Cerebrospinal Fluid Biomarkers for the Differential Diagnosis of Normal Pressure Hydrocephalus and Alzheimer's Disease

by

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Abstract

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Differential diagnosis of Idiopathic Normal Pressure Hydrocephalus (NPH) is complicated by symptomatic overlap with neurodegenerative conditions such as Alzheimer's Disease (AD). Efforts to improve diagnosis through the use of cerebrospinal fluid (CSF) biomarkers have led to the identification of more than a dozen potential diagnostic markers for NPH. However, no single biomarker has proven sufficient for differential diagnosis in clinical practice. The current study uses proteomic analysis of CSF to identify sets of protein markers that are expressed differentially in NPH and AD. Two-dimensional gel electrophoresis was used to analyze the CSF of 8 probable NPH and 8 probable AD patients. Gels were stained with SYPRO Ruby and the percentage volume of over 1339 spots was determined. The Random Forest statistical method was used to identify proteins that optimally segregated NPH cases from AD. Protein identification was achieved by the use of a previously published CSF map and mass spectrometry. Eleven protein spots were found to optimally distinguish the groups, correctly classifying 100% of all NPH and AD samples. Of the 11 proteins of interest, six were identified and include the following: β-trace, serum albumin A, serum albumin B, apolipoprotein A-IV precursor, pigment epithelium-derived factor, and complement component 3 precursor. The current study identifies CSF biomarkers that differentiate between NPH and AD cases. The

highly successful separation of cases obtained in this study suggests that multiplexed CSF markers have the potential to improve the differential diagnosis of NPH from one of its most common competing diagnoses.

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General Introduction and Background

Idiopathic Normal Pressure Hydrocephalus (NPH) is a neurogeriatric disorder of unknown etiology characterized by enlargement of the cerebral ventricles accompanied by disturbances in gait and balance, control of urination, and cognitive function. While the precise incidence and prevalence is uncertain, estimates suggest that 1-6% of all dementia in the elderly may be related to NPH, and approximately half of one percent of the population over 65 may be suffering from the disease (Caruso, Cervoni, Vitale, & Salvati, 1997; Trenkwalder, et al., 1995). It is the second most common cause of hydrocephalus, next to subarachnoid hemorrhage (Chahlavi, El-Babaa, & Luciano, 2001), but unlike secondary hydrocephalus, no apparent precipitating factor, such as head trauma or meningitis, is identified. Unfortunately, many cases of NPH are unrecognized until late stages or are misdiagnosed as having other disorders (P. Klinge, Marmarou, Bergsneider, Relkin, & Black, 2005). Additionally, differential diagnosis is complicated by symptomatic overlap with conditions such as Alzheimer's disease (AD), vascular dementia (VD), and Parkinson's disease (PD) (Holodny, et al., 1998; J. A. Vanneste, 2000), and similar symptoms in older adults (changes in gait, cognition, and urinary symptoms). Recognition has been improved by the use of tools such as magnetic resonance imaging (MRI), temporary lumbar drainage, and neuropsychological assessment (Gallia, Rigamonti, & Williams, 2006; Huckman, 1981; Jacobs, Conti, Kinkel, & Manning, 1976), but there is still considerable diagnostic uncertainty. When a diagnosis is made, surgical placement of a ventricular shunt is implemented, often times reducing or reversing debilitating symptoms (Benzel, Pelletier, & Levy, 1990; J. A. Vanneste, 2000). Nevertheless, the alleviation of symptoms does not alter the underlying pathology, which may contribute to the relatively inconsistent outcome and high rate

of treatment complications.

A molecular biomarker for NPH would increase the accuracy of clinical diagnosis, and make earlier diagnosis possible. Additionally, examining biomarkers for NPH cerebrospinal fluid (CSF), which contains an abundance of proteins and other biochemical products that can reflect pathogenic processes in the brain (Rohlff, 2000), could improve the general understanding of NPH's relatively unknown pathophysiological mechanisms. Thus, examining biomarkers could potentially facilitate the conception of novel treatment, and assist in the prognostication of cognitive and motor changes after treatment. Although recent studies examining target proteins have found some associations between CSF biomarkers and a diagnosis of NPH (Brettschneider, Riepe, Petereit, Ludolph, & Tumani, 2004; Kapaki, et al., 2007; Malm, Kristensen, Ekstedt, Adolfsson, & Wester, 1991; Mase, et al., 2003; Nooijen, Schoonderwaldt, Wevers, Hommes, & Lamers, 1997; Tullberg, et al., 2000), none of these studies have yielded a biomarker with sufficient sensitivity and specificity to warrant routine clinical use.

Clinical Manifestations of NPH Include the "Clinical Triad"

Traditionally, the symptoms of NPH most commonly described, often called the "clinical triad," included disturbance of gait, control of urination, and deficits in cognition (Adams, Fisher, Hakim, Ojemann, & Sweet, 1965). While the majority of patients diagnosed with NPH present with a gait or balance disturbance, features of dementia or urinary changes are not always evident at the time of diagnosis. The presence of the classic clinical triad, previously considered essential for the diagnosis of NPH, is now considered representative of the more severe end of the spectrum (Relkin, Marmarou, Klinge, Bergsneider, & Black, 2005).

Gait disturbance is typically the most common clinical feature, and is often the first symptom

to develop and resolve with treatment (Fisher, 1980; Krauss, et al., 2001; Petersen, Mokri, & Laws, 1985). The gait pattern in NPH is often described as "magnetic" and "shuffling." Identifiable features include slow, short steps with reduced foot-floor clearance, reduced arm swing, broad based stance, and overall difficulty with balance (Stolze, et al., 2000); postural instability and falls are frequently observed. NPH patients also have demonstrated other motor symptoms. Motor slowing of the upper extremities has been reported in as many as half of NPH cases (Krauss, et al., 1997).

Urinary incontinence can be a relatively inconsistent symptom among NPH patients but may become evident as the disease progresses. Urinary symptoms more typically seen in the initial stages of the disease include increased urinary frequency and urgency. Urinary dysfunction in NPH has been hypothesized to result from the stretching of the frontal horns of the lateral ventricles, which results in a partial loss of bladder contraction inhibition (Tsakanikas & Relkin, 2007).

The cognitive impairments commonly observed in NPH patients tend to be consistent with a cognitive profile typically associated with subcortical dysfunction, and include slowing of information processing, reduced acquisition of information, executive dysfunction, psychomotor slowing, and some visuospatial deficits (Iddon, et al., 1999; Merten, 1999). Cortical deficits, including aphasia, agnosia, apraxia, are not typically seen, however these syndromes might develop at later stages or may be evident as a result of a comorbid cortical dementia.

Differential Diagnosis of NPH is Challenging

Diagnosis of NPH can be challenging due to considerable overlap or co-occurrence with symptoms of other neurodegenerative disorders as well as the variability in the nature and

severity of the presenting symptoms (Relkin, et al., 2005). Aspects of the symptom triad of gait disturbance, urinary incontinence, and impaired cognitive functions can be associated with aging and age-related conditions (i.e., functional limitation of ambulation, prostate-related urinary incontinence), and may falsely be attributed as such (Coppola, et al., 2002). Neuroimaging, usually computed tomography (CT) or MRI, is an obligatory element of the diagnostic process. Documentation of enlarged ventricles disproportionate to cerebral atrophy, in the absence of obstruction to CSF flow, is required to make a diagnosis of NPH. Specifically, enlargement of the frontal and temporal horns is relatively uniform and symmetric, and the cerebral aqueduct and third ventricle may be rounded. Enlarged lateral ventricles may mimic the appearance of cerebral atrophy on neuroimages, and NPH patients are sometimes misdiagnosed with AD based on a brain scan that shows ventriculomegaly (Holodny, et al., 1998). Accordingly, if the specific characteristics of ventricular enlargement are not considered, brain scans of non-NPH patients with diffuse cerebral atrophy may be misdiagnosed as having ventricular enlargement.

Some patients have NPH and AD simultaneously, and the prognosis for long-term positive response to treatment is reduced in such cases. As such, it is important to be able to distinguish AD from NPH and ultimately to identify when these two disorders co-exist. In fact, Bech-Azeddine and colleagues (2007) discovered that 25% of their NPH sample studied had concomitant biopsy-confirmed AD. The cohort with a comorbid diagnosis also represented over half of the patients who did not respond to treatment, suggesting that NPH may not have been the primary cause of symptoms. When using neuropsychological assessment as a differential diagnostic aid, studies have demonstrated that cognitive profiles may sometimes differentiate NPH from AD (Ogino, et al., 2006). In general, NPH patients have been shown to have greater impairment on frontal lobe tasks such as attention, working memory, and executive functions,

and relatively less dysfunction in the domain of memory. The profile of cognitive impairment in NPH could be attributed to damage of the frontal lobe, or potentially subcortical-frontal connectivity, rather than the medial temporal region affected by AD. However, neuropsychological testing alone may be insufficient to diagnose NPH with certainty or to conclusively distinguish it from the panoply of other disorders that can disrupt cognitive functions in the elderly.

An additional challenge in the differential diagnosis is the consideration of a unifying theory of disease, suggesting that NPH shares a common pathophysiology with other neurodegenerative disorders. For example, some researchers propose that although AD, VD, and NPH are distinct entities, they have overlapping clinical and pathological features suggesting an interrelated pathogenesis (Bateman, 2004). In addition, approximately 40% of the brains meeting clinical criteria for VD also have pathology consistent with AD, including senile plaques and neurofibrillary tangles (Skoog, Kalaria, & Breteler, 1999). While this gives rise to possible theories of a unified underlying pathophysiology, the diseases are treated with different approaches. Therefore, the differential diagnosis, which can be both clinically and pathophysiologically challenging, is essential to providing appropriate treatment. In addition, it should be noted that the current form of treatment for NPH does not address its underlying pathophysiology, as it redirects the flow of CSF without altering the source of CSF dynamic disruption, which is yet unknown.

Although the diagnosis of NPH is challenging due to the variability of symptoms and potential overlap with other disorders, a set of evidence-based guidelines was recently introduced (Marmarou, Bergsneider, Relkin, Klinge, & Black, 2005). These guidelines classify “Probable”, “Possible” or “Unlikely” based on history, brain imaging, clinical assessment of impairment, and

physiological findings (Relkin, et al., 2005). However, there are still no criteria for postmortem confirmation of NPH diagnosis, as is the case with AD and several other neurodegenerative diseases. Shunt responsiveness has been considered by some to be the gold standard of diagnostic verification (Ojemann, Fisher, Adams, Sweet, & New, 1969), but this approach can lead to an unacceptable number of false negative diagnoses. While shunt treatment is a relatively straightforward neurosurgical intervention, the challenge in treating NPH often lies in selecting appropriate surgical candidates by identifying those who are most likely to show shunt responsiveness. In many cases symptoms are only partially alleviated, and the benefits may be noticeable for only a short period of time due to the high rate of comorbidity of neurodegenerative disorders (J. Vanneste, Augustijn, Dirven, Tan, & Goedhart, 1992). Furthermore, disease duration, severity of symptoms, and concomitant cerebrovascular disease have also been shown to negatively impact treatment outcome (Holodny, et al., 1998). As such, early detection of the disease and more accurate differential diagnosis using novel tools such as biomarker panels may contribute to improved outcome in some cases. In addition, the relatively high rate of shunt complications makes patient selection of significant importance, as NPH morbidity must outweigh the potentially considerable risks. Complications associated with shunting include infection, seizures, subdural fluid collection, overdrainage headaches, shunt underdrainage, and most significant, subdural hematoma (Bergsneider, Black, Klinge, Marmarou, & Relkin, 2005).

