

This is an Open Access document downloaded from ORCA, Cardiff University's institutional repository:<https://orca.cardiff.ac.uk/id/eprint/109894/>

This is the author's version of a work that was submitted to / accepted for publication.

Citation for final published version:

Zelek, Wioleta M., Watkins, Lewis M., Howell, Owain W., Evans, Rhian, Loveless, Sam , Robertson, Neil P. , Beenes, Marijke, Willems, Loek, Brandwijk, Ricardo and Morgan, B. Paul 2019. Measurement of soluble CD59 in CSF in demyelinating disease: Evidence for an intrathecal source of soluble CD59. *Multiple Sclerosis* 25 (4) , pp. 523-531. 10.1177/1352458518758927

Publishers page: <http://dx.doi.org/10.1177/1352458518758927>

Please note:

Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher's version if you wish to cite this paper.

This version is being made available in accordance with publisher policies. See <http://orca.cf.ac.uk/policies.html> for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.





Measurement of soluble CD59 in CSF in demyelinating disease: evidence for an intrathecal source of soluble CD59.

Journal:	<i>Multiple Sclerosis Journal</i>
Manuscript ID	MSJ-17-0638
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	24-Aug-2017
Complete List of Authors:	Zelek, Wioleta; Cardiff University School of Medicine, Infection and Immunity Watkins, Lewis; Swansea University, Swansea University Medical School Howell, Owain; Swansea University, Swansea University Medical School Loveless, Samantha; Cardiff University, Department of Infection, Immunity and Biochemistry Robertson, Neil; Cardiff University, Neurology Beenes, Marijke; Hycult Biotech, Research and Development Willems, Loek; Hycult Biotech, Sales Brandwijk, Ricardo ; Hycult Biotech, Research and Development Morgan, B; Cardiff University, Department of Infection, Immunity and Biochemistry
Keywords:	CD59, MS, choroid plexus
Abstract:	<p>Background: CD59, a broadly expressed glycosylphosphatidylinositol (GPI) anchored protein, is the principle inhibitor of complement membrane attack on cells. In the autoinflammatory demyelinating disorders, multiple sclerosis (MS) and neuromyelitis optica spectrum disorder (NMOSD), elevated complement protein levels were reported in cerebrospinal fluid (CSF), including soluble (s)CD59 in CSF.</p> <p>Objectives: We compared levels of sCD59 in CSF and matched plasma in controls and patients with MS, NMOSD, and clinically isolated syndrome (CIS), investigated the source of sCD59 in CSF and whether it was microparticle-associated.</p> <p>Methods: sCD59 was quantified using ELISA (Hycult Biotech; HK374-02). Patient and control CSF was subjected to western-blotting to characterise anti-CD59-reactive materials. CD59 was localised by immunostaining.</p> <p>Results: CSF sCD59 levels were double those in plasma (sCD59 in CSF= 30.2ng/ml, plasma= 16.3ng/ml; n= 91). sCD59 levels in plasma, but not CSF, differentiated MS from NMOSD, MS from CIS, and NMOSD/CIS from</p>

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

	<p>controls. Centrifugation to remove microparticles demonstrated that >85% of CSF CD59 was not membrane anchored. Conclusions: High levels of sCD59 in CSF relative to plasma suggest an intrathecal source; CD59 expression in brain parenchyma was low but expression was strong on choroid plexus (CP) epithelium, immediately adjacent the CSF space, suggesting that this is the likely source.</p>

SCHOLARONE™
Manuscripts

For Peer Review

1
2
3 **Measurement of soluble CD59 in CSF in demyelinating disease: evidence for an intrathecal**
4 **source of soluble CD59.**
5
6
7
8
9

10
11 **Names of Authors:**

12
13 Wioleta M. Zelek¹, Lewis M. Watkins³, Owain W. Howell³, Sam Loveless², Neil P. Robertson²,
14
15 Marijke Beenes⁴, Loek Willems⁴, Ricardo Brandwijk⁴, B. Paul Morgan¹.
16
17

18
19
20 **Affiliations:**

21
22 ¹Systems Immunity Research Institute and ²Division of Psychological Medicine and Clinical
23
24 Neurology, School of Medicine, Cardiff University, Cardiff CF14 4XN, UK.
25
26

27
28 ³Institute of Life Sciences (ILS 1), Swansea University Medical School, Singleton Park Campus,
29
30 Swansea, SA2 8PP, UK.
31

32
33 ⁴Hycult Biotech, Frontstraat 2a, 5405 PB, Uden, The Netherlands.
34
35
36

37 **Keywords:** complement; CD59; MS; choroid plexus.
38
39
40
41

42 **Correspondence author:**

43
44 Prof. B. Paul Morgan; Henry Wellcome Building, School of Medicine, Cardiff University,
45
46 Cardiff CF144XN. Telephone: (44) 2920 687096. Email: Morganbp@cardiff.ac.uk
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Abstract

Background: CD59, a broadly expressed glycosylphosphatidylinositol (GPI) anchored protein, is the principle inhibitor of complement membrane attack on cells. In the autoinflammatory demyelinating disorders, multiple sclerosis (MS) and neuromyelitis optica spectrum disorder (NMOSD), elevated complement protein levels were reported in cerebrospinal fluid (CSF), including soluble (s)CD59 in CSF.

