Elevated CSF protein in *POLG* related epilepsy: diagnostic and prognostic implications

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Summary

Objective: Epilepsy is common in individuals with mutations in *POLG*, the gene encoding the catalytic subunit of the mitochondrial DNA polymerase gamma. Early recognition and aggressive seizure management is crucial for patient survival. Disruption of the blood brain barrier (BBB) is implicated in various neurological disorders including those with epilepsy. The aim of this study was to assess whether *POLG*-related disease is associated with BBB dysfunction and what clinical implications this has for patients.

METHODS: Our retrospective study used data from 83 patients with pathogenic *POLG* mutations from four countries - Norway, Sweden, Finland, and the United Kingdom. Data were collected using a structured questionnaire. We used the presence of raised cerebrospinal fluid (CSF) protein and a raised CSF/serum ratio of albumin (Q-alb) to evaluate the integrity of the blood-CSF barrier.

RESULTS: Raised CSF protein was found in 70% of patients (*n*:58/83) and appeared associated with the most severe phenotypes. In those in whom it was measured, the Q-alb ratio was markedly elevated (*n*:18). The majority of those with epilepsy (*n*:50/66, 76%) had raised CSF protein and this preceded seizure debut in 75% (*n*:15/20). The median survival time from symptom onset for those with raised CSF protein was decreased (13 months) compared to those with normal CSF protein (32 months).

Significance: Our results indicate that there is disruption of the BBB in *POLG*-related disease as evidenced by a raised CSF protein and Q-Alb ratio. We also find that raised CSF protein is a common finding in patients with *POLG* disease. Our data suggest that the presence of BBB dysfunction predicts a poorer outcome and elevated CSF protein may therefore be an additional biomarker both for early diagnosis and to identify those at high risk of developing epilepsy.

Keywords: Blood- brain-barrier (BBB), CSF protein, CSF/serum ratio of albumin (Q-alb), epilepsy, mitochondria, *POLG*.

Key points:

- Epilepsy is very common in individuals with *POLG* disease and associated with high morbidity and mortality.

- Mutations in *POLG* are associated with progressive disruption of blood brain barrier.

- Raised CSF protein is commonly seen in patients with *POLG* disease, especially in those with severe phenotypes.

- CSF protein can be used as a biomarker to identify those with high risk to develop epilepsy.

INTRODUCTION

Mitochondria perform a number of important functions including a major role in energy metabolism. Oxidative phosphorylation (OXPHOS), the enzyme pathway responsible for ATP production, contains thirteen subunits encoded by mitochondrial DNA (mtDNA), while the remaining subunits, together with more than a thousand other proteins required for mitochondrial structure and function are encoded in the nuclear DNA (nDNA)[1]. The diagnosis of mitochondrial disorders remains challenging and currently, no effective treatments exist for the majority of these disorders.

POLG is a nuclear gene that encodes the catalytic subunit of DNA polymerase γ (Polγ), the enzyme responsible for mtDNA replication and repair [2]. Mutations in *POLG* cause disease that varies in severity from devastating infantile phenotypes such as Alpers syndrome and myocerebrohepatopathy spectrum (MCHS), to juvenile syndromes comprising epilepsy and ataxia [3-5] and to late onset myopathies with progressive external ophthalmoplegia (PEO) [6-7]. Epilepsy is particularly common in the early childhood and juvenile groups [3-4,8-10]; focal seizures, commonly evolving into bilateral convulsive seizures[11-12] are the most common seizure types in both adult and paediatric patients, with epileptiform discharges predominantly occurring over the occipital regions [13]. The majority of the patients develop therapy resistant epilepsy [9-10].

The blood-brain-barrier (BBB) is formed by a continuous layer of cerebral endothelial cells held together by tight junctions (Figure 1), separating the blood components from the brain microenvironment, regulating the entry and exit of ions, nutrients, macromolecules, and energy metabolites. The BBB is a part of the neurovascular unit that includes blood vessels, neurons, astrocytes, pericytes and microglia, and which is divided into several compartments; the BBB, the Blood-Cerebrospinal fluid (CSF) barrier, the arachnoid barrier, neuroependyma

(the fetal-CSF brain barrier) and adult ependyma (free exchange) [14]. Accumulating research suggests that disruption of the BBB contributes to the pathophysiology of various acute and chronic neurological disorders (Table 1) [15-19] .

