

# Complement protein isoforms in CSF as possible biomarkers for neurodegenerative disease

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**Abstract.** It has been suggested that the activation of the complement system is involved in the pathogenesis of several neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS). Here, the CSF expression levels of complement proteins C3b, C4b, factor B, and factor H were compared between normal subjects and patients diagnosed with AD, PD, MS, and neurosyphilis. The CSF proteins were initially separated using two-dimensional gel electrophoresis, which allowed the comparison of some of the individual complement isoforms. Patients with AD, PD, and MS all showed more than one complement isoform with a significant change ( $p < 0.05$ ) in CSF expression level compared to normal subjects. PD patients were found to have the greatest number of significantly changed isoforms, all showing a decreased expression level in PD CSF. The complement isoforms examined were able to distinguish between some, but not all, of the diseases studied. The data suggest that when investigating a protein as a possible biomarker, it may be useful to compare individual protein isoform expression levels in addition to the more commonly measured total protein expression level.

## 1. Introduction

There is a growing interest in the use of proteomic technologies to identify proteins that can act as disease biomarkers. These biomarkers could be used to improve disease diagnosis, monitor disease progression, or examine the effects of therapy [1]. One area of active biomarker research is related to the differential diagnosis of neurodegenerative diseases. For some neurodegenerative diseases, such as Alzheimer's disease (AD), a definitive antemortem diagnosis cannot be made using current diagnostic methods [2]. There is also a need to identify protein markers that can discriminate between different neurodegenerative diseases that have overlapping symptoms.

The complement system is an integral part of the innate immune system. It participates in the body's defense against pathogens and can initiate an inflamma-

tory response. The complement system has also been implicated in the pathology of various neurodegenerative disorders including Alzheimer's disease (AD) [3, 4], Parkinson's disease (PD) [5,6], and multiple sclerosis (MS) [7,8]. The system is composed of approximately 30 soluble and membrane bound proteins and can be activated by two pathways, the classical and alternative pathways (see Fig. 1). The classical pathway is primarily activated by antibody-antigen complexes, while the alternative pathway is antibody independent. Both the classical and alternative pathways result in the formation of the membrane attack complex (MAC), which can cause lesions in targeted cell membranes and lead to cell lysis. In addition, complement activation results in the deposition of complement opsonins (C3b) on the surface of targeted cells and the release of protein fragments (C3a and C5a) that act as anaphylatoxins and mediate inflammation [9]. Studies indicate that the complement system is also involved in remodeling and repairing the brain tissue, clearing toxic protein deposits, and mediating the phagocytosis of necrosed/apoptotic neurons by microglia (reviewed in [3]).

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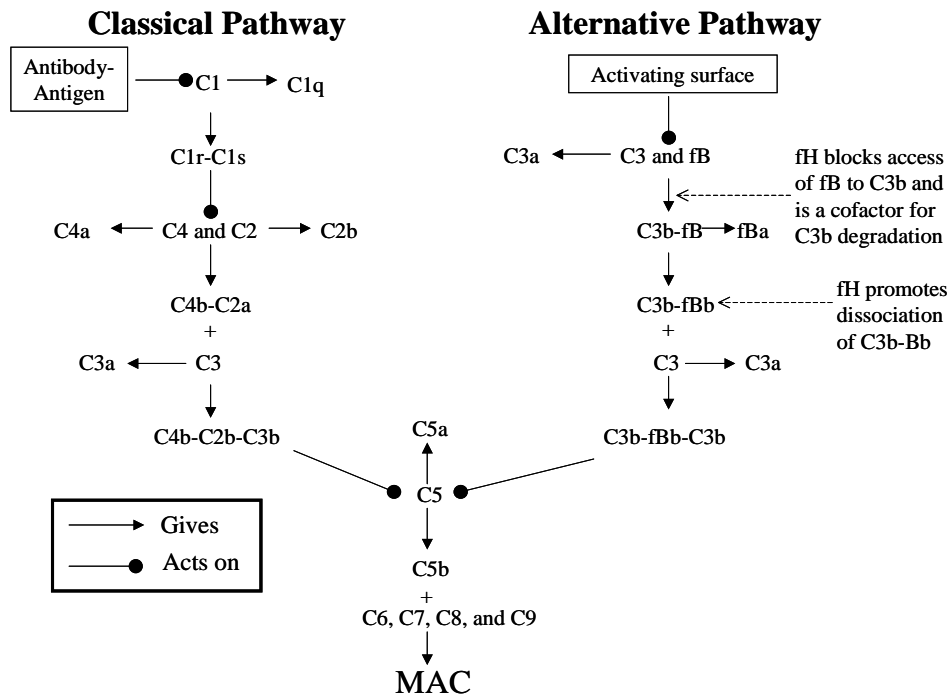


