate controls [24]. These changes are not detected in the *ashen* mouse, a Rab27a mutant, indicating that the changes are a result of loss of prenylation of a Rep1 target protein, other than Rab27a. Although the authors suggested that this could be a result of dysfunction of another or multiple Rab proteins, it is also possible that the changes are a result of loss of prenylation of an alternative Rep1 target protein, involved in degradation pathways.

Therapies for choroideremia

There are currently no approved treatments for CHM, however a number of clinical trials are underway (Figure 3), and much progress has been made in recent years.

Gene therapy

With the recent success of voretigene neparovec (Luxturna) for Leber congenital amaurosis (LCA) caused by *RPE65* mutations, considerable research has gone into the development of a gene therapy for other monogenic inherited retinal dystrophies, including CHM. The most used vector for gene therapy is adeno-associated viral (AAV) vector, which can easily

accommodate the 1959 bp REP1 cDNA. The first phase 1/2 clinical trial (NCT01461213[I]) for CHM started in 2011 using the AAV2/2 vector encoding REP1, under the control of CAG promoter, in 14 CHM patients via subretinal injection. Follow up at two years revealed that patient's median visual acuity had improved by 4.5 letters in best corrected visual acuity (BCVA). However, two patients experienced severe adverse effects, one with retinal stretching caused by surgical complication and one with intraocular inflammation, which led to the development of an automated injection system [25-27]. Three more phase 2 clinical trials (NCT02671539[II] [28, 29], NCT02077361[III] [30] and NCT02553135[IV] [31]) using a higher dose of the same vector were completed, with similar outcomes. A phase 1/2 clinical trial with the AAV2 vector by Spark Therapeutics (NCT02341807[V]) failed to report any differences in visual acuity between injected and uninjected eyes at two years post-surgery [32].

There was promise with the commencement of a phase 3 multicentre randomised clinical trial by Biogen for timrepigene emparvovec (BIIB111/AAV2-REP1) (NCT03496012[VI]). However, in a recent update, Biogen reported that the study did not meet its primary endpoint of improvement of at least 15 letters in BCVA at 12 months post treatment (https://investors.biogen.com/news-releases/news-release-details/biogen-announces-topline-results-phase-3-gene-therapy-study).

An alternative approach is currently under investigation, with intravitreal injection. A phase 1/2 dose escalation clinical trial for intravitreal injection of an AAV capsid variant carrying transgene encoding codon-optimised *CHM* gene (4D-110) by 4D Molecular Therapeutics (4DMT), in collaboration with Roche (NCT04483440[VII]). However, Roche recently withdrew

funding as a result of their assessment of a change in the risk-benefit profile. 4DMT however stated that they have not changed their position and will be continuing with the clinical trial (https://4dmt.gcs-web.com/news-releases/news-release-details/4d-molecular-therapeutics-announces-rare-disease-ophthalmology).

Gene therapy is by far the most investigated and clinically progressed treatment under development for CHM. Although gene therapy showed early promising results and good safety profiles, concerns regarding efficacy and inflammation are raised. Whilst gene therapy clinical trials are still ongoing, investigation and development of other alternative therapies is required.

Nonsense suppression therapy

Approximately 30% of *CHM* mutations, are nonsense mutations resulting in a premature termination codon (PTC) [2], therefore an alternative approach for patients with these mutations is nonsense suppression therapy. During normal translation, when a PTC is encountered eukaryotic release factors bind to the A-site of the ribosome and translation is terminated. Nonsense suppression agents, or translational readthrough inducing drugs (TRIDs), bind to the ribosomal subunit and increase the ability of a near-cognate tRNA, which has 2 out of 3 complementary bases, to compete with eukaryotic release factors for binding to the A-site. A near cognate amino acid is added to the peptide chain, effectively allowing read-through of the PTC and leading to production of a full-length functional protein [33]. The small molecule drug, PTC124 (Ataluren), has shown promise in preclinical studies with the *chm*^{ru848} zebrafish model, which harbours a nonsense mutation, resulting in a premature UAA stop codon [15]. Zebrafish only possess one isoform of rep, which is

196 homologous to human REP1. Due to lack of a REP2 homologue, *chm*^{ru848} zebrafish display a
197 systemic phenotype, with widespread degeneration, including oedema, shortened body
198 length, smaller eyes and are embryonic lethal, surviving up to 4.8±1.2 days post fertilisation
199 (dpf). Treatment of *chm*^{ru848} fish with PTC124 increased survival to 10.1±1.6 dpf, significantly
200 reduced oedema and increased body length and eye diameter. Treatment also resulted in
201 marked improvement in retinal lamination and significantly reduced cell death. PTC124
202 dosing restored rep1 protein expression and prenylation activity [15].
203
204 Although significant rescue of phenotype is observed in the zebrafish model, these results
205 were not reflected in patient cells. Treatment with PTC124 did not restore REP1 protein
206 expression in *CHM*^{Y42*} fibroblasts, which has a premature UAG codon, although level of
207 unprenylated Rabs were reduced [15]. In p.K258* fibroblasts and iPSC-derived RPE, with a
208 premature UAA stop codon, PTC124 dosing did not restore REP1 protein expression or
209 function [34].
210
211 However, effectiveness of nonsense suppression therapy is limited by nonsense mediated
212 decay (NMD), the cells natural surveillance mechanism. Premature termination transcripts
213 that are more that 50-55 nucleotides upstream of the final exon-exon boundary are
214 degraded by NMD, however some transcripts escape NMD, making them available for
215 readthrough [35]. ~~We recently showed that~~ *CHM* mRNA expression is significantly reduced
216 in CHM patients, and does not correlate with mutation position, with 40% variability in *CHM*
217 mRNA expression between patients with the p.R239* mutation [36]. mRNA levels may
218 therefore be a useful predictor of readthrough efficiency, with higher transcript levels more
219 likely to respond better to nonsense suppression, as more substrate is available for

