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Skogholt's disease—A tauopathy precipitated by iron and copper?

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ABSTRACT

Background: It has been suggested that Skogholt's disease is a new neurological disease entity. The disease, confined to a family line in Hedmark county, Norway, usually affects both the brain and peripheral nerves. Typical findings are white matter lesions in the brain, myelin damage in peripheral nerves, and discolored cerebrospinal fluid with high concentrations of protein, copper, and iron. Little is known about the natural progression of the disease and its underlying cause, but the high level of copper and iron in the cerebrospinal fluid may cause or exacerbate inflammation in the central nervous system.
Methods: The present clinical study further explores the disease progression with clinical chemistry analyses and mass spectrometry of blood and cerebrospinal fluid (CSF) from patients and controls. Findings are corroborated with cognitive assessments.
Results: Pathological changes in CSF with low amyloid-β_{42} and high levels of tau proteins, total protein, copper, and iron, were discovered among Skogholt patients. The Montreal Cognitive Assessment identified 36 % of the patients as below normal range, while most patients performed slower than the norm mean time on the Trail Making Test. Mini-Mental Status Examination disclosed only minor deviations.
Conclusion: The findings in the present study strengthen our initial suggestion that Skogholt's disease most likely is a new neurological disorder and provide new clues to its cause: The disease may belong to the family of neurodegenerative disorders termed tauopathies. The increased level of copper and iron may contribute to neuroinflammation as these metals also have been associated with other neurodegenerative disorders. Although the causes of neurodegenerative disorders are currently largely unknown, studies on rare disease entities, such as the present one, may increase the understanding of neurodegeneration in general.

1. Introduction

Neurodegenerative disorders such as Alzheimer's (AD) and Parkinson's disease make up a substantial part of the neurological global burden of disease [1]. Despite decades of research, the knowledge of neurodegenerative mechanisms is limited [2] and sometimes disputed [3]. Studies on rare diseases may contribute to the understanding of neurodegeneration [4,5].

A local community physician in the southeastern region of Norway, Jon Skogholt, identified in the 1980s a family line with several cases of what then appeared to be a new dys-myelinating disorder [6], later referred to as Skogholt's disease [7]. The condition affects both the central and peripheral nervous system. It is characterized clinically by slowly progressing distal sensory loss, distal muscle weakness, unsteady gait, and dysarthria, as well as some complaints of reduced cognitive ability [6]. Brain imaging with CT and MRI scans have demonstrated white matter lesions in many of the patients [6]. Laboratory investigations of blood and cerebrospinal fluid (CSF) have revealed greatly elevated levels of total protein in CSF, indicating a defect in the blood-brain barrier (BBB) or the blood-CSF barrier (BCB). A genetic cause has been assumed since the disease seemingly only appears in the maternal line, but neither mitochondrial DNA sequencing nor coupling analysis have revealed any causal mutations [8,9]. Trace element analysis in the CSF has shown increased levels of copper and iron [7].

As these two metals are physiologically important redox-active compounds, it is tempting to assume that one or both is central to the

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disease initiation and/or propagation in Skogholt patients [10]. Disturbed homeostasis in copper and iron metabolism have already been implicated in several neurodegenerative disorders [11,12]. Free copper and iron can catalyze formation of reactive oxygen species, and thus cause oxidative stress and inflammation.

However, knowledge of pathogenesis and progression in Skogholt's disease is still meager and clinical findings are unspecific; more detailed analyses are needed. The aim of the present study is to expand upon previous findings of increased CSF metals in Skogholt's disease using a wider array of laboratory tests including analyses of CSF levels of tau proteins and amyloid-β_{42} ($A\beta_{42}$), as well as a thorough cognitive screening.

2. Materials and methods

2.1. Subjects

Patient characteristics have been described in previous studies [6–10]. The present investigation includes 11 Skogholt patients and 14 controls (Table 1).

During 2017 and 2018, the Skogholt patients were invited to a two-day neurological workup at the Department of Neurology at Innlandet Hospital Trust, Norway. This included sampling of blood and CSF, and a cognitive evaluation by an occupational therapist. Self-reported disease duration at the time of examination ranged from 8 to 22 years with a median (IQR) of 19 (14, 20) years.

Since lumbar puncture is rarely justified for healthy individuals, the controls in this study had to be recruited from among neurological patients already scheduled for the procedure. This resulted in a number of different diagnoses in the control group: Cerebral small vessel disease, cervical spinal stenosis, chronic intractable pain, clinically isolated syndrome (cervical myelitis), cranial neuralgia, frontotemporal dementia, hereditary spastic paraparesis, hydrocephalus (unspecified), migraine with aura (and white matter lesions), normal pressure hydrocephalus, normal pressure hydrocephalus and cerebrovascular disease, mild organic cognitive impairment (possible early AD), polyneuropathic paraplegia (possibly paraneoplastic), and polyneuropathy.

2.2. Ethics

Approval of the study protocol was obtained by the Regional Committee for Medical and Health Research Ethics, Region South-East, Norway, ref. no. 556-04224 plus 2013/1017. The study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients before enrollment. The study participants were allowed to withdraw at any time.

Table 1
Baseline demographical characteristics.