Fig. 1. A simplified schematic of the interaction between the complement proteins in both the classical and alternative pathways [6,9]. The classical pathway is primarily activated by antibody-antigen complexes while the alternative pathway is antibody-independent. Both pathways result in the formation of the membrane attack complex (MAC) and the release of anaphylatoxins (C3a and C5a).

Previous studies have investigated complement proteins in cerebrospinal fluid (CSF) as possible biomarkers of central nervous system disease [8,10–14]. In these studies, the concentration of complement components or factors was measured using antibody-based techniques such as ELISA or other immunoassays. The results of these studies suggest that the CSF concentrations of complement proteins C3, C4, and the C5b-9 complex can differentiate between normal subjects and patients with MS [10–12]. For AD, previous studies have found inconclusive results when comparing the total amount of C3a [15] and mixed results comparing total amount of C1q [14,16] in the CSF between AD patients and controls. For PD there are no studies comparing concentrations of complement proteins to normal subjects. However, there is a study indicating that the CSF concentration of C4d is lower in PD patients than patients with progressive supranuclear palsy and is similar between patients with PD and patients with cervical spondylosis [13].

We have previously built a database of CSF proteins after separation by two-dimensional gel electrophoresis (2DE) [17]. In these electrophoresis gels, several proteins from the complement cascade were identified including complement components C3b and C4b and

complement factors B and H. All of these complement proteins were identified in several 2DE protein spots indicating that multiple isoforms of each protein are present in CSF. This is probably caused by post-translational modifications (PTM). It is possible that a comparison of the individual isoform expression levels between disease states, in addition to a comparison of the total protein expression level, may improve the diagnostic utility of that protein. From Figure 1 it can be seen that C3 participates in both the alternative and classical complement pathways, C4 only participates in the classical pathway, and factor B (fB) only participates in the alternative pathway. Factor H (fH) is a regulator of the complement pathway; it degrades C3b and accelerates the decay of the C3 convertase enzyme. Therefore, an examination of these four proteins (C3b, C4b, fB, and fH) can give information on both of the complement pathways and the regulation of the complement system. Here, we present a comparison of these four proteins on CSF 2DE gels from normal subjects and patients diagnosed with AD, PD, MS and neurosyphilis (NS) to determine if the individual complement protein isoform expression levels and the total protein expression levels may be useful as biomarkers of neurodegenerative disease.

## 2. Methods

### 2.1. CSF samples

Antemortem lumbar CSF samples from several CSF tissue banks and other sites in the United States were shipped on dry ice and stored at  $-70^{\circ}\text{C}$  until needed. A total of 34 CSF samples were used: nine from patients diagnosed with Alzheimer's disease (AD diagnosis confirmed by autopsy); ten from patients diagnosed with Parkinson's disease; three from patients diagnosed with multiple sclerosis; three from patients diagnosed with neurosyphilis; and nine from normal subjects with no indication of dementia or neurodegenerative disease.

