Proteomics analysis of cerebrospinal fluid in patients with idiopathic normal pressure hydrocephalus

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3.3.3 Protocol IMAC-CU assay

3.3.4 H50 Array

3.3 Analysis in the ProteinChip SELDI Reader

4 Results

4.1 CM10 ProteinChip array

4.1.1 High mass analysis

4.1.2 Low mass analysis

4.2 Q10 ProteinChip array

4.2.1 High mass analysis

4.2.2 Low mass analysis

4.3 IMAC ProteinChip array

4.3.1 High mass analysis

4.3.2 Low mass analysis

4.4 H50 ProteinChip array

4.4.1 High mass analysis

4.4.2 Low mass analysis

5 Discussion

6 Future work

7 Conclusion

8 References

8.1 Journals and Publications

8.2 Web references and personal communication

3
Abbreviations

AD  Alzheimer’s disease
BIH  Benign intracranial hypertension
CJD  Creutzfeld-Jacob disease
CHAPS  3-[(3-Cholamidopropyl) dimethylammonio]-1-propane-sulfonate
CM10  Weak cation exchange surface
CNS  Central nervous system
CSF  Cerebrospinal fluid
CT  Computer tomography
Da  Daltons
DIGE  Different gel electrophoresis
DNA  De-oxyribonucleic acid
2D  Two dimensional
EAM  Energy absorbing molecules or matrix
ELISA  Enzyme-Linked ImmunoSorbent Assay
IMAC  Immobilized metal affinity capture
H50  Hydrophobic coated array
INPH  Normal pressure hydrocephalus
MRI  Magnetic resonance imaging
MS  Mass spectrometry
M/z  Mass to charge ratio
NaCl  Sodium chloride
PD   Parkinson’s disease
PAGE Polyacrylamide gel electrophoresis
QTOF Quadrupole time-of-flight
Q10  Strong anion exchange surface
RNA  Ribosomal nucleic acid
SELDI-TOF MS Surface enhanced laser desorption ionization time of flight mass
Spectrometry
SPA  Sinapinic acid
TFA  Trifluoroacetic acid
TOF  Time of flight
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1 Abstract.

Background

Idiopathic normal pressure hydrocephalus (INPH) is now recognized as a treatable cause of dementia. Recent estimates show that it accounts for 1-10% of patients with the diagnosis of dementia. Although, surgical treatment by inserting a ventriculo-peritoneal shunt is the only known procedure to treat the symptoms, the selection of patients for surgery has always been problematic. Therefore, a pilot project was undertaken to see if by using the method of proteomics biomarkers for NPH could be identified for patients with this condition.

Objectives

The aim of the project was to use surface enhanced laser desorption ionisation time of flight mass spectrometry (SELDI-TOF MS) to look at the expression of protein in idiopathic NPH and control groups. If there are any changes in the expression of proteins between the two groups, the SELDI-TOF MS and sophisticated biomarker wizard software will detect these and identify the statistical relevance of the biomarker. Once a biomarker has been 'flagged', further studies will be used to obtain the identity of the protein.

Methods and materials

Sixteen CSF samples were analysed in this study. Five patients with trigeminal neuralgia and four patients with benign intracranial hypertension were used as controls; seven patients with idiopathic normal pressure hydrocephalus comprised the study group. SELDI-TOF MS analysis of all 16 samples was performed using four different ProteinChip arrays. Nine (high molecular mass proteins) potential biomarkers of NPH have been detected by using high mass CM10 proteinChip with significant statistic results. No biomarkers were detected using the other arrays used in this study.

Conclusions

In this study, the SELDI-TOF MS technique has been used to study the proteins in the CSF of patients with INPH, and compare them with the proteome maps of the control groups. This has resulted in the detection of several significant changes in the CSF proteins of the patients with INPH when compared to the other two groups used as controls.
2 Introduction

2.1 Normal pressure hydrocephalus:

Normal pressure hydrocephalus (NPH) is a term commonly used to describe chronic adult-onset hydrocephalus. Typically, patients with NPH have a characteristic triad of clinical findings: a slowly progressive gait disorder is usually the earliest feature, followed by impairment of mental function and urinary incontinence (Walter G. et al., 2004).

![CT head scan and T2-weighted MRI](http://www.emedicinehealth.com/normal_pressure_hydrocephalus/page16_em.htm)

**Figure 1.** a) CT head scan of a patient with normal pressure hydrocephalus showing dilated ventricles. The arrow points to a rounded frontal horn. b) T2-weighted MRI showing dilatation of ventricles out of proportion to sulcal atrophy in a patient with normal pressure hydrocephalus. The arrow points to transepidual flow.

NPH can develop secondary to trauma, infection or subarachnoid haemorrhage. However, in about one third of cases no cause is found (Joakimsen O. et al., 1987). In spite of enlarged ventricles seen on CT or MRI, in the majority of cases a lumbar puncture generally reveals a normal CSF pressure (Boon AJ. et al. 1999). For this reason “normal pressure” is an unfortunate
The presenting symptoms may be related to gait or to mental impairment. NPH causes an apraxic gait, which is an inability to lift the legs as if they were stuck to the floor. In most cases the gait disturbance takes the form of unsteadiness and impairment of balance. Weakness and tiredness of the legs are also frequent complaints (Bugalho P. et al., 2006; Fisher C.M. 1982). Moreover, the motor strength is intact, reflexes are usually normal, and Babinski’s sign is absent. (Walter G. et al., 2004) Patients may be misdiagnosed as having Parkinson’s disease, by exhibiting short steps and stooped, forward-leaning posture, but there is no rigidity, slowness of alternating movement, or tremor (Miodrag A. et al., 1987). Therefore, when the condition remains untreated, the steps become shorter, with frequent shuffling and falls; eventually standing and sitting and even turning over in bed become impossible (Walter G. et al., 2004).

The mental changes in most patients with NPH are frontal in character with dullness in thinking and actions, and slight inattention. The dementia is of the subcortical type, and involves slowing of verbal and motor responses with preservation of cortical functions, such as language and spatial resolution (Iddon JL. et al., 1999). Moreover, patients are apathetic and may appear depressed. Neuropsychological testing determines the reduction in intellect and the degree of dementia (Thomas G. et al., 2005).