readthrough. NMD inhibitors in combination with nonsense suppression drugs may be beneficial in patients with low baseline mRNA levels. Treatment of CHM fibroblasts with caffeine, an NMD inhibitor, significantly increased expression of *CHM* transcripts [36]. Recently, treatment of fibroblasts from patients with Bardet-Biedl and Alström syndromes, with amlexanox, a drug with dual function of NMD inhibition and readthrough, was shown to restore protein expression, and ciliary function [37].

Antisense oligonucleotides

Another therapy that is being explored for CHM is antisense oligonucleotides (AON). AON are small RNA sequences that bind to cryptic splice sites in pre-mRNA, and interfere with splicing, such as exon skipping or redirecting splicing to restore the normal transcript. AON have shown success in both *in vitro* and *in vivo* models of LCA caused by the frequent deep intronic *CEP290* variant, c.2991 + 1655A > G [38-40]. ~~Promising results were reported from phase I clinical trials, with improved vision at 3 months and no adverse side effects [41].~~ However, in a recent update ProQR announced that the Phase 2/3 trial did not meet its primary endpoint of BCVA at 12 months (https://www.proqr.com/press-releases/proqr-announces-top-line-results-from-phase-23-illuminate-trial-of-sepofarsen-in-cep290-mediated-lca10). Garanto et al [42] generated an AON for *CHM* targeting the deep intronic splice mutation, c.315-4587 T>A, which results in the introduction of a 98 base pair pseudo exon and PTC. AON treatment in lymphoblast cells from two CHM patients corrected the mutation and redirected splicing, however expression of REP1 was not restored. As AON therapy is mutation dependent, and a common splice variant has not been reported for CHM, this may not be as widely applicable as other therapies described.

Statins versus fibrates

With lipid profiles in both human and zebrafish studies being disrupted, it is fair to assume that CHM patients may be taking lipid lowering drugs like statins. In the study by Zhou at al., of the CHM patients surveyed, there was a significantly higher number of patients without functional vision taking statins in comparison to those with functional vision, with the authors suggesting that statin use may have a negative effect on visual function in CHM patients [9]. Statins block the mevalonate pathway required for cholesterol synthesis; however, this pathway is also required for prenylation and REP1 function. Statins have previously been shown to inhibit Rab prenylation [43]. Whereas fibrates act independently of the mevalonate pathway to regulate lipid levels. Therefore, it was hypothesised that fibrates would be more beneficial for CHM patients over statins. The effects of statins vs fibrates was therefore tested in *chm*^{ru848} zebrafish. Treatment with both simvastatin and fenofibrate lowered cholesterol levels and increased survival by 2.3 and 3.3 days, respectively [12]. Fenofibrate treatment improved retinal lamination and lens structure, which was not observed in simvastatin treated fish, therefore fenofibrate may have higher therapeutic benefit in CHM patients with hyperlipidemia compared to statins, and further studies in patients would be beneficial.

Concluding remarks

Significant advances over the past decade have led to the development of gene therapies with a number entering into clinical trials in a relatively short time period. Early data suggested good safety profiles, however with disappointing results from the recent phase 3 clinical trial, questions are raised over the effectiveness of gene therapy for CHM (see outstanding questions box). Currently the primary endpoint for CHM clinical trials has been

set as an improvement in number of letters in BCVA, however this is known not to change over decades with well-preserved central acuity until late stages of the disease. In a recent study, investigating retinal changes in CHM patients over a 12 month period, visual acuity did not change in patients over a 12 month period [44]. The most useful parameters for monitoring disease progression were progressive loss of subfoveal choroidal thickness and areas of preserved choriocapillaris, ellipsoid zone using spectral domain optical coherence tomography (SD-OCT) and fundus autofluorescence (FAF) over a 12-month period. Disease progression also correlated with age, with slower progression in patients ≥50 years, despite their more advanced stage [44]. However, due to the high levels of inter- and intra- observer measurement variability, highly skilled graders and photographers are required [45]. Therefore, a multimodal approach is recommended for deep phenotyping of patients, including microperimetry, SD-OCT and FAF and a more appropriate endpoint for monitoring effectiveness of gene therapy is ability to slow progression of field loss. In addition, age of patients should be taken into consideration, when determining disease progression and response to treatments.