OXPHOS deficiency in central nervous system microvasculature, including vascular smooth muscle cell layer and endothelial cells, has been reported previously [20-22]. Evidence of BBB dysfunction, with plasma protein extravasation, has been suggested by the findings of raised CSF protein [3], but only been demonstrated on post-mortem examination in a single patient with *POLG* disease [22].

Given that *POLG* related disease causes both a progressive encephalopathy and aggressive epilepsy, the aims of this study were to investigate whether BBB dysfunction occurs in *POLG* disease and, if so, what are the consequences for affected patients. Our rationale was that greater understanding of the mechanisms driving *POLG* related disease has the potential to provide us with both new biomarkers to facilitate earlier diagnosis and novel therapeutic targets.

PATIENTS AND METHODS

Study design and population:

We conducted a multinational, retrospective study of patients from nine centres in four European countries; Norway (Haukeland University Hospital, Oslo University Hospital, St. Olav's Hospital and University Hospital of Northern Norway); United Kingdom (Great Ormond Street Hospital, London); Sweden (Centre for Inherited Metabolic Diseases, Karolinska University Hospital and The Queen Silvia Children's Hospital, University of Gothenburg), and Finland (Children's Hospital, Helsinki University Hospital and Department of Children and Adolescents, Oulu University Hospital, Institute of Clinical Medicine,

University of Oulu). Patients from these centres with biallelic, pathogenic *POLG* mutations were included.

Patients were classified phenotypically into six groups; (a) Alpers syndrome, (b) myocerebrohepatopathy spectrum (MCHS), (c) myoclonic epilepsy, myopathy, sensory ataxia (MEMSA), (d) ataxia neuropathy spectrum (ANS), (e) autosomal dominant and autosomal recessive progressive external ophthalmoplegia. Classification was based on previously described criteria [6,23]. Patients who did not fulfil the clinical criteria for the above phenotypes were reported as (f) others. The study cohort as whole was also classified into two groups according to the presence or absence of epilepsy. Therapy resistant epilepsy was defined using the International League Against Epilepsy (ILAE) definition[24].

Ethical approval was obtained from the Regional Committee for Medical and Health Research Ethics, Western Norway (REK 2014/1783-4). Each participating centre also obtained approval from their local ethical committee. The study was registered as an audit at Great Ormond Street Hospital, London, United Kingdom.

Clinical and laboratory data:

Patient's data were collected using an electronic-Case Report Form (e-CRF). Clinical data included: age at onset of symptoms requiring medical evaluation, detailed medical history, phenotype, and survival status or date of last follow up.

Laboratory data included: hepatic aminotransferase, blood and CSF lactate, muscle respiratory chain enzyme activities, muscle histology findings and genetic findings. CSF protein and/or albumin values at disease onset and during the disease course were recorded. Since the reference range for CSF protein may be age dependent, we have looked at the values according to age[19,25-26] and the cut-off values used in this study are reported in supplementary file 1. CSF samples artificially contaminated with blood as a complication of lumbar puncture

were not included and none of the individuals included in this study had clinical or laboratory evidence of meningitis or encephalitis.

Since albumin is produced exclusively in the liver, all albumin detected in CSF originates, by definition, from blood. The level of CSF albumin provides, therefore, a parameter with which to evaluate the permeability of the blood-CSF barrier. The ratio of CSF to serum albumin (Q-alb) corrects for the individual's albumin level and provides a reflection of the diffusion gradient of albumin [27-28]. This ratio was calculated as CSF albumin (milligram (mg)/L)/ serum albumin (g/L). The reference range for Q-alb is age dependent, the cut-offs used in this study were reported in supplementary file2 [19,27-29].

Statistical analysis:

Data were analysed using SPSS (Statistical Package of Social Sciences), Version 23.0. Results were considered statistically significance with a two-sided P value of less than 0.05. Survival was defined by the end-point - time to death, which was defined as the interval in months from the onset of symptom to the date of death. A comparison of survival time between clinical categories was performed using log-rank test (Kaplan Meier).