### 2.2. Two-dimensional gel electrophoresis

The details of the protocols used for performing two-dimensional gel electrophoresis (2DE) have been previously published [18]. Briefly, 250  $\mu\text{L}$  of CSF (containing approximately 100  $\mu\text{g}$  of protein) were precipitated using ice-cold ethanol and the resulting protein pellet was dissolved in a solution of 9M urea, 2% 2-mercaptoethanol, 2% IGEPAL, and 0.8% carrier ampholytes. The sample was then hydrated into 18 cm, 3–10 nonlinear immobilized pH gradient (IPG) isoelectric focusing gels (Amersham Biosciences). Isoelectric focusing was then performed at  $20^{\circ}\text{C}$  using the Protean IEF unit (Bio-Rad Laboratories) for a total of 100 kVh to separate proteins in the first dimension by isoelectric point. The IPG gels were equilibrated in solutions containing dithiothreitol and subsequently iodoacetamide for reduction and alkylation of the focused proteins. To separate proteins in the second dimension by protein size, polyacrylamide gel electrophoresis was performed using 12–15%T vertical gradient slab gels. The separated proteins were fixed in the gel, stained with SYPRO Ruby Protein Gel Stain (Molecular Probes), and destained for 24 hours in a solution of 10% methanol and 7% acetic acid following the manufacturer's instructions. The gels were scanned on a FLA-3000 Fluorescent Image Analyzer (Fuji Photo Film Company).

The proteins in the 2DE spots have been previously identified using mass spectrometry [17]. The spots were excised from the 2DE gel and the proteins were digested in-gel with trypsin using previously published methods [19]. The resulting peptides were analyzed on a matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometer (MALDI-TOF/TOF, 4700 Proteomics analyzer, Applied Biosys-

tems) as described in [17]. The peptide mixture was co-crystallized on a stainless steel MALDI plate with 5 mg/mL  $\alpha$ -cyano-4-hydroxycinnamic acid/ $\sim 1$  mM ammonium phosphate in 50% acetonitrile/0.1% trifluoroacetic acid using the dried droplet method. Peptide mass fingerprint data were collected in positive reflector mode in the range of 900 to 4000 mass to charge ratio ( $m/z$ ) and calibrated internally using trypsin autolysis peptides. Several of the highest intensity non-trypsin peaks were selected for tandem mass spectrometry analysis. The selected peptides were isolated and then fragmented using air, at  $1\text{E}-6$  torr, as the collision gas. The  $m/z$  of the peptide fragments were measured and calibrated using a default calibration.

The spectra were analyzed using GPS Explorer (Version 1.1, Applied Biosystems), which acts as an interface between the Oracle database containing raw spectra and a local copy of the Mascot search engine (Version 1.8 [20]). The peptide mass fingerprint spectra and the tandem mass spectra were searched together against a locally stored copy of the NCBI human protein sequence database [21] using the Mascot search engine. The search included all peaks with a signal to noise ratio greater than 5 and allowed for up to one missed trypsin cleavage site, carbamidomethylation of cysteine, and oxidation of methionine. A mass tolerance of 25 ppm was used for the peptide mass fingerprint data and of 0.2 Da for the tandem mass spectrometry data. To be considered a match, a confidence interval (%CI) calculated by GPS Explorer (GPS), of at least 95% was required. The GPS %CI is calculated from the Mascot Mowse score<sup>1</sup> with the significance threshold removed. The closer a GPS %CI is to 100%, the higher the probability that the identification is correct.

The gel images were imported into the Melanie software package (Version 4.0, GeneBio). Spots were auto-detected by the software and the detected spots were manually edited to remove technical artifacts. The spots that contained complement proteins were determined in each of the sample gels. The percent integrated optical density (% volume) of each of these 2DE spots was then measured. The % volume data were used in a series of Student t-tests to determine if the protein isoform concentrations were statistically different ( $p < 0.05$ ) between the different disease states. To determine the total % volume of a particular protein, the % volume for each of the isoforms of that protein were added.

<sup>1</sup>The Mascot Mowse score is equal to  $-10*\text{Log}(P)$  where  $P$  is the probability that the observed match is a random event [20].