The most widely used diagnostic test to assess candidacy for shunt placement is the CSF Tap Test, also called a large volume (40 to 50 ccs) lumbar puncture (LP). While this test is not required for diagnosis, it is commonly used to prognosticate about shunt responsiveness. In fact, clinical improvement following Tap Test is one of the few established prognostic indicators of a

positive response to shunting in patients with NPH (Marmarou, Bergsneider, Klinge, Relkin, & Black, 2005). Although neuropsychological assessment is often part of a comprehensive diagnostic workup, few studies have systematically examined whether cognitive tests are sensitive to change following Tap Test.

Theories of NPH Pathophysiology are Variable

The clinical symptoms of NPH are thought to be associated with the stretching of the periventricular white matter resulting from enlarged ventricles, possibly leading to reduction of blood flow and metabolism and altered neuronal conduction (Momjian, et al., 2004). While this mechanism explains the clinical manifestation of the NPH presentation, it does not address the underlying pathophysiology which caused ventriculomegaly.

CSF dynamics-absorption deficit is a potential cause of enlarged ventricles. The theory of CSF circulation states that CSF is generated from blood in the choroid plexus of the cerebral ventricles in a process that involves the bulk flow of water and solutes and the diffusion/active transport of macromolecules. CSF flows through the aqueduct of Sylvius and fourth ventricle into the subarachnoid space, and then flows over the surface of the brain to the arachnoid villi, which are considered the primary source for CSF absorption. The mechanism of CSF transport across the villi has been considered by some to involve one-way valves, whereas other theories propose the role of transendothelial channels (Castro, Portnoy, & Maesaka, 1991). Disrupted CSF transport and/or absorption can lead to an increased CSF outflow resistance (R_{out}), which is known to be characteristic of most forms of hydrocephalus and may be secondary to obstruction in CSF in the ventricles of subarachnoid space (obstructive hydrocephalus), aqueduct of Silvius (aqueductal stenosis), or alterations in arachnoid villi (communicating hydrocephalus). One

theory of NPH pathogenesis suggests that chronic inflammatory meningeal disease contributes to reduced CSF reabsorption by causing meningeal thickening over the convexity of the cerebral hemispheres (Adams, 1975).

Cerebrovascular changes have been associated with NPH. White matter changes and reduction in cerebral blood flow (CBF) have been noted in NPH, although the underlying mechanisms of these physiological abnormalities are of some debate. CBF has been shown to be lower in NPH than in healthy controls, and evidence suggests that some areas of CBF increase following successful shunting and CSF removal via lumbar puncture (Owler & Pickard, 2001).

Some hypothesize that vascular disease associated with ischemia causes the ventricular dilation by damaging the deep white matter. It is reported that tissue loss results from watershed ischemia that may exist in the deep white matter in NPH, between the boundary from the middle cerebral artery perforators and the deep medullary pial branches (Mathew, Meyer, Hartmann, & Ott, 1975). However, it has been suggested that the ischemia is likely a result rather than a cause as a consequence of reduced nutrients due to deafferentation and reduced neuronal activity (Bateman, 2008). It has also been suggested that ischemia occurs secondary to the stagnation of vasoactive peptides (stagnation occurs in the CSF and the peptides are reabsorbed through the deep white matter) and that these may interfere with cerebrovascular reactivity (Marmarou, Takagi, & Shulman, 1980).

Mechanisms of Altered CSF Protein Expression in NPH

NPH is a form of communicating hydrocephalus, defined as ventriculomegaly in the absence of an obstruction to normal CSF circulation and, thus, can be viewed as a disorder of CSF dynamics (Gleason, Black, & Matsumae, 1993). CSF is in direct contact with the brain, and

examining the molecular composition of CSF via biomarkers can reflect not only biochemical changes in the brain, but also alterations in flow dynamics. Alterations in these dynamics, including resistance to outflow, reduced production and turnover of CSF, and blood-brain barrier (BBB) permeability have been well documented in NPH. Changes in CSF circulatory physiology have been noted in the aging process, although the level of CSF resistance to outflow seen in NPH leads to enlarged ventricles not typically documented in normal aging. CSF production has been shown to decrease by up to 50% with normal aging (May, et al., 1990), and evidence suggests that patients with NPH have significantly lower CSF production when compared to normal aging and other patients groups, including acute hydrocephalus and PD (Silverberg, et al., 2002). Reduced CSF turnover has also been documented in patients with NPH. Silverberg and colleagues (Silverberg, Mayo, Saul, Rubenstein, & McGuire, 2003) hypothesized that decreased turnover results in reduced clearance of toxic molecules that may contribute to the cerebral dysfunction in NPH patients. Finally, isolated BBB dysfunction (i.e., elevated albumin CSF/serum quotient) has been found in patients with NPH in the absence of additional pathological CSF findings (Brettschneider, Claus, Kassubek, & Tumani, 2005). One theory postulates that decreased CSF flow rate and subsequent changes in molecular flux as occurs in NPH lead to pathological dysfunction of BBB (Reiber, 2001). Reiber (2001) proposes that the altered CSF flow rate leads to changes in molecular concentration of macromolecules from blood to CSF.

Biological Markers of NPH

Previous biomarker studies of NPH use target marker approach. Most CSF biomarker studies of NPH have used an approach in which a single protein is selected for study based on

mechanisms related to NPH symptomatology. Several studies have examined target proteins, finding some associations between blood and CSF biomarkers and a diagnosis of NPH (Brettschneider, et al., 2004; Galard, et al., 1997; Kapaki, et al., 2007; Laske, et al., 2007; Lins, et al., 2004; Malm, et al., 1991; Mase, et al., 2003; Nooijen, et al., 1997; Tullberg, et al., 2000). Nevertheless, most of these studies examined nonspecific markers commonly associated with other neurodegenerative disorders such as AD and VD, rather than proteins that are hypothesized to be unique to a hypothesis of the NPH disease process (see Table 1 for summary). While target biomarker studies can be useful for testing markers based on a theory regarding pathophysiology or physiological mechanisms, they do not allow for new biomarker discovery, which is particularly important for a disease like NPH of which the etiology is still relatively unknown.

A recent review of target biomarker studies concluded that TNF-alpha, tau, lactate, β-trace, and sulfatide are some of the most promising markers of NPH (Tarnaris, 2006). However, comparison across several studies revealed some inconsistent results. For example, studies measuring products of neuronal degeneration indicated that a significantly higher level of tau was noted in NPH CSF relative to controls (Kudo, et al., 2000), whereas others have not found differences between the groups (Lins, et al., 2004). Similarly, one biomarker analysis of lactate revealed that NPH samples had reduced levels compared to normal controls, whereas a separate study found levels increased when compared to normal controls and AD patients (Malm, et al., 1991; Nooijen, et al., 1997). More importantly, review of the literature reveals that most studies made comparisons to neurologically healthy control groups, which does not aid in the understanding of how a particular protein might be useful in the differential diagnosis of NPH and other neurological disorders. Thus far, all such biomarkers identified have not demonstrated sufficient specificity or sensitivity for use in routine clinical practice.

Proteomic analysis is highly applicable to the study of NPH. Proteomics refers to the analysis of the total protein content from tissues or living systems. It establishes a comprehensive analysis of molecular interactions required to create or maintain a biological system. A cell or organism responds to changes by regulating protein levels and activities, which is then reflected in the proteome as a dynamic entity. Comparative proteomics takes an empirical approach to new biomarker discovery that is highly applicable to the study of an idiopathic disorder such as NPH whose pathophysiology is currently unknown, as it uses quantitative expression profiling technologies to measure changes in protein expression from different CSF samples with the goal of identifying statistically significant changes.

Before understanding the application of comparative proteomics, one must examine the paradigm shift of molecular biology. Originally stated, a gene is transcribed into RNA and then translated into a protein. The new paradigm describes the model as the genome giving rise to a transcriptome, which includes the complete set of mRNA in any given cell, which translates into the proteome, or complete set of proteins in a given cell (Twyman, 2004). Both the transcriptome and proteome are dynamic, in contrast to the relatively static genome, and change in response to different conditions. Thus, analysis of proteins reveals the biochemical activity of a cell, and can provide information regarding qualitative and quantitative differences between protein profiles of healthy and disease samples.

The powerful comparative proteomic technique has been successfully applied to other neurodegenerative diseases, including PD, frontotemporal dementia, and Huntington's disease (Abdi, et al., 2006; Dalrymple, et al., 2007; Davidsson, et al., 2002; Zhang, et al., 2008). Moreover, the identification and validation of a panel of CSF protein markers for AD has been accomplished (Finehout, Franck, Choe, Relkin, & Lee, 2007). However, only one published

study, to date, has taken the comparative proteomic analysis approach to identify a pattern of altered expression in NPH versus normal controls. Li and colleagues (2006) used two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis and mass spectrometry (MS) to separate and identify the changes in protein expression of CSF from 15 NPH patients and 12 normal controls. Seven proteins were reported to distinguish NPH patients from the normal control group. The proteins identified included α1-antichymotrypsin, leucine-rich α-2-glycoprotein, apolipoprotein J, apolipoprotein D, haptoglobin α1, serum albumin, and α-1-microglobulin/bikunin precursor, which are related to processes such as inflammation and neuronal development and repair. While this study demonstrated the feasibility of identifying NPH biomarkers using proteomic analysis, the comparison of NPH patients to normal controls by Li and coworkers does not fully address the issue of differential diagnosis, which is most relevant to clinical identification of NPH.

Although recent research has offered evidence that biomarkers relevant to NPH pathogenesis, progression, and differential diagnosis can be identified, studies are limited and have been plagued by inconsistent results. Single protein concentrations have not been established as useful biomarkers for diagnostic purposes, as none of these studies have yielded a biomarker with sufficient sensitivity and specificity for routine clinical use. Furthermore, examining single observed change in protein expression between patient groups cannot take interactions among proteins or unique biochemical pathways into account.

Differential diagnosis of NPH has been complicated by both symptomatic overlap with neurodegenerative conditions such as AD, and heterogeneity in symptom presentation. Efforts to improve diagnosis through the use of CSF biomarkers have led to the identification of more than a dozen potential NPH diagnostic markers through single biomarker studies. However, these

target biomarker methods cannot take interactions among proteins or biochemical pathways into account. The effect of changes in CSF dynamics that are established in NPH may not be apparent from the examination of single biomarkers but more readily discerned from studying the CSF proteome in its entirety. In addition, multivariate statistical methods can be used to combine information from multiple variables to improve disease diagnosis. This is possible given that the sensitivity of current proteomic techniques are adequate to measure levels of thousand of proteins simultaneously, and CSF dynamic changes known to be associated with NPH are expected to alter protein concentration in CSF.

The current study uses proteomic analysis of CSF to identify sets of protein markers that are expressed differentially in NPH and AD. The present study hypothesized that alterations in protein expression will provide a fingerprint specific to NPH potentially permitting development of a differential diagnostic test and thereby improving the general understanding of the pathophysiological mechanisms of NPH.

Specific Aims

Primary Aim: To identify patterns of CSF protein expression associated with NPH using CSF specimens from well-characterized NPH and AD patients using two-dimensional gel electrophoresis (2DE) and MS.

Hypothesis 1: A multiplexed panel of CSF protein biomarkers will distinguish a majority of NPH from AD patients.

Secondary Aim: To identify NPH specific proteins that can be measured as a function of disease severity and cognitive functions, providing further evidence that distinguishing proteins are unique to the NPH disease process.