Objectives: We compared levels of sCD59 in CSF and matched plasma in controls and patients with MS, NMOSD, and clinically isolated syndrome (CIS), investigated the source of sCD59 in CSF and whether it was microparticle-associated.

Methods: sCD59 was quantified using ELISA (Hycult Biotech; HK374-02). Patient and control CSF was subjected to western-blotting to characterise anti-CD59-reactive materials. CD59 was localised by immunostaining.

Results: CSF sCD59 levels were double those in plasma (sCD59 in CSF= 30.2ng/ml, plasma= 16.3ng/ml; n= 91). sCD59 levels in plasma, but not CSF, differentiated MS from NMOSD, MS from CIS, and NMOSD/CIS from controls. Centrifugation to remove microparticles demonstrated that >85% of CSF CD59 was not membrane anchored.

Conclusions: High levels of sCD59 in CSF relative to plasma suggest an intrathecal source; CD59 expression in brain parenchyma was low but expression was strong on choroid plexus (CP) epithelium, immediately adjacent the CSF space, suggesting that this is the likely source.

Introduction

The complement system is a central component of innate immunity responsible for recognition and killing of bacteria by lysis and promoting phagocytosis through opsonisation. The main source of most complement proteins is the liver, however other cells, including fibroblasts, epithelial and endothelial cells, glia and neurons, also produce complement proteins.¹ Activation of complement via the classical, lectin or alternative pathway leads to the formation of C3 convertase complexes that cleave C3 to C3a and C3b. C3b is the nidus for the C5 convertase that cleaves C5 to C5a and C5b, the latter initiating formation of the membrane attack complex (MAC) responsible for osmotic cell lysis. CD59, an ~18–20 kDa GPI anchored protein, regulates formation of MAC by preventing recruitment of C9.² Little is known about expression of CD59 in the brain; in one study neurones were reported to lack CD59 and expression of CD59 on glia was increased in inflamed brain.³ The blood–brain barrier (BBB) restricts entry of molecules into the central nervous system (CNS), providing a natural defence against circulating toxic or infectious agents.⁴ The choroid plexus (CP) plays a crucial role in the production of CSF, and may be a site of immune activation; for example, in a mouse model of human systemic lupus erythematosus (SLE), dense deposits of immunoglobulin G (IgG) and C3 were found in the CP.⁵ More recently the role of CP in cellular immunology became more evident with the demonstration that CP was responsible for controlling and development of immune responses in the CNS.⁶ Recent immunohistochemical (IHC) data on human CP showed deposition of complement proteins; C1q, C3d, and C9 in stromal concretions and IgG in epithelial cells, suggesting significant involvement of CP in clearance of complement-tagged immune complexes.⁷ Multiple sclerosis (MS), neuromyelitis optica spectrum disorder (NMOSD) and clinically isolated syndrome (CIS) are autoinflammatory demyelinating brain disorders in which

1
2
3 dysregulation of complement has been noted in CSF, plasma and brain.⁸⁻¹⁵ It was recently
4 reported that soluble (s) CD59 is increased in CSF in MS and NMOSD.¹⁶ This finding prompted
5 us to further investigate levels of sCD59 in CSF and plasma from MS, NMOSD and CIS cases
6 and healthy controls with the aim of gaining insight into underlying pathology. sCD59 was
7 measured using a commercial ELISA and the nature of sCD59 in CSF, microparticle-associated
8 or free, was explored. To discover the source of sCD59 in CSF we analysed brain CD59
9 expression by IHC.
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26

27 **Materials and methods**

28
29 All chemicals, except where otherwise stated, were obtained from either Fisher Scientific UK
30 (Loughborough, Leicestershire, UK) or Sigma Aldrich (Gillingham, Dorset, UK) and were of
31 analytical grade.
32
33
34
35
36
37
38

39 **Subjects**

40 *CSF Samples*

41
42
43
44
45
46 CSF Samples from 50 MS patients, 11 NMOSD patients, 17 CIS patients, and 34 controls and
47 matched Ethylenediaminetetraacetic acid (EDTA) plasma samples from 41 MS patients, 10
48 NMOSD, 14 CIS patients and 26 controls were obtained from the Welsh Neuroscience Research
49 Tissue Bank, Institute of Neurosciences and Mental Health, Cardiff University. Mean age and %
50 Female were as follow; MS; 45, 68%, NMOSD; 49, 80%, CIS; 43, 78%, Controls; 32, 77%.
51
52
53
54
55
56
57
58
59
60

