Characterization of arachnoid cysts using clinical chemistry, qualitative and quantitative proteomics

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Scientific environment

The candidate has been affiliated to the Institute of Medicine, Department of Surgical Sciences and Department of Biomedicine at the University of Bergen. Professor Dr.med Rune J. Ulvik has been the main supervisor, PhD MSc Frode Berven and PhD MD Christian Helland have been co-supervisors.

The present work has been carried out at the Proteomics Unit at the University of Bergen (PROBE) with exception of clinical chemistry analysis in Paper I, performed at the Laboratory for Clinical Biochemistry at Haukeland University Hospital.

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Table of content

Abstract

Arachnoid cyst are benign intracranial lesions with a reported prevalence up to 1.1 % in the population. The origin of such cysts and the mechanisms of filling and sustaining are poorly understood. The aim of the thesis was to characterize the arachnoid cyst fluid and compare it with cerebrospinal fluid from the same individuals to evaluate the content of arachnoid cysts, as well as to gain further knowledge of the mechanisms of filling and sustaining of such cysts. Patients were recruited prior to elective surgery for fenestration of symptomatic arachnoid cysts in the temporal fossa and arachnoid cyst fluid and cerebrospinal fluid was collected with written informed consent from 19 patients. In Paper I the content of arachnoid cyst fluid and cerebrospinal fluid from the same patients were compared by clinical chemistry. The protein content of arachnoid cyst fluid is reduced relative to cerebrospinal fluid, while the concentration of phosphate is elevated. The results from this evaluation indicated that arachnoid cyst fluid is not identical to cerebrospinal fluid. In Paper II the protein content in arachnoid cyst fluid from 15 patients was evaluated by qualitative proteomics and the findings were compared with published databases of plasma and cerebrospinal fluid. These comparisons indicated that the arachnoid cyst fluid proteome was similar to cerebrospinal fluid, but dissimilar to plasma. In Paper III we performed a quantitative comparison of the proteomes of arachnoid cyst fluid and cerebrospinal fluid for five patients. 348 proteins were quantified in individual patients, and 1425 proteins in a pool of the same patients using an iTRAQ-strategy combined with extensive fractionation. We identify differences between the fluids, but currently we are not able to elute the biological significance. Searched against DNA and mRNA-data, we find some differences, but not in patterns of biological significance. This is the first quantitative proteomics comparison of AC fluid and CSF. In conclusion, the work presented in this thesis indicates that AC fluid is similar, but not identical, to CSF. Results do not support oncotic filling or valves as mechanisms for filling and sustaining of arachnoid cysts but rather an active or selective mechanism for filling.

List of publications

Paper I

Berle M, Wester KG, Ulvik RJ, Kroksveen AC, Haaland OA, Amiry-Moghaddam M, Berven FS, Helland CA: Arachnoid cysts do not contain cerebrospinal fluid: A comparative chemical analysis of arachnoid cyst fluid and cerebrospinal fluid in adults. Cerebrospinal Fluid Res 2010, 7(1):8

Paper II

Berle M, Kroksveen AC, Haaland OA, Aye TT, Opsahl JA, Oveland E, Wester K, Ulvik RJ, Helland CA, Berven FS: Protein profiling reveals inter individual protein homogeneity of arachnoid cyst fluid and high qualitative similarity to cerebrospinal fluid. Fluids Barriers CNS 2011, 8(1):19.

Paper III

Berle M, Kroksveen AC, Garberg H, Aarhus M, Haaland OA, Wester K, Ulvik RJ, Helland CA, Berven FS: Evaluation of intraoperatively collected arachnoid cyst fluid and cerebrospinal fluid from arachnoid cyst patients with quantitative proteomics. Submitted.

List of figures and tables

List of abbreviations

AC – Arachnoid cyst (s/pl)

ACN – Acetonitrile

AM – Arachnoid mater

CID – Collision-induced dissociation

CSF – Cerebrospinal fluid

DDNTPs – Dideoksynuclotide tri-phosphates

DTT – dithiothreitol

FA – Formic acid

FDR – False discovery rate

HCD – Higher Energy Collision Dissociation

HPLC – High pressure liquid chromatography

IAA – Iodoacetamide

ICMJE –International Committee of Medical Journal Editors

IEX – Ion exchange chromatography

iTRAQ – Isobaric tag for relative and absolute quantification

LC-MS – Liquid chromatography (coupled to) Mass spectrometry

LTQ – Linear trap quadropole

MALDI-TOF – Matrix assisted laser desorption/ionisation - Time of Flight

MIDAS – Multiple reaction monitoring (MRM) initiated detection and sequence analysis

MMR – Measles-mumps-rubella vaccine

MRM – Multiple reaction monitoring.

MS – Mass spectrometer

MW – Molecular weight

NKCC1 – Na+ K+ 2Cl- cotransporter gene

NOG – N-octyl-β-D-glycopyranoside

NSE – Gamma-enolase

PCA – Principal component analysis

PPM – Parts per million

PTM – Post-translational modification

qTOF – Quadropole Time of Flight

REK – Regional Committee for Medical and Health Research Ethics

RP-WAX – Reverse phase weak anion exchange

SAX – Strong anion exchange

SCX – Strong cation exchange

SELDI – Surface enhanced laser desorption/ionisation

SRM – Selected reaction monitoring

TFA – Trifluoroacetic acid

TOF –Time of Flight

Introduction

> *'There are no hard distinctions between what is real and what is unreal, nor between what is true and what is false. A thing is not necessarily either true or false; it can be both true and false.'*
>
> - Harold Pinter, Art, Truth and Politics. Nobel Prize Lecture in Literature 2005.

In this introduction, I will focus on the previous and up-to-date knowledge of arachnoid cysts (AC), as well as the historical and scientific foundation of a project of translational science between the basal sciences of clinical chemistry and molecular biology, and neurosurgery. As this thesis is based on a relatively complex selection of methods, emphasis will be given to the theoretical basis and the technical basis behind the articles. I will also describe the basics of molecular biology, although familiar to readers trained in biology in general. I will mention but not go in to the subject of optimal treatment, patient selection or cut-off limits for indication of surgery. I will not go in to specific casuistic for clinical presentation of AC. My objective of this thesis is to contribute to the understanding of AC, in particular knowledge of the composition of the AC fluid might increase the understanding on the mechanisms for formation and filling.