Hypothesis 2: Proteins considered most statistically important to the distinguishing classification of NPH from AD CSF samples will correlate with NPH disease severity (as measured by gait impairment on the total gait scale score).

Hypothesis 3: Proteins considered most statistically important to the distinguishing classification of NPH from AD CSF samples will correlate with cognitive and motor tasks that best distinguish between NPH and AD (specific tasks of complex attention and motor speed).

Results from this exploratory analysis will be used as pilot data to inform future studies examining biological and cognitive markers of shunt outcome.

Method

Participants

NPH subjects. Eight well characterized patients with clinically diagnosed Probable NPH (Relkin, et al., 2005) were recruited from the Department of Neurology at Weill Cornell Medical College and informed consent was obtained. A diagnosis of NPH was made if the following criteria were met: 1) gradual onset of gait disturbance in both legs that is not explained by other conditions; 2) the presence of at least one of the two other cardinal features of NPH (mild to moderate cognitive impairment or urinary symptoms) that is unexplained by other conditions; and 3) radiographic evidence (CT or MRI) confirming the presence of ventriculomegaly disproportionate to cerebral atrophy and an absence of obstruction to CSF flow.

AD subjects. Eight patients with mild to moderate stage clinically diagnosed Probable AD provided consent as part of an approved ongoing study that permitted analysis of previously collected CSF and allowed access to records that provide information regarding demographics and diagnosis confirmation. All subjects met the National Institute of Neurological and Communicative Disorders and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria for Probable AD.

Procedure

Institutional Review Board approval was obtained for human subject data collection at all institutions (Weill Cornell Medical College, Queens College of the City University of New York, and Delaware Biotechnology Institute).

Neuropsychological and gait assessments. Two specific tests measuring aspects of frontal systems functioning, Symbol-Digit Modalities and Trail Making Test B, and a measure of gait

functioning were correlated with NPH-specific protein markers to collect pilot data for future studies examining biological predictors of shunt outcome. These specific tests were chosen based on results of an ongoing study of cognitive recovery in NPH which employs the identical neuropsychological and gait measures. Recent results demonstrated that Trail Making Test B, Symbol-Digit Modalities, and the Gait Scale were sensitive to change when comparing pre and post shunt performance. In addition, a more comprehensive neuropsychological battery was used to characterize the cognitive functioning and disease severity of the NPH sample. The battery consisted of tests that were chosen based on their clinical and empirical sensitivity to the spectrum of cognitive and behavioral functions known to be compromised in NPH.

Modified Mini Mental Status Exam [3MS (Teng & Chui, 1987)]: The 3MS has been shown to be a reliable, valid, and stable cognitive screening instrument (Grace, et al., 1995; McDowell, Kristjansson, Hill, & Hebert, 1997). Scores range from 0-100. Scores for the Mini Mental State Exam [MMSE (Folstein, Folstein, & McHugh, 1975)] can also be derived from this test.

Dementia Rating Scale [DRS (Mattis, 1988)]: The DRS is a tests to determine the general level of cognitive impairment in neurologic patients. It is comprised of a total score and five subscales (attention, initiation/perseveration, conceptualization, construction and memory). Total scores range from 0-144.

Geriatric Depression Scale [GDS (Yesavage, et al., 1982)]: This self report measure is a well-established screening tool to assess depressive symptoms in the elderly. Scores range from 0-30.

Wechsler Adult Intelligence Scale-III- Digit Span subtest (Wechsler, 1997): Digit Span is a measure of auditory attention. Number correct as well as the largest span forward and backward will be recorded. Total scores range from 0-30, forward span ranges from 0-9 and backward

span from 0-8.

Trail Making Tests (TMT) A and B (Reitan, 1985)]: TMT A and B is a measure of complex attention, sequencing, mental tracking and speed of processing. Amount of time taken to complete sequencing is recorded.

Controlled Oral Word Association [COWAT (Spreen, 1969)]: COWAT is a measure of verbal fluency. This test measures number of words produced in three minutes to phonemic categories (F,A,S) and number of words generated in one minute to a semantic category (animals), as well as preservation and intrusions.

Hopkins Verbal Learning Test-Revised [HVLT-R (Benedict, Schretlen, Groninger, & Brandt, 1998)]: HVLT-R is a 12-word verbal list learning task with three learning trials, delayed recall trial, cued recall trial and recognition trials (hits and false positives).

Symbol-Digit Modalities Test (Smith, 1973): Symbol-Digit is a measure of sustained attention and processing speed. Subjects are given 90 seconds to transpose digits for nine different symbols shown in a key at the top of the page.

Grooved Pegboard Test (Reitan, 1985): Grooved Pegboard is a measure of motor speed, coordination and dexterity. Time to place all the pegs, as well as time to complete the first row, are measured for each hand.

Finger Tapping Test (Reitan, 1985): Finger Tapping is a measure of simple motor speed. The mean number of taps in 10 seconds over five trials is measured for each hand.

Line Tracing Test (P Klinge, 2001): Line Tracing is a measure of motor precision in which the subject traces a maze without lifting their pencil. Time to completion and accuracy are measured.

Serial Dotting Test (P Klinge, 2001): Serial Dotting is a measure of motor speed in which the

subject places dots in the middle of 100 circles as quickly as possible.

Gait Scale (Boon, 1997): Ratings of gait disturbance are quantified by a scale that evaluates the presence of 10 features of gait known to be characteristic of NPH Total score ranges from 2 (normal) to 40 (severely impaired) and includes a Walking, Time, and Step Score. The Walking Score is a measure of eight qualitative aspects of gait rated dichotomously as either normal (0 points) or disturbed (2 points), and scores range from 0 to 20. The Step Score is based on the number of steps required to walk 10 m. The Time Score is based on the amount of time required to walk 10 m.

CSF sample collection. As part of standard clinical care, NPH patients underwent a Tap Test for shunt prognostication, during which 50 ccs of CSF are removed by gravity drainage LP and sequentially collected in 10 cc aliquots. AD subjects underwent an LP procedure as part of an ongoing approved study, during which 30 ccs of CSF were removed by gravity drainage and sequentially collected in 10 cc aliquots. CSF samples with significant blood contamination owing to traumatic LPs were identified by cell counts and excluded from further analysis. CSF was coded immediately after collection with an identification number; all personal identifiers were removed.

Protein separation: Two-dimensional gel electrophoresis (2DE). Approximately 250 microliters of CSF were precipitated using ice-cold ethanol. The first dimension separation is isoelectric focusing, which was used to separate proteins on the basis of net charge (carried out in a pH gradient 3-10). The resulting protein pellet was dissolved in a solution of 9 M urea (Bio-Rad), 2% 2-mercaptoethanol (J.T. Baker), 2% IGEPAL (Sigma), and 0.25% carrier ampholytes (Bio-Rad). The sample was then hydrated directly into 18 cm, 3-10 nonlinear immobilized pH gradient (IPG) isoelectric focusing gels (Amersham Biosciences). Isoelectric focusing was then

performed at 20 ºC 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 (Bio-Rad) and subsequently iodoacetamide (Fluka) for reduction and alkylation of the focused proteins. Polyacrylamide gel electrophoresis was performed using 12-15% vertical gradient slab gels to separate proteins in the second dimension by protein size. The separated proteins were fixed, stained with SYPRO Ruby Protein Gel Stain (Molecular Probes), and destained for 24 hours in a solution of 10% methanol and 7% acetic acid resulting in a collection of protein-stained spots against a clear background that can be visualized with a laser-based fluorescence imaging system. The gels were scanned on a FLA-3000 Fluorescent Image Analyzer (Fuji Photo Film Company). Gels created from samples were used to establish protein spots of interest.

Image analysis. The resulting gel images were imported into the Melanie software package (Version 4.0, GeneBio). The location and boundaries of the spots were auto-detected by the software, and the detected spots were manually edited to remove technical artifacts. CSF gels from both NPH and AD patients were used to create the master gel (combining all spots from all gels created) to account for the fact that there were spots that only appeared in CSF gels from one of these patient groups. The spots from each of the sample gels were matched to spots on the master gel to allow inter-gel spot comparison. Matching was performed using the automatic matching function then manually edited by a single individual to correct for obvious missed or incorrect matches. The percent-integrated optical density (percentage volume) of each matched 2DE spot in each gel was exported to a spreadsheet file. The percentage volume is a quantitative measure related to the amount of a given protein in the CSF sample gel, and not a direct quantitative measure of protein concentration. If a spot is not detected on a gel, it is assigned

zero percentage volume for subsequent statistical analysis.

Protein identification. Protein spots determined to best distinguish the group (see Statistical Analysis section below) were compared to a previously published CSF map (Finehout, Franck, & Lee, 2004). Remaining unidentified spots were identified using tryptic digestion followed by tandem mass spectrometry [MS/MS (Finehout & Lee, 2003)]; CSF map identified spots were also confirmed via MS. Peptide mass fingerprint data was collected in positive reflector mode in the range of 900 to 4000 mass to charge ratio (m/z). Several of the highest intensity non-trypsin peaks were selected for MS/MS analysis. The selected peptides were isolated and then fragmented. The spectra were analyzed and searched against a sequence database using combined MS and MS/MS data. For a match to be considered a valid identification, a confidence interval of at least 95% was required (i.e. $p<0.05$). The identification of proteins was examined across spots to determine if any functional pattern emerges.

Statistical Analysis

Demographic and neuropsychological characterization. Chi-squared (nominal variables) and independent sample t-tests (continuous variables) were employed in order to determine whether there were statistically significant differences in demographics between the groups. In order to characterize the neuropsychological profile of the NPH group, *z*-scores were calculated based on normative data on the following measures: MMSE, DRS, Digit Span, TMT, COWAT, HVLT, Symbol-Digit, Grooved Pegboard, and Finger Tapping.

Power analysis for Random Forest. To determine quantitative changes in protein expression, technical and biological variation must be considered. When examining numerous 2DE gels, this variation is determined by the coefficient of variation [CV (SD/mean x 100)] for all spot

volumes on a gel. A review of quantitative variation in proteomic studies revealed that a typical range of biological variation is 39-47% (Choe & Lee, 2003; Molloy, Brzezinski, Hang, McDowell, & VanBogelen, 2003). With even a 100% coefficient of variation assumed, 16 participants are sufficient to have at least 80% power at the 0.05 alpha level (see Figure 1). Thus, it can be reasonably anticipated that in the current study, eight subjects per group will ensure sufficient power to test our hypotheses.

Random Forest analysis. The Random Forest (RF) method, (Breiman, 2001) which is based on classification trees, can be used to analyze underspecified systems when there are significantly more variables than samples (e.g., more proteins than CSF samples). It can be used even when a large number of the variables are irrelevant to the classification of the samples (Izmirlian, 2004). This is important since only a small percentage of proteins may show an expression change in response to a disease. There is also a smaller effect from noise in the variables with an RF analysis compared to some other methods because the RF method does not concentrate weight on any subset of samples. Another feature of RF is the ability to measure the importance of individual variables in sample classification.

RF method was used to determine if any protein spots show a pattern of changes in their percentage volume that can be used to differentiate between NPH and AD CSF gels (Hypothesis 1). Classification trees were built where each tree represents a randomly selected and independent subset (approximately two-thirds) of the samples. To build a tree, the RF program chose a random subset of variables and determined which variable in the subset can best separate the classes (e.g., NPH and AD gels). The tree was grown until the two classes of samples were separated. After a tree was constructed, the program ran the remaining one-third of samples (termed the out-of-bag samples) down the classification tree and predicted what class each

sample belongs to based on the percentage volume data. To determine the overall predicted class for a sample the votes were tallied over all N trees. For each sample, the class that gets the most votes was the predicted class for that sample. The predicted class for the out-of-bag samples was then compared to the actual class and the out-of-bag error (oob error) was calculated. This error is a statistical prediction of the ability of the forest to classify future data sets. It has been shown that the predicted error using this method is unbiased and equivalent to using one-half of the samples as a training set and one-half of the samples as a validation set.