1
2
3 Ethnicity was reported in the majority of patients as Caucasian (two cases were non-Caucasian;
4 one each of MS and NMOSD). Informed consent was obtained from all patients, and ethical
5 approval was gained from South East Wales Ethics Committee (ref no. 05/WSE03/111).
6
7
8
9
10 Diagnosis for MS cases was made according to established criteria¹⁷; all had clinically definite
11 disease and had exhibited a relapsing disease course for which comprehensive follow-up clinical
12 data was available. In addition all MS cases had undergone diagnostic and follow-up magnetic
13 resonance imaging (MRI) as part of routine clinical care. All patients with NMOSD were
14 reviewed and diagnosis confirmed according to established criteria¹⁸ by a neurologist with
15 experience in the diagnosis and management of neuroinflammatory disorders including
16 NMOSD. All NMOSD cases had undergone diagnostic and follow-up MRI as part of routine
17 clinical care. AQP4-Ab and MOG-Ab status was assessed at the Clinical Neuroimmunology
18 Service, John Radcliffe Hospital Trust, Oxford, UK.^{19, 20}
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33

34 *Neuropathological tissue*

35
36 MS (n=5 cases) and non-neurological control (n=7 cases) post-mortem tissue was provided by
37 the UK Multiple Sclerosis Society Tissue Bank at Imperial College and the Oxford Brain Bank
38 at Oxford University, with research ethical approval (South West Wales REC study
39 12/WA/0292). Six micron thick sections were prepared from a single region-matched paraffin-
40 embedded block per case of the anterior hippocampus at the level of the lateral geniculate
41 nucleus. All sections assessed contained intact meninges and choroid plexus (of the inferior horn
42 of the lateral ventricle). Case details are listed in Table 1.
43
44
45
46
47
48
49
50
51
52
53
54

55 *CD59 Enzyme-Linked Immunosorbent Assay (ELISA)*

1
2
3 The concentration of sCD59 in CSF and plasma samples was measured using a commercial
4 ELISA (Hycult Biotech, HK374-02, Uden, The Netherlands). Levels of sCD59 in CSF,
5 unfractionated and following high-speed centrifugation (21200 rcf for 5 minutes at room
6 temperature) to remove microparticles, were compared in a subset of 23 samples.
7
8
9
10
11
12
13

14 *SDS-PAGE and Western Blotting*

15
16 Patient and control CSF was subjected to SDS/PAGE and western blotting to further characterise
17 anti-CD59-reactive materials in CSF. Prior to analysis the patient CSF samples (MS and
18 NMOSD) were 10-fold concentrated using Vivaspin sample concentrators (GE Healthcare,
19 Amersham Place, Little Chalfont, UK, cut-off size; 10 kDa). As a positive control, human
20 erythrocyte membranes, prepared by lysing erythrocytes in deionised water, centrifuging and
21 washing the membrane pellet in PBS, were solubilised in non-reducing sample buffer (0.1M
22 Tris, 10% w/v glycerol, 2% w/v SDS, bromophenol blue, pH 6.8 in deionised water). Undiluted
23 plasma or CSF samples were mixed 1 in 5 with non-reducing sample buffer. All samples were
24 resolved on 4 – 20% gradient SDS-PAGE gels (Biorad; 456-1094); then electrophoretically
25 transferred onto 0.45 µm nitrocellulose membrane (GE Healthcare, Amersham, UK, 10600002)
26 at a constant voltage of 90 V in 25mM Tris, 19.2mM Glycine buffer with 20% methanol. After
27 transfer, non-specific sites on the membrane were blocked with 3% BSA in PBS for 1 h at room
28 temperature (RT). All subsequent incubations were carried out for 1 hour at RT and followed by
29 three washes with PBS containing 0.1% Tween-20 (PBS-T). Post-blocking, the membrane was
30 incubated with the primary antibody, monoclonal anti-human CD59 (MEM43 at 1µg/ml in PBS-
31 T). After washing, the bound antibodies were revealed by incubation with donkey anti-mouse
32 IgG-HRP conjugate (Jackson Immunoresearch, 715-035-151) at an optimized dilution of 1:2000
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 in PBS-T. The blot was developed with ECL (GE Healthcare, Amersham, UK, RPN 2106) and
4
5
6 visualised by autoradiography.
7
8
9