Characteristic[a]		Skogholt *(n=11)*	Control *(n=14)*	p-values[f]
Sex:	Female	6 (55 %)	11 (79 %)	.39
	Male	5 (45 %)	3 (21 %)	
Age *(y)*		57 (45, 67)	64 (56, 70)	.29
Coffee[b] *(cups/d)*		3.0 (2.2, 4.2)	2.5 (<1, 4.0)	.28
Smoking[c] *(pky)*		9 (4, 29)	<1 (0, 22)	.10
Alcohol[d] *(u/m)*		9 (3, 14)	3 (1, 6)	.21
Exercise[e] *(h/w)*		≥3 (1–2, ≥3)	1–2 (1–2, ≥3)	.65
Education *(y)*		9 (8, 11)	12 (12, 16)	.005

[a] Statistics presented: n (%), median (IQR).
[b] Coffee or caffeine equivalent beverage.
[c] 1 pack year (pky) = 20 cigarettes per day for one year.
[d] Alcohol consumption in Norwegian units per month.
[e] Hours per week.
[f] P-values from Fisher's exact test and Mann-Whitney *U* tests.

2.3. Sampling of blood and cerebrospinal fluid

Blood samples were obtained from a peripheral vein using standard needles with vacutainers. Samples for trace element analysis were drawn into heparinized vacutainer tubes certified for this use (Becton, Dickinson & Co.) and poured directly into ultraclean vials (vwr.com art. no. 525-04061). Cerebrospinal fluid was obtained by lumbar puncture with Spinocan 0.55–0.90 × 88 mm needles and collected in one standard polystyrene universal container, a polypropylene NUNC-cryo-vial for Aβ and tau protein analyses and two ultraclean polypropylene vials for trace element analyses. Polypropylene vials have the advantage of minimizing loss of analytes through adsorption to the vial wall.

Blood and CSF sampling of the Skogholt patients was done early on the second day after hospitalization while fasting. The control group was not fasting, and sampling were completed about two hours later in the day. Blood samples were collected as soon as possible after completed CSF sampling. All samples from both controls and cases were prepared for immediate analysis or long-time storage within about one hour after blood sampling.

2.4. Clinical chemical analyses

Routine methods at the Department of Medical Biochemistry, Innlandet Hospital Trust, Norway, were used for clinical chemical analyses. Total protein values in blood, serum, and CSF were quantified colorimetrically [7] and fractionated by isoelectric focusing. Concentrations of albumin and IgG were determined by immunonephelometry on a BN ProSpec analyzer (Siemens).

$A\beta_{42}$, total tau (T-tau) and phospho-tau (P-tau) were determined using the ELISA technique with Innotest kit (Innogenetics, Ghent, Belgium) at Akershus University Hospital, Norway. The laboratory is a part of the Alzheimer's Association QC program for CSF biomarkers. The cut-off values for abnormal CSF were $A\beta_{42}$ <700 ng/L, P-tau >80 ng/L and T-tau >300 ng/L for patients below 50 years; >450 ng/L for patients aged 50–69 years; and >500 ng/L for patients aged above 70 years [13].

2.5. Trace element analysis

CSF and whole blood samples of approximately 1700 mg and 700 mg were digested with 0.5 mL and 1 mL nitric acid, respectively, and diluted with ultrapure water. The samples were digested using a high-performance microwave reactor (UltraCLAVE, Milestone, Italy) before multielement analysis using high-resolution inductively coupled plasma mass spectrometry (HR-ICP-MS, Thermo Finnigan Element 2, Thermo Finnigan, Bremen, Germany), as previously described by Gellein et al., [7]. The accuracy was checked by analyzing Seronorm Serum Level 1 and 2 reference material (Sero, Norway), showing values within 85–115 % of the certified concentrations. The precision was checked by repetitive analyses of the same sera, showing coefficients of variation lower than 10 %.

2.6. Cognitive evaluation

For cognitive evaluation we used verbal fluency tests [14], the Norwegian Revised Mini-Mental State Examination (MMSE-NR3) [15,16], the Clock Drawing Test (KT-NR3) [17,18], the Montreal Cognitive Assessment (MoCA) [19] and Trail making tests A and B [20]. Difficulties in activities of daily life was assessed through questionnaires [21].

2.7. Statistics

The statistical package R (version 4.0.3) was used for all calculations and statistical graphics [22–24]. Central tendency and dispersion were described by the mean and standard deviation (SD), and the median and interquartile range (IQR). Differences between the groups were tested

using Student's two-tailed two-sample *t*-test as well as Mann-Whitney *U* test and Yuens *t*-test of trimmed means because some variables had extreme outliers or non-normal distributions according to Shapiro-Wilk normality testing. At $p < 0.05$ differences were judged significant.

3. Results

3.1. Laboratory findings in CSF

The mean CSF levels of total protein and albumin in Skogholt patients was elevated by factors of 3.5 and 3.4 respectively, compared to controls. All successfully sampled CSF samples from Skogholt patients were visually clear, but with a distinct yellow color compared to the normal clear and colorless appearance (Fig. 1). Spectrophotometry on Skogholt samples revealed a broad peak with maximum absorbance in the 406−410 nm range, whereas the bilirubin absorbance at 476 nm was minute and likely within normal range considering the total protein concentration (Supplemental material, Fig. 1b–d).

CSF concentrations of markers of neurodegeneration are shown in Fig. 2. All Skogholt patients had tau levels more than twice the upper limit of the normal reference range and most had $A\beta_{42}$ levels below the normal range [13]. The differences were statistically significant as shown in Table 2. Note that $A\beta_{42}$ levels were significantly different *only* when outliers were removed; as with Yuen's *t*-test on trimmed means ($p < 0.001$); or with Mann-Whitney *U* test when control patients with $A\beta_{42} < 700$ ng/L, T-tau > 500 ng/L, or P-tau > 80 ng/L were excluded ($p = 0.017$).