Analyses identify the optimum number of variables that minimize classification error. After the classification error was calculated, the value of each variable was then individually modified (e.g., the percentage volume measurement for a specific spot) and the modified samples were re-classified based on the existing forest. Based on the magnitude of the change in the classification error after modification, the importance of that particular variable in classifying the samples was determined.

To visualize the classification, a plot of canonical functions indicating the scaled distances between samples was constructed. The functions were calculated using the proximity of each possible pair of samples. The proximity of the two samples was based on the number of times the two samples are placed in the same terminal node of a classification tree. The relative importance of each spot identified in classifying the 2DE gels was indicated by a *z*-score calculated based the raw importance score divided by the standard deviation; the higher the *z*-score the greater the importance of the spot.

Correlation analysis. Using the Pearson product-moment correlation coefficient, the relationship between *z*-scores of specific proteins found to be most important in gel classification and NPH-specific neuropsychological (TMT B and Symbol-Digit raw scores) and gait measures

(Gait Scale total raw score) were assessed (Hypotheses 2 and 3).

Results

Demographics

Participants include 8 NPH and 8 AD patients. NPH and AD patients were comparable with respect to age, gender, handedness, education, and disease duration (see Table 2 for comparison of demographic characteristics). Overall, patients in both groups had been experiencing symptoms for an average of 1.5 to 2.5 years. Independent sample *t*-tests for mental status (MMSE) revealed that NPH patients ($M = 27.00$, $SD = 2.65$) performed significantly better than AD patients ($M = 21.13$, $SD = 2.64$), while level of self-reported depression (GDS) was mild and comparable between the groups.

Neuropsychological Characterization of NPH group

Overall mental status on a screening measure was in the normal range (mean MMSE = 27), although average score on a screening measure for dementia was measured slightly above the cut-off (mean DRS Total = 128). As a group, the NPH subjects demonstrated inefficiencies in list learning, psychomotor speed, and upper extremity motor functioning (see Table 3 for a comprehensive lists of *z*-scores).

Proteomic Analysis

The percentage volume data were analyzed using the RF method (R environment, Version 2). The CSF 2DE gels had an average of 1100 detected spots. The master gel had a total of 1339 spots. Student's *t*-test analysis comparing spots from NPH and AD gels identified 109 spots with a significant change in expression level ($p < 0.05$), 34 of which had a $p < 0.01$, suggesting that 2DE can measure statistically significant differences in protein levels among samples.

Random Forest 1. The initial variable list consisted of the percentage volume of all of the spots present on the 2DE gels from 8 NPH and 8 AD patients. The value of m (the number of variables in the random subsets) that minimized the oob error was then determined and subsequently used to create a forest of 2000 trees. This number of trees was found to be sufficient for a stable oob error rate (i.e., the error rate did not decrease further if additional trees were built). This initial forest was able to correctly classify 6 of 8 NPH samples and 7 of 8 AD samples, with an oob error rate of 18.75%. As a control experiment, the same set of spots, with half of the disease classifications reversed, was then used to build another forest. This second forest correctly classified only 2 of the NPH gels and 2 of the non-NPH gels with an oob error rate of 75%. This observation is consistent with the existence of a spot pattern present on the gels that differentiates the NPH and AD gels.

The 100 variables determined by this forest to have the highest statistical importance in classifying samples were then used to build another forest. Because the error rate continued to decline, the protein spots determined to be less statistically important were sequentially removed from the analysis, and the oob error rate was measured. Spots were removed from the analysis until too many spots are removed to effectively segregate the NPH and AD gels. The variable list was reduced to 15 spots that could correctly classify 8 of the 8 NPH samples and 8 of the 8 AD samples with an oob error rate of 0%. See Figure 2 for scaled plot of distances between groups. The relative importance of each of the 15 spots in classifying the 2DE gels, as indicated by the *z*-score calculated based on the RF analysis, is shown in Table 4. The *z*-score is defined as the raw importance score divided by the standard deviation; the higher the *z*-score the greater the importance of the spot.

Random Forest 2. Examination of Group (NPH and AD) x Variable (all 1339 matched

spots) histograms to determine spots with greatest difference in percentage volume between AD and NPH gels revealed 231 variables with group separation of means and minimal overlap in standard deviations. Of these 231 variables, 204 spots were present in only one group: 136 spots were present only in NPH gels and 68 spots were present only in the AD gels. A second forest was built using the 231 variables, which was able to correctly classify 8 of 8 NPH samples and 8 of 8 AD samples with an oob error rate of 0%. Less important variables were then removed until additional removal of variables increased the oob error rate, in order to obtain the fewest number of variables while maintaining a 0% error rate. Ultimately, a panel of 11 spots correctly classified 8 of the 8 NPH samples and 8 of the 8 AD samples. See Figure 3 for scaled plot of distances between groups. The relative importance of each of the 11 spots in classifying the 2DE gels, as indicated by the *z*-score calculated based on the RF analysis, is shown in Table 5.

Variable lists of both RFs 1 and 2 were compared and 6 of the variables were identified in both analyses. A third forest was created using only these 6 spots, resulting in a classification of 7 of 8 NPH samples and 8 of 8 AD samples with an oob error rate of 6.75%. Examination of the scaled distance plots of RFs 1 and 2 revealed more significant separation between groups using variables established from RF 2. Thus, the panel of 11 spots from this analysis was considered most valuable in separating NPH from AD gels. The gel locations of the 11 spots that were identified by the RF 2 analysis are indicated in Figure 4.

Protein identification. Spots of interest were identified on the gels and compared to a previously published 2DE CSF map in order to identify proteins. Three of the 11 spots were identified using the map: β-trace [prostaglandin D2 synthase (AD percentage volume greater than NPH)], serum albumin A (NPH percentage volume greater than AD), and serum albumin B (NPH percentage volume greater than AD). Three of the 8 remaining spots were identified using

tryptic digestion followed by MS and by MS/MS: apolipoprotein A-IV precursor (NPH percentage volume greater than AD), pigment epithelium-derived factor [PEDF (NPH only)], and complement component 3 precursor [C3 (NPH only)]. See Figures 5 through15 for distribution of percentage volume differences between groups for each spot of interest.

Secondary analyses. Correlational analyses between a measure of disease severity (Gait Scale) and the 11 proteins of interest did not reveal significant correlations. Additionally, analyses correlating measures of psychomotor functioning (TMT B and Symbol-Digit) with the 11 proteins did not reveal significant correlations.

Discussion

The present results indicate that there are significant differences in the expression of certain proteins in the CSF of patients with NPH and AD. More specifically, examination of the NPH and AD proteomes identified a multiplexed panel of 11 CSF protein biomarkers that distinguished 100% of NPH from AD patients, supporting the primary hypothesis. In many cases, analysis of the 11 proteins of interest revealed that if the single protein was used as an individual marker, one or more patient would be misdiagnosed. Thus, use of all 11 proteins yielded optimal categorization, even when a specific patient's protein profile did not entirely fit the pattern. This is particularly important given the relative heterogeneity of the NPH clinical presentation as a result of comorbid conditions and presentation at differing stages of the disease, which may have potential impact on the CSF protein expression.

Of the 11 proteins found to distinguish between AD and NPH, 8 were found to be more abundant in NPH than AD (see Figure 16). These results were somewhat surprising, as the effects of neuronal degradation and inflammation seen in neurodegenerative diseases such as AD often lead to an increased protein concentration in CSF as a result of increased turnover. However, it is hypothesized that alteration in CSF circulatory physiology results in reduced clearance of toxic molecules that may contribute to the cerebral dysfunction in NPH patients (Silverberg, et al., 2002), and ultimately the overall increase in CSF protein expression currently observed.

Gel comparison with a published CSF map in conjunction with mass spectrometry identified six proteins, some of which have been implicated in previous NPH biomarker studies. Of the six identified, β-trace, serum albumin, PEDF, and C3 have possible mechanistic associations with the differential diagnosis of NPH and AD. Nevertheless, it is critical to note that it is likely the

interactions between proteins and their function that ultimately contributes to its effectiveness in distinguishing between NPH and AD patients.

β-trace is Shown to be Reduced in NPH

β-trace (lipocalin-type prostaglandin D synthase) is an enzyme considered to be produced mainly in the spinal leptomeninges and arachnoid membrane, and secreted into CSF, making it particularly relevant to a disorder of the CSF such as NPH. β-trace protein has been shown to support the survival and growth of neuronal cells and to stimulate production of neurotrophic factors. While the leptomeninges have been a major site of β amyloid deposition in AD, leptomeninges are often affected in NPH, and leptomeningeal fibrosis has been seen in cases of NPH. Additionally, target biomarker studies examining the association of β-trace and NPH have shown reduced levels of the protein in NPH CSF samples as compared to control groups. Evidence suggests that NPH levels of β-trace are consistently reduced compared to healthy controls (Brettschneider, et al., 2004; Mase, et al., 2003), although the data comparing NPH samples to those of other forms of dementia reveal less consistent results. Mase and colleagues (2003) report that β-trace levels were significantly lower in NPH than a dementia control group that included cases of AD, VD, and dementia with Lewy Body. However, when Brettschnieder and colleagues (2004) compared β-trace levels between NPH, AD, VD, PD, and healthy controls, no statistically significant difference were noted between NPH and VD samples. Consistent with previous reports, NPH CSF samples were shown to have lower levels of β-trace than samples of patients with AD, depression, and healthy controls. It is challenging to determine whether the reduced level of β-trace is a cause or an effect of the NPH disease process. We hypothesize that damage to the arachnoid membrane and subsequent disturbance of

CSF circulation caused by enlarged ventricles may reduce β-trace production by the trabecular cells of the arachnoid membrane.

Serum Albumin Pattern Suggests a Complex Interaction

Two of the relevant spots differentiating NPH from AD gels contained serum albumin. Studies have suggested that albumin interacts with Aβ albumin that has been found in senile plaques (Elovaara, Maury, & Palo, 1986). In vitro, albumin prevents the formation of Aβ macroaggregates and protects red blood cells from lysis by Aβ, and evidence indicates that in plasma the majority of Aβ (approximately 89%) is bound to albumin. Thus, it is anticipated that albumin may be revealed as a potential marker of AD pathology.

In terms of NPH pathology, albumin is often used as a primary measure of BBB integrity, which can be potentially impacted by disrupted CSF dynamics seen in NPH. Blood derived proteins such as albumin may pass through different brain structures, such as the choroid plexus, ventricular surface, leptomeninges, and subarachnoid space, before diffusing into CSF. Therefore, an increased CSF to blood albumin ratio suggests a breakdown in the BBB barrier, as increased blood-derived proteins have reached the lumbar CSF. Some suggest that the weakening of this barrier is related to the regulation of specialized intercellular junctions of the brain capillary endothelial cells (Rubin & Staddon, 1999), while a more recent theory postulates that decreased CSF flow rate and subsequent changes in molecular flux are primarily responsible for pathological blood-brain function (Reiber, 2001). Reiber (2001) concludes that the CSF flow rate is responsible for the change in molecular concentration from blood to CSF, and suggests that reduced CSF flow results from reduced CSF production, restriction of flow in the subarachnoid space, and/or blocked flow through arachnoid villi. Isolated barrier dysfunction

has been found in patients with NPH in the absence of additional pathological CSF findings.