Scientific method is the tool of the trade of science. Oxford English Dictionary [1] defines scientific method as “method of procedure that has characterised natural science since the 17th century, consisting in systematic observation, measurement, and experiment, and the formulation, testing, and modification of hypotheses.” Scientific method is the continuous verification by peers that experiments show the results they claim, that policy and understanding of the world is based on real knowledge. The need for the scientific method is exemplified by the sad story of the measles-mumps-rubella (MMR) triple vaccine scandal where Wakefield et al [2] published an article, later fully retracted, suggesting a relation between the MMR vaccine and autism [3].

The consequence of this article, even though it was fully retracted and refuted, is a large degree of scepticism in parts of the general population to vaccines. From a public health perspective, unjustified fear of vaccines causing parents to refrain from vaccination of their children from diseases that can be prevented is a mere tragedy. Scientific method is thus not only what is wrong, not only what is right, but also rather a tradition on how to figure that out. The scrutiny in which science is performed is not only from the scientist, but also from the required openness in methodology as for other scientists to themselves verify the results.

> *The real purpose of the scientific method is to make sure Nature hasn't misled you into thinking you know something you don't actually know.*
>
> Robert M. Pirsig, Zen and the Art of Motorcycle Maintenance

A personal experience of the strength of academic arguments was lectures in Bioethics by Professor Boman at the University of Bergen in a lecture on eugenetics for the sake of the argument chose a supporting position, this as a challenge to young students unfamiliar to defending their ethics to a by far more knowledgeable debater. Science and university tradition is not only about the mere production of knowledge, but also the reflection and sense of criticism to refute a false argument. In the sense of ethical barriers, the scientific tradition has implemented demands of ethical regulations on the conduct of science, in form of the Helsinki declaration from World Medical Assosiation [4].

For historical reasons, in particular the Nuremberg trials concerning war crimes for medical research purposes, the governance of ethics in medical research has been formalised in the period after the Second World War. This formalization of research is

more strict and transparent than only moral imperatives such as the Declaration of Geneva, based on the Hippocrates oath of moral conduct of medical professionals. Ethics committees have been implemented to evaluate research projects before the project is undertaken. Concerning publication of results, an increasing number of biomedical journals demand adherence to a strict set of rules set from the International Committee of Medical Journal Editors (ICMJE) [5], usually referred to as the Vancouver rules.

In the general optimism of science, taking a step back to consider the theoretical foundation in which modern natural science is built may cause reflections on objective, methods and results. The popular science article “The truth wears off” in the magazine The New Yorker [6], is an example on such reflections, discussing decline in strength on a multitude of scientific experiments over time. The same argument is presented in formal form in the Ioannidis’ 2005 article “Why most published research findings are false” in PLOS Medicine [7], discussing several obvious weaknesses in the ordinary perspective of the scientific method. The general argument from Ioannidis for this is the lack of reproduction of published results, lack of independent research teams for verification of results, bias and specific interests in study design and publication, as well as lack of large-scale studies or qualified meta-analyses.

Premises of science are a combination of tradition and consensus culture, not necessarily with further thought than what other researchers have done in the past. The ubiquitous boundary line of significance at 95 % set by Ronald Fischer in 1922 [8], was rumoured set there by no more specific reason than 5 % being a simple figure for pencil and paper calculations.

With this in mind, I will refer the foundation for the research of this thesis.

1. Arachnoid cysts

1.1 Epidemiology

Arachnoid cysts (AC) are relatively common benign lesions of the arachnoid, with reported prevalence of up to 1.1 % in the general population [9-11] – a prevalence with large variance between studies [10, 12].

The aetiology and pathogenesis of AC has been controversial and still remains unclear [13, 14]. Primary AC is believed to be congenital malformations or developmental anomaly in the architecture of the arachnoid mater (AM) [14-16]. These should be differentiated from other types of cysts that result from CSF sequestration resulting from inflammation or following traumatic processes, haemorrhage or tumours; these can be called secondary cysts [13, 14, 16].

The first report of AC is the classic work of Bright from 1831 [17], reporting a cystic malformation of the arachnoid layer. The 1879 report by Cunningham on the autopsy result of a young acromegalic patient who died from diabetes insipidus described the coincidence of a right hemispheric AC with a pituitary adenoma. Cunningham described a right hemispheric AC with a pituitary adenoma, that when opened, "a large quantity of thin sero-sanguinolent fluid escaped". This finding after a previous episode of strong headache suggested intracystic haemorrhage [14, 18]. The condition was named the "temporal lobe agenesis syndrome", by Richard Robinson in a publication from 1964 [19]. Although later retracted, the term is a misnomer based on the wrong assumption that the temporal lobe has been underdeveloped on the affected side [20]. Robinson himself withdrew this.

Arachnoid cysts can be located in relation to all parts of the central nervous system, but three out of four appear in relation to the cerebrum. Intracranial AC in adults are

most common in relation to the middle fossa / Sylvian fissure where two thirds are found, with prevalence for the left side and in males [21] [22-25]. In children, one of three is found in relation to the Sylvian fissure.

1.2 Through the microscope and beyond

AC was examined by Starkman et al in 1958 [26] observing that AC are truly intra-arachnoid in origin, a finding confirmed by electron microscopy by Rengachary et al in 1978 [27]. Rengachary et al performed a histopathological study on four selected cases of AC [28], observing several structural features distinguishing AC membranes from normal arachnoid: the splitting of the arachnoid at the margin of the cyst, a thick layer of collagen in the wall, the absence of trabecular processes in the cyst as well as hyperplastic arachnoid cells in the cyst wall. The collagen is believed to be reactive, as a consequence of pressure while clear arachnoid cells in the wall are suggested to be involved in the production of collagen. A striking feature of this article is the observation of the inner layer of arachnoid cysts consisting of clear arachnoid cells. The authors observe these clear arachnoid cells to be hypertrophic and hyperplastic with a resemblance to observed human foetal arachnoid cells. Rengachary et al observed a nearly invariable association of AC with normal subarachnoid cisterns, suggesting AC as a congenital anomaly in the developing subarachnoid cisterns. Prior to the availability of CT, there was less systematic investigation of the prevalence of arachnoid cysts [29]. Wester [30] suggested on basis of the observed predilection for the middle fossa as localization of AC, a mechanism that involved a defect in the early folding of the brain and meningeal anlage. Sequestration of spinal fluid during foetal development, misfolding in development from the neural tube, the association to subarachnoid cisterns as well as immature arachnoid cells suggest a developmental defect [26, 28, 31], but the causalities remains to be unveiled. The folding pattern and development of structural abnormalities have been studied by several authors, but the timeline of appearance of arachnoid cysts is unclear [32, 33]. The observed left dominance of AC, especially in males, suggests a genetic mechanism of formation [24]. Some authors observe an association with other heritary conditions such as