Cell counts (leucocytes) in CSF from patients and controls were all normal and below the detection limit of the instrument ($<5{\cdot}10^6$/L) except for one control who had pleocytosis with 68 cells per μL and turned out to have clinically isolated syndrome (cervical myelitis) suggestive of demyelinating disease. CSF protein separation by isoelectric focusing did not show monoclonal or oligo-clonal bands in Skogholt patients.

Analysis with HR-ICP-MS showed that iron, copper, selenium, sulfur, zinc and manganese were the elements in CSF with the most marked differences between Skogholt patients and controls (Table 3). These were also the only elements (out of the 56 elements determined) that remained significantly different in cases compared with controls after Bonferroni multiple testing correction of the *p*-values. Adjusted *p*-values from the Mann-Whitney *U* tests were .016 for iron, copper, selenium, sulfur, and zinc, and .039 for manganese, while adjusted *p*-values from t-tests for the same elements were <.001 except for manganese with $p = .037$.

Fig. 1. Fresh sample of discolored cerebrospinal fluid from a Skogholt patient (left) compared to the normal appearance (right).

Table 3 shows that the CSF levels of iron, copper, and selenium were increased in the patients compared with controls by a factor of 8.5, 5.1 and 4.9 respectively. The concentrations of sulfur, zinc, and manganese were also increased by a factor of 3.2, 2.8 and 2.7, respectively. Only small or non-significant differences were seen for the other elements determined. Borderline significance was seen for sodium, rubidium, phosphorus, silicon, magnesium, calcium, potassium, cesium, and mercury. Mean mercury levels in Skogholt patients was 63 % of the control value, but this difference was not significant after Bonferroni correction for multiple testing.

When the element concentrations in CSF were expressed relative to the total protein content (Fig. 3), the differences between the cases and controls decreased. However, the relative iron and copper levels remained convincingly high in cases, but for sulfur and zinc the apparent excess was reversed showing a markedly lower relative element concentration. When expressed as element/albumin ratio the values for copper, iron, and sulfur were increased in cases compared with controls.

3.2. Laboratory findings in blood

In Skogholt patients, the levels of total cholesterol, LDL, triglycerides, and HbA1c were in the upper part of the physiological range, or marginally increased (Table 2). Even though they were fasting, their triglyceride and HbA1c levels were significantly higher than in the control group. Other routine clinical chemical analyses, including hemoglobin and CRP, were usually within the reference interval, and did not show any significant differences between the groups.

Apart from a marginal increase in sulfur concentrations in the blood of Skogholt patients (Table 4), probably due to slight dehydration, trace element levels were not significantly different from the controls after Bonferroni correction (not shown).

3.3. Cognitive evaluation

All Skogholt patients reported at least one subjective cognitive difficulty. As many as 36 % experienced some difficulty either in finding words or presenting normal rate of speaking, 73 % reported that they were thinking more slowly than previously, while 64 % reported some memory problems. Phonemic and categorical verbal fluency testing with the FAS and Animal Naming tests did not show any significant deviation from education and age stratified norm-data [14]. Cognitive evaluation using the Norwegian Revised Mini-Mental State Examination (MMSE-NR3) [15,16] was usually in the normal range (≥28), with a median score of 29 and IQR from 28 to 30. Mental arithmetic contributed the most to decreased MMSE-scores. Only two cases had scores below the normal range.

The results from Montreal Cognitive Assessment (MoCA) showed greater variation in test-scores with a median education-adjusted score of 26 and IQR from 24.5 to 29. Four cases scored below the suggested normal range (≥26) [19]. When adjusted against norm-data [19], the MoCA-scores showed that 64 % of the patients had negative z-scores; their median z-score was -0.62 with IQR from -1.30 to 0.74. Delayed recall contributed the most to decrease in raw MoCA-scores, but only mental arithmetic ($p = 0.056$) and abstraction ($p = 0.038$) deviated substantially from the norm-data (Fig. 4).

The Trail Making Tests showed that median (IQR) time in seconds to completion was 37 (33–45.5) for TMT-A and 155 (83.5–189) for TMT-B. Only one case was above the normal range (≤ 60) on TMT-A, and four were above the normal range (≤ 170) on TMT-B [20]. Standardizing the scores with norm-data [25] gave median (IQR) z-scores of -0.30 (-1.04 to 0.09) for TMT-A and -3.17 (-3.85 to -1.19) for TMT-B (negative z-scores

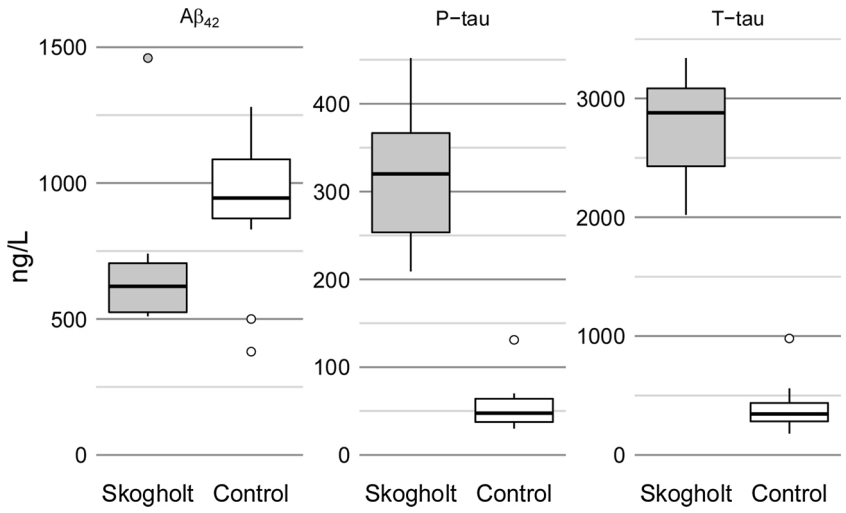

Fig. 2. CSF biomarkers of neurodegeneration. Boxplots of amyloid-β_{42}, phosphorylated tau and total tau in cerebrospinal fluid (CSF) from Skogholt patients and controls. Shaded areas of the scales represent normal ranges. Boxes represent the interquartile range (IQR, 1st to 3rd quartile), horizontal lines are medians (2nd quartile) while vertical whiskers represent most extreme observation within 1.5 × IQR from the box.