RESULTS:

Demography:

Eighty-three patients, (39 male, 44 female) were identified. Fifty patients were diagnosed in Norway, nineteen in Sweden, nine in the United Kingdom and five in Finland. Median age of onset for the group as a whole was 9 months (range 3 weeks – 67 years). The majority of patients were Northern European (*n*: 78), with three patients from Iraq and two from Cyprus.

Phenotype spectrum:

Thirty-seven patients fulfilled diagnostic criteria for Alpers, thirty for MEMSA, eight for ANS, three for PEO, two for MCHS. Three patients did not fulfil the diagnostic criteria for the above phenotypes and were classified as “others” (one with a Leigh-like phenotype, one with a mitochondrial neurogastrointestinal encephalopathy (MNGIE)-like phenotype, and one with epilepsy and transient mild elevation in liver function tests) (Table 2).

Laboratory findings:

Laboratory investigations (Table 3) revealed that 59% (*n:* 42/71) had elevated serum lactate, 61% (*n*: 27/54) had elevated CSF lactate and 65% (*n*: 54/82) had elevated hepatic aminotransferase levels. Muscle biopsy and respiratory chain analysis showed that 53% (*n*:35/66) had abnormal histological findings, defined by the presence of ragged red fibres and/or COX-negative fibres, and 47% (*n*: 14/30) had abnormal respiratory chain activities; the majority (71%, *n*:10/14) were in individuals with raised CSF protein / albumin.

Raised CSF protein and/or albumin was found in 70% (*n*:58/83) at some point during the disease course and in 65% (*n*:48/73) it was present at disease onset. Seventy-one percent (*n*:30/42) had abnormal CSF protein and/or albumin measured later in the disease course. The level of CSF protein increased with disease duration; a median value of 0.83 g/L (range 0.53 - 6.00) at disease onset rose to 1.04 g/L (range 0.74 - 4.24) later. In 12 patients, longitudinal data showed that CSF protein increased with time; median CSF protein at onset was 0.94 g/L (range 0.57-2.5) rising to 1.18 (range 0.8-4.2) later in the disease. The frequency of abnormal elevated CSF protein according to each specific phenotype is provided in table 2.

CSF protein and epilepsy:

Eighty percent (*n*: 66/83) of the study cohort had epilepsy (focal onset, focal to bilateral tonic-conic, generalized onset, myoclonic, epilepsia partialis continua and status epilepticus) and in 76% (*n*: 50/66) of these, CSF protein was raised regardless of seizure type. Median total CSF

protein for those with status epilepticus was 1.04 g/l (range 0.8 - 2.6). Further analysis showed that 75% (*n*: 15/20) of those who developed epilepsy later in the disease course had raised CSF protein at disease onset and that the majority of those with abnormal raised CSF protein, 85 % (*n*: 45/53), had therapy resistant epilepsy.

Evidence of Blood-CSF barrier dysfunction:

Serum albumin and CSF albumin values taken simultaneously were available for 18 patients allowing calculation of the Q-alb ratio. All cases showed a markedly raised Q-alb ratio with median value 21.5 x 10^{-3} (range 14.2 x10^{-3} - 56.8x 10^{-3}).

Genetics findings:

Pathogenic *POLG* variants for each case were identified either by targeted mutation analysis for specific common mutations (c.1399G>C, p.Ala467Thr and c.2243G>C, p.Trp748Ser) or by sequence analysis of all coding regions of *POLG*. Further analysis showed that there was no clear association between specific genotype and raised CSF protein.

Survival analysis:

Median survival time from symptom onset for those with normal CSF protein was 32 months, compared to 13 months for those with abnormal CSF protein. Although not statistically significant (*P*= 0.08), Kaplan Meier curve analysis showed a clear trend of worse survival for those with abnormal CSF protein (supplementary file 3).

DISCUSSION

Our study evaluated the question of whether *POLG* disease is associated with BBB dysfunction as had been suggested by a previous histological study [22]. Using clinical and laboratory data from 83 individuals, we analysed the frequency of abnormally raised CSF

protein and evaluated the integrity of the brain barrier by measuring CSF/serum ratio of albumin (Q-alb) [27-28]. We found that more than two thirds of patients (70%) had raised CSF protein and/or albumin. The CSF/serum ratio of albumin (Q-alb) was markedly raised in the 18 patients in whom this was measured (median value of 21.5 x 10^{-3}), indicating a major dysfunction in the BBB. When compared with other disorders in which BBB dysfunction occurs, the Q-alb ratio was among the highest recorded suggesting that *POLG*-related disease profoundly affects BBB integrity (Table 1). Further, our results showed that there was a progressive deterioration in the BBB integrity as both the proportion of those with abnormal raised CSF protein and the actual CSF protein value increased with time.