Of note, and consistent with potential mechanisms described, there is a complex interaction among protein expression, as one of the spots identified was increased in NPH, while the other was increased in AD. Additionally, albumin was previously identified as a protein of importance in a proteomic study comparing NPH to healthy controls, suggesting that albumin may perhaps be considered a meaningful component of the NPH biomarker fingerprint.

PEDF Increase in NPH May be a Response to Edema

PEDF is a glycoprotein that is a member of the serine protease inhibitor gene family, and has been recently shown to have neuroprotective and anti-oxidant qualities. In addition, it has been shown to inhibit abnormal blood vessel growth, thus playing a pivotal role in controlling angiogenesis (Chader, 2001). Yamagishi and colleagues examined the expression of PEDF in AD brains to observe the effect of PEDF in the prevention of oxidative stress involved in the pathogenesis of AD (Yamagishi, Inagaki, Takeuchi, & Sasaki, 2004). PEDF was found to have stronger immunoreactivity in cortical neurons and astrocytes in the brain of AD than in those of healthy controls. This increased PEDF expression is thought to reflect the protein's compensatory mechanism against the oxidative stress that can result in neuronal cell injury in AD. The increased expression of PEDF found in NPH CSF compared to AD may also reflect the increase in oxidative stress likely caused by damaged ependyma and degenerative changes in axons and myelin sheath as a result of ventricular enlargement and stretching. However, the glutamatergic neurotransmission, which is associated with deposition of Aβ peptides in AD brains, is also associated with oxidative stress. Therefore, it is uncertain why levels of PEDF demonstrate a significant increase in NPH samples. Post-mortem histological studies comparing

the location of PEDF expression in AD and NPH brains may help localize the areas of neuronal cell death and quantify the differences.

PEDF has also been shown to reduce brain edema in a model of cold induced brain injury (Jinnouchi, et al., 2007). A rat model demonstrated that PEDF could inhibit the cold injury-induced brain edema by counteracting the biological effects of vascular endothelial growth factor. Thus, one might speculate that an increased expression of PEDF found in NPH CSF compared to AD may be a response to altered brain water content in NPH brains, as periventricular hyperintensities noted in many NPH cases are thought to be associated with periventricular edema (Tullberg, Jensen, Ekholm, & Wikkelso, 2001). It is assumed that the diffusion of water in the periventricular region is altered in NPH patients due to transependymal resorption of CSF. This fluid diffuses away from the ventricular surface, producing increased extracellular water in the periventricular white matter (Chun, 2000). In support of this theory, use of diffusion coefficient measures has confirmed that periventricular lucency seen on MRI represents increased water content in the extracellular space in NPH brains (Aygok, Marmarou, Fatouros, & Young, 2006), and suggested that increased water content is seen throughout the entire brain (Chun, 2000).

C3 Contributes to a Unique Inflammatory Response

C3 is a component of the complement system, mechanism of the immune system responsible for identifying and clearing pathogenic substances. It is also known that the activation of the complement system initiates a cascade that contributes to an inflammatory response that can lead to destructive events, including tissue damage generated by the release of toxic products by activated phagocytic cells. It is hypothesized that Aβ fibrils found in AD can activate both the

classic complement and alternative complement pathway via an interaction with C3, leading to formation of activation and chemotactic fragment C5a and the membrane attack complex C5b-9. In support of this theory, several studies have shown the association of complement proteins (C1q, C3, C4d, and C5b-9) with AD brain pathology (Akiyama, et al., 2000; Tenner, 2001). Therefore, C3 and its precursor are consumed in conditions that activate the alternative complement pathway, such as the chronic inflammatory state of AD, and it is hypothesized that the higher levels of C3 seen in the CSF of NPH samples may reflect greater complement consumption in AD compared to NPH.

Nevertheless, inflammation has often been a suggested mechanism in the pathogenesis of hydrocephalus. Secondary hydrocephalus caused by conditions such as injury, infection, and meningitis, often results from inflammation which affects the CSF pathways, thus impeding CSF flow. However, the role of inflammation in idiopathic NPH is unclear. Proinflammatory cytokines such as tumor necrosis factor-alpha have been shown to be increased in NPH, and may be associated with periventricular white matter lesions and demyelination (Tarkowski, Tullberg, Fredman, & Wikkelso, 2003). Whereas this offers evidence that an inflammatory process may be associated with NPH pathology, it is not necessarily a process that activates the complement system seen in AD.

Neuropsychological Correlates of NPH biomarkers

Examining the proteins as a function of a neuropsychological performance associated with NPH or AD may provide information regarding the role of the biomarkers in symptom presentation and disease expression. In addition, correlation of markers with specific neuropsychological tests sensitive to disease specific symptoms (e.g., performance on tests of

upper extremity motor speed is impaired in patients with NPH, and sensitive to change after treatment) may contribute information regarding the proteins' relationship with disease progression and treatment outcome. However, correlational analyses of the protein spot percentage volume and measures of disease severity and psychomotor functioning for both AD and NPH patients did not reveal a significant relationship. The relatively small sample size, although large enough to perform the proteomic analysis, restricted the ability to identify significant associations with the neuropsychological variables. The reduced statistical power for these analyses likely contributed to the null finding when exploring the relationship of specific proteins and gait impairment and psychomotor functions (Hypotheses 2 and 3). The fact that proteins were analyzed independently during the correlation analyses, and not as one value representing the "fingerprint," may also explain the null findings, as the multivariate statistical methods used to discover the proteins of importance do not rely on variable independence.

While lack of significant findings for the secondary analyses may be a result of reduced power and lack of multivariate techniques, alternative explanations should be explored. It is possible that the biomarkers of interest may not be representative of disease state or symptom presentation, but more linked to the actual existence of the condition. Thus, identification of the 11 proteins used to differentiate AD from NPH may help detect different diseases regardless of a patient's severity of symptoms or stage of disease.

The diagnosis of the NPH and AD groups are limited by the current clinical and neuroradiologic techniques available, which is, in part, the reason the outcome of this study is so valuable to improved differential diagnosis. Thus, it is possible that an NPH patient had comorbid neurodegenerative process, and similarly, an AD patient may hydrocephalus superimposed on cortical atrophy that was not differentiated via neuroimaging. Although groups

were able to be differentiated with no error, clinical misdiagnoses could impact the number and type of proteins that ultimately comprised the panel. It should also be noted that the AD and NPH gels were created at different times, which introduces the possibility of systematic group differences due to technical variation. In attempts to control for possible error the same person created all 16 gels using the same set of equipment, significantly minimizing technical variability.

Clinical Significance of Biomarkers for Improved Differential Diagnosis

An effective diagnostic assay may potentially aid the accurate differential diagnosis of NPH and AD, which is particularly important to the treatment outcome of both disorders. Evidence has shown that surgical intervention during early stages of NPH results in improved outcome (P. Klinge, et al., 2005), and this type of early detection is reliant upon advanced diagnostic techniques. Additionally, a misdiagnosis of NPH in an AD patient may result in an unnecessary surgical procedure with a relatively high morbidity rate. Furthermore, initiating a surgical intervention may prevent the early administration of medications shown to delay the progression of symptoms in mild to moderate AD. Conversely, failure to diagnose a patient with NPH could delay surgical intervention and the symptoms are then allowed to progress to a stage that becomes more treatment resistant. Thus, a delay in accurate diagnosis leads to a significant reduction in the window of time to achieve optimal treatment effects.

The issue of differential diagnosis and NPH often includes other forms of dementia, including VD, PD and other movement disorders, and frontotemporal dementia, as they frequently share cognitive and/or motor symptomatology. However, the current focus of differential diagnosis of NPH and AD is of vital importance as AD is the most common form of

dementia and most often observed in medical settings with a prevalence rate of 3% versus a prevalence rate of 0.5% for NPH cases. Consequently, the extensive research and information regarding characterization and clinical presentation of AD is well-established in comparison to other neurodegenerative conditions, thus establishing a more homogenous control group with which to compare NPH CSF samples.

Finally, collection of CSF is already a part of the routine evaluation of suspected NPH by many physicians to predict shunt responsiveness via a Tap Test (Damasceno, Carelli, Honorato, & Facure, 1997; Sand, et al., 1994; Wikkelso, Andersson, Blomstrand, Lindqvist, & Svendsen, 1986). It will therefore be a relatively straightforward matter to incorporate CSF biomarker analysis into the NPH clinical evaluation process.

Future Directions

The next step of analysis includes validation of the panel of proteins using alternative protein quantitation techniques such as immunoassays. Additionally, the panel of proteins must be tested with a validation cohort of NPH and AD samples in order to assess sensitivity and specificity of the biomarkers. Furthermore, these results should also be tested using a broader and more diverse sample population (e.g., mild to moderate AD, early and late stage NPH) to get a more accurate estimate of the prediction error. The current study established biomarkers of differentiation between NPH and AD, and not biomarkers of the disease in isolation. And while this does not produce a set of independent biomarkers for NPH or AD, it addresses the critical issue of differential diagnosis of NPH and AD, and suggests the possibility of eventually developing clinically relevant diagnostic assays based on CSF proteomic analyses.

Table 1

Summary of NPH Target Biomarker Studies

NPH Biomarkers	Function	Findings in NPH Patient Samples	References
Neurofilament light protein (NFL)	neuronal degeneration	elevated level; correlated with more extensive periventricular hyperintensities; increased compared to HC, associated with severe symptoms; high preoperative level associated with favorable shunt outcome	Tisell, 2004; Tullberg, 2007; Tullberg, 1998
Thiobarbituric acid-reactive material (TBAR)	free radical peroxidation, damage to cytoplasmic membranes	increased compared to HC	Fersten, 2004
Leucine-rich alpha 2 glycoprotein (LGR)	possible interaction with Tbeta R-II	increased compared to HC	Xianfeng Li, 2006; Li et al., 2006
TGF-BI	cell development, disease, and repai; in choroids plexus/meninges	increased compared to HC	Xianfeng Li, 2006
Tbeta R-II	receptor for synthesized TGF-beta	increased compared to HC	Xianfeng Li, 2006
Tau	marker of neuronal/axonal damage	increased compared to controls, but not as high as AD; no significant increase compared to vascular dementia, PD, and controls	Kapaki 2007; Lins, 2004; Tisell, 2004
Glial fibrillary acidic protein	structural basis of astrogliosis	increased compared to HC, no association with particular sign or symptom	Tullberg, 1998
Vasoactive intestinal peptide	cerebrovascular vasodilator	normal levels but pre-operative levels correlated inversely with improvement; not correlated with periventricular hyperintensities	Tisell, 2004; Tullberg, 2007
Sulfatide	demyelination	normal levels but pre-operative levels correlated inversely with improvement; not correlated with periventricular hyperintensities; higher levels in NPH patients with cerebrovascular aetiology, significantly higher in subcortical arteriosclerotic encephalopathy	Tisell, 2004; Tullberg, 2007
Serum albumin	blood-brain barrier function	normal levels but pre-operative levels inversely correlated with improvement	Tisell,2004
Cholecystokinin	implicated in neurotransmitter modulation	reduced compared to HC	Galard, 1997
Brain derived neurotrophic factor (BDNF)	neuronal survival, differentiation, and synaptic plasticity	reduced compared to controls and in the same range as AD	Laske, 2006
Beta amyloid1-42	main components of amyloid plaques; neuronal injury	reduced compared to HC and in the same range as AD; reduced compared to VD and PD	Kapaki 2007; Lins, 2004
Lactate	end product of anaerobic glycolis	reduced compared to HC; increased relative to controls and AD	Malm, 1991; Nooijen, 1997
Acetylcholinesterase	enzyme breaking down acetylcholine	reduced in both NPH and AD compared to HC	Malm, 1991
Beta trace protein	meningeal function; level increases with age	reduced relative to HC, AD, depression; reduced relative to HC and dementia	Brettschneider, 2007; Mase, 2003
Phospho-tau$_{181}$	potential marker for neurofibrillary degeneration	within the range of HC	Kapaki, 2007

Note. AD = Alzheimer's disease; HC = healthy control; NPH = Idiopathic Normal Pressure Hydrocephalus; PD = Parkinson's disease; VD = vascular dementia.