10 *Immunohistochemistry and analysis*

11
12 Sections were de-waxed, rehydrated, and subjected to heat-induced epitope retrieval in citraconic
13
14 anhydride (0.05%), then quenched in H₂O₂ and blocked with normal serum before overnight
15
16 incubation with monoclonal (MEM43) or rabbit polyclonal anti-CD59 antibody. Bound primary
17
18 antibody was detected using the appropriate biotinylated goat secondary antibody (Vector Labs)
19
20 antibody was detected using the appropriate biotinylated goat secondary antibody (Vector Labs)
21
22 prior to incubating with the Avidin-biotin HRP complex (ABC Elite; Vector Labs). Staining was
23
24 visualised using diaminobenzidine (Impact DAB; Vector) and all sections were counterstained
25
26 with haematoxylin before dehydrating, clearing and mounting in DePex (Fisher Scientific). No-
27
28 primary-antibody and irrelevant mouse or rabbit antibodies were included as staining controls
29
30 and all sections were immunostained in the same experimental run. Sections were viewed and
31
32 images captured using a Zeiss Axio Imager II fitted with an AxioCam HR camera (Carl Zeiss
33
34 Ltd.). The relative intensity of CD59 immunoreactivity (mouse monoclonal antibody MEM43) in
35
36 the choroid plexus, ependyma, meninges, vasculature and brain parenchyma was evaluated as: 0,
37
38 absent; +, mild (very few stained cells or weakly stained structures); ++, moderate (30-70% of
39
40 cells immunopositive or structures showing intermediate immunopositivity); +++, substantial
41
42 (majority of cells or tissues are intensely immunopositive).
43
44
45
46
47
48
49

50 **Results**

51 52 53 54 55 *Levels of sCD59 in CSF and plasma*

1
2
3 Soluble CD59 was detected in all CSF and plasma samples tested; levels in CSF were
4
5 approximately double those in matched plasma (Figure 1A; $p < 0.0001$). There was no significant
6
7 difference in CSF sCD59 levels between the various disease groups (Figure 1B); however,
8
9 measurement of sCD59 in plasma differentiated MS from NMOSD ($p < 0.005$), MS from CIS
10
11 ($p < 0.003$) and NMO/CIS from controls ($P < 0.003$) (Figure 1C). The data confirm that the
12
13 observed ratios between CSF and plasma sCD59 levels were consistent across the groups (Figure
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

The majority of the CD59 in CSF is not lipid-anchored to microparticles.

sCD59 can exist in biological fluids either as an anchor-free soluble small molecule or as a GPI-
anchor intact lipid/microparticle-associated molecule.²¹ To test the nature of sCD59 found in
CSF, those samples available in sufficient volume (23) were subjected to high speed
centrifugation to remove microparticles and sCD59 concentration was measured prior to and
after centrifugation. Levels of sCD59 in centrifuged CSF were not significantly different
compared to uncentrifuged (mean concentration before centrifugation 32.5ng/ml; after
centrifugation 28.1ng/ml), confirming that majority of the CD59 (>85%) in CSF is not anchored
to microparticles (Figure 2). As an independent verification of this finding, CSF was passed
through a 0.2 μ M filter; sCD59 level was not significantly altered by filtration (data not
included).

sCD59 detected in patients CSF samples resembles cell-associated CD59.

The nature of the sCD59 reactivity in MS, NMOSD and control CSF samples was tested by
western blot analysis. In each sample tested, CSF sCD59 comprised a broad band of 19-23 kDa,

1
2
3 slightly higher in MW and more compact than the band obtained in erythrocyte lysates and
4
5 resembling published data on nucleated cell-derived CD59.²² (Figure 3 A, B).
6
7
8
9

10 *The choroid plexus and brain vasculature are enriched in CD59.*

11
12 Using a mouse monoclonal antibody to CD59 we demonstrated strong anti-CD59
13 immunoreactivity at the brain-CSF and brain-blood barriers of the human brain. Anti-CD59
14 immunoreactivity was comparable between MS (Fig 4A, B) and non-inflammatory control brains
15 (Fig 4C, D) and was notable in the parenchymal vasculature (both arteries and veins), meningeal
16 vasculature, ependymal lining of the ventricles and the choroid plexus epithelium (Figure 4). In
17 contrast to the modest to substantial immunoreactivity of these structures, cell-specific staining
18 within the hippocampus and parahippocampal grey and white matter was absent or weak. This
19 pattern of CD59 expression was verified using a polyclonal anti-CD59 antibody raised in rabbit
20 (Fig 4K).
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37

38 **Discussion**

39 Measurement of complement proteins, regulators and activation products in CSF has been
40 described in several studies and proposed as contributory biomarkers in demyelinating and
41 degenerative neurological diseases. Levels of C3 and FH were reduced in Alzheimer's disease
42 CSF and correlated with disease staging.²³ The soluble terminal complex sC5b-9 was increased
43 in CSF in both MS and NMOSD, more so in the latter, indicating more complement activation.²⁴
44 Soluble complement receptor 2 (sCR2) levels were increased in MS CSF and suggested to be of
45 functional relevance but its source was not explored.²⁵ A recent study reported that levels in
46 CSF of the soluble form of the GPI-anchored cell surface complement regulator CD59 (sCD59)
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 were significantly elevated in MS and NMOSD compared to non-inflammatory neurological
4 disease controls provoked our interest.¹⁶ We set out to replicate these findings in a larger sample
5
6 and to explore the source of sCD59 in CSF.
7
8
9