When we compared the proportion of individuals with abnormal CSF protein and/or albumin to those having abnormalities of more commonly used biomarkers, we found that the proportion of those with elevated protein/albumin was higher (Table 3). This suggests that elevated CSF protein has a higher diagnostic sensitivity for *POLG*-related disease than lactate, or indeed the other commonly used biomarkers.

The presence of raised CSF protein was particularly common in patients with early onset, severe disease phenotypes e.g. Alpers, MCHS and MEMSA phenotypes (Table 2). Indeed, the median survival time from symptom onset for those with raised CSF protein was markedly decreased (13 months) compared to those with normal CSF protein (32 months). Elevated CSF protein showed a clear association with disease severity and with the presence of epilepsy, another major prognostic factor. Our analysis showed that 76% of *POLG* patients with epilepsy had raised CSF protein irrespective of seizure type, i.e. since patients with focal seizures also develop status epilepticus, it is not possible from our data to assess whether one seizure type was associated with higher CSF protein. Interestingly, the presence of elevated CSF protein and thus the possibility of BBB dysfunction, preceded the onset of the seizures (*n:* 15/20). Our study demonstrates therefore, not only the diagnostic relevance of measuring

CSF protein, but also its clinical importance as a biomarker in the early identification of patients with a high risk of developing epilepsy.

The relationship between BBB disruption and epilepsy has been described both in animal models and in patients [30-34]. Large molecular proteins such as albumin that cross an impaired BBB can be taken up by neurons or astrocytes; uptake into neurons occurs by unknown mechanisms, but entry into astrocytes is via the TGF-β receptor [35]. Uptake is followed by down-regulation of extracellular potassium buffering capacity [36], which facilitates N-methyl-D-aspartate receptor mediated neuronal hyperexcitability [34,37], and the release of proinflammatory cytokines [35,38-39]. All of these mechanisms may either lead to seizures or lower the threshold for seizure initiation [30,34,40]. In addition, manipulating mitochondrial function *in vitro* by inhibition complex I with rotenone, impairing ATP production using carbonyl cyanide 4- (trifluoromethoxy) phenylhydrazone (FCCP) or inhibiting complex V with oligomycin, can also produce rapid changes in BBB permeability [41]. Further, the same study demonstrated that inhibition of mitochondrial respiratory chain activity in mice, by epidural administration of rotenone, impaired BBB integrity and was associated with marked increase in BBB permeability [41].

Based on our findings and work by others [30,34-35,41], we suggest a potential mechanism; *POLG* mutations induce a mitochondrial dysfunction that leads to disruption of the BBB tight junctions and leakage of proteins such as albumin. Uptake of proteins by astrocytes and neurons initiates a cascade of events that contribute to seizure development (Figure 1) and subsequent worsening of the BBB dysfunction. Further, we believe that the raised CSF protein is itself evidence of impaired BBB integrity and a valuable biomarker that can identify those at high risk of developing epilepsy and, by extrapolation, those with a poor prognosis. Based on our findings, we recommend that CSF protein and Q-alb status are measured in all patients presenting with undiagnosed encephalopathy, and that those with *POLG* related

disease with raised CSF protein are closely monitored with frequent EEG recordings to facilitate early recognition and immediate seizure treatment.

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Ethical Publication Statement: All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. The ethical approved for the study was obtained from the Regional Committee for Medical and Health Research Ethics, Western Norway (REK 2014/1783-4). Each participating country had obtained approval by the local ethical committee. The study was registered as an audit at Great Ormond Street Hospital, London, UK (Registration Number 1675). We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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Figure legend:

Figure 1. Schematic representaion of events that we hypothesise are involved in blood brain barrier dysfunction and epilepsy in individuals with *POLG* disease.