Table 2
Clinical chemical analyses in CSF and blood.

	Mean (SD)		Median (IQR)			
Analytes[a] *(normal range)*	Skogholt *(n = 11)*	Control *(n = 14)*	Skogholt *(n = 11)*	Control *(n = 14)*	*p*-value[b]	Ratio[c]
CSF-Total protein *(0.15–0.45 g/L)*	1.57 (0.33)[8]	0.45 (0.22)	1.57 (1.34, 1.83)[8]	0.36 (0.32, 0.53)	<.001	3.5
CSF-Albumin *(mg/L)*	985 (239)[8]	292 (159)	982 (830, 1173)[8]	238 (190, 368)	<.001	3.4
CSF/S-Albumin ratio *(0–10)*	23.9 (5.7)[8]	7.2 (3.5)	25.8 (17.7, 28.4)[8]	6.4 (4.6, 9)	<.001	3.3
CSF- $A\beta_{42}$ *(700–1200 ng/L)*	721 (337)[7]	936 (253)	620 (525, 705)[7]	945 (870, 1088)	.09	0.8
CSF-Total Tau *(0–450 ng/L)*	2753 (476)[7]	396 (203)	2880 (2430, 3085)[7]	345 (283, 438)	<.001	6.9
CSF-Phospho Tau *(<80 ng/L)*	317 (88)[7]	54 (26)	320 (254, 367)[7]	48 (38, 64)	<.001	5.9
CSF-IgG *(mg/L)*	99.4 (33.5)[8]	38.9 (36.3)	97 (75, 121.8)[8]	25.6 (19.8, 37.1)	.002	2.6
CSF-Total Bilirubin *(μmol/L)*	1.02 (0.04)[6]	0.66 (1.19)[11]	1 (1, 1)[6]	0 (0, 0.6)[11]	.06	1.5
S-Iron *(9–34 μmol/L)*	20 (8)	15 (5)	18 (15, 26)	16 (13, 19)	.12	1.3
S-Transferrin *(1.9–3.3 g/L)*	2.7 (0.5)	2.5 (0.3)	2.5 (2.3, 3)	2.5 (2.2, 2.7)	.34	1.1
S-Transferrin saturation *(15–50 %)*	30 (11)	25 (9)	28 (23, 36)	25 (18, 34)	.37	1.2
P-Copper *(12–25 μmol/L)*	20 (4)	18 (3)	19 (19, 21)	18 (16, 19)	.20	1.1
P-Ceruloplasmin *(0.24–0.55 g/L)*	0.29 (0.04)	0.26 (0.04)	0.3 (0.27, 0.31)	0.25 (0.23, 0.3)	.13	1.1
B-HbA1c *(20–42 mmol/mol)*	59 (6)	55 (8)	60 (58, 62)	57 (55, 59)	.04	1.1
S-Cholesterol *(3.9–7.8 mmol/L)*	5.8 (1.5)	4.8 (1.1)	5.3 (5.1, 7.1)	4.8 (4.1, 5.4)	.15	1.2
S-HDL-cholesterol *(1.0–2.7 mmol/L)*	1.3 (0.4)	1.7 (0.5)	1.3 (1, 1.6)	1.7 (1.3, 2.2)	.10	0.8
S-LDL *(2.0–5.4 mmol/L)*	4.1 (1.4)	2.9 (0.9)	3.9 (3.3, 5.4)	3 (2.4, 3.5)	.07	1.4
S-Triglycerides *(0.45–2.60 mmol/L)*	1.96 (1.03)	1.19 (0.33)	1.83 (1.29, 2.19)	1.23 (1.01, 1.42)	.02	1.7

Numerical superscripts indicate the value of n when other than stated in column header.
[a] Analyte prefixes: CSF = cerebrospinal fluid, S = serum, P = plasma, B = blood.
[b] *p*-values from Mann-Whitney *U* tests with continuity correction. Not adjusted for multiple testing.
[c] Ratio of means between Skogholt and control patients.

on TMT indicate poorer performance than norm). While 64 % of the cases had negative z-scores on TMT-A, as many as 91 % had negative z-scores on TMT-B. The mean standardized time-score for completion of TMT-B was significantly lower ($p < 0.001$) than the norm.

Self-maintaining activities of daily life showed median (IQR) sum-score of 7 (6–7), and instrumental activities of daily life showed median (IQR) sum-score of 8 (8–9), thus both instruments showed a 3rd quartile of only one point above normal.

4. Discussion

The most intriguing findings in the present study are the unusually high CSF levels of tau proteins together with persistently raised levels of iron, copper, and total protein in the Skogholt patients. As most cases had previous records of clearly elevated levels of these analytes compared to controls [6,7]. Some cases had a medical history of cerebral stroke, which could have precipitated a transient lesion in the BBB and could partly explain the previously observed white matter lesions [6]. However, the persistently increased level of total protein together with the white matter lesions may indicate a slowly progressing neurodegeneration [6].