Table 1  
Identification information for each of the complement isoforms

	NCBI nr accession #	Mascot mowse score	GPS % CI	Amino acid sequence coverage from mass spectrum
<b>Factor B</b>				
Isoform1	4502397	95	100	51-74, 309-319, 338-348, 547-564, 566-581, 669-679
Isoform2	4502397	125	100	51-74, 183-193, 309-319, 338-348, 547-581, 588-605, 609-639, 754-764
Isoform3	4502397	152	100	51-74, 151-176, 300-319, 338-348, 420-445, 447-454, 547-564, 566-581, 754-764
<b>C3b</b>				
Isoform1	4557385	84	99.9	105-148, 208-249, 264-281, 291-304, 344-359, 387-425, 440-478, 531-544, 567-584, 634-657
Isoform2	4557385	182	100	36-66, 105-119, 137-148, 162-176, 208-258, 264-281, 290-304, 323-359, 387-425, 428-478, 489-497, 509-544, 567-600, 634-657
<b>Factor H</b>				
Isoform1	758073	86	99.9	157-175, 212-232, 296-303, 332-351
Isoform2	758073	52	97.9	157-175, 332-351
<b>C4b</b>				
Isoform1	14577919	106	100	1475-1495, 1508-1530, 1602-1610, 1644-1652
Isoform2	14577919	102	100	1455-1462, 1475-1495, 1508-1561, 1602-1610, 1613-1627, 1644-1652
Isoform3	14577919	67	99.9	1451-1462, 1475-1495, 1508-1530, 1563-1570, 1602-1610, 1613-1627, 1644-1652
Isoform4	14577919	88	99.9	1475-1495, 1508-1530, 1563-1574
Isoform5	14577919	95	100	1475-1495, 1508-1530, 1644-1652

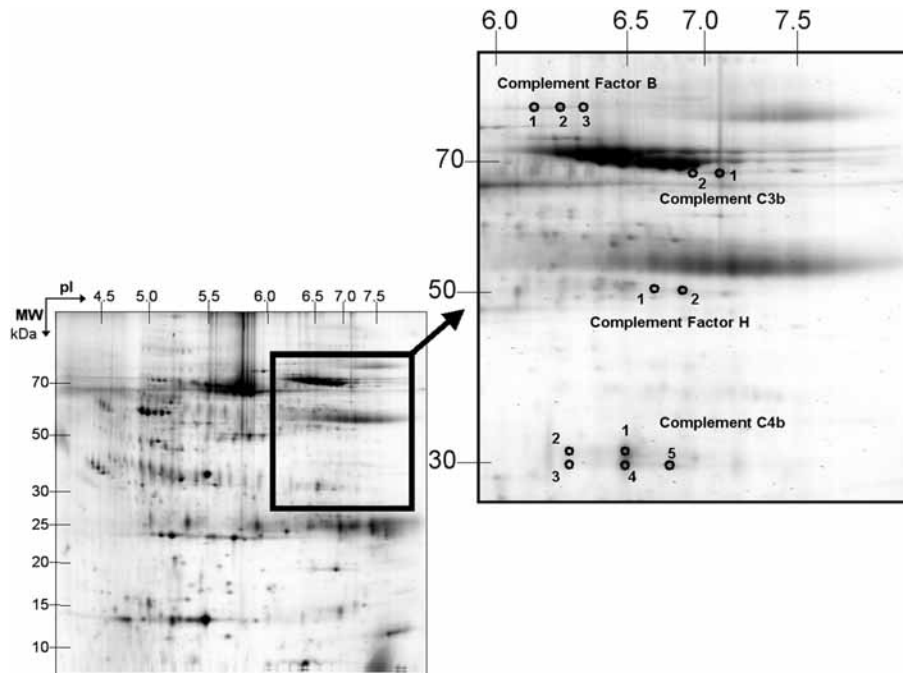


Fig. 2. An example of a 2DE gel from a normal subject. For each of the complement proteins identified in the 2DE gel, multiple isoforms were present. The complement protein isoforms used in this study are indicated by circles and are numbered.