POLG mutations induce a mitochondrial dysfunction that leads to disruption of the BBB tight junctions and leakage of proteins such as albumin. Uptake of proteins by astrocytes and neurons initiates a cascade of events (down-regulation of extracellular potassium buffering

capacity, N-methyl-D-aspartate receptor mediated neuronal hyperexcitability and the release of proinflammatory cytokines) that contribute to seizure development.

Table 1**.** Neurological conditions associated with raised CSF protein / Q-albumin ratio

Neurological disorder (reference)	**Q-albumin X10^{-3} median (Range)**
Multipe sclerosis[15]	
Pattern I	4.8(3.0-10.8)
Pattern II	7.3(2.1-33.9)
Pattern III	9.0(3.3-20.7)

Alzheimer [16] Early onset Late onset	 5.4 (4.1-7.7) 6.3(4.9-8.2)
Amyotrophic lateral sclerosis[17]	7 (3–15)
Parkinson[16]	7.4 (5.6-10.5)
Viral meningitis[18]	9.1 (7.2-14.7)
Ischaemic stroke[19] Territorial Lacunar	 11.1 (7.8–14.4) 9.7 (7.9–18.5)
Guillain-Barre syndrome[17]	16 (9–55)
Lyme disease[18]	17.2 (9.7-28.4)
Bacterial meningitis[17]	18 (6–69)
POLG related disorders*	21.5 (14,2-56,8)

* study data

Table 2. Percentages of normal and abnormal CSF total protein according to *POLG* related phenotypes.

Phenotype	**Abnormal CSF protein *n* (%)**	**Normal CSF protein *n*(%)**	**Total**
Alpers	30 (81%)	7(19%)	37
MCHS	2 (100%)	0(0%)	2
MEMSA	20 (67%)	10(33%)	30

ANS	4(50%)	4(50%)	8
PEO	1(33%)	2(67%)	3
Other	1(33%)	2(67%)	3
Total no.(%)	58 (70%)	25(30%)	83

Table 3. Major laboratory findings in patients with *POLG*-related diseases.

Laboratory test	**Percentage (%)**
Elevated blood lactate	59% (*n*:42/71)
Elevated hepatic aminotransferase	65% (*n*:54/82)
Elevated CSF lactate	61% (*n*:27/54)
Elevated CSF protein	70% (*n*:58/83)

Muscle pathology (RRF, COX-)	53% (*n*:35/66)
Abnormal RC activities	47% (*n*:14/30)

CSF: Cerebrospinal fluid, RC: respiratory chain, RRF: ragged red fibres.

Supplementary files:

- Supplementary file 1**:** Age-dependent normal cut-off value of CSF total protein and CSF total protein values in individuals with *POLG* disease.

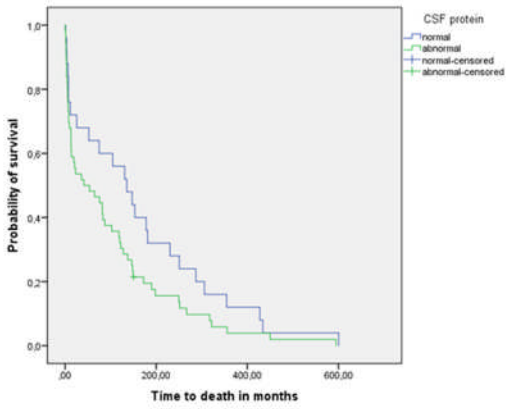
CSF: Cerebral spinal fluid, TP: Total protein, single patient *.