4.1. Does Skogholt's disease represent a neurodegenerative process?

The present study confirms the conclusion of previous studies [6,7] that Skogholt's disease represents a neurodegenerative disease entity which differs from previously described neurological diseases. Considering common biomarkers of neurodegeneration, the patients are characterized by substantially elevated levels of T-tau and P-tau proteins in the CSF, and the values for $A\beta_{42}$ are modestly decreased compared with controls and established cut-off limits [13]. Although some of the patients had previously been hospitalized with a diagnosis of cerebral stroke, the persistent decrease in CSF $A\beta_{42}$ and increase in the tau proteins is not expected several years after an acute cerebrovascular event

Table 3
Elements in cerebrospinal fluid.

	Mean (SD)		Median (IQR)				
Elements[a] *(unit)*	Skogholt (n = 7)	Control (n = 14)	Skogholt (n = 7)	Control (n = 14)	*p*-value[b]	Ratio[c]	LOD_{CSF}
Iron *(μg/L)*	77.6 (9.25)	9.15 (3.41)	81.5 (71.7, 82.2)	8.57 (6.94, 12.3)	<.001	8.49	0.325
Lead *(ng/L)*	227 (473)	40.4 (14.1)	54.9 (38.9, 63.3)	38.8 (32.9, 46.0)	.17	5.62	20.1
Copper *(μg/L)*	48.7 (3.23)	9.64 (2.45)	49.1 (46.0, 50.2)	9.62 (7.72, 11.7)	<.001	5.05	0.301
Selenium *(μg/L)*	8.54 (1.61)	1.75 (0.672)	7.85 (7.54, 9.47)	1.82 (1.28, 2.29)	<.001	4.88	0.502
Sulfur *(mg/L)*	34.8 (4.31)	10.8 (3.34)	34.3 (31.0, 39.0)	10.2 (8.16, 12.4)	<.001	3.22	0.201
Zinc *(μg/L)*	34.4 (2.55)	12.4 (2.92)	33.2 (32.9, 35.9)	12.2 (9.97, 13.8)	<.001	2.78	0.251
Manganese *(ng/L)*	826 (241)	302 (149)	830 (756, 991)	285 (209, 331)	<.001	2.74	60.2
Tin *(ng/L)*	72.0 (54.7)	54.4 (37.3)	52.8 (43.2, 72.4)	38.4 (31.6, 64.6)	.25	1.32	10.0
Sodium *(g/L)*	1.46 (0.243)	1.15 (0.280)	1.55 (1.30, 1.58)	1.12 (0.999, 1.19)	.01	1.27	0.000100
Rubidium *(μg/L)*	53.1 (6.93)	42.2 (10.9)	53.2 (49.8, 55.6)	40.9 (39.4, 44.6)	.01	1.26	0.120
Phosphorus *(mg/L)*	15.2 (1.09)	13.6 (0.869)	15.0 (14.6, 15.1)	13.8 (13.0, 14.2)	.002	1.12	0.00401
Strontium *(μg/L)*	11.2 (3.53)	10.2 (2.07)	10.4 (8.07, 14.4)	10.4 (8.78, 11.2)	.79	1.10	0.251
Silicon *(μg/L)*	541 (15.8)	500 (46.2)	544 (532, 554)	512 (486, 520)	.004	1.08	100
Magnesium *(mg/L)*	22.3 (0.803)	21.1 (0.703)	22.2 (21.6, 22.8)	21.2 (20.6, 21.6)	.006	1.06	0.00502
Calcium *(mg/L)*	42.4 (1.63)	40.0 (2.62)	42.2 (41.7, 43.2)	39.6 (38.2, 42.1)	.05	1.06	0.0201
Potassium *(mg/L)*	104 (2.94)	101 (5.71)	104 (102, 104)	100 (98.4, 102)	.05	1.03	0.0100
Caesium *(ng/L)*	213 (98.1)	248 (78.3)	179 (168, 189)	236 (210, 302)	.22	0.86	5.02
Boron *(μg/L)*	24.5 (8.41)	31.3 (14.7)	21.0 (19.5, 30.9)	28.3 (23.8, 38.1)	.31	0.78	0.502
Lithium *(ng/L)*	125 (56.8)	174 (133)	104 (87.5, 154)	136 (107, 152)	.39	0.72	50.2
Mercury *(ng/L)*	798 (245)	1270 (344)	692 (632, 878)	1220 (977, 1490)	.003	0.63	20.1

[a] Elements excluded when cases and controls had median concentration below limit of detection (LOD), or high intra analytical variance (median RSD above 30 %).
[b] *p*-values from Mann-Whitney *U* tests with continuity correction. Not adjusted for multiple testing.
[c] Ratio of means between Skogholt and control patients.