Urinary symptoms appear relatively late in the illness. Initially, they consist of urgency and frequency. Later, the urgency is associated with incontinence. However, incontinence of urine may occur early in the course, especially in patients with prominent gait disturbance (Allan H. et al, 2005)
NPH may follow subarachnoid haemorrhage, or head trauma, resolved acute or chronic meninitis (Edwards. et al., 2004), Paget disease of the base of the skull (Roohi F. et al., 2005), and mucopolysaccharidosis of the meninges (Taccone A. et al., 1993). However, in most cases, no cause is found; it is thought to be due to asymptomatic fibrosing meningitis and the mechanical effect of ventricular enlargement on the adjacent brain is responsible for the syndrome (Hakim S. et al., 1995).

Diagnosis of NPH is principally clinical, with structural brain imaging typically showing ventricular enlargement with ventriculosulcal disproportion. As enlarged ventricles, with normal CSF pressure can be seen in many cases, such as neurodegenerative diseases and infectious diseases (Walter G. et al., 2004), diagnosis of adult-onset NPH and selection of patients for placement of a ventriculo-peritoneal shunt has been difficult (Boon AJ. et al., 2000). Many of these patients have hypertensive vascular disease with lacunar infarcts (Krauss JK, et al. 1996) and features of Parkinson’s disease (Michel AW. et al, 2004), therefore it is recommended that all patients with Parkinson’s disease have imaging to rule out hydrocephalus (Allan H. et al, 2005). Although CT and MRI have helped in separating Parkinson’s disease, vascular dementia and NPH, NPH may occasionally coexist with these diseases (Vanneste J, et al, 1990. Spanu G. et al, 1989). For this reason, a lumbar puncture should be performed for diagnostic purposes and CSF pressure measured carefully. Drainage of large amounts of CSF (20 to 30 mL or more) by lumbar puncture often results in clinical improvement in gait and mental state for a few days (Vanneste JA, 2000).
As mentioned previously, the selection of patients for shunting requires a combination of clinical findings and diagnostic test results, because no test can predict with high enough sensitivity whether a patient is likely to benefit from an operation (McGirt M, et al, 2005). When the clinical picture is not entirely clear, or there has been doubt in diagnosis which is based on improvement due to spinal drainage, the patient should be admitted to the hospital and have an insertion of a lumbar drain for three days, draining approximately 360mL/L of CSF daily and observing the response in gait and mentation (Brecknell JE., 2004)

Neuroimaging is an essential part in the evaluation of patients with idiopathic NPH. Magnetic resonance imaging (MRI) and computer tomography (CT) are considered the methods of choice because of the information they provide. An essential feature is the demonstration of ventriculomegaly and the absence of any obstruction to CSF flow to rule out any other pathology (Marmarou A. et al., 2005).

Many other neuro-imaging techniques have been employed in the past in the investigation of INPH, including Positron Emission Tomography (PET), CSF flow studies (phase-contrast MRI), single-photon emission CT (SPECT) and nuclear cisternography. At present these tests are not routinely used in work up of patients with suspected INPH due to their poor diagnostic accuracy (Bradley WG. 2001).

The treatment of NPH is by insertion of a ventricular peritoneal shunt to divert the CSF. Due to the fact that the aetiology of NPH is heterogeneous, and unknown in some cases, good clinical response to shunt has been found to range from 25% to 80% (Klinge P. et al., 2005). However,
30% to 40% of the patients with NPH suffered post-operative complications, such as infection, haemorrhage, shunt malfunction or blockage and anaesthetic complications (Vanneste et al. 1992). For this reason, careful evaluation must be made to weigh up the risk/benefit ratio of the treatment for these patients, particularly the elderly with co-morbidities. Furthermore, enlarged ventricles secondary to diffuse brain atrophy, does not respond to shunting (Takeuchi T. et al., 2000), while ventricular enlargement with little or no observed cortical atrophy is more likely to be associated with NPH.

The potential failure of shunting should be expected in patients who do not conform to the typical syndrome or whose disease has advanced to the stage of long-standing incontinence or dementia (Spanu G. et al, 1989). In some cases, a lack of improvement is explained by inadequate decompression using a shunt with a valve that drains at higher pressures. In contrast, overdrainage causes headaches that may be chronic or orthostatic and in many cases associated with small subdural collections of blood or fluid (hygromas). These hygromas consist of CSF and blood products and usually no drainage is required, unless they enlarge and cause focal neurological symptoms such as seizures (de Jong DA, et al, 2000).

Cerebrospinal fluid biomarkers may be more useful because they give an insight into changes that happen in the brain that are associated with the condition. The composition of CSF is a key sample in the research for novel molecular biomarkers of neurodegenerative disorders and consequently more research has been done on the composition of cerebrospinal fluid. A recent review of the literature has suggested that tumour necrosis factor, tau protein, lactate, sulfatide and neurofilament triple protein could act as biomarkers for NPH (Tarnaris et al. 2006).
However, the molecular mechanisms underlying many CNS pathologies remain poorly understood and so far no reliable disease-related markers are available (Jin T. et al. 2007). The definition of a biomarker as given by the Biomarkers Definition Working Group was "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention. (Clin Pharmacol Ther 2001).

The use of proteomics in CSF analysis for diagnostic and therapeutic purposes in many CNS pathologies has been recently reported and in many cases it has showed promising results (D’Aguanno S. et al. 2007). There are a number of diseases, for example, Alzheimer’s disease (AD) and other forms of dementia (German DC. et al., 2007), demyelinating diseases (Terzi M. et al., 2007), Parkinson’s disease (PD) (Berg D. 2006) and Creutzfeldt-Jakob disease (CJD) (Van Everbroeck B. et al., 2005) that proteomics has been used for the purpose of biomarker discovery.

2.1.1 Summary

The pathogenesis of idiopathic normal pressure hydrocephalus (INPH) is unknown, and the diagnosis or appropriate treatment of patients with NPH is not easy. It is known that, coexistent Alzheimer’s disease or cerebrovascular disease is generally a predictor of poor outcome following CSF diversion (Andereasen N. et al, 2001).

Several methods have been used for the diagnosis of NPH. However, many cases of this disease are underestimated or given a wrong diagnosis (Stolze H. et al., 2000). For this reason many
efforts have been made to find a new approach for diagnosis. Proteomics as a new technique used for analysis of CSF to discover new biomarkers may show potential results. SELDI-TOF MS uses non-gel based research methods to detect significant changes in protein expression and peptides of CSF, based on their molecular weight as opposed to genomic studies using DNA or RNA (Volker S et al, 2004). Proteomic analysis using SELDI-TOF MS has provided a valuable tool in detecting change in protein expression profiles in different sample groups, for instance, diseased and healthy individuals (Anderson, et al, 1998).