10
11
12 Expression of complement regulators, including CD59, in the human brain has been the subject
13 of previous work. CD59 expression in the normal, non-inflamed brain is very low with minimal
14 expression on glia and negligible expression on neurones.³ The only sites in the normal brain
15 where CD59 is abundantly expressed are the vascular endothelium and the choroid plexus where
16 CD59 is reported to decorate the apical surface of the ependymal cells. In the inflamed brain,
17 expression of complement regulators, including CD59, is increased, although levels remain low
18 compared to the periphery.²⁶ It is likely that this increase in regulator expression is a homeostatic
19 response as levels of complement proteins are increased in the inflamed brain due either to
20 intrathecal production or transudation of complement proteins across the leaky BBB.²⁷
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35

36 We measured sCD59 levels in CSF from 50 MS, 11 NMOSD and 17 CIS patients, and 34 non-
37 inflammatory controls. CSF sCD59 levels were ~30ng/ml in the samples and were not different
38 between the groups; in particular, levels were not higher in MS and/or NMO compared to
39 controls in contrast to recent report.¹⁶ In 91 of the available cases, matched plasma taken at the
40 time of lumbar puncture was available; in these we measured plasma sCD59 levels in the same
41 assay. Plasma sCD59 levels were, on average, around half that in matched CSF samples (mean
42 sCD59 in CSF = 30.2ng/ml, plasma = 16.3ng/ml (n = 91). Plasma sCD59 levels were
43 significantly higher in MS and controls compared to NMOSD and CIS.
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 Our findings do not support the published work describing CSF sCD59 as a powerful biomarker
4 of NMOSD and MS.¹⁶ The reasons for this failure to replicate are uncertain. Our cohort was
5
6 overwhelmingly comprised of Caucasians with well-characterised disease followed up over a
7
8 prolonged period; the published study was from Japan and thus was very different in terms of
9
10 ethnicity. We used a commercial assay using validated anti-CD59 monoclonal antibodies and a
11
12 recombinant protein standard; the published study used a different commercial CD59 ELISA
13
14 assay (by MyBioSource, Inc., detection range; 31.25–2000 pg/ml) with no details of its
15
16 architecture. Our measured values were approximately 100-fold higher than those in the previous
17
18 report (~30ng/ml cf. ~300pg/ml); of note, our ability to detect sCD59 by western blotting in 10-
19
20 fold concentrated CSF supports our finding of higher levels.
21
22
23
24
25
26
27
28

29 CD59 is a GPI-anchored membrane-associated molecule and its appearance in the fluid phase
30
31 requires either shedding on lipid vesicles with an intact GPI anchor or enzymatic anchor
32
33 cleavage and release as a soluble, unanchored molecule; sCD59 in urine has been shown to be
34
35 anchor-free, likely derived by enzymatic cleavage from renal tubular cells.²⁸⁻³⁰ In order to
36
37 investigate the nature of sCD59 in CSF we performed high-speed centrifugation to remove
38
39 microparticles and re-measured sCD59 in a subset of CSF samples. Measured levels were little
40
41 affected by centrifugation, indicating that CSF sCD59 was anchor free; this was further
42
43 confirmed by showing that passage of the few CSF samples available in sufficient quantities
44
45 through a 0.2µM filter did not reduce measured sCD59 levels. Characterisation of sCD59 by
46
47 western blotting showed that it ran as a 19-23kDa band closely resembling published blots of
48
49 urine-derived CD59.³¹
50
51
52
53
54
55
56
57
58
59
60

1
2
3 To explore the source of sCD59 in CSF we stained human brain sections; as previously reported,
4
5 CD59 staining in brain parenchyma was restricted to vessel endothelium and lumen. In sharp
6
7 contrast, choroid plexus staining was abundant, co-localised with vessels and with ependyma,
8
9 specialised glial cells responsible for production of the CSF. We suggest that enzymatic shedding
10
11 from this surface represents the likely source of CSF sCD59.
12
13
14
15

16
17
18 A surprising finding was that CSF levels of sCD59 were much higher than in matched plasma. A
19
20 possible explanation of this is that although sCD59 might be generated in peripheral tissues, its
21
22 small size when anchor-free means that it is excreted first-pass through the kidney where it
23
24 accumulates in urine; in CSF, sCD59 accumulates because the BBB is impermeant to molecules
25
26 larger than 600 Da.³² Whether the (relatively) high amounts of sCD59 in CSF are of any
27
28 functional relevance remains to be demonstrated. In principle, sCD59 might contribute to
29
30 homeostasis by dampening down complement activation in the CSF; however, in other
31
32 biological fluids sCD59 is a poor regulator because it associates with other proteins.³³
33
34
35
36
37
38