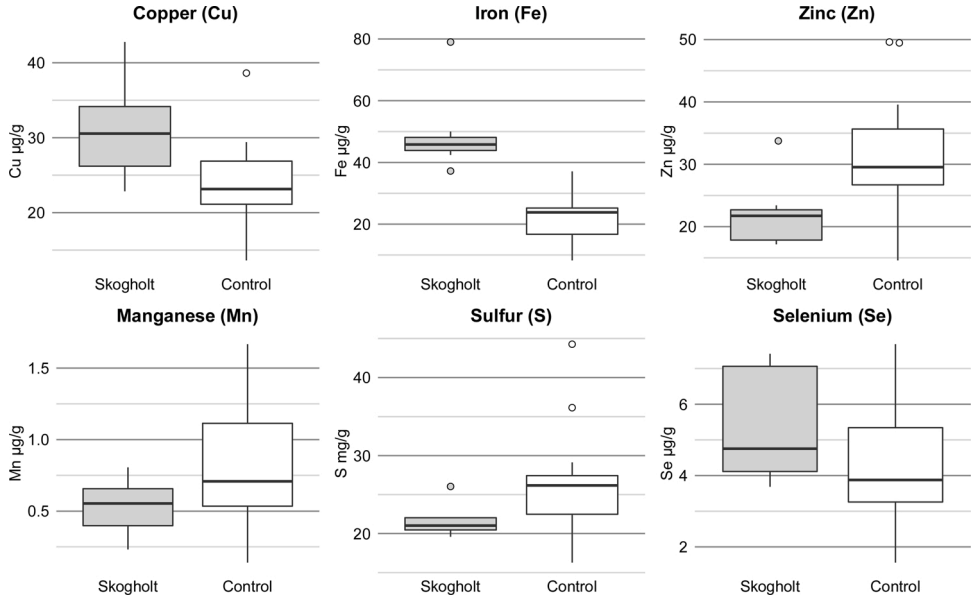

Fig. 3. Boxplots of elements in CSF expressed by concentration relative to total protein concentration in CSF (i.e., element/protein ratio). Boxes show the IQR (distance from 1 st to 3rd quartile). The horizontal line dividing the box represents the median (2nd quartile). Whiskers extend to the most extreme observation within 1.5 IQR from the box. Observations beyond the whisker range (outliers) are shown as small circles. Shaded figure elements represent the Skogholt patients.

[26–28]. Both the tau proteins and variants of $A\beta_{42}$, though lacking in specificity, have been widely used as biomarkers of dementia or progressive neurodegeneration [29]. Therefore, we find it surprising that the MMSE-scores were only modestly reduced in the Skogholt cohort. More thorough examination, however, using the MoCA test panel, identified four patients below the suggested normal range (26–30). The median z-score of -0.62 is consistent with some cognitive difficulties in the Skogholt group; the low scores on arithmetic and abstraction were the most striking deviations from the norm. The Trail Making Tests suggested that a principal difficulty lies within the mental flexibility or executive functional domains. The general impression of scores decreasing with age, even with age and education-adjusted scores, suggests the possibility that our patient group is in an early stage of a clinically mild neurodegenerative process. However, this does not mean that the condition is beyond reach of intervention. Symptom relief has been reported after lifestyle change with emphasis on healthy diet, weight management and exercise.

4.2. *Persistent abnormalities of CSF in Skogholt patients*

Previous findings of substantially increased levels of copper and iron in CSF [7] have been confirmed in the present study, apparently representing lasting physiological changes in Skogholt patients. Normally, the transport of copper and iron from blood to cerebrospinal fluid is tightly regulated [30]; even after a stroke these functions are restored within few weeks [28]. In contrast, the Skogholt patients' CSF levels of

Table 4
Elements in whole blood.

	Mean (SD)		Median (IQR)				
Elements[a] *(unit)*	Skogholt (n = 11)	Control (n = 14)	Skogholt (n = 11)	Control (n = 14)	*p*-value[b]	Ratio[c]	LOD_{Blood}
Rubidium *(mg/L)*	2.10 (0.243)	1.64 (0.479)	2.02 (1.98, 2.23)	1.63 (1.48, 2.00)	.01	1.28	0.000310
Phosphorus *(mg/L)*	327 (19.4)	290 (34.6)	330 (312, 338)	293 (278, 312)	.003	1.13	0.0103
Zinc *(mg/L)*	5.70 (0.408)	5.05 (0.618)	5.54 (5.45, 5.98)	4.79 (4.58, 5.44)	.01	1.13	0.000645
Sulfur *(g/L)*	1.46 (0.0715)	1.34 (0.0811)	1.46 (1.44, 1.51)	1.35 (1.29, 1.40)	<.001	1.09	0.000516
Iron *(mg/L)*	461 (49.6)	423 (52.2)	456 (430, 499)	419 (393, 460)	.12	1.09	0.000516
Selenium *(μg/L)*	104 (9.39)	95.3 (19.2)	106 (99.4, 111)	95.1 (85.5, 106)	.16	1.09	1.29
Potassium *(g/L)*	1.69 (0.0960)	1.58 (0.152)	1.69 (1.60, 1.74)	1.56 (1.48, 1.69)	.06	1.07	0.0000258
Magnesium *(mg/L)*	33.5 (2.29)	31.5 (4.66)	33.9 (32.1, 34.5)	32.1 (29.7, 33.8)	.22	1.06	0.0129
Copper *(μg/L)*	804 (112)	767 (109)	771 (749, 851)	745 (700, 860)	.49	1.05	0.774
Silicon *(mg/L)*	1.29 (0.187)	1.24 (0.267)	1.36 (1.19, 1.40)	1.23 (1.05, 1.38)	.53	1.04	0.258
Antimony *(μg/L)*	1.24 (0.165)	1.21 (0.440)	1.23 (1.15, 1.31)	1.15 (0.958, 1.27)	.20	1.03	0.0516
Calcium *(mg/L)*	52.1 (5.71)	53.9 (4.06)	52.4 (47.2, 55.8)	53.8 (51.0, 56.1)	.43	0.97	0.0516
Lead *(μg/L)*	11.8 (5.90)	12.3 (4.50)	11.0 (6.80, 15.1)	10.9 (9.50, 13.1)	.72	0.96	0.0516
Tin *(ng/L)*	113 (65.3)	119 (83.0)	81.9 (71.7, 124)	93.0 (74.2, 120)	.64	0.95	25.8
Sodium *(g/L)*	1.76 (0.121)	1.88 (0.121)	1.74 (1.72, 1.81)	1.84 (1.80, 1.93)	.02	0.94	0.000258
Caesium *(μg/L)*	3.09 (1.08)	3.28 (1.18)	2.95 (2.59, 3.30)	3.23 (2.52, 3.81)	.64	0.94	0.0129
Manganese *(μg/L)*	7.62 (1.36)	8.61 (3.68)	7.18 (6.57, 8.70)	7.71 (7.24, 8.64)	.60	0.88	0.155
Strontium *(μg/L)*	15.7 (4.24)	18.1 (3.38)	13.5 (12.6, 18.5)	18.9 (15.1, 19.9)	.15	0.87	0.645
Mercury *(μg/L)*	1.87 (1.42)	2.24 (1.63)	1.36 (1.23, 1.78)	1.90 (1.05, 3.02)	.64	0.84	0.0516
Cadmium *(ng/L)*	377 (289)	478 (462)	267 (198, 478)	289 (174, 610)	1.0	0.79	51.6
Titanium *(ng/L)*	<516 (658)	727 (596)	<516 (<516, 569)	688 (<516, 916)	.13	0.71	516
Lithium *(ng/L)*	305 (91.4)	475 (274)	291 (247, 381)	421 (309, 515)	.04	0.64	129
Boron *(μg/L)*	15.1 (7.57)	28.0 (11.6)	13.9 (8.77, 20.9)	27.8 (19.3, 35.2)	.01	0.54	1.29
Arsenic *(μg/L)*	1.93 (1.70)	6.37 (11.5)	1.61 (<0.645, 2.93)	3.53 (1.60, 5.43)	.08	0.30	0.645