2.2 Proteomics

Overview

Proteomics is a method used to study and perform analysis of protein expression, particularly their structure and functions. The concept of proteome (PROTEins complement to a genOMe) is formed by fusion between proteins and genome, study of genes, which has been used to describe proteins expressed by a genome (Sarah A. et al., 2004). Therefore, this term has been coined to contain all the expressed proteins in a cell type or tissue at a given time point. In other words, the term proteome is the complete protein complement of that cell or tissue type (Daniel, et al 2002). The proteome and peptidome maps can provide us with what is called ‘a bird’s eye view’ of physiological and pathological products and of processes occurring at any one time. Moreover, it provides a ‘snapshot’ of what proteins are present at a given time within a tissue or cellular system.

The genome tells us nothing about the 3-dimentional structure of a protein or any post-translated modifications they may have undergone and which are important for their transport, half-life or
action. This is why proteomics is important as proteins, and not genes, do the cellular work of the body.

Proteomics can be defined as the large-scale study of protein properties such as expression levels, post-translational modifications and interactions with other molecules to obtain a global view of cellular processes at protein level (Daniel, et al 2002). “To really understand biological processes, we need to understand how proteins function in and around cells since they are the functioning units,” says Hanno Steen, Director of the Proteomics Centre at the Children’s Hospital Boston, USA.

Proteomics is often considered the next step after genomics in the study of biological system, and is regarded as a complementary technology and approach to genomics and mRNA expression mapping using microarrays. Moreover, a proteome differs from cell to cell and constantly changes through its biochemical interaction with the genome, which is rather constant. As a result, studying proteomics is much more complicated than genomics. The 35,000 or so genes in the human genome can code for at least ten times as many proteins (Timothy P, 2002).

Proteomics can be generally divided into two areas of research:

(i) Protein expression mapping, and

(ii) protein-protein interaction mapping.
(i) Protein expression mapping (Biomarker discovery)

Nearly all cellular functions are determined by the activity of proteins. However, many cellular processes are performed by complexes of several different proteins. It is crucial that the protein components of these complexes be expressed at the same time and the same place for the cell to function efficiently (Rasmussen et al. 1996). For this reason, an understanding of cellular function at the molecular level requires knowledge of the patterns of expression of all of the component proteins (Oda et al., 2001; Zhou et al., 2001).

(ii) Protein-protein interaction mapping involves determining the interaction patterns for each of the encoded proteins of a cell. The majority of the proteins within a cell are thought to work with other proteins via direct physical interactions to carry out cellular processes (Ito et al., 2001; Uetz et al., 2000). Therefore, a great deal can be inferred about the function of proteins through knowledge of their interacting partners.

Proteomic analysis has become a valuable tool in determining the presence of proteins within a tissue. Moreover, it can also play an important role in mapping protein profiles in different sample groups, such as, diseased and healthy individuals to look for variety of protein expression patterns (Zhen X. et al., 2004). In the end, the final result to this experiment is a list of proteins which are down- or up-regulated between diseased and healthy individuals. Furthermore, the main role of proteomics in medicine is primarily in the discovery of new biomarkers for early detection of diseases, such as cancer to aid early diagnosis, screening and monitor treatment.
2.2.1 Proteomics applications.

The proteomics technique used for biomarker discovery in this pilot project was SELDI-TOF MS. The successful use of the very sensitive ProteinChip Array depends on a high quality of biological samples and on a good design of experiments (Anderson et al., 1998). Therefore, sample preparation factors appear to influence quality. Furthermore, many of the significant clinical applications of SELDI rely on its ability to provide many biomarker patterns that distinguish diseased from control states (Seibert V. et al., 2005). In addition, research focusing on bio-molecular interactions has benefited from the accuracy of the SELDI technology (Zhen X. et al., 2004). This includes studies related to protein-nucleic acid interactions and protein-protein interactions.
2.2.2 SELDI-TOF MS/ ProteinChip Array technology

ProteinChip™ Arrays:

Surface Enhanced Laser Desorption Ionisation TOF MS

![ProteinChip Array Technology](image)

Figure 2. Illustrates ProteinChip array Technology

Personal communication with Dr Kevin Mills (www.ich.ucl.ac.uk/mass_spectrometry)

Surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF MS) or ProteinChip Array technology was developed in 1993 (Hutchens and Yip, 1993) and commercialised in 1997 by Ciphergen Biosystems Inc. As previously mentioned, this high-throughput, array based technology, is primarily used to study changes in protein expression for biomarker discovery. It can also bring us closer to a better understanding of cellular functions at the protein level. Moreover, the SELDI technology is designed to perform mass spectrometry (MS) analysis on mixtures of proteins separated using chromatographic array surfaces (Weinberger, et al, 2001). This method produces spectra of complex protein mixtures.
dependant on the mass-to-charge ratio of the proteins and on the binding affinity to the chip surface. Proteins are purified or fractionated into classes depending on the array used and then analysed in the SELDI-TOF MS. Proteins are weighted very accurately according to their time of flight (TOF) across the flight tube of the mass analyser. Smaller proteins travel faster than larger proteins and by measuring the TOF, the mass can be accurately determined.

Expressed proteins of different profiles may be determined by comparing peak intensities. These comparisons of the peak protein patterns obtained from the samples represent different states and are thought to give detailed diagnostic patterns classifying pathological or cellular states, and can also provide new ideas to the control and function of biological processes (Daniel, et al 2002). In other words, this method has already given clinically and biologically significant results in the detection of biomarkers.

2.2.3 Technology: the ProteinChip Biomarker System

For Expression Difference Mapping applications with ProteinChip Biomarker System the basic procedure is directly straightforward. Any solutions containing proteins can be directly applied to the spots of ProteinChip Arrays. The spots of ProteinChip Arrays contain either a chromatographic surface which has certain physical and chemical characteristics (anionic, cationic, hydrophobic, hydrophilic, or metal ion presenting), or are pre-activated for the coupling of capture molecules (RNA, DNA, or protein) before sample loading (Seibert V. et al., 2005) (Figure. 3).
Protein Array Technology (SELDI)

Figure 3. Shows addition of the sample to the spot array

Personal communication with Dr Kevin Mills (www.ich.ucl.ac.uk/mass_spectrometry)

The chromatographic surfaces are used for expression difference mapping experiments. In this method sample requirements is low (1-10ug total protein per spot); after a short incubation period on a shaker, contaminants and proteins that do not bind to the spot surfaces are removed by washing. Then a solution of laser energy absorbing molecules (EAM) or matrix is applied to each of the spots and the ProteinChip Array is ready for the analysis in the ProteinChip Reader, SELDI-TOF MS. Results are seen in spectra, on the x-axis mass-to-charge ratio of the sample, and on y-axis corresponding signal intensities. Sophisticated computer software enables the user to manipulate the automated detection process and use a wide range of software tools for comparative analysis of higher numbers of samples (Volker et al., 2004).
2.2.4 Advantages of the SELDI process

One of the most important advantages of this surface enhanced laser desorption ionisation process is that materials, such as detergents and salts which commonly cause problems with mass spectrometry, are removed prior to the analysis. Therefore, only proteins actively interacting with spot surfaces are analysed in the mass spectrometer.

Moreover, because each analysis is automatically linked to an on-spot fractionation step, the complexity of the samples is reduced. Subsequently, the probability of detecting biomarkers that are present in lower concentrations increased. Furthermore, the proteins are equally distributed on the spot surface of the chip, so the signal intensities obtained correspond well to the concentration of the proteins. The SELDI-principle has unique characteristics which constitute
of the ProteinChip technology an enabling a wide range of different applications on a single integrated platform (Weinberger et al., 2002).

2.2.5 Automated biomarker discovery

When starting a biomarker discovery process, the first runs of each group can contain between 8-96 samples which result in multiple spectra depending on the number of array type combinations applied. Moreover, even with small data sets, the user is able to generate hundreds of spectra in a short time; during the following validation period the data output will be much higher. In order to meet these demands, special software tools are provided to ensure a reliable and fast evaluation process (Volker et al., 2004; Biomek 2000 laboratories). Furthermore, after an automated normalisation of the profiles, the “Biomarker Wizard” can be used for the fast detection of single biomarkers.

Potential Biomarkers

A clear group of biomarkers are observed between 8-9kD. These are shown here in the trace and gel views as well as by using the biomarker wizard.

Figure 5. Illustrates biomarkers group seen in gel views and biomarker wizard (Volker S. et al., 2004).
In this step the program summarizes signals of equal masses into clusters, and all different clusters of a given range of profiles, returning p-value calculations and graphical presentations. From the results it can be easily visualised which cluster intensities are statistically different between the sample groups i.e., which proteins are up or down regulated in one of the groups, and which of these proteins can be considered as significant biomarker for especial type of disease (Volker et al., 2004; Kodadek. T. 2002).

2.2.6 Protein identification

Identification of the important proteins and peptides is the final step in the SELDI-TOF MS based expression difference mapping technique. This process is performed after the importance of a marker protein has been recognised (Shiwa. M.et al., 2003). In addition, this way of analysis incorporated with ProteinChip technique, also has a beneficial effect of eliminating the unnecessary effort made when identifying nondifferentially regulated, common proteins (Shiwa. M. et al., 2003). Normally, each protein needs to be purified and enriched for subsequent identification experiments. Moreover, for protein identification, the enriched or purified protein is proteolytically cleaved and the peptide fragments are determined by another type of mass spectrometer (QTOF) either by sequencing or mass-mapping database searches (Weinberger et al, 2002).

2.2.7 Summary

Proteomics is a rapidly developing field that is mainly focused on technology development. However, the proteomics provides an effective method to screen a list of disease specific
biomarkers. It is hoped that this study of the CSF proteins for patients with NPH using SELDI-TOF MS, may help in finding certain CSF proteins that differ from the control group and may also be useful adjuncts in the clinical diagnosis of NPH.
**Materials and Methods**

Sample collection

\[\rightarrow\]

SELDI analysis

\[\rightarrow\]

1. CM 10 protocol

3. IMAC-CU protocol

\[\rightarrow\]

2. Q 10 protocol

4. H 50 Array

\[\rightarrow\]

Analysis in the ProteinChip Reader

\[\rightarrow\]

1. Low mass spot protocol
2. High mass spot protocol

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Data analysis using Biomarker Wizard software

\[\rightarrow\]

Detection of possible biomarker

Figure 6. The method design
3 Materials and methods.

3.1 Sample collection

A total of sixteen CSF samples were used in this project from prospectively recruited participants from the hydrocephalus research programme in progress at the Institute of Neurology, Queen Square, London. The criteria for inclusion of the patients into the programme were the feature of ataxic gait, combined with dementia and/or urinary incontinence. All patients had preoperative imaging that demonstrated ventriculomegaly with non-obstructive cause and an Evans Index > 0.3. They also demonstrated improvement following external lumbar drainage for 72 hours in a timed-gait assessment and neuropsychological profile. Furthermore, all patients had at least one measurement of CSF pressure performed to demonstrate normal lumbar CSF pressure (5-15 mm H$_2$O). The ventricular CSF samples were obtained intraoperatively during catheterisation. The first 5 mL of the ventricular CSF were discarded. This was done to avoid the brain debris obtained during the ventricular catheterisation causing spurious results.

CSF samples were obtained as follow: 1) 5 patients suffering from trigeminal neuralgia (TGN) as a control group, 2) 7 patients with idiopathic normal pressure hydrocephalus (INPH)-group A and 3) 4 patients with benign intracranial hypertension (BIH)-group B. In the TGN group, which was used as control group, the samples were obtained from the cisternal subarachnoid space following the opening of the dura during the retromastoid approach in microvascular decompression. In the BIH group, we used CSF obtained from lumbar catheterization during the insertion of a lumbo-peritoneal shunt. The inclusion criteria for the latter group were more than
two measurements of increased lumbar CSF pressures, no evidence of a space-occupying lesion on cerebral imaging and preoperative papilloedema in neuro-ophthalmological examination. The study was approved by the Local Research Ethics Committee and all participating patients signed a consent forms.

All CSF samples were centrifuged within 20 min of collection and the supernatant aliquoted, frozen and stored at -80 °C until analysis.

3.2 SELDI-TOF MS

SELDI-TOF MS technology consists of 3 main components: The ProteinChip array, the reader and the software. The ProteinChip array has eight of 2mm spots comprised of a specific chromatographic surface (figure 2). Each surface is designed to select proteins from extracts according to specific properties. Each spot contains chemically (cationic, anionic, hydrophobic and metallic arrays) treated surface. Ideally, surfaces are treated with a biochemical agent (affinity reagent) and designed to interact specifically with a single target protein. Therefore, any sample can be applied to the surface to promote interactions with the protein molecule (Issaq HJ. et al., 2002).

Detection of protein biomarkers is a simple procedure. A few amount of the sample is dispensed into the ProteinChip surface under specific binding conditions that determine which proteins will be retained by the surface. The protein specificity is obtained by the application of washes with an appropriate buffer which prepared to remove unbound proteins and interfering substances, such as salts. After the ProteinChip array has dried, energy absorbing molecule (EAM) solution
is added. Finally, the array is inserted into the mass spectrometry to measure the molecular weight of the bound proteins.

Statistical reports obtained by SELDI-TOF MS are based on a comparison of the mean between the patient group and the control. i.e., significant difference (P value) should be less than 0.05

3.3 SELDI analysis.

Each CSF sample was liquated and analysed using four different chromatographic array surfaces: a weak cation exchange surface (CM10), a strong anion exchange surface (Q10), metal binding (IMAC), a reversed-phase surface and hydrophobic (H50) coated array. All ProteinChip arrays were prepared according to the standard protocols of the manufacturer. Mass spectrometry was performed using a linear SELDI time of flight mass spectrometer fitted with delayed extraction (Ciphergen, Surrey, UK). All samples were analysed in positive ion mode.

ProteinChip arrays were analysed on the mass spectrometer using the ProteinChip software 3.2 (Ciphergen Biosystem, Inc). Each ProteinChip array was analysed in the spectrometer at two laser intensities: high intensity optimised for high molecular mass proteins and low intensity optimised for lower molecular weight proteins (Franz H, et al. 2004).
3.3.1 SELDI CM10 Protocol (detection of net positively charged proteins)

The mechanism of ionic exchange of CM10 array is the reversible binding of charged molecules to the surface of arrays. The surface of the CM10 array contains predominantly negative charges and is termed a cat-ion exchange array. The array surface is equilibrated in a buffer of net negative charge and also the samples. This facilitates sample preparation (Bio-Rad Laboratories, 2006).

10 µl of each CSF sample was added to the 90 µl of [9M] urea/ [2%] CHAPS, and the samples were incubated for 30 min on a shaker at room temperature in an urea/CHAPS solution to unfold the proteins to expose the internal charges of the amino acids inside each spots and increase their binding efficiency to the SELDI chip array surface. To prime the surface of the ProteinChip arrays, each chip was placed into a separate 15 ml Falcon tube and shaken with 5 ml of binding...
buffer (100 mM Na Acetate, ph 4.0). Tubes were then inverted for a few times to ensure all spots were coated with binding buffer. The reason for doing this is to eliminate bubbles forming on the chip surface.

Arrays were washed with 150 µl binding buffer three times. 10 µl of CSF sample was added to each well and followed by the adding of 90 µl of binding buffer to each well. Samples were incubated for an hour (hr) on a shaker, after the samples were removed and the arrays were washed three times as in the previous steps to remove non-specifically bound proteins. Arrays were then removed from the bioprocessor, washed gently by placing into 15 ml Falcon tubes with 5mM ammonium acetate pH 4 to remove any residual salts, and allowed to air dry. Sinapinic acid (SPA (2 µl) was applied twice to each sample spot, the spots were allowed to dry between applications. Once the array was fully air dried, each array was placed in the mass spectrometer and analysed. Immediately all samples were analysed using a low and a high mass pass for detecting low and high molecular mass proteins.

3.3.2 SELDI Q 10 Protocol

Similarly to the CM10 array, this ProteinChip array acts by reversible binding of charged molecules to the surface of the array and the binding of proteins and peptides are controlled by the surface charge. The surface of the Q10 array contains predominantly positively charges and is termed an anion exchange array. The array surface is equilibrated in a buffer of net positive charge and also the samples. This facilitates binding of negatively charged proteins to the array surfaces. All the steps were the same as in the previous protocol (SELDI CM 10 Protocol) except the binding buffer was exchanged for 100 mM Tris-HCl pH 9.0.
Figure 8. Schematic of how the CM10 ProteinChip array works

Personal communication with Dr Kevin Mills (www.ich.ucl.ac.uk/mass_spectrometry)
3.3.3 Protocol IMAC-CU assay

IMAC array (Immobilised Metal Affinity Capture) works by the reversible binding of proteins to the surface of array through a metal interaction between the proteins and their affinity to these proteins towards a metal ion (Cu²⁺) immobilised into the array surface. The use of this array is primarily in the analysis of phosphorylated and metal binding proteins (Bio-Rad Laboratories, 2006).

The binding buffer was 100 mM NaPO₄, 500 mM NaCl. 150 μl of CSF sample was added to 10μl 9M Urea/2% CHAPS and 50 μl of binding buffer. The sample was then vortexed and incubated for 30 min on a shaker. Following incubation, chips were washed with binding buffer and rinsed twice with 100μl H2O (2x1 min); 150μl binding buffer was added to the chips, followed by the addition of samples and incubated for an hr at room temperature on a shaker (1200rpm). Arrays were washed with binding buffer twice followed by a final water wash to remove residual salts which would interfere with the subsequent mass spectrometry analysis, and removed from the bioprocessor and allowed to air dry. SPA was applied twice with air dry in between the applications. Once the arrays were air dried they were analysed in the SELDI-TOF MS.

3.3.4 H50 Array

The H50 ProteinChip array surface acts by binding to proteins through reversible-phase hydrophobic interaction chromatography. The array works via the more hydrophobic protein bind to the array surface, whilst the less hydrophobic protein will not bind to the array surface.

Sample preparation
40 µl of CSF was added to 10 µl of [9M] urea / [2%] CHAPS. The sample was incubated for 30 min at room temperature on a shaker. Following incubation of the sample, 100µl of binding buffer (10% acetonitrile, 150 mM NaCl, 0.1% TFA (low stringency)) was added, followed by vortexing and centrifugation to remove any particulate.

A H50 array was put into a 10 ml glass tube containing 10 ml of 50% methanol and mixed for 5 min on a shaker at room temperature. This step was repeated twice, and samples were left to air-dry for 20 min. 150µl of binding buffer was added to each well and mixed for 5 minutes at room temperature on a shaker. Care was taken to make sure no air bubbles were in the wells. The binding buffer was then removed from the well and the step was repeated twice.

The prepared sample was added to the well (40 µl CSF, 10 µl [9M] urea / [2%] CHAPS and 100 µl binding buffer and incubated for an hr at room temperature on a shaker 1200rpm. Then the samples were removed from the wells by inverting the bioprocessor and 150 µl of binding/washing buffer was added to each well. Each sample was then washed by shaking for 5 min and removed. This step was repeated for a total of three times. A final wash with 150 µl of deionised water on each spot for 10 seconds was necessary to remove any salt residue from the spot surfaces that would affect subsequent analysis in the mass spectrometer. SPA was added to each spot, with spots being allowed to air-dry between applications. Once the array was dry, it was analysed in the SELDI-TOF MS.
3.4 Analysis in the ProteinChip SELDI Reader

Each array was read twice using a ‘low mass’ and ‘high mass’ spot protocol to detect high and low molecular weight proteins.

**Low mass spot protocol:**

A low intensity laser setting was used on all chips, with high mass set to 30,000 Daltons and optimised between 2,000 Da to 30,000 Da. Starting laser intensity was determined manually with detector sensitivity of 8, focused by optimization centre, and the deflector was setting to auto. The data acquisition method was set to SELDI Quantitation with parameters 20, delta to 2 transients per to 5 ending position to 80. In the final step, warming positions were setting with 2 shots at intensity +20. Warming shots were not included in the analysis.

**High mass spot protocol**

A high intensity laser setting was used on all chips, with high mass to 200,000 Daltons and optimised from 20,000 Da to 160,000 Da. Starting laser intensity was determined manually with detector sensitivity started of 8, focused by optimisation centre, and deflector setting to auto. Data acquisition method was setting to SELDI Quantitation with parameters 21. delta to 2. Transients per to 5 ending position to 81. In the final step, warming positions were setting with 2 shots at intensity +20 and were not included in the analysis.
4 Results

4.1 CM10 ProteinChip array

4.1.1 High mass analysis

Control vs. NPH group

Results (spectra) obtained using SELDI-TOF MS showed many proteins were detected (figure 9, overleaf). A total of 45 proteins of mass to charge ratio (M/Z) were observed. Nine proteins were observed to have significantly elevated expression in the NPH group relative to the control group.Statistic analysis showed their changes were significant with a p-value < 0.05. (Figure 10, p37)

Control vs. BIH group

Results (spectra) obtained using SELDI-TOF MS showed lesser amount of lower mass proteins compared to that in the NPH group, with a total number of proteins detected being approximately 32. No significant differences in protein expression were detected between the control group and the BIH group (Figure 13, p40). This result was supported statistically as analysis showed no significant changes in the mean of the BIH group compared to the mean of control group, p value was > 0.05.
CM10 High mass analysis control vs. NPH group

Potential biomarker

Figure 9. a) High mass spectra obtained from SELDI-TOF MS analysis show potential biomarker (protein) whose expression was significantly elevated (some are reduced in b) between control and NPH group.
CM10 High mass analysis control vs. NPH group

<table>
<thead>
<tr>
<th>M/Z</th>
<th>p</th>
<th>Mean - a</th>
</tr>
</thead>
<tbody>
<tr>
<td>13839.4964453373</td>
<td>0.0495346152</td>
<td></td>
</tr>
<tr>
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<td>7859.0690073102</td>
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</table>

Figure 10. Statistic report for CM10 high mass control vs. NPH group obtained from SELDI-TOF MS analysis. The report show significance changes in the mean NPH group compared to the mean of control group, P value NPH group <0.05

Table 1. Summary of results (potential biomarkers)

<table>
<thead>
<tr>
<th>Array type</th>
<th>Mass of potential biomarker (da) M/Z</th>
<th>Statistical significance P value</th>
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<tr>
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<td>33554</td>
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<td>0.04</td>
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<td>0.04</td>
</tr>
<tr>
<td></td>
<td>82545</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Table 1. Illustrates potential biomarkers obtained by using CM10 high mass analysis with their masses (M/Z) and statistic results
Figure 11. Low mass spectra obtained from SELDI-TOF MS analysis; there was no difference in protein expression observed between NPH group and control group.

Figure 12. Low mass spectra obtained from SELDI-TOF MS analysis; there was no difference in protein expression observed between BIH group and control group.
4.1.2 Low mass analysis

Control vs. NPH group

Results (spectra) obtained using SELDI-TOF MS showed fewer amount of proteins were detected (less than 30) compared to the CM10 high mass analysis. No significant changes in the protein expression between control and NPH group were detected (Figure 11, p38). Statistic analysis showed no significant changes in the mean of NPH group compared to that of control group.

Control vs. BIH group

The number of proteins detected was 30 and the results were similar as in NPH group. No significant changes in the protein expression were seen on the spectra, nor were statistically significant changes observed (figure 12, p38).
CM10 high mass analysis control vs. BIH group

Figure 13. High mass spectra obtained from SELDI-TOF MS analysis, there was no difference in protein expression observed between control group and BIH group.
4.2 Q10 ProteinChip array

4.2.1 High mass analysis

Control vs. NPH group

Results obtained using the SELDI-TOF MS analysis showed a total number of proteins detected of 13 (figure 14 a, p43). No significant changes in protein expression between control and NPH group were evident after statistical analysis.

Control vs. BIH group

The total number of proteins was approximately 73, and the results obtained using SELDI-TOF MS showed no significant changes in the protein expression were seen on the spectra nor were statistically significant changes in the mean seen on statistic analysis (Figure 15 a, p44).

4.2.2 Low mass analysis

Control vs. NPH group

The total number of protein detected by using SELDI-TOF MS analysis was 85 proteins. However, no significant changes in protein expression were observed between the control and NPH group were detected on the spectra (Figure15 b, p44). Statistical analysis showed no significant changes in the mean of the control group compared to the mean of NPH group.
Control vs. BIH group

Large numbers of proteins were detected in this analysis (> 75 proteins) (Figure 15 b, p44). However, no significant changes in protein expression were observed between the control and BIH group and statistic analysis was unremarkable.
Q10 high mass control vs. NPH group

Figure 14 a. Q10 high mass spectra obtained from SELDI-TOF MS analysis show no significant changes in protein expression between control and NPH group.

Q10 low mass control vs. NPH group

Figure 14 b. Q10 low mass spectra obtained from SELDI-TOF MS analysis show no significant changes in protein expression between control and NPH group.
Q10 High mass analysis control vs. NPH group

Figure 15 a. Q10 high mass spectra obtained from SELDI-TOF MS analysis show no significant changes in protein expression between control and NPH group.

Q10 High mass analysis control vs. BIH group

Figure 15 b. Q10 high mass spectra obtained from SELDI-TOF MS analysis show no significant changes in protein expression between control and BIH group.
4.3 IMAC ProteinChip array

4.3.1 High mass analysis

Control vs. NPH group

Results (spectra) obtained using SELDI-TOF MS analysis illustrated that the total number of proteins detected was 13 (Figure 16a, p47). However, no significant changes in protein expression between control and NPH group were observed. Statistical analysis showed no significant changes between the NPH group and the control group.

Control vs. BIH group

Results (spectra) obtained using SELDI-TOF MS showed that total number of proteins was 15. There were no significant differences in protein expression between the control group and the BIH group (Figure 17a, p48). This result was supported statistically as the P value of BIH was >0.05.

4.3.2 Low mass analysis

Control vs. NPH

Results obtained using SELDI-TOF MS analysis illustrated a large number of proteins, 47, were detected. No significant changes in protein expression between the control and patients group were detected (Figure 16b, p47). Statistical analysis showed no significant changes in the mean of control group compared to NPH group.
Control vs. BIH

Spectra obtained using SELDI-TOF MS analysis showed the total number of purified proteins detected was 35. As in the NPH group there were no significant changes in protein expression between the control group and BIH group. This was confirmed by showing no significant changes in the mean of BIH compared to the mean of control group (Figure 17 b, p48).
Figure 16 a. IMAC high mass spectra obtained from SELDI-TOF MS analysis show no significant changes in protein expression between control and NPH group.

Figure 16 b. IMAC low mass spectra obtained from SELDI-TOF MS analysis show no significant changes in protein expression between control and NPH group.
Figure 17 a. IMAC low mass spectra obtained from SELDI-TOF MS analysis, there was no difference in protein expression between NPH group and control group in CSF samples.

Figure 17 b. IMAC low mass spectra obtained from SELDI-TOF MS analysis, there was no difference in protein expression between BIH group and control group in CSF samples.
4.4 H50 ProteinChip array

4.4.1 High mass analysis

Control vs. NPH group

Spectra obtained using SELDI-TOF MS analysis illustrated a total of approximately 40 proteins detected with no significant changes protein expression between control and NPH group. This was confirmed by showing no significant changes in the mean of NPH compared to the mean of control group (Figure 18 a, p52).

Control vs. BIH group

The results obtained using SELDI-TOF MS analysis showed that the number of proteins detected was 46 proteins. There were no significant changes in protein expression between the control and BIH groups (Figure 19 a, p51). Statistical analysis confirmed no significant changes in the mean of BIH group compared to the mean of control group.

4.4.2 Low mass analysis

Control vs. NPH group

Spectra obtained using SELDI-TOF MS analysis showed an excess of 40 proteins were detected (Figure 18 b, p52). However, no significant changes in the protein expression between patients group and control group were confirmed by statistical analysis.

Control vs. BIH group

Results obtained from SELDI-TOF MS analysis presented that a total of an excess of 40 proteins were observed. No significant changes in the protein expression between the control group and
BIH group were detected. Statistic analysis showed no significant changes in the mean of control compared to mean of BIH group.
Figure 19 b. H50 low mass spectra obtained from SELDI-TOF MS analysis illustrate significant changes in protein expression between control group and BIH group in CSF samples.

Figure 18 a. H50 high mass spectra obtained from SELDI-TOF MS analysis show no significant changes in protein expression between control and NPH group.
Figure 18 b. H50 low mass spectra obtained from SELDI-TOF MS analysis show no significant changes in protein expression between control and NPH group.
5 Discussion

The recent increase of the interest in, and search for new biomarkers for diseases of the central nervous system has become possible because of new sophisticated techniques in the field of proteomics. The detection of biomarkers or biomarker patterns enables the scientific and medical community to develop reliable diagnostic tools and allow monitoring of disease progression or therapeutic intervention (Petricoin, et al. 2002; Zhang, et al. 2004).

In this study, the proteomic techniques surface-enhanced laser disorption ionisation time of flight/ mass spectrometry (SELDI-TOF MS), was used to look for specific and novel biomarkers of Idiopathic Normal Pressure Hydrocephalus (INPH). This was the first use of SELDI-TOF MS to look for biomarkers for NPH, by quantitating proteins in the CSF of different sample groups in the search for potential biomarkers. This method required using very small amounts of sample, with rapid analysis and utilising four different types of ProteinChip array (Ciphergen Biosystems, Inc.) surfaces.

CM10 ProteinChip array was the most successful array used in this study, in terms of the most proteins and significant biomarkers. High mass spectral analysis showed clear and significant changes in the protein expression between control group and NPH group (nine high molecular mass proteins were detected). These proteins detected in the analysis have net positive charges using low stringency. Further studies will be necessary to repeat the analysis only using high stringency conditions in the hope of finding more biomarkers. In other words, because the CM10 ProteinChip array is a weak cation exchanger (Franz H, et al 2004) these interactions between the proteins in the CSF and the binding buffer on the chip surface suggested that these peptides and proteins were mostly positively charged molecules. Although, according to the
spectra obtained from SELDI-TOF MS analysis high mass analysis showed significant changes in the protein expression between control group and NPH group, no such changes were seen for low mass analysis. These results were supported by statistical analysis obtained using SELDI-TOF MS analysis biomarker wizard software, as the largest P value results were yielded by applying high mass spectra.

On the other hand using the CM10 ProteinChip array with other pathologic groups, such as BIH, no such significant changes were observed in protein expression between the control group and BIH group in both the high and low mass spectra. It could be speculated that there were few positive charged proteins in this patient group.

Furthermore, in the present study when we used the Q10 ProteinChip Array (strong anion exchanger) protocol which has the same mechanism of action to the CM10 Array (ion exchange), no significant changes were detected in the protein expression between the control group and NPH group either in the low or high mass analysis. The reason might be due to the proteins present in CSF being predominantly positively charged and few negative charged proteins present.

In addition, no significant changes in protein expression between control group and BIH group for neither high and low mass spectra, nor statistically significant changes in the mean between control and BIH group were detected confirming the hypothesis that most proteins found in the CSF were predominantly positively charged.
The third array we used in this study was the ProteinChip H50 in which its surface binds to hydrophobic proteins i.e., proteins tend to be hydrophobic (water-hating) or membrane-bound will bind more efficiently to the array surface. The results obtained from SELDI-TOF MS analysis showed that there were no significant changes in the protein expression between the control and NPH groups. However, these results using low and high mass spectra indicated that there is a significant number of hydrophobic proteins that may or may not be secreted from the brain are present in the CSF, since we do not usually get many proteins binding to H50 array during plasma analyses. Furthermore, this result was supported by statistical analysis obtained from SELDI-TOF MS which showed no significant changes in the mean of NPH group compared to the mean of the control group. When we compared the control and BIH groups the results obtained from SELDI-TOF MS analysis were almost the same as that in the NPH group. As mentioned previously these results indicated that both patient groups have few hydrophobic or membrane-bound proteins present in the CSF that probably mean they were not brain derived. This is because the brain is predominantly a hydrophobic environment which mainly consists of lipid and the membrane bound proteins are hydrophobic.

The fourth array we used was the IMAC (Immobilised Metal Affinity Capture) array surface which binds to proteins through coordinated metal interaction. Metal ions interact with Nitrilotriacetic Acid (NTA) to form stable complexes that interact with phosphorylated groups or proteins that are metal binding. As with the Q10 and H50 arrays, no significant changes in protein expression between control group, BIH and NPH group were detected. These negative results might be due to the CSF not containing many metal binding proteins or phosphorylated proteins.
Using the SELDI-TOF MS it was possible to characterise very small amounts of proteins as seen in CSF. It was possible to perform proteomic screens in less than 30μl of CSF. The primary objective of this study was to find a biomarker or significant differences in the proteins of CSF of patients with NPH compared to control group and another group of patients with BIH. Nine potential biomarkers were detected using SELDI-TOF MS in this study.

We did not find any difference in the protein profile in the CSF of the BIH group when compared to the NPH or TGN groups. That might be due to the different source of sampling (i.e. ventricular/cisternal CSF compared to lumbar CSF). It has been postulated that the concentrations of neuropeptides using lumbar instead of ventricular samples is different. This is due to the effect of the gravity which may influence the concentration of the peptides in the samples (Gjerris et al. 1998; Reiber H. 2001). This is the first study to our knowledge to study the proteome of CSF in patients with BIH. This study is ought to be replicated by using a bigger number of samples.

In summary this pilot project demonstrated that SELDI-TOF MS is a powerful tool for the equal screening and detecting of biomarkers in biological fluids.
6 Future work

At this stage several potential biomarkers have been identified between the control group and NPH group by using SELDI-TOF MS. However, SELDI-TOF MS biases analyses of proteins to those of mass below 30KDa, therefore protein above this were not analysed optimally (Rodland K.D. 2004). The best way to analyse higher mass proteins is using DIGE/2D PAGE. If more time was available, the next stage of research would have been to perform a screen on the same samples using the above technique.

Different Gel Electrophoresis (DiGE) is a relatively direct application of differential labelling designed of protein samples fluorescent dyes. Cuanin dyes are used in this technique to labelled different proteins prior to analysis using 2 D electrophoresis (Garfin D.E. 2003).

This allows proteins to be analysed at very low amounts and eliminates any experimental variations. Using sophisticated computer software, small charges in protein expression or post-translated modifications can rapidly be detected.

In addition, time permitting; the next stage of the research would be to try to identify the biomarkers discovered by, firstly, isolating them i.e. making them pure, digesting them with trypsin and sequencing the peptides for identification purposes using the QTOF mass spectrometer. Once they have been identified, antibodies would be raised against these proteins and a simple Enzyme-Linked ImmunoSorbent Assay (ELISA) test could be developed. Then the ability to develop a simple, affordable test for NPH would facilitate a method of diagnosis for many labs and reduce the need for expensive and complicated mass spectrometers.
Although this pilot project was successful, further studies using more samples and different control groups should be undertaken. i.e., CSF from patients with vascular dementia or Alzheimer disease, to provide the sensitivity and specificity of the biomarkers identified in this project.
7 CONCLUSIONS

In this study, a novel proteomic method has been used to study proteins of CSF samples obtained from patients with INPH, and two more control groups. This has resulted in the detection of several significant changes in the CSF proteins among the samples obtained from known cases of INPH compared to the two other groups.

As discussed in my initial summary of the literature, (2.1.1), early diagnosis is the only promising approach to significantly improve the surgical outcome in patients with INPH. For this reason, the purpose of this study was to identify new biomarkers within CSF samples in patients with INPH.

In the course of my research, using SELDI-TOF MS analysis, I worked with four different ProteinChip array techniques: CM10, Q10, IMAC and H50. Of the 4 techniques, we discovered that CM10 is a suitable technique for finding even a small difference in the proteins and peptides between CSF samples of patients with INPH compared to control and other pathological groups. Table 1 summarises these findings. Furthermore, these changes in the proteins seen on the spectra were confirmed by the statistical report obtained by SELDI-TOF MS analysis. As discussed in the section on Future Work (6.), new CSF biomarkers for INPH have been potentially identified and detected in CSF sample sets.
8 References:

8.1 Journals and publications:


Krauss J K., MD; Jens P. Regel, MD; Werner Vach, PhD; Dirk W. Droste, MD; Jan J. Borremans, MD Thomas Mergner, MD (1996) Vascular Risk Factors and Arteriosclerotic Disease in Idiopathic Normal-Pressure Hydrocephalus of the Elderly *Stroke.* ;27:24-29.


8.2 Web References and personal communication

http://www.emedicinehealth.com/normal_pressure_hydrocephalus/page16_em.htm

Personal communication with Dr Kevin Mills (www.ich.ucl.ac.uk/mass_spectrometry)

(Ciphergen Biosystem, Inc).