39 In conclusion, we show that a non-anchored soluble form of sCD59 is present in CSF in health
40
41 and neuroinflammatory diseases at similar levels, that CSF levels of sCD59 are consistently
42
43 higher than in matched plasmas and that the likely source of CSF sCD59 is the choroid plexus, a
44
45 site of abundant expression in the normal and inflamed brain.
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

References

1. Morgan BP, Gasque P, Singhrao S, et al. The role of complement in disorders of the nervous system. *Immunopharmacology* 1997; 38: 43–50.
2. Morgan BP, Berg CW, Harris CL. Homologous restriction'' in complement lysis: roles of membrane complement regulators. *Xenotransplantation* 2005; 12: 258–265.
3. Canova C, Neal JW, Gasque P. Expression of innate immune complement regulators on brain epithelial cells during human bacterial meningitis. *J Neuroinflammation* 2006; 3: 22.
4. Veerhuis R, Nielsen HM, Tenner AJ. Complement in the brain. *Mol Immunol* 2011; 48: 1592–603.
5. Lampert PW, Oldstone MB. Host immunoglobulin G and complement deposits in the choroid plexus during spontaneous immune complex disease. *Science* 1973; 180: 408–10.
6. Meeker RB, Williams K, Killebrew DA, et al. Cell trafficking through the choroid plexus. *Cell Adh Migr* 2012; 6: 390–396.
7. Moore GRW, Laule C, Leung E, et al. Complement and Humoral Adaptive Immunity in the Human Choroid Plexus: Roles for Stromal Concretions, Basement Membranes, and Epithelium. *J Neuropathol Exp Neurol* 2016; 75: 415–28.
8. Barnett MH, Parratt JDE, Cho E-S, et al. Immunoglobulins and complement in postmortem multiple sclerosis tissue. *Ann Neurol* 2009; 65: 32–46.
9. Compston DA, Morgan BP, Campbell AK, et al. Immunocytochemical localization of the terminal complement complex in multiple sclerosis. *Neuropathol Appl Neurobiol*; 15: 307–16.
10. Ingram G, Hakobyan S, Hirst CL, et al. Complement regulator factor H as a serum biomarker of multiple sclerosis disease state. *Brain* 2010; 133: 1602–11.

11. Ingram G, Hakobyan S, Hirst CL, et al. Systemic complement profiling in multiple sclerosis as a biomarker of disease state. *Mult Scler* 2012; 18: 1401–11.
12. Ingram G, Loveless S, Howell OW, et al. Complement activation in multiple sclerosis plaques: an immunohistochemical analysis. *Acta Neuropathol Commun* 2014; 2: 53.
13. Michailidou I, Willems JGP, Kooi E-J, et al. Complement C1q-C3-associated synaptic changes in multiple sclerosis hippocampus. *Ann Neurol* 2015; 77: 1007–26.
14. Storch MK, Piddlesden S, Haltia M, et al. Multiple sclerosis: in situ evidence for antibody- and complement-mediated demyelination. *Ann Neurol* 1998; 43: 465–71.
15. Watkins LM, Neal JW, Loveless S, et al. Complement is activated in progressive multiple sclerosis cortical grey matter lesions. *J Neuroinflammation* 2016; 13: 161.
16. Uzawa A, Mori M, Uchida T, et al. Increased levels of CSF CD59 in neuromyelitis optica and multiple sclerosis. *Clin Chim Acta* 2016; 453: 131–3.
17. Polman CH, Reingold SC, Banwell B, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol* 2011; 69: 292–302.
18. Wingerchuk DM, Banwell B, Bennett JL, et al. International consensus diagnostic criteria for neuromyelitis optica spectrum disorders. *Neurology* 2015; 85: 177–189.
19. Waters P, Woodhall M, O'Connor KC, et al. MOG cell-based assay detects non-MS patients with inflammatory neurologic disease. *Neurol Neuroimmunol neuroinflammation* 2015; 2: e89.
20. Kitley J, Leite MI, Küker W, et al. Longitudinally Extensive Transverse Myelitis With and Without Aquaporin 4 Antibodies. *JAMA Neurol* 2013; 70: 1375.
21. Rudd PM, Morgan BP, Wormald MR, et al. The glycosylation of the complement regulatory protein, human erythrocyte CD59. *Adv Exp Med Biol* 1998; 435: 153–62.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
22. Marchbank KJ, Morgan BP, van den Berg CW. Regulation of CD59 expression on K562 cells: effects of phorbol myristate acetate, cross-linking antibody and non-lethal complement attack. *Immunology* 1995; 85: 146–52.
 23. Hu WT, Watts KD, Tailor P, et al. CSF complement 3 and factor H are staging biomarkers in Alzheimer’s disease. *Acta Neuropathol Commun* 2016; 4: 14.
 24. Wang H, Wang K, Wang C, et al. Increased soluble C5b-9 in CSF of neuromyelitis optica. *Scand J Immunol* 2014; 79: 127–30.
 25. Lindblom RPF, Aeinehband S, Ström M, et al. Complement Receptor 2 is increased in cerebrospinal fluid of multiple sclerosis patients and regulates C3 function. *Clin Immunol* 2016; 166–167: 89–95.
 26. Singhrao SK, Neal JW, Rushmere NK, et al. Differential expression of individual complement regulators in the brain and choroid plexus. *Lab Invest* 1999; 79: 1247–59.
 27. Singhrao S., Neal J., Morgan B., et al. Increased Complement Biosynthesis By Microglia and Complement Activation on Neurons in Huntington’s Disease. *Exp Neurol* 1999; 159: 362–376.
 28. Jacob A, Alexander JJ. Complement and blood–brain barrier integrity. *Mol Immunol* 2014; 61: 149–152.
 29. Lehto T, Honkanen E, Teppo AM, et al. Urinary excretion of protectin (CD59), complement SC5b-9 and cytokines in membranous glomerulonephritis. *Kidney Int* 1995; 47: 1403–11.
 30. Wakabayashi M, Ohi H, Tamano M, et al. Acquired loss of erythrocyte complement receptor type 1 in patients with diabetic nephropathy undergoing hemodialysis. *Nephron Exp Nephrol* 2006; 104: e89-95.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
31. Gonzalez-Calero L, Martin-Lorenzo M, de la Cuesta F, et al. Urinary alpha-1 antitrypsin and CD59 glycoprotein predict albuminuria development in hypertensive patients under chronic renin-angiotensin system suppression. *Cardiovasc Diabetol* 2016; 15: 8.
32. Pajouhesh H, Lenz GR. Medicinal chemical properties of successful central nervous system drugs. *NeuroRX* 2005; 2: 541–553.
33. Väkevä A, Jauhiainen M, Ehnholm C, et al. High-density lipoproteins can act as carriers of glycoposphoinositol lipid-anchored CD59 in human plasma. *Immunology* 1994; 82: 28–33.