### 3. Results

An example of a 2DE gel from a normal subject is shown in Fig. 2. The spots that were previously identified to contain complement proteins are marked with a circle and each complement protein isoform is numbered. An example of a peptide mass spectrum and a

tandem mass spectrum for Isoform 2 of complement C4b is shown in Fig. 3. The tandem mass spectra from a MALDI-TOF/TOF instrument typically includes y-ions, b-ions, a-ions, immonium ions, and internal fragments. In Fig. 3 the b-ion series has been labeled and the corresponding amino acid sequence is shown. The

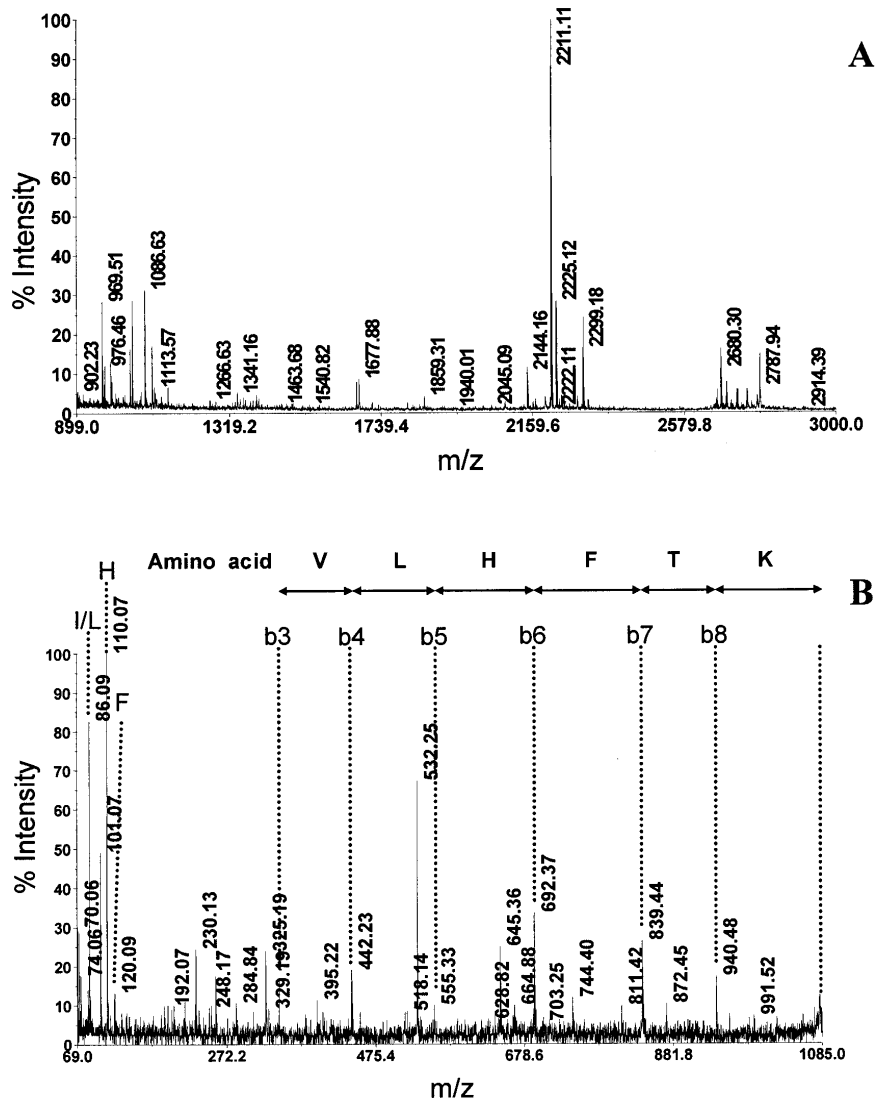


Fig. 3. Mass spectra for the 2DE spot labeled as complement C4b Isoform2 in Fig. 2. A) Peptide mass fingerprint spectrum, B) Spectrum resulting from the isolation and fragmentation of the peptide with  $m/z = 1086.63$ . This peptide covers amino acids 1644–1652 of the C4 protein and has the amino acid sequence ITQLHFTK. The immonium ions and b-ions detected in the spectrum are labeled and the corresponding amino acid sequence is shown.

amino acid sequence coverage for each of the complement isoforms (based on the peptides identified in the MS spectra) are shown in Table 1 along with the NCBI accession numbers, the Mascot Mowse scores, and the GPS %CI.