Table 2

Demographic Characteristics of NPH and AD Groups

	NPH		**AD**		
	Mean	**SD**	**Mean**	**SD**	**p-value**
Age (years)	78.62	4.62	74.88	8.10	0.31
Education (years)	15.88	4.36	15.38	3.06	0.80
Months since diagnosis	21.33	10.01	30.24	23.82	0.36
Gender	7 male		4 males		0.11
Race	7 white		8 white		0.30
MMSE±	27.00	2.65	21.12	2.64	.035
GDS	6.29	7.06	2.88	2.75	.266

Note. NPH = Normal Pressure Hydrocephalus; AD = Alzheimer's disease; SD = standard deviation; MMSE = Mini Mental State Exam; GDS = Geriatric Depression Scale; ±= *p*-value < .05

Table 3

Neuropsychological Characteristics of the NPH Sample (n = 8): Z-Scores Based on Normative Data

	Minimum	Maximum	Mean	SD
MMSE	25	30	27.00	2.65
3MS	72	89	81.00	8.54
DRS Total	122	137	128.00	5.83
Symbol Digit z-score**	-3.25	-0.31	-1.99	0.93
Semantic Fluency z-score*	-1.7	-0.18	-1.15	0.61
HVLT Total z-score**	-3.31	-1.31	-2.01	0.72
HVLT Delay z-score**	-3.11	-0.61	-1.94	0.84
HVLT Hits z-score**	-3.67	-0.33	-1.76	1.24
HVLT False Positives z-score±	-4.78	0.78	-2.56	2.23
Finger Tapping Dom z-score**	-3.9	-0.3	-1.86	1.31
Finger Tapping NonDom z-score**	-3.5	0.9	-1.70	1.61
Line tracing z-score	-5.81	0.8	-0.96	2.38
Serial dotting z-score±	-14.11	-1.44	-4.79	4.38
Gait Scale±	13	22	17.20	3.70

Note. NPH = Normal Pressure Hydrocephalus; SD = standard deviation; MMSE = Mini Mental State Examination; 3MS = Modified Mini Mental State Examination; DRS = Dementia Rating Scale; HVLT = Hopkins Verbal Learning Test; * = low average; ** = borderline; ± = impaired

Table 4

Z-scores (Relative Importance) of 15 Spots Derived from Random Forest 1

Protein	%vol	Importance
Beta-trace	AD>	2.5633405
Apolipoprotein A-IV Precursor	NPH>	2.5255724
Serum Albumin A	NPH>	2.4102657
Complement Component 3 Precursor	NPH only	2.4081814
Serum Albumin B	AD>	2.2889011
no ID	NPH>	2.2740349
Apolipoprotein A1	NPH>	2.2346423
no ID	NPH>	1.9486371
no ID	AD only	1.9428969
no ID	AD>	1.8165762
no ID	AD>	1.7900825
Serum Albumin C	AD>	1.6765466
no ID	AD>	1.5017408
Transferrin	NPH>	1.4162833
no ID	NPH>	0.4473031

Note. %vol = percentage volume; AD = Alzheimer's disease; NPH = Normal Pressure Hydrocephalus; no ID = protein was not identified via CSF map comparison or mass spectrometry; NPH > = NPH gels had increased expression of protein compared to AD gels; AD > = AD gels had increased expression of protein compared to NPH gels

Table 5

Z-scores (Relative Importance) of 11 Spots Derived from Random Forest 2

Protein	%vol	Importance
no ID	NPH>	5.4042450
Beta-trace*	AD>	5.2213050
Apolipoprotein A-IV Precursor*	NPH>	5.2190120
no ID	NPH only	4.6754970
Serum Albumin A*	NPH>	4.6583370
Pigment Epithelium-Derived Factor/Albumin	NPH only	4.4243560
Complement Component 3 Precursor*	NPH only	4.3123840
Serum Albumin B*	AD>	3.9820150
no ID	NPH>	3.9668290
no ID	AD>	3.8210800
no ID*	NPH>	3.8137000

Note. %vol = percentage volume; AD = Alzheimer's disease; NPH = Normal Pressure Hydrocephalus; * = spots also identified in the top 15 spots of importance in the original Random Forest analysis

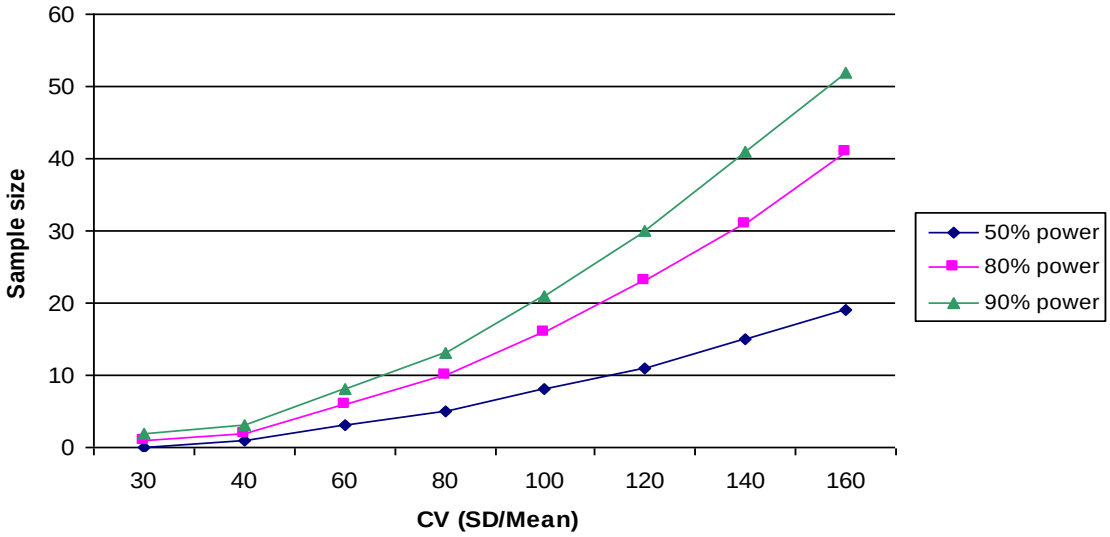

Figure 1. The relationship between sample size, coefficient of variance (CV) and statistical power (0.05 *p*-value, 2-tailed) for the RF analysis.

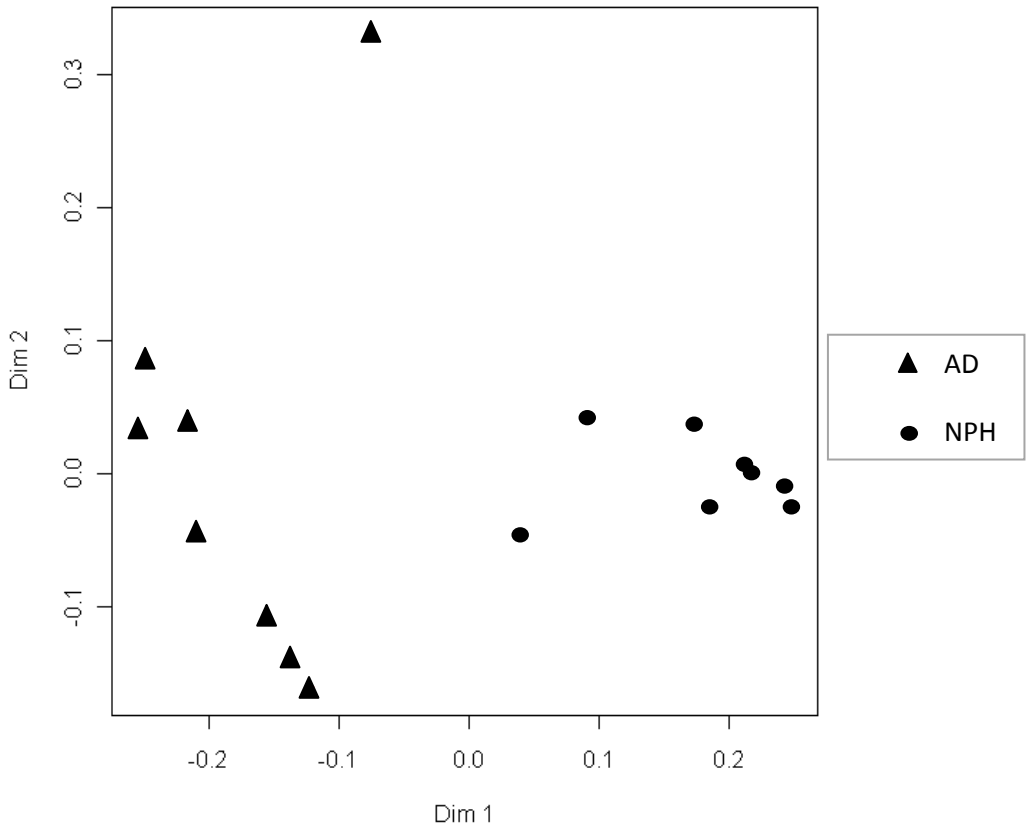

Figure 2. Scaled plot of distances between the NPH and AD sample gels based on the 15 spots of interest identified in the original Random Forest.

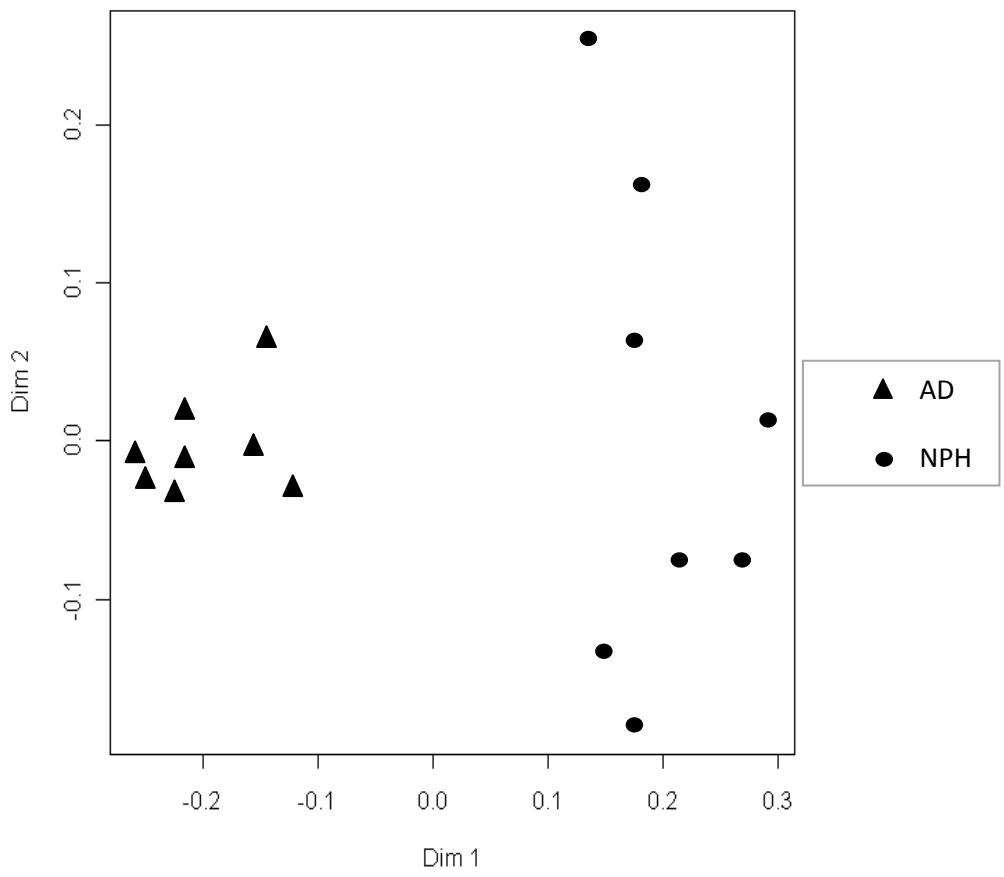

Figure 3. Scaled plot of distances between the NPH and AD sample gels based on the 11 spots of interest identified in the Random Forest 2 analysis.