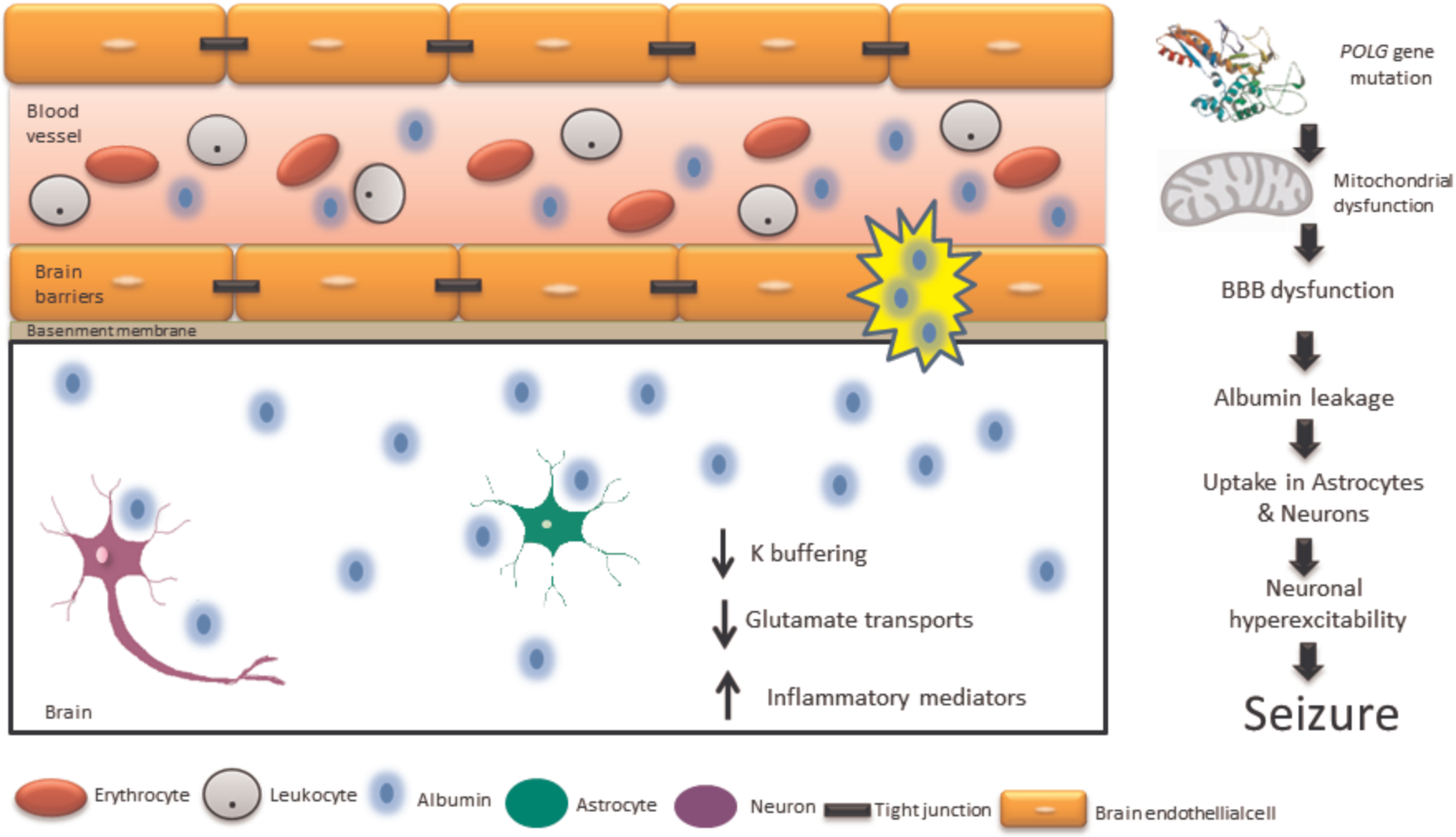
- Supplementary file 2: Reference intervals of normal Q-alb values according to the age.

Age, years	Normal CSF TP g/L	Median (Range) CSF TP g/L individuals with *POLG* disease At disease onset	Median (Range) CSF TP g/L individuals with *POLG* disease Later during disease course
< 15	< 0.35	0.82(0.53-2,9)	0.95(0.8-1.65)
16-60	< 0.45	0.78(0.61-2.5)	1.84(0.8-2.6)
>60	< 0.46	0.75*	4.24*

Age (years)	**CSF/serum ration of albumin (Q-alb)x 10 $^{-3}$**
<1	<28
1-15	4-5
15-60	5-8
60-90	8-10

- Supplementary file 3**:** Survival analysis**:**
  Kaplan Meier curve analysis showed a clear trend of worse survival for those with abnormal CSF protein




Blood vessel
Brain barriers
Basenment membrane
Brain
K buffering
Glutamate transports
Inflammatory mediators
POLG gene mutation
Mitochondrial dysfunction
BBB dysfunction
Albumin leakage
Uptake in Astrocytes & Neurons
Neuronal hyperexcitability
Seizure
Erythrocyte
Leukocyte
Albumin
Astrocyte
Neuron
Tight junction
Brain endothellialcell