There were three isoforms of complement factor B (fB). The predicted properties of fB (MW = 85 kDa and pI = 6.6) and the mass spectra sequence coverage indicate that these spots contain intact fB.

Two isoforms of complement component 3 were identified at an approximate molecular weight of 70 kDa and approximate pIs of 6.9 and 7.1. Com-

plete component 3 has a molecular weight of 185 kDa, which indicates that these 2DE spots contain a fragment. Complement component 3 has two primary fragments: C3a and C3b. C3b is composed of two chains linked by disulfide bonds [9]. The amino acid sequence coverage of the peptide mass fingerprint spectra for these 2DE spots corresponds to the beta chain of complement component 3b (which includes amino acids 23 through 667 of the complement 3 protein). The position of the spots also matches the predicted MW and pI of the beta chain of C3b (MW = 71 kDa and pI = 6.8).

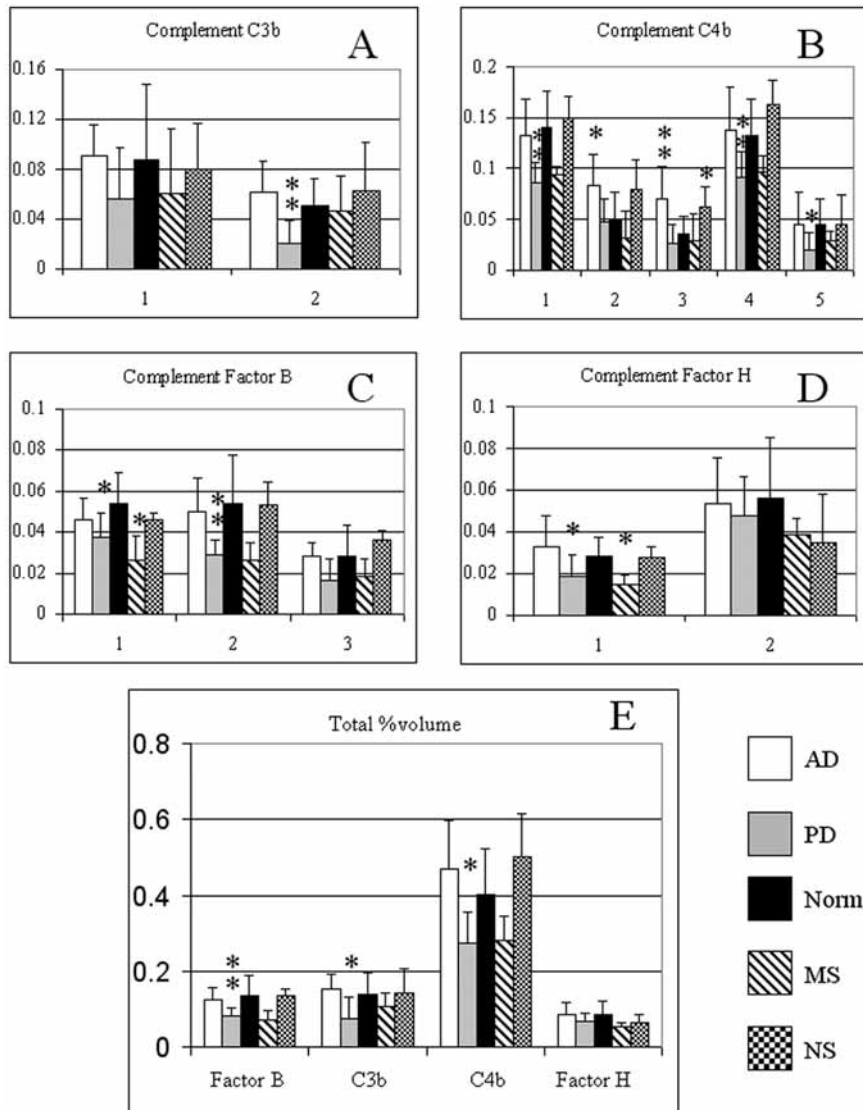


Fig. 4. Plots indicating the average % volume for complement proteins in CSF samples from each disease state. Asterisks indicate the isoforms that show a significant change in % volume for the disease state compared to normal state. A) Average % volume for C3b isoforms. B) Average % volume for C4b isoforms. C) Average % volume for fB isoforms. D) Average % volume for fH isoforms. E) Average total % volume for all four proteins in A-D.

Complement factor H (fH) was identified as being present in two spots at a MW of approximately 50 kDa and pIs of approximately 6.6 and 6.8. The mass spectra sequence coverage, the MW, and the pI suggest that the 2DE spots contain the alternative splice variant of fH also known as reconectin [22]. This splice variant, which contains amino acids 1 through 445 of complete complement factor H, has a predicted MW and pI of 51 kDa and 6.8.

Five isoforms of complement component 4b (C4b) were identified. C4b is a fragment of complement com-

ponent 4 (NCBI accession #14577919) that contains three chains linked by disulfide bonds [9]. The locations of the C4b spots (MW and pI) as well as the mass spectra sequence coverage correspond to the gamma chain of C4b. The gamma chain includes amino acids 1454 through 1744 of the intact complement 4 protein and has a predicted MW of 33 kDa and a predicted pI of 6.4.

The average % volume data for each of the isoforms in each of the disease groups are shown in Fig. 4. Table 2 indicates which individual isoform % volume and