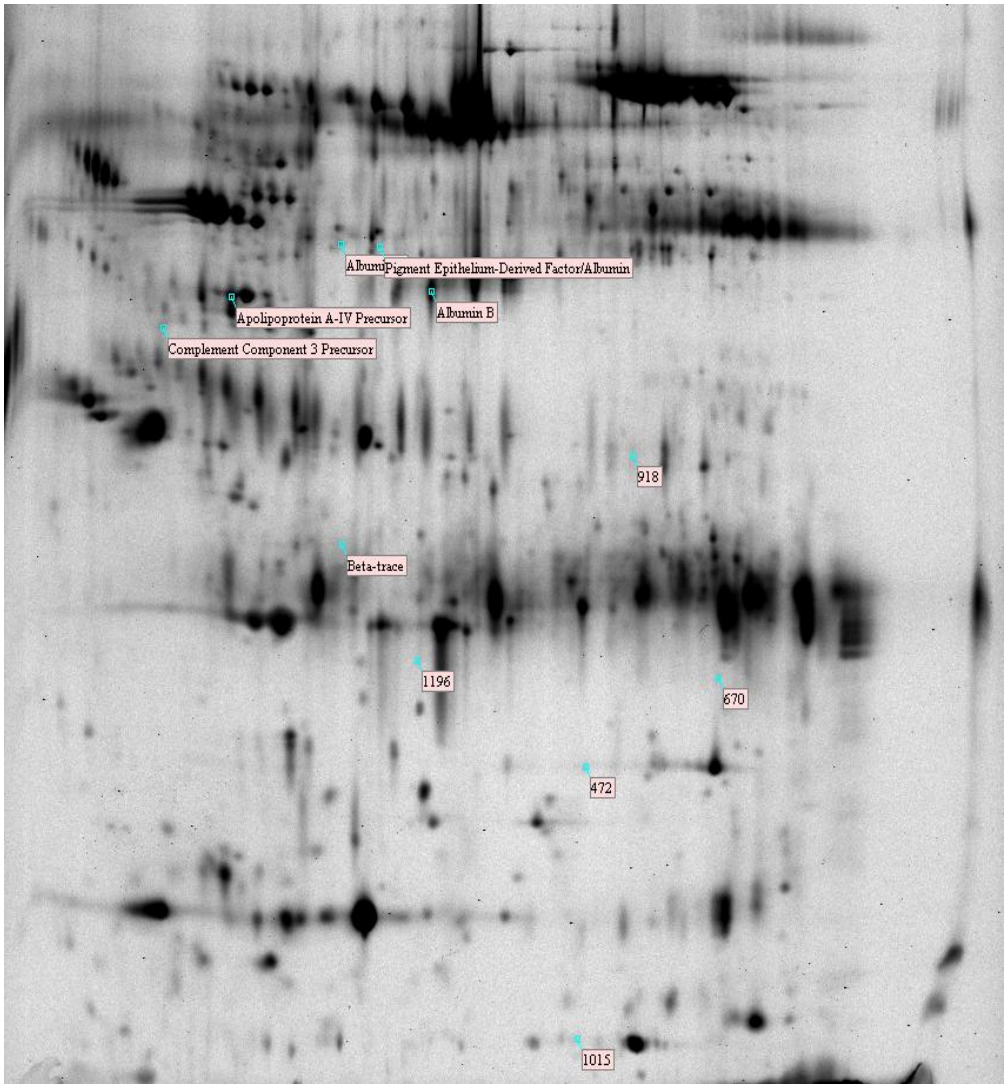

Figure 4. Two-dimensional electrophoresis gel image of an NPH CSF gel with 11 proteins of interest.

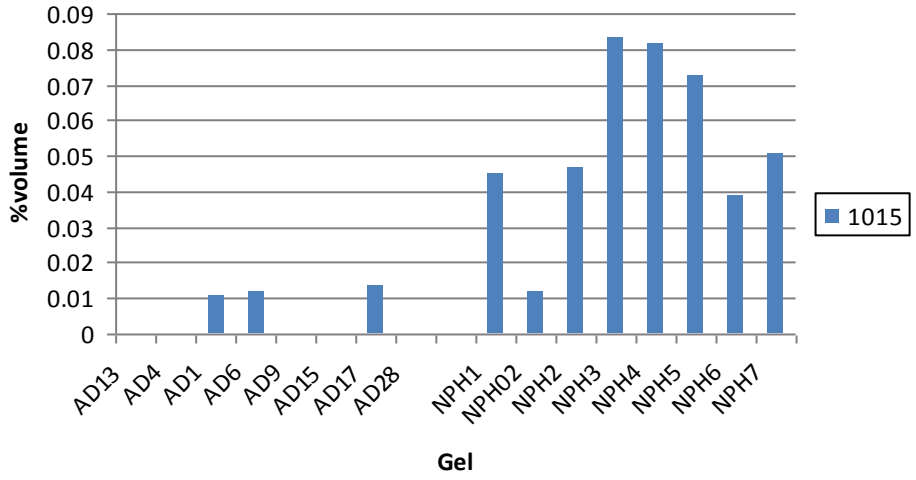

Figure 5. Overall percentage volume of spot 1015 was greater in NPH than AD CSF gels.

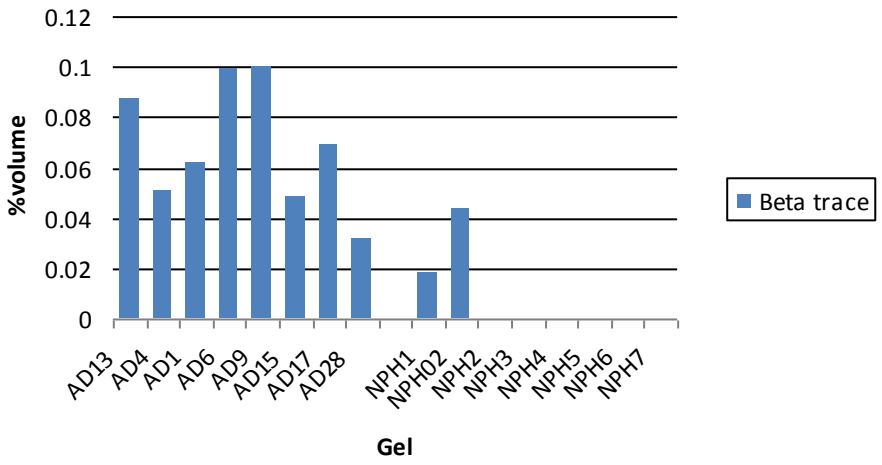

Figure 6. Overall percentage volume of β-trace was greater in AD than NPH CSF gels.

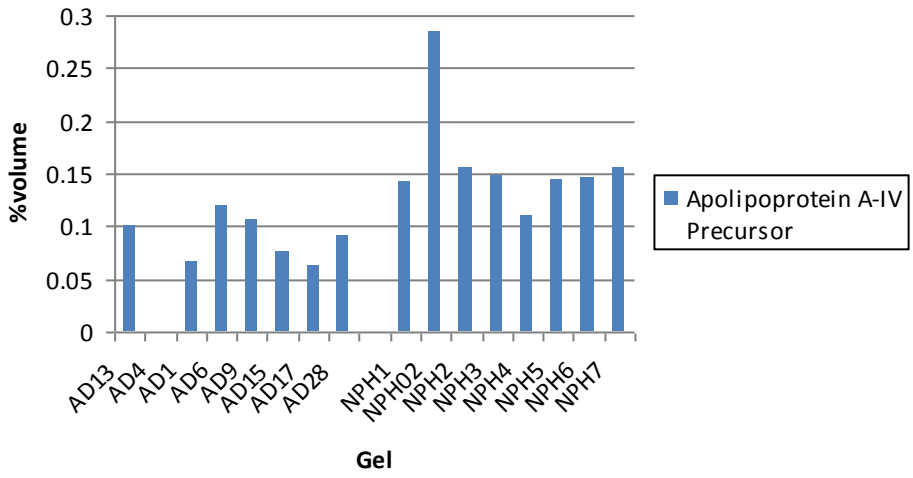

Figure 7. Overall percentage volume of apolipoprotein A-IV precursor was greater in NPH than AD CSF gels.

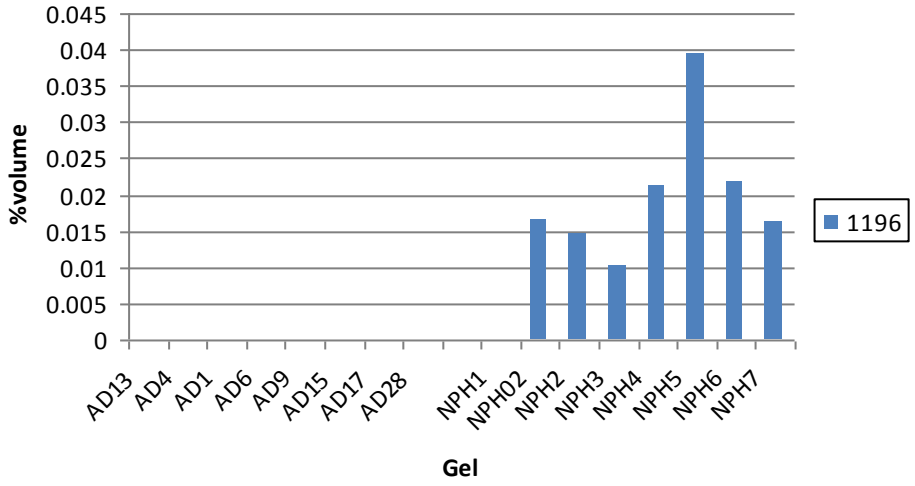

Figure 8. Overall percentage volume of spot 1196 was greater in NPH than AD CSF gels.