All clinical trials for CHM to date have utilised AAV2/2 vector however alternative serotypes may facilitate increased efficiency. Cereso et al. assayed a panel of AAV vector serotypes in iPSC-derived RPE cells from a CHM patient and showed that AAV2/5 was the most efficient [46]. Additionally, an intravitreal approach may be advantageous as it could theoretically treat the whole retina. Alternatively, non-viral gene therapy may be more appropriate to prevent inflammation and immune response. Despite the recent results from clinical trials, gene therapy is still the most promising therapeutic avenue for CHM and as we progress, research continues to improve transduction efficiencies and surgical techniques, and data

from ongoing clinical trials will inform the next steps. In addition, recent evidence suggests possible systemic manifestations, further highlighting the need for close monitoring of patients and natural history studies (see clinician's corner). *in vitro* and *in vivo* studies are required to investigate the extent and significance of these metabolic changes and identify potential therapeutic targets. One potential target is oxidative stress; N-acetylcysteine (NAC), an antioxidant, is currently in clinical trials for retinitis pigmentosa (NCT03063021[VIII]), with promising early results [47], and may be beneficial for CHM, particularly as it is administered orally and could therefore have systemic benefits.

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Resources

[I] https://clinicaltrials.gov/ct2/show/NCT01461213

[II] https://clinicaltrials.gov/ct2/show/NCT02671539

[III] https://clinicaltrials.gov/ct2/show/NCT02077361

[IV] https://clinicaltrials.gov/ct2/show/NCT02553135

[V] https://clinicaltrials.gov/ct2/show/NCT02341807

[VI] https://clinicaltrials.gov/ct2/show/NCT03496012

[VII] https://clinicaltrials.gov/ct2/show/NCT04483440

[VIII] https://clinicaltrials.gov/ct2/show/NCT03063021

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Glossary

Choroideremia: an X-linked chorioretinal dystrophy characterised by the progressive degeneration of photoreceptors, choroid and retinal pigment epithelium, caused by mutations in the *CHM* gene.

REP1: Rab escort protein 1 is an enzyme encoded by the *CHM* gene and is responsible for the prenylation of Rab proteins.

Prenylation: a post translational addition of one or more farnesyl or geranygeranyl groups to the C-terminus of a protein.

Lipofuscin: autofluorescent material that accumulates in the retina with ageing

Subretinal injection: drug is injected into the subretinal space between the retinal pigment epithelium and photoreceptors

Intravitreal injection: drug is injected into the vitreous, the gel-like fluid in the eye

Statins: a group of lipid lowering drugs that inhibit HMG-CoA reductase, the rate limiting enzyme of the mevalonate pathway

Fibrates: a group of drugs that reduce plasma triglyceride levels through activation of peroxisome proliferator activated receptor α (PPARα).

Clinician's corner

- A number of clinical trials for gene therapy for choroideremia have been undertaken and currently ongoing, however the most recent phase 3 clinical trial failed to meet primary and secondary endpoints.

- Alternative therapies are under investigation, including nonsense suppression and antisense oligonucleotides.
- Choroideremia may have potential systemic manifestations, in particular, related to lipid metabolism and oxidative stress.
- Natural history studies are required to determine rate of disease progression and identification of possible systemic phenotypes.

Figure legends

Figure 1: (A) Colour fundus image of the left retina of a 24 year old male with choroideremia showing extensive chorioretinal atrophy with a preserved central retinal island. (B) Corresponding fundus autofluorescence (AF) image showing the preserved retinal island with scalloped edges but abnormal speckled hyperAF, and surrounding loss of macular AF. (C) Spectral-domain optical coherence tomography (SD-OCT) showing boundaries of the preserved retinal island with extrafoveal loss of the ellipsoid zone, cystoid macula oedema and loss of the outer nuclear layer, together with choroidal and RPE atrophy in the same patient. (D) Pathogenic *CHM* variant types. Three hundred unique pathogenic variants are reported on LOVD: 51% are frameshift, 34% are nonsense and 8% are missense; 2% are in-frame deletions, 1% are in frame insertions and 1% are in frame indels. Others include variants where no protein is produced, silent changes and deep intronic; 2% are unknown. Data from https://databases.lovd.nl/shared/genes/CHM/graphs.

Figure 2: Proposed targets of REP1. (A) REP1 is involved in the prenylation of Rab proteins and is essential for intracellular trafficking of vesicles. We propose a number of potential

targets for REP1; (B) FALDH, which oxidises fatty aldehydes, a downstream product of S1P, requires prenylation for correct function; (C) Serotonylation of Rab27a, which is a known target of REP1, regulates insulin secretion in pancreatic β-cells and (D) Cytochrome P450 enzymes are also proposed targets of REP1, and metabolise a number of compounds including cholesterol, xanthines, fatty acids and steroids.

Figure 3: Gene therapy clinical trials for choroideremia. BCVA: best corrected visual acuity; SAE: serious adverse effects.