Table 1: Details of the cases used for IHC in this study.

Case	Gender	Age at death	Cause of death	PMD (hours)
MS 1	F	37	Pulmonary Embolism	13
MS 2	F	39	Bronchopneumonia, MS	9
MS 3	M	51	MS, Respiratory Failure	17
MS 4	F	45	MS	17
MS 5	M	50	Pneumonia	12
Ctrl 1	F	62	Metastatic Colorectal Cancer	24
Ctrl 2	F	60	Metastatic Breast Cancer	48
Ctrl 3	M	59	Infective Exacerbation	24
Ctrl 4	M	56	Cardiac Arrest	40
Ctrl 5	M	68	Cardiac Arrest	48
Ctrl 6	M	56	Intra-Operative Death	48
Ctrl 7	M	69	Drug Overdose	36

All cases were pathologically confirmed as progressive MS or as non-neurological, non-neuroinflammatory controls (Ctrl). PMD; post-mortem delay.

Figure legends

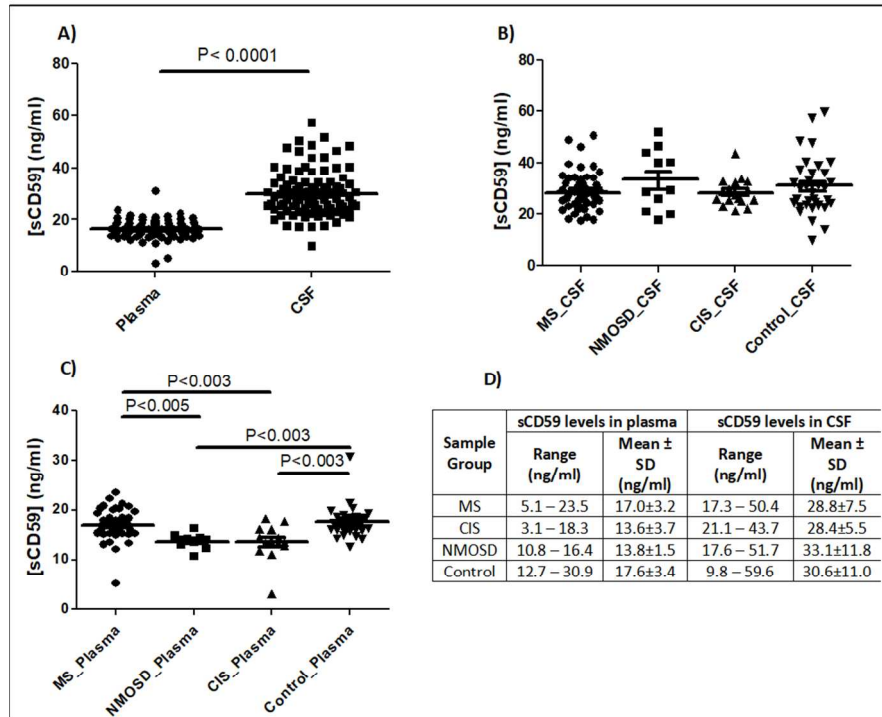
Table 1. Details of the cases used for IHC in this study. All cases were pathologically confirmed as progressive MS or as non-neurological, non-neuroinflammatory controls (Ctrl). PMD; post-mortem delay.