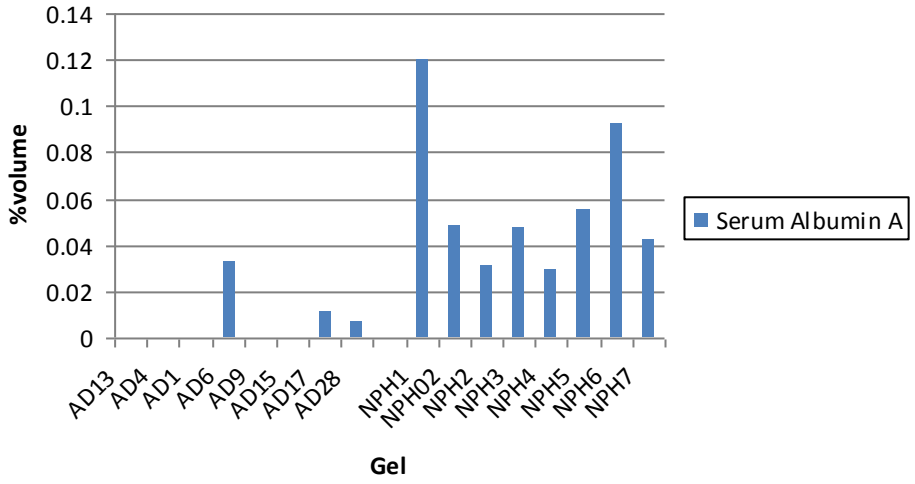

Figure 9. Overall percentage volume of serum albumin A was greater in NPH than AD CSF gels.

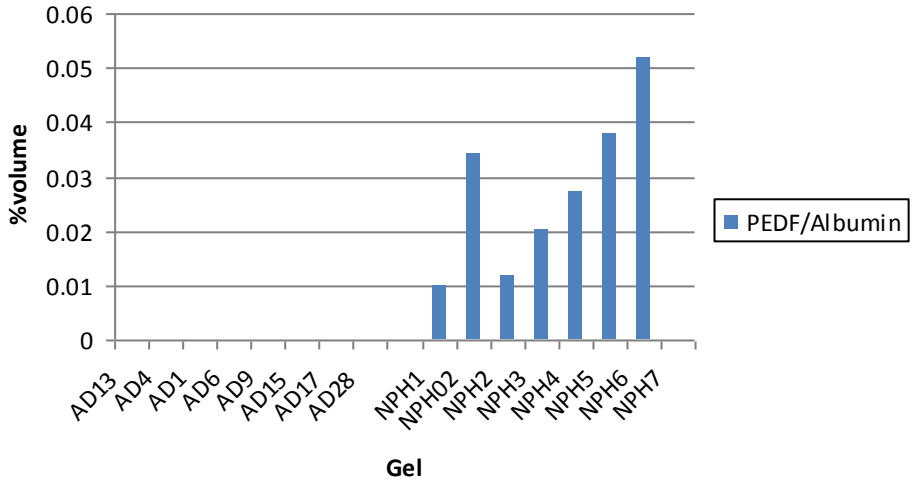

Figure 10. Overall percentage volume of PEDF was greater in NPH than AD CSF gels.

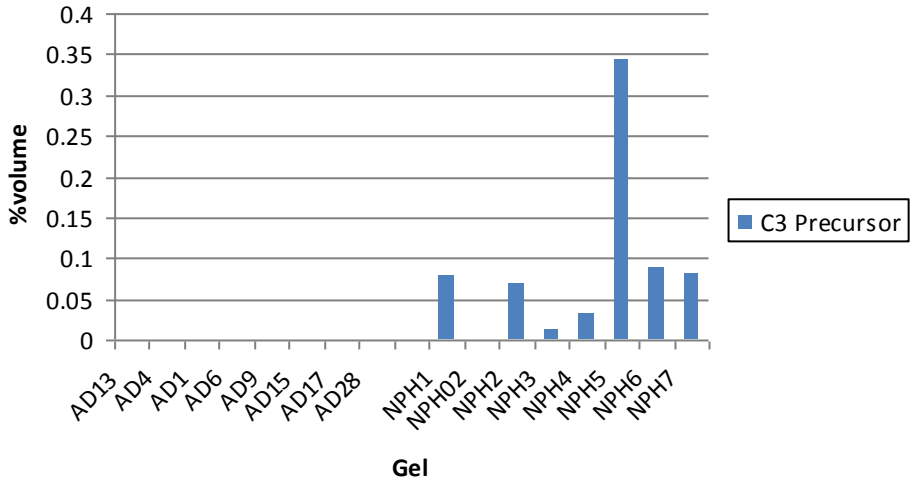

Figure 11. Overall percentage volume of C3 was greater in NPH than AD CSF gels.

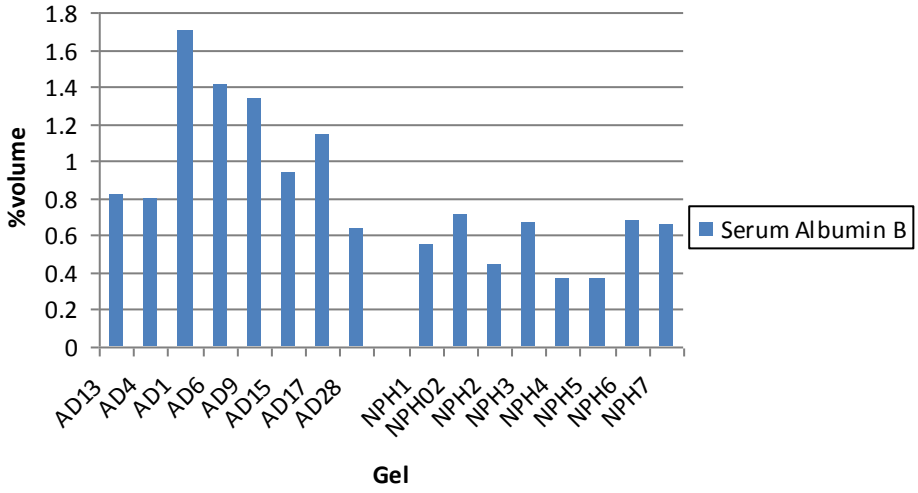

Figure 12. Overall percentage volume of serum albumin B was greater in AD than NPH CSF gels.

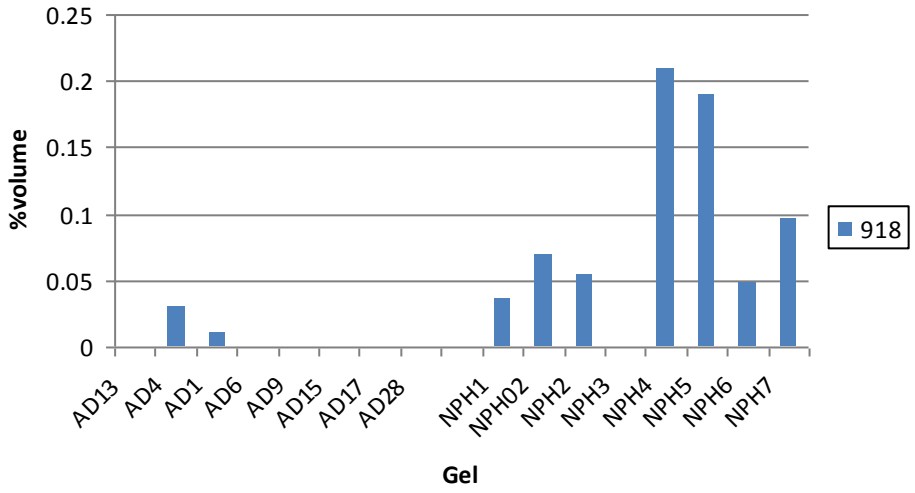

Figure 13. Overall percentage volume of spot 918 was greater in NPH than AD CSF gels.

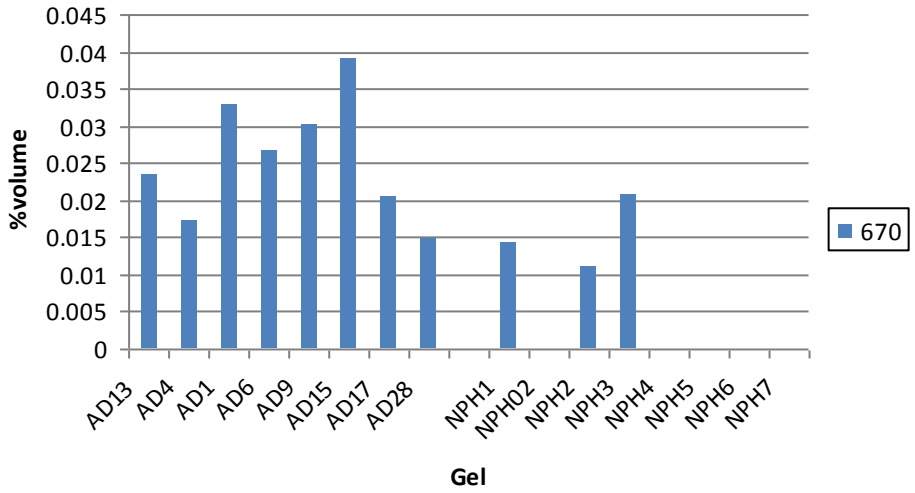

Figure 14. Overall percentage volume of spot 670 was greater in AD than NPH CSF gels.

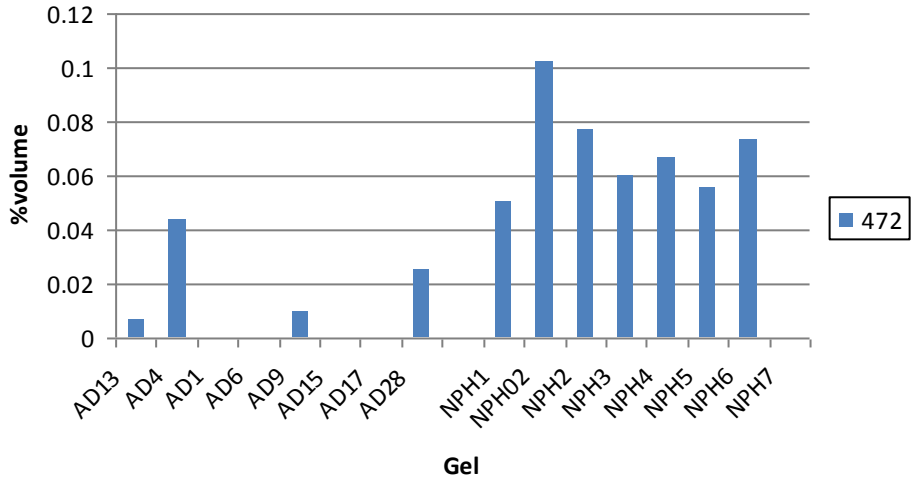

Figure 15. Overall percentage volume of spot 472 was greater in NPH than AD CSF gels.

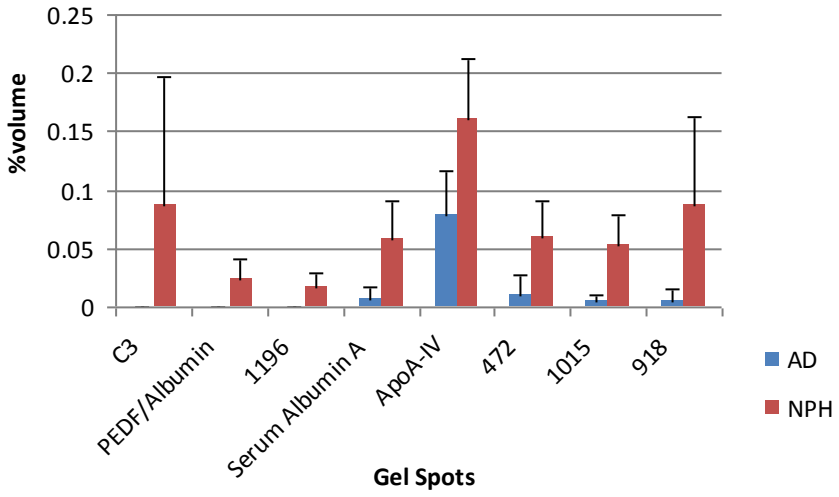

Figure 16. Eight of the 11 spots of interest are significantly increased in NPH compared to AD CSF samples.

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