[a] Elements excluded when cases and controls have median concentration below limit of detection (LOD), or high intra analytical variability (median RSD > 30 %).
[b] *p*-values from Mann-Whitney *U* tests with continuity correction. Not adjusted for multiple testing.
[c] Ratio of means between Skogholt and control patients.

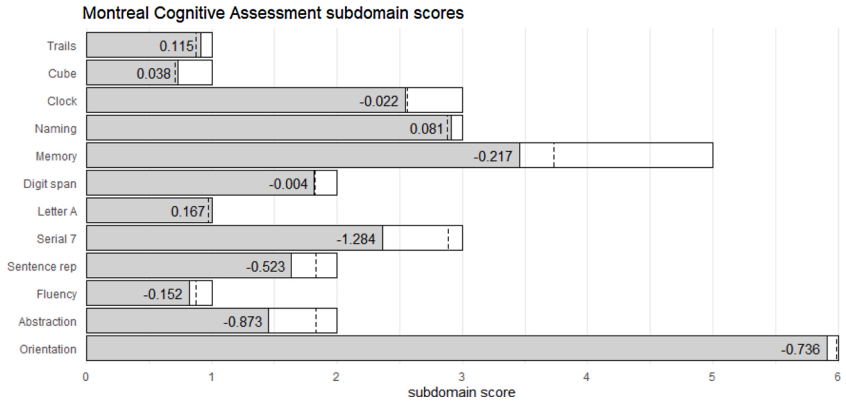

Fig. 4. Bars showing maximum possible score for each subdomain. Grey parts indicate mean scores for Skogholt patients (normalized z-scores provided). Dashed lines represent the means of norm-data (z = 0).

total protein, iron, and copper have not significantly changed from the investigation presented in 2008 until the present study [7]. Furthermore, the persistently increased CSF levels of iron and copper could not be explained from external exposure leading to changed blood levels, since the latter values were not different from control values. Consequently, it is tempting to assume that a continuous leakage of metal-protein or metal-peptide complexes from blood to CSF takes place in the Skogholt patients; either through defects in the endothelial lining of brain capillaries, or across dysfunctional layers in the choroid plexus. The leakage could potentially be due to clinically silent cortical cerebral microhemorrhages associated with cerebral amyloid angiopathy (CAA), but the several fold increase in tau-protein concentration suggests otherwise. We would also expect a marked increase in bilirubin absorbance in Skogholt CSF if chronic microhemorrhages was the cause, but the adjusted bilirubin absorbance was normal (Supplemental material, Fig. 1d). Since red blood cells and any siderophages in the CSF would be included in automated cell counts, the absence of pleocytosis also speaks against recent subarachnoid hemorrhages into the CSF. The possibility of CAA or other vasculopathies cannot be completely ruled out, but previous genetic testing for CADASIL was negative [6], and the markedly increased tau protein levels in CSF far exceeds the typical findings in CAA [31]. Thus, the hypothesis of a primary biochemical lesion in the BBB causing the persistent leakage of components from blood to CSF in Skogholt's disease seems plausible.

4.3. Suggested mechanisms of iron and copper transfer into CSF

We have suggested in a previous publication that the neurological symptoms of Skogholt's disease is initiated by an inherited defect that affects BBB integrity, leading to selectively increased transfer of iron and copper complexes of low molecular weight plasma proteins or peptides to CSF [10]. An alternative hypothesis is that an ongoing cerebrovascular disorder with associated inflammation has led to an acquired deterioration of the BBB integrity as a component of Skogholt's disease, although vascular lesions usually give rise only to transient increases in tau protein levels [32–34]. Irrespective of whether the underlying cause

is genetic or acquired, it is evident from the persistent increase of the CSF-serum albumin ratio that a dysfunctional BBB or BCB is a persistent characteristic in the Skogholt patients [10]. It is, however, not known whether the transport of the elements in question is mainly occurring through passive filtration or if there is dysregulation of physiological transport mechanisms across the barrier systems. Zheng and Monnot point to an active physiological transport of Cu and Fe into the brain across the BBB and a likely regulatory role of the BCB as the metals are actively transported against their gradients by divalent metal transporter-1 (DMT1) and copper transporter-1 (CTR1) at the choroidal plexus epithelium [30].