Figure 1. Measuring sCD59 in CSF and matched plasma samples. A) sCD59 is measurable in all plasma and CSF samples tested and are ~2-fold lower in plasma compared to CSF. B) CSF levels of sCD59 were not significantly different between the individual groups; MS, NMOSD, CIS or Controls. C) Plasma levels of sCD59 in MS, NMOSD and CIS patients compared with normal controls differentiate MS from NMOSD ($p < 0.005$), MS from CIS and NMOSD/CIS from controls ($p < 0.003$). D). Summary of the data.

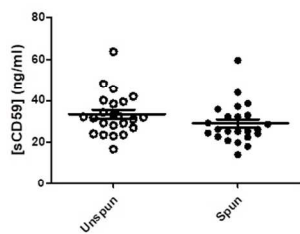
Figure 2. Measurement of sCD59 in CSF; samples from 23 individuals (MS=9, NMOSD=2, CIS=4, control=8) were measured before (unspun) and after (spun) high-speed centrifugation; no significant difference seen in sCD59 levels between both groups (mean; unspun: 28.1ng/ml, spun: 32.5ng/ml).

Figure 3. Detection of sCD59 in CSF patient samples using western blot; CSF samples were concentrated x 10. (MS=3, NMOSD=3, Control=6). A) Samples with higher concentrations of sCD59 as measured in the ELISA (NMOSD, Control), B); Samples with lower concentration of sCD59 in ELISA (MS and Control). In all cases, 10-fold concentrated sample (25 μ l per line) was resolved on 4-20% gradient PAGE under non-reducing conditions and processed for western blot with MEM43 (monoclonal anti-CD59). MS; Multiple Sclerosis, NMOSD; Neuromyelitis Optica Spectrum Disorder, CIS; Clinically Isolated Syndrome, CTR; Control, Hu EA(+)/Ctr; CD59 positive human erythrocyte lysate.

1
2
3 Figure 4. CD59 is expressed at vessel luminal surfaces and by cells lining the choroid plexus
4 and ventricle wall. Immunohistochemistry of formalin-fixed paraffin embedded MS and
5 control hippocampus revealed strong staining with monoclonal anti-CD59 in the vasculature
6 of the meninges (A- D), parenchymal vessels (E, F), cells of the ventricle wall (ependyma of
7 the inferior horn of the lateral ventricle (G, H) and epithelial cells of the choroid plexus (I, J).
8 An identical pattern of staining was seen with a polyclonal anti-CD59 antibody (K). Staining
9 with an irrelevant mouse (L) or rabbit (not shown) IgG was negative. All images captured
10 with a 10x (A, C, E, G, I) or 40x (B, D, F, H, J, K, L) objective. Scale bar= 50µm shown in A
11 and B.
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



243x268mm (120 x 120 DPI)

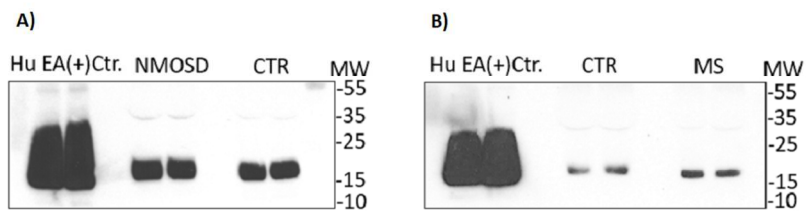


243x137mm (120 x 120 DPI)

Peer Review

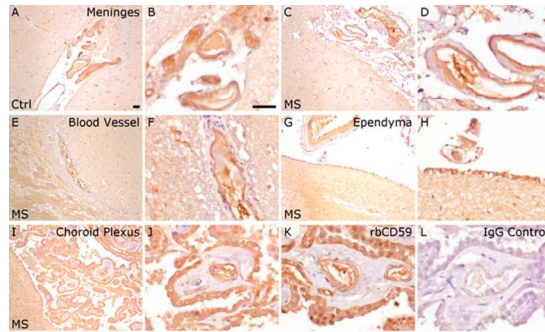
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



243x137mm (120 x 120 DPI)

Peer Review



243x137mm (120 x 120 DPI)

er Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60