Table 2

Complement isoforms showing a significant ( $p < 0.05$ ) change in %volume compared to normal controls<sup>1</sup>

AD ( $n = 9$ )	MS ( $n = 3$ )	NS ( $n = 3$ )	PD ( $n = 10$ )
C4b: Isoform2, <b>Isoform3</b>	fB: Isoform1 fH: Isoform1	C4b: Isoform3	C3b: <b>Isoform2</b> , total C4b: <b>Isoform1</b> , <b>Isoform4</b> , Isoform5, total fB: Isoform1, <b>Isoform2</b> , total, fH: Isoform1

<sup>1</sup>Bold type indicates  $p < 0.01$ .

total protein % volume show a statistical difference between the normal and diseased states. CSF from PD patients was found to have the largest number of complement isoforms showing a change in %volume while CSF from NS patients showed the smallest number of %volume changes. It should be noted for NS, MS, and AD that although there are no total complement % volume data which show a significant change as compared to normals, some individual isoforms do show a significant change. For AD one of these differentially expressed isoforms has a  $p < 0.01$ .

To determine if these complement proteins could differentiate between the disease states, a series of t-tests were performed. Table 3 indicates which isoform or total complement % volumes showed a difference in expression between each pair of disease states. None of the isoforms or total complement proteins show a significant % volume change between the NS and AD samples or between the PD and MS samples.

#### 4. Discussion

Numerous studies have indicated that there is a change in complement activity in the brains of patients with AD (see review in [3,4]). Both the mRNA and protein expression levels of complement proteins have been found to increase in AD brain tissue [23]. Although none of the total % volume indicate a difference between the AD and normal patients, two isoforms of the C4b protein (Isoform2 and Isoform3) show a significant increase in %volume ( $p < 0.05$  and  $p < 0.01$  respectively). These two isoforms migrate to the same pI, however, isoform2 has a slightly larger molecular weight as measured by 2DE. The fact that these isoforms exhibit a significant change in % volume may suggest that the presence of a post-translational modification could be disease-dependent. Therefore, a method that can simultaneously monitor the expression levels of individual isoforms (such as 2DE) may be advantageous in disease biomarker studies.