Active absorption from the blood of ferric transferrin by transferrin receptor-mediated endocytosis is likely the main physiological entry route for iron into the brain [35–37]. However, with its molecular weight of only about 80 kDa, transferrin has also been considered a possible passive carrier of iron into the CSF across a modestly deteriorated BBB [10,35,38]. As several vascular and neuronal constituents including mitochondria are rich in transferrin receptors, cerebral vessels or neurons might be especially susceptible to iron overload in Skogholt patients [39]. In contrast, zinc and manganese, commonly incorporated in proteins with higher molecular weight proteins [40], do not penetrate the BBB to the same extent in Skogholt patients.

The most important copper transporter in blood, ceruloplasmin, has a molecular weight above 150 kDa. This metalloprotein is not expected to easily pass the BBB in its intact form [41]. The strong correlation between copper and sulfur previously reported in CSF of Skogholt patients [10], suggests that sulfur-containing ligands bind copper in the CSF of these patients. This is consistent with a possible involvement of low molecular weight ceruloplasmin fragments in the copper dysregulation in Skogholt's disease, and reminiscent of the proposed role of copper-containing ceruloplasmin fragments in AD [42]. Alternatively, glutathione or other sulfur-containing peptides might act as copper chelators or carriers [43].

Copper levels in whole blood were not increased in Skogholt patients when compared with the reference group in a study by Syversen et al. [44] or in other studies [45]. Comparison of iron levels and other elements determined by Syversen et al. [44] do not give indications of high metal exposure in Skogholt patients.

Reference levels of trace elements in CSF from healthy individuals is generally lacking because CSF sampling is rather invasive and usually only done on clinical indication. Available data on trace element concentrations in CSF is usually from patients with AD or other neurodegenerative disorders. The average CSF levels for copper reported from controls in the scientific literature, are usually lower than those reported here for Skogholt patients, while the average CSF levels in AD patients were usually higher than in the Skogholt patients [46].

4.4. *Hypothetic mechanisms of metal-mediated neurodegeneration*

Complexing peptides or proteins carrying iron and copper from blood to CSF do not bind and detoxify the metal ions irreversibly [47]. Low-molecular-weight chelates of copper and iron, or the free cations, are most probably taken up intracellularly and may exceed the detoxifying capacity of cellular chaperones, resulting in minor or major injuries affecting cerebrovascular as well as neuronal structures and functions [48]. Generation of reactive oxygen species (ROS) has been implicated in cerebrovascular diseases [49,50], and oxidative stress has been observed in several neurodegenerative disorders [51,52]. Neuroinflammation, reported to accompany AD [53], might be precipitated by iron and copper dysmetabolism [30,48]. Excess of free iron or copper ions in the tissues and cells will accelerate conversion of nontoxic oxygen species to ROS through the Fenton reaction [54], and thereby promote ferroptosis [55] and formation of advanced glycation end products [56]. Iron and copper may through these mechanisms contribute to cerebrovascular lesions as well as to neuronal axonal degeneration leading to tauopathy and finally to a neurodegenerative cascade with $A\beta_{42}$ retention and plaque formation.

Recent studies have reported peroxidative processes, also in conjunction with metal exposure, during the progression of neurodegenerative diseases [57]. If ROS toxicity is involved in the reaction cascade in tauopathies [58], metal induced ROS toxicity may be a common mechanism in the pathogenesis of progressive neurodegenerative diseases, including the cerebrovascular components. In addition to adequate therapy and follow-up of metabolic disturbances, such as e.g. in metabolic syndrome [59], a therapeutic benefit in neurodegenerative disease could plausibly be obtained by applying antioxidants or antidotes against iron and copper; a hypothesis that deserves further exploration [60–62].

5. Limitations

The small number of cases and controls in this study is a major limitation. The heterogeneity of the control group, particularly the inclusion of controls that turned out to have cerebrovascular disease or cognitive impairment, was also unfortunate, but had little effect on our conclusions. Multiple testing also increases the probability of false discoveries, but our main findings remained statistically significant after multiple testing correction. Cognitive evaluation of cases with a comprehensive neuropsychological test battery might provide more information than the screening tests used in this study. Positron emission tomography (PET) with labelling of tau protein could potentially resolve whether our hypothesis that Skogholt's disease is a tauopathy is correct or not, while amyloid PET could shed light on the nature of potential cerebral amyloid depositions. Specialized MRI-sequences might also provide useful diagnostic insights.

6. Conclusion

We have demonstrated high levels of tau proteins, and persistent elevation of total protein, copper, and iron in the cerebrospinal fluid from patients with Skogholt's disease. The increased levels of copper and iron, metals associated with other neurodegenerative disorders, may contribute to neuroinflammation. At present we believe the diagnostic criteria should be a familial history of Skogholt's disease together with the described CSF abnormalities; yellow discoloration, high total-protein, high tau-proteins, and low or normal $A\beta_{42}$ levels. Since Skogholt's disease is a potential source for new insights into neurodegenerative processes, we believe further study is warranted.

Data availability

The data that has been used is confidential.

Author statement

With the submission of this revised version of the manuscript, the authors confirm that the above-mentioned manuscript has not been published totally or partly, nor is under editorial review for publication elsewhere.

Author contributions

KTA: Conceptualization, investigation, software, visualization and writing original draft.

TH: Conceptualization, writing, review, and editing.

TPF: Writing, review, and editing.

JEW: Writing, supervision, review, and editing.

JOA: Conceptualization, supervision, project administration, writing, review, and editing.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jtemb.2021.126915.

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