polycystic ovarial disease (PCOS), was suggested by Schievink et al, Leung et al and Alehan et al [34-36] – implying a possible genetic mechanism. The observation of mirror image cysts in monozygotic twins also indicates a genetic mechanism of formation [37].

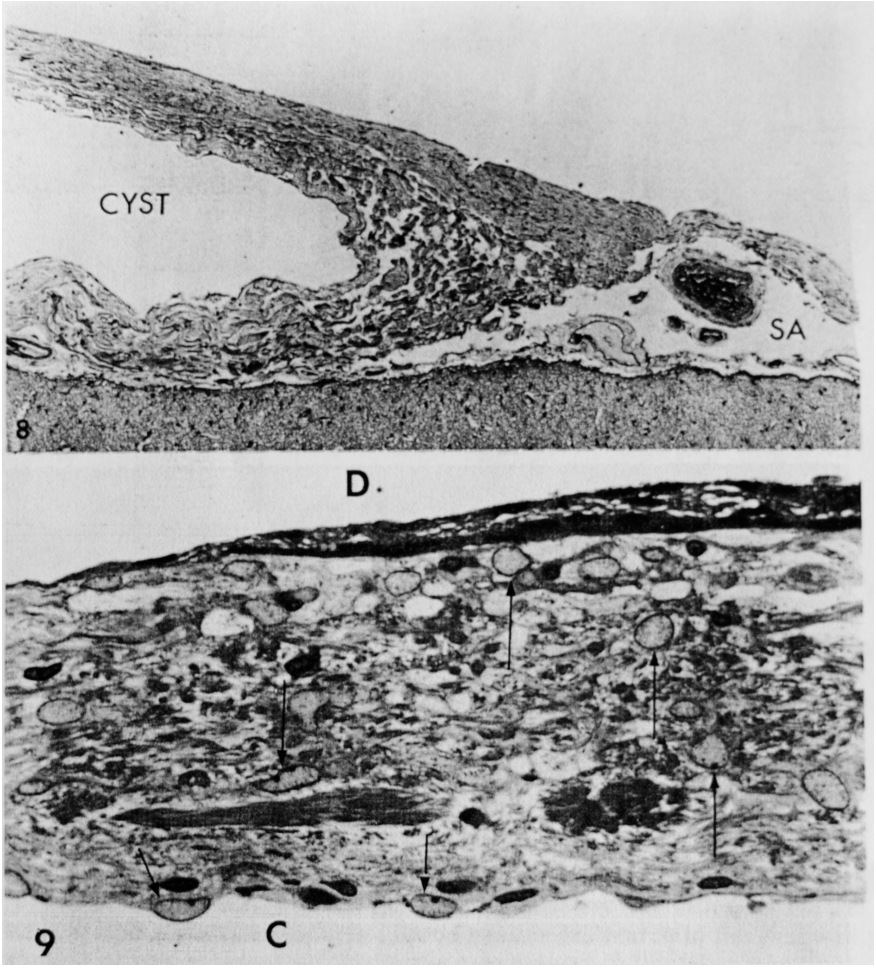

Figure 1. Electron microscopy from arachnoid cyst membrane. (From Rengachary SS, Watanabe I (1981) [28], reprinted with permission)

Upper figure: Margin of the AC showing the splitting of the arachnoid membrane to enclose the cyst.

Lower figure: The cyst wall is made up of dense connective tissue covered on the outside by thick tiers of subdural arachnoid cells. There are numerous hyperplastic arachnnoid cells (long arrows) especially beneath the subdural layer. There are also many hyperplastic arachnoid cells (short arrows) close to the cyst lumen (C). The dural side of the membrane is denoted by D. Semi-thin section stained with toluidine blue – x 510.

There are three prevailing theories on the mechanisms of filling and sustaining of such cysts [13]; active pumps, a valve mechanism or oncotic pressure. Go et al [38] suggested a mechanism of fluid secretion. Based on cytochemical identification of an active pump in the cyst membrane, more precisely a transport ATPase in the luminal surface membrane, it was interpreted as evidence of secretory function. Dyck and Gruskin [39] suggested that osmotic pressure gradients were involved in the filling of the cysts, as also implied by Schachenmayr and Friede [31]. Smith and Smith[40], later supported by among others Santamarta D et al [41], have suggested several types of one-way valves as a mechanism by which fluid enters the cyst.

Sandberg et al [42] performed chemical analyses of arachnoid cyst fluid and reported similar results as reference values for CSF, except for some unexplained with elevated protein content. They suggested elevated protein content as explaining factor in cyst filling. Helland et al [43] identified up-regulation of the Na+ K+ 2Cl- cotransporter NKCC1- gene in AC membrane when compared with normal arachnoid membrane. Several authors have by cytochemistry identified Na+ K+ adenosine triphosphatase (ATPase) in the membranes of AC, suggesting fluid transport [13]. Proteins co-transporting water with electrolytes or small molecules is reviewed by Zeuthen [44], stating that up to 590 water molecules can be co-transported with a single molecule of

NaCl [45]. Thus a considerable transport of water takes place with a relatively small amount of electrolytes. Aarhus et al [46] analysed gene expression of the AC membrane relative to normal arachnoid membrane through mRNA-analyses. The expression profiles of 33096 gene probes were similar except for a small subset of nine genes, as well as some duplicated DNA regions. The authors suggested further analyses in larger sample sets for verification.

Temporal ACs are classified according to Galassi et al [47]. Briefly, a type I cyst is small, biconvex, and located at the anterior temporal pole with no distortion or displacement of the midline of ventricle stuctures. A type II cyst involves the anterior and middle segments of the Sylvian fissure, extends superiorly and leaves the Sylvian fissure open. A type III cyst is large, involves the entire Sylvian fissure and has a marked radiological mass effect with displacement of not only the temporal lobe, but also the frontal and parietal lobe.

1.3 Clinical presentation

Most symptoms caused by an AC are non-specific and without sudden deterioration, in contrast to acute haemorrhages or stroke. Neither are the symptomatology necessarily progressing, such as might be the case for intracranial tumours.

In children, AC cause signs of increased intracranial pressure, such as headache, vomiting, lethargy, papilloedema, abnormally increasing head circumference, and seizures [16, 20, 25, 48, 49]. Signs of elevated intracranial pressure occur in more than 50 % of cases and are the leading symptom on admission. The other symptoms are mostly related to the cyst location; seizure disorders are reported in 18.2 % of patients and focal signs corresponding to the cyst are found in 31.9 % [16].

The most common complaint in adults is headache, followed by seizures, dizziness, dyscognition and focal neurological deficits. [14, 20, 25, 50-53]. In adults, clinical improvement after surgical decompression is *not* related to cyst size [20].

AC may grow over time, which may increase clinical symptoms due to increased intracranial pressure [54, 55], but most cyst don't seem to grow [56].

1.4 Surgical treatment

Treatment options for AC have included cyst aspiration, cyst excision, cystoperitoneal shunting, cyst fenestration, cystocisternostomy, marsupialisation in the subarachnoid space and ventriculocystostomy [53]. Currently, the treatment options of AC are fenestration or ventriculoperitoneal shunting. In fenestration, the cyst is operated either by craniotomy or by endoscopy [22, 57] creating an opening to the subarachnoid space surrounding the cyst. A ventriculoperitoneal shunt is a system draining the content of the cyst to the peritoneum with the objective of reducing the size of the cyst.

Most authors prefer fenestration as treatment of choice, either endoscopically or by open surgery, rather than shunt-related complications [23, 58, 59]. Although still debated, cystoperitoneal shunting is readily performed due to low invasiveness and safety [58, 60].

There is a significant controversy concerning when, if at all, surgical treatment for arachnoid cysts is indicated [25][52][53].There is also a controversy on which procedure is the optimal treatment [61]. In principle, the controversy of surgical indication is a question on when the symptoms are sufficient for the patient to be operated, as well as to which degree more diffuse symptomatology can be used as indication for surgery but it is also a discussion of whether or not the benefits from treatment overcome the risk of the procedure.

2. Proteomics, laboratory methodology, medical research and molecular biology

2.1 Cerebrospinal fluid

The cerebrospinal fluid (CSF) is the clear liquid surrounding the brain, protecting the brain from shock and preventing strain from the weight of the brain. The CSF is bordered between the arachnoid mater and the pia mater, the middle and the inner of the three meninges covering the brain, filling ventricles of the brain, around the spinal cord and in the central canal. The CSF is primarily produced in the choroid plexus in the lateral ventricles. The production of CSF is about 500 ml /day, while the CSF space is at about 130-150 ml. The resorption of CSF is primarily in the arachnoid granulations; bulky ports to the sinus sagittalis superior, a part of the cerebral vein system.

The protein concentration in CSF typically ranges from 0.2 to 0.8 mg/mL, which is 0.3 – 1 % of the plasma concentration [62, 63]. Around 20 % of the proteins in CSF are CNS derived, and the remaining 80 % derives from blood [63, 64]. The CSF both gains and loses content during its circulation through the CSF space in and around the CNS, representing challenges and opportunities for research. It is a general consensus that markers of disease in an organ will be found in higher concentrations in body fluids in close proximity to the organ in question [65] than elsewhere. The CSF with its close passage to the brain tissue is suggested as a body fluid suitable for investigating cerebral pathology [66]. The blood-brain barrier (BBB) is relatively impermeable to a multitude of substances, causing the passage of liquids, salts, proteins and other substances to the CSF to be selective. The brain-CSF barrier (BCSFB) is a much more permeable membrane than the BBB, allowing a higher pace of diffusion of substances between brain parenchyma and CSF. The mechanisms of passage in these barriers are not fully understood. The total protein content and

composition is dependent on several variables. Location of sample collection is thus not necessarily indifferent [62].

2.2 Ethics

Laws and science consensus regulate the involvement of human specimens in research. In Norway, studies like the present require informed consent. The project, with description of objective, as well as how the study is performed and procedures for written informed consent is supervised and approved by a Regional Ethics committee (REK). The Data Protection Authority approves the creation of biobanks of patient sample material. Publication of results is regulated by the International Committee of Medical Journal Editors (ICMJE) Uniform Requirements for Manuscripts Submitted to Biomedical Journals [5]. The current study is in accordance with the regulations on ethics and biobanking as well as the uniform requirements by ICMJE.

2.3 Sample handling

A current challenge in providing good analytic data is the collection of representative sample material. This should be handled in such a way that no variation is introduced to the sample material before analysis. The analysis setup should be planned using the same instrument with the same instrumental settings and interpret the result in the same manner for all samples or patients included.

Before a set of sample is analyzed, the collection, aliquotation and sample handling happens at different times, and the time of storage is different. It is important to standardise the collection methods as well as storing of samples in such a manner that degradation is reduced to a minimum. An example of good sampling procedure is the consensus protocol of the European Network for Biomarkers in MS, BioMS-eu, by Teunissen et al [67]. The work by Berven et al [68] concerning degradation in the low molecular weight proteome with different storage conditions demonstrate the need for adhering to standardised collection methods. The work by Kraut et al [69] illustrates

the importance of standardised selection of storage tubes, especially on hydrophobic peptides over time.

Standardization of sample handling should cover every step from the method of sample collection, centrifugation speed, time limits before ultra freezer or dry ice, the use of refrigeration before centrifugation and the choice of storage tubes and equipment [70].

2.4 Clinical chemistry

The term ”clinical chemistry” refers in medicine to analyses of the major electrolytes, minerals, cells and some abundant relevant proteins and enzymes in body fluids. In patient diagnostics analyses are performed in a standardised manner, mostly by hospital laboratories. Clinical chemistry is readily available in bedside diagnostics and plays an important part in clinical decision-making. For both clinical and research purposes, clinical chemistry makes up a significant complement to a multitude of problems. It is a readily available way to explore biology as well as it for most clinical chemical analyses, represents a limited invasive sampling of patient material.

2.5 Molecular Biology

Warren Weaver coined the name Molecular Biology in 1938 on basis of the ideal of physical and chemical explanations of life. Molecular Biology is a branch of biology that deals with the molecular basis of biological activity and consists of studies of form and function of the molecular machinery and blueprint of life itself. In practice, molecular biology studies the various forms of cells and cell compartments, as well as the study of DNA, RNA and proteins [71].

The central dogma of Molecular Biology was first stated publically in a rather brilliant article by Francis Crick in 1958 [72] and briefly revised in a Nature paper in 1970

[73]. This central dogma describes the information transfer between DNA, RNA and proteins, more specifically that information once it has reached the protein, cannot be transferred back to either nucleic acids.

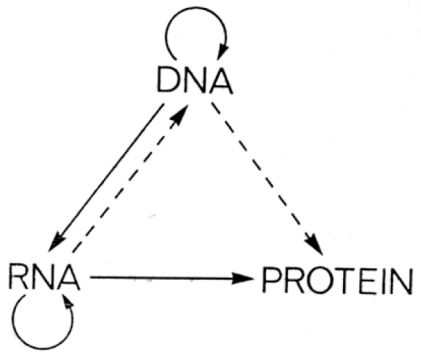

Figure 2. Central dogma of Molecular Biology (Illustration adapted from Crick, Nature 1970 [73])

2.5.1 DNA

DNA - deoxyribonucleic acid is based on a relatively simple chemical backbone of phosphate and the sugar deoxyribose with attached one of four nucleobases adenine, thymine, cytocine and guanine – with specific pairing of adenine to thymine, cytocine to guanine creating the double helix. DNA is the building material, the code in which our genetic material is written. The segments of DNA that contain specific information are called genes. Although not all DNA is genes, large segments of DNA have a regulatory function on the expression of the actual genes. The DNA is organised as rings in relatively simple organisms such as bacteria, while it in more complex organisms is folded up and organised by histons, separated on different chromosomes [71]. Although the code of DNA consists of only four possible inputs, the nucleobases, the possible information output is immense.

In the history of genetics, some experiments have made a leap in the understanding of the way information transfer is performed. Avery–MacLeod–McCarty [74] demonstrated in 1944 that genes are made up from DNA and that DNA is the heredity material of bacteria. Watson and Crick [75] presented the organisation of DNA in the double helix DNA model. The understanding of the copying machinery of DNA was demonstrated in the Meselson-Stahl experiment, showing copying of DNA as a semi-conservative process [76]. The understanding of structure did not make the information available for analysis straight away. This did not happen before Sanger and co-workers developed the method using modified nucleic acids (dideoksynuclotide tri-phosphates (ddNTPs)) to terminate chains in duplication for sequencing [77].

Before this, several scientists did experiments on inheritance and specific traits. Charles Darwin published his On the Origin of Species in 1859 [78], exploring natural selection and adaptation to environment. Gregor Mendel presented his results from garden crossbreeding in 1865 [79], although Mendel's rules were not recognised until after the pattern of inheritance was rediscovered in 1900.

Optimism of understanding the human genome was at its greatest at the time of the first publications about the sequence of the complete human genome. Two separate projects, one from The International Human Genome Mapping Consortium and one private from Celera Genomics and Craig Venter were published just a day apart, February 15th 2001 in Nature[80] and February 16th 2001 in Science [81], respectively. Since then, the understanding of further complexity has increased with the recognition of the role of epigenetics.

Epigenetics refers to inheritable information encoded by modification of the genome and chromatin components that affect gene expression. Thus, epigenetic changes do

not modify the DNA sequence. Epigenetic modification does not follow Mendelian inheritance and may be reversible. Epigenetics in DNA refer to key areas such as histone and chromatin remodelling and methylation of DNA. As a phenomenon, epigenetics is not restricted in DNA, but might also be seen in RNA and proteins – the complexity is only beginning to be unveiled. As Taft et al state in their 2010 review [82]:

> *"The absolute number of protein-coding genes encoded by a genome is essentially static across all animals from simple nematodes to humans, indicating that additional genetic elements must be involved in the development of the increasingly complex cellular, physiological and neurological systems"*

2.5.2 RNA

The understanding of RNA as a separate entity evolved in parallel with the research on DNA. RNA differs from DNA in some simple features. The RNA is single-stranded and may be folded and bent in to complex structures, an effect which is partially caused by the lack of rigidity in the structure created from its backbone sugar, ribose. The protein translation mechanism from RNA in three separate entities; transfer RNA, messenger RNA and ribosomal RNA, is complex. The understanding of how the mechanism works was delayed several decades until the recent advent of structural biology. As Alexander Rich states in his 2009 review paper [83]

> *"The hybrid DNA–RNA helix remains the bedrock of information transfer in biological systems. The existence of a hybrid helix seems so obvious today that young researchers simply take it for granted. There is little realization of the extent to which scientists once wrestled with the question of how helical*

polynucleotides with different conformations could react together to make a stable hybrid duplex structure"

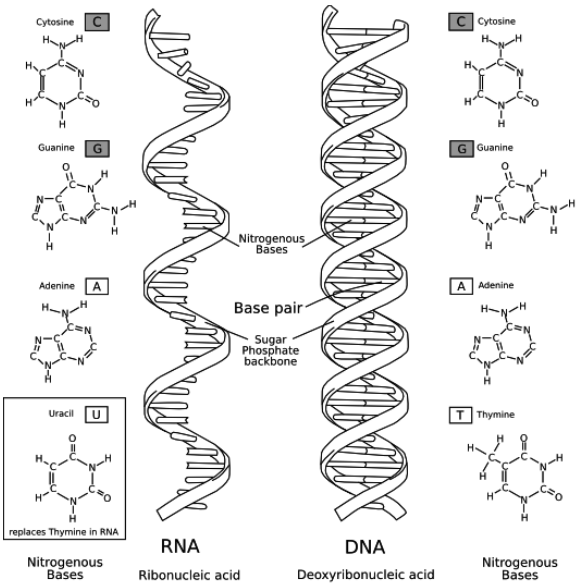

Figure 3. Schematic overview of the structure of DNA and RNA with its backbone of nucleobases. (from Wikimedia.org, free commons licence)

Transcription and regulation of ribosomal RNA, messenger RNA, and transfer-RNA from DNA is not direct and one-to-one, but rather modified caused by introns and exons from the first transcript of RNA. The ribosomal RNA forms the ribosome, which is the protein transcription machinery. The messenger RNA works as a template for proteins, while the transfer RNA collects amino acids as building blocks in to the ribosome and "bridges" to achieve a correct and precise translation of RNA to protein. Less than 2 % of the total RNA transcripts from DNA is transcripted into coding mRNA. Previously the remaining 98 % of DNA was given the misnomer term "junk-DNA". Several authors point out a consensus in the scientific community, that there is

no logic to maintain such an energy demanding process without a function. Now, the function of “non-coding DNAs” is just beginning to be discovered [71, 82, 84]. Until 2010, 30 people have been rewarded Nobel Prizes for research on RNA, the latest in 2009, where Ada Yonath, Venkatraman Ramakrishnan, and Thomas Steitz were awarded the Nobel Prize in Chemistry for their structural work on the ribosome and translation mechanisms [85, 86].

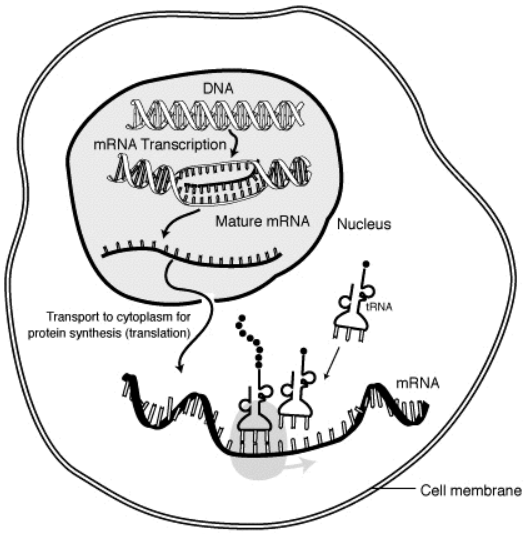

Figure 4. From DNA to protein, transcription from DNA and translation from mRNA to protein. Proteins are built from amino acids represented as dots, by tRNA.

(from Wikimedia.org, free commons licence)

2.5.3 Proteins

Proteins are long chain molecules consisting of one or more polypeptides composed of 20 different amino acids as building blocks, encoded by the mRNA. The peptide is a single polymer of amino acids linked with amide bonds, where the –NH-C(R)-(C=0)- amino acid common part is a small and repeating flexible backbone which provides a

flexible and foldable structure. The residues of the amino acid backbone create a potential for folding and union of several peptides into proteins. The folding is either spontaneous or assisted by the cellular machinery. The folding of proteins happens in such a manner that specific sequences of amino acids fold dependent on amino acid sequence. These regions again fold up such as polar groups fold towards each other or towards polar solutes, apolar groups towards each other or end up in lipid membranes, folding is dependent on amino acid polarity. The structure is also stabilised by thiole (-SH) groups create a potential for sulfur bridges (-S-S-) fixing superstructures of proteins. These processes create a multitude of protein structures, from the simple cellular structures such as alfa-helixes or beta-sheets to more complex metal ion binding proteins such as zink fingers.

The functions of proteins are thus not solely explained by the sequence of amino acids, but rather depends on a three dimensional structure built through folding and linkage of several peptides. The structure of a protein is differentiated in levels, where the primary structure is the amino acid sequence, the secondary structure is mainly the composition of alfa-helixes, beta-sheets and loops, and the tertiary structure is the spatial folding of these structures fitting together with polar attraction as van-der-Waal forces, hydrogen bonds and disulfide bridges. The quarternary structure of a protein is the union of several peptides forming the protein, sometimes also with cofactors [71].

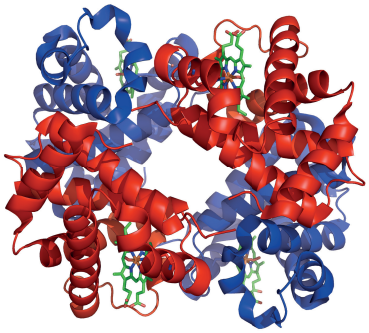

Figure 5. Quarternary structure of haemoglobin, of four haemoglobin (2 alpha, 2 beta) chains each with heme cofactors.

(Adapted from Wikimedia.org under creative commons licence)

Proteins with its small and flexible backbone as well as a much larger variation of substituent groups than RNA and DNA, as well as the huge versatility in structure and folding, is the reason how proteins perform most of the cellular functions. Proteins construct receptors and signal substances, ion channels and mechanisms for metabolism and energy generation, cellular transport, machinery for cell division, movement and linking. The flexibility in structure and function is thus much larger in proteins than in RNA. While life itself may have started as RNA, it has most certainly evolved through proteins.

2.6 Proteomics

Wasinger and Wilkins introduced the term proteome in 1995, referring to “the PROTEin complement expressed by a genOME” [63, 87, 88]. As a field of research, this includes tracking of single proteins’ structure and function, of groups of proteins

present or not present in a sample material, appearance of proteins specific for a condition as a biomarker of a biologic process or measuring specific changes in concentration of proteins as markers of biologic changes. An organism's proteome is determined in part by the genome encoding the primary amino acid sequence. Other factors affecting a proteome include alternative splicing variants, post-transitional modification (such as phosphorylation and ubiquitylation) and protein cleavage. Taken all possible variations into account, the potential number of protein species in a cell is far greater than the number of genes. Adding to the complexity, the proteome of a cell is not static, but may change in response to both internal cellular state and the external environment [89]. Proteins, or polypeptides in general have traditionally been sequenced by cutting off one amino acid at a time from the peptide chain, and thereafter identified each amino acid identified by UV spectroscopy, a method termed Edman degradation [90]. This field of research is in rapid development both in science and with respect to innovative instrumentation and methods that enable the research to be performed. The development of advanced mass spectrometers coupled with high-pressure chromatography over the last ten years has enabled a leap in detection limits and possibilities for specific detection of proteins present in low concentrations in a mixed matrix.

2.7 Gel electrophoresis

Until a few years ago, most separation science as well as staining and identification has been performed by gel-electrophoresis, both one and two-dimensional experimental setup. The principle of gel-electrophoresis is utilizing a gel based on agarose or polyacrylamide to separate denatured proteins; during electrophoresis the drift is roughly proportional to the protein mass. 2D electrophoresis applies a separation on isoelectric focusing before ordinary electytophoresis. This creates a pI gradient along the x-axis, and a MW gradient along the y-axis. Although a separation is performed, this method has limitations both on the minimal amount of proteins that has to be loaded for analysis, as well as the ability to identify, select and extract proteins from the gel. There are several advanced procedures for staining and

identification on gel electrophoresis, such as coomassie brilliant blue [91], Sypro ruby [92] and Silver staining [93] each with its advantages and drawbacks [94]. The proteins present have to be compared between different gel sheets for different samples, creating a challenge concerning reproducibility to the matching between samples, as well as challenges to missing values in samples [95] There is also a limitation on the dynamic range of the analysis, defined as the difference in concentration between the weakest and strongest "spot" on the gel.

2.8 Trypsin / tryptic digest

Trypsin is a protease, a pancreas enzyme that cleaves peptide chains mainly at the carboxyl site of the amino acids lysine and arginine, except when either is followed by the amino acid proline [96]. The principle of cleaving proteins to peptides is to make the sample available for mass spectrometry - a necessity for mass spectrometry as the polypeptides is analyzed as ions. Another advantage of cleaving proteins to polypeptides is the relatively equal size between different protein fragments, allowing reproducible and reliable chromatography. Tryptic cleavage is an essential part of the proteomics workflow and the enzyme has become a workhorse in proteomics.

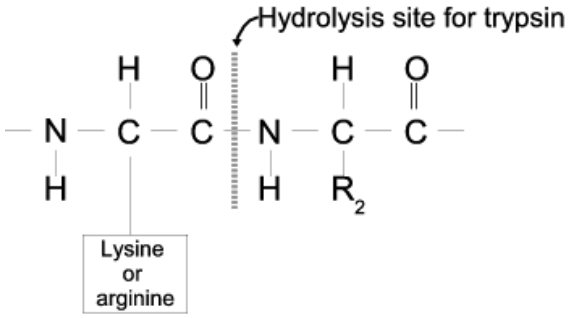

Figure 6. Hydrolysis of proteins by trypsin (figure from clcbio.com under creative commons licence).

2.9 Chromatography

Chromatography is a term describing separation science of molecules, and is performed with a multitude of materials, solvents and analytes. For proteomic purposes, liquid chromatography has taken over for the separation function previously performed by gel electrophoresis [94, 97-99]. Reverse phase (RP) miniaturised high-pressure liquid chromatography (HP-LC) is the predominant separation technique in proteomics, both offline and coupled to mass spectrometers. These are columns with an apolar column material, typical C-18 on a silica backbone. Its miniaturization, implying reduced volumes and reduced inner diameters for the columns, causes a more precise separation without wasting sample material in the separation columns, supported by high-pressure pumps to overcome the resistance in the system. The principle for reverse phase chromatography is to use a non-polar stationary phase at the inner covering material of the chromatography columns and elute a gradient from polar to non-polar solvents consisting of water and water miscible organic solvents such as acetonitrile (ACN), acidified by formic or tetrafluoroformic acid (TFA). In such a column, the very polar substances, such as salt and impurities, will often leave first, following a gradient of peptides based on size and polarity.

The ion exchange chromatography (IEX), in particular strong cation exchange (SCX) chromatography and strong anion exchange (SAX) chromatography are useful techniques, as they are mostly orthogonal to reverse phase chromatography. The principle in cation exchange is that the stationary phase is negatively charged so positively charged molecules (peptides) interact by ionic interaction to the solid support. A mobile phase, with a pI/pKa between the charged molecule and the solid support will elute the peptides based on a change in pI gradient on basis of acid buffered salts. Equivalent for strong anion exchange, a positively charged column bind negatively charged peptides, which are eluted on basis of pI gradient. Disadvantages of such orthogonal chromatography are the need for removal of large amounts of salt-containing solvent. A relatively new solution to this problem has been combination columns, termed mix-phase columns such as reverse phase-weak anion

exchange (RP-WAX) combined columns [100]. This is a system set-up with a mobile phase changing on both polarity and pH, such that peptides are separated better than either of the single modalities, without the need to desalt the sample before analysis. The principal advantage here is separation with less introduced complexity.

2.10 Mass spectrometry

During the last ten years, proteomics has switched the analytical platform from gel-based to mass spectrometry-based techniques [63, 89, 99]. In the years just after 2000, optimism was great to "high-throughput methods" such as MALDI-TOF, short for matrix assisted laser desorption/ionisation – time of flight mass spectrometry, and SELDI, which is a version of the same with specific surfaces to improve ionization. MALDI is a method to make ions enter a mass spectrometer, introduced by Karas et al [101, 102]. Briefly, the sample is placed on a surface and mixed with a matrix for ionization, often consisting of small organic acids such as sinapinic acid or other substituent molecules from cinnamic acid. While the precise mechanism is not fully understood [103], the principle is that the dried sample-matrix spot is ionised with a laser, resulting in vaporised charged particles able to enter the mass spectrometer. MALDI is a stable, quick and simple method for analyzing samples. As a method, it does not permit chromatography in itself; this must be performed off-line before spotting. In general the mass resolution, meaning the instrument specific ability to separate two specific similar masses, of a MALDI-TOF is not high enough for high confidence peptide identification [103].

Several research groups developed over many years MALDI as a principle and method for ionization. Koichi Tanaka was awarded a part of the 2002 Nobel Prize in chemistry for demonstrating that a combination of laser wave length and energy to a matrix with corresponding physical and chemical properties could cause a soft ionization [104], a prize John Fenn also took a part in for the development of electrospray ionization (ESI).

Electrospray ionization mass spectrometry (ESI-MS), introduced by Fenn et al in 1989 [105], has enabled analyzes of samples in a mass spectrometer without being dependent on matrixes or specific surfaces for ionization. The principle is as for MALDI to perform a soft ionization of peptides, where soft meaning ionization without breaking structural chemical bonds – as opposed to hard ionization with fragmented ions. ESI-MS is in use for many different types of mass spectrometers, both offline and coupled to high-pressure liquid chromatography (HPLC). The advantages for coupling to liquid chromatography (LC), especially at low flow in small bore columns for HPLC, is the increase in sensitivity when allowing instruments to analyze fractions of one sample separated over time, still allowing for the use of small sample volumes. A typical simple HPLC setup for a proteomics mass spectrometer is a reverse phase non-polar column of silica –C18, with a mobile phase being polar to nonpolar gradients of water and acetonitrile (ACN), added a minute amount of formic acid. The chromatography column ends directly to the ESI, and the chromatographed sample is continuously injected in the mass spectrometer. The typical gradient time is some 60-80 minutes, gaining a separation over time for the content of the sample – thus allowing the MS instrument to thoroughly analyze, fragment and identify peptides as they appear eluted from the chromatography column.

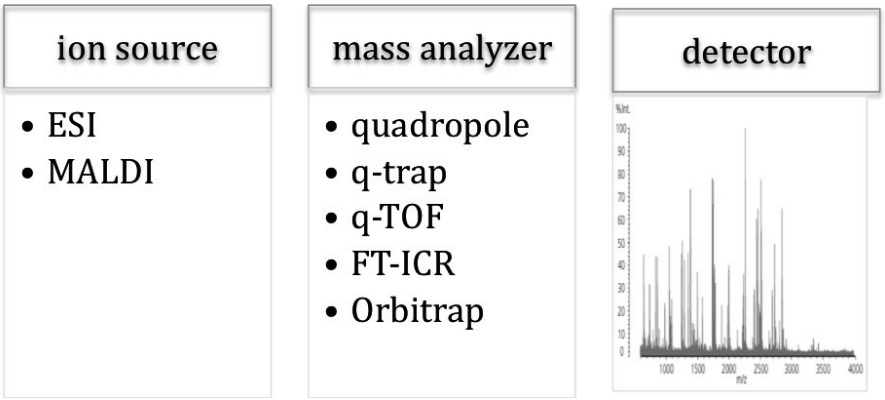

Figure 7. Mass spectrometry principle

There are several different forms of mass spectrometers, with different qualities [99, 106, 107]. In principle, the mass spectrometer consists of three parts, the ion source, the mass analyzer and the detector (see figure X).

ESI and MALDI are ion sources, converting a peptide from a solid or solubilised form to a gaseous charged molecule capable of "flying" in electric fields in vacuum. There are several other modalities than ESI and MALDI in ion sources, but for most mass spectrometers in proteomics, ESI is by far the most predominant.

Mass analyzers separate ions depending on mass per charge (m/z) in an electric field and can perform different functions, depending on architecture. The range is from simple separators of ions based on mass per charge to the more complex combined units where a mass spectrometer is coupled to collision cells selecting and fragmenting separated ions (MS-MS). This fragmentation divides the peptide in smaller random fragments, mainly with the purpose on basis in statistics to reconstitute the sequence of

amino acids in the peptide, as well as to free of reporters for labelled tags for analysis, further discussed under “quantitative proteomics”.

The detector is recording individual *m/z* from the separated peptides, and also the number ions hitting the detector, thus providing grounds for determining relative intensity of ions present.

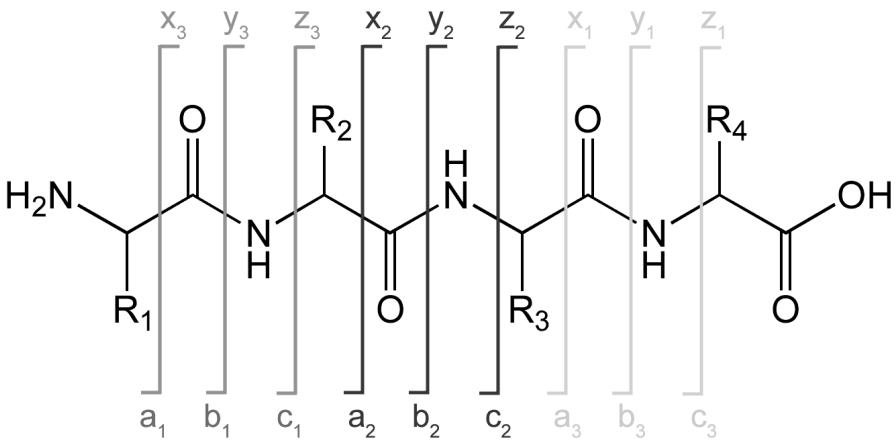

Figure 8. Peptide fragmentation patterns. The figure represents fragmentation patterns in principle in an amino acid chain, where b/y – ions, separation in the (C=O)-N-H amide bond is the most common (figure from Wikimedia.com under creative commons licence, adapted from Roepstorff [108]).

The principle of quadropole mass spectrometers [107] is that a filter passes ions through chambers limited of charged rods, where ions can be selected to pass through on basis of ion resonance of m/z. Only specific m/z molecules are able to pass, and can therefore be separately measured by a detector behind the quadropole. A quadropole

cell in this instrumental set-up can also be used as a trap, confining selected ions to a limited space before being passed further within the instrument.

Single quadropoles itself are not very specific, but they are often combined with other modalities or placed in sequence. Linear trap quadropole mass analyzers (LTQ) consist in principle of three quadropoles in a row. LTQ instruments are able to select for specific masses in the first quadropole, colliding and fragmenting in a second quadropole and again analyzing the fragments of masses selected in a third. This gives information of the content of specific peaks identified in the mass spectrum, giving opportunities to follow specific fragments of peptides – a very useful feature for selected reaction monitoring (SRM) [109, 110], further discussed in the section "quantitative proteomics". SRM is a label free technique to follow specific fragments; it is not necessary to "scan" the whole mass spectrum. This gives an increase in instrument sensitivity of one to two orders of magnitude [109].