Previous studies also indicate that there are changes in the complement activity in the brain of PD pa-

tients [5,6]. The mRNA expression levels of several complement components were found to be increased in the PD brain [6]. Lewy bodies in substantia nigra of PD patients were positive for C3d, C4d, C7 and C9 [5] when tested using immunoassays. The % volume data in Fig. 4 indicate that there is a change in CSF complement expression in PD patients. In general, the %volume of the C3b, C4b, and factor B isoforms are lower than those in normal subjects and many of these changes are statistically significant. A decrease in these components could indicate that they are being depleted by over-activation of the complement system.

For MS, these preliminary results, found using 2DE to measure the relative concentration of individual isoforms, are similar to the results that have previously been found using antibody-based methods. The pathology of multiple sclerosis has been linked to a change in complement activity [24,25] and previous comparisons of the C3 and C4 concentrations in the CSF of MS and normal patients have found a decrease in one or both components [11,12]. However, not all studies found the decrease to be statistically significant. The data in Fig. 4 and Table 2 show that all of the complement protein isoforms demonstrate a lower % volume in MS patients compared to normals, but that this decrease is only significant for one factor H isoform and one factor B isoform.

In the case of neurosyphilis, although the complement system may be part of the body's immune response to the invading bacteria *Treponema pallidum*, there is no published evidence to link the over-activation of the complement system to the pathology of the disease. It is found that only one isoform (C4b Isoform3) shows a change in % volume in NS patients.

The ability of the complement proteins to differentiate between neurodegenerative diseases is shown in Table 3. The data indicate that although the complement proteins studied here can differentiate between the diseases that have a larger change in complement expression (i.e. PD and MS) and lower change in complement expression (i.e. NS and AD) they cannot differentiate within these two groups (i.e. differentiate PD

Table 3

Complement isoforms that were found to have a significant ( $p < 0.05$ ) difference in %volume between two disease states<sup>1</sup>

	AD	MS	NS
MS	fB: Isoform1, Isoform2, total C4b: Isoform2, total	NA	—
NS		fB: Isoform2, Isoform3, total C4b: Isoform1, Isoform4, total fH: Isoform1	NA
PD	fB: <b>Isoform2, Isoform3, total</b> , C3b: Isoform1, <b>Isoform2, total</b> C4b: <b>Isoform1, Isoform2, Isoform3, Isoform4, Isoform5, total</b> fH: Isoform1		fB: Isoform2, Isoform3, <b>total</b> C3b: Isoform2 C4b: <b>Isoform1, Isoform3, Isoform4, total Isoform4, total</b>

<sup>1</sup>Bold type indicates that  $p < 0.01$ .

from MS). A differential diagnosis is therefore not possible using only these complement proteins. The proteins can differentiate between some disease states (i.e. AD and PD) therefore combining them with other established disease markers (such as  $\beta$ -amyloid in AD) may result in a higher overall diagnostic accuracy.

The results of this study suggest that isoforms of complement proteins may be useful as biomarkers. However, to confirm these preliminary results, follow-up studies need to be performed with a larger number of CSF samples. Further, the complement protein fragments and isoforms compared in this study are those that had been identified in a previous 2DE CSF database. Other complement associated proteins and fragments are known to be present in CSF but have not yet been identified in the 2DE CSF database and therefore are not included in this preliminary analysis.

## 5. Conclusions

The complement proteins C3b, C4b, factor B, and factor H were found to be present as multiple isoforms within CSF. Changes in complement isoform concentrations were found in the CSF samples of AD, MS, and PD patients compared to normal subjects. This is the first report of a change in the CSF complement expression between Parkinson's disease patients and normal subjects. These initial results also indicate that, when searching for disease biomarkers, it would be useful to measure the expression level of individual protein isoforms in addition to the total protein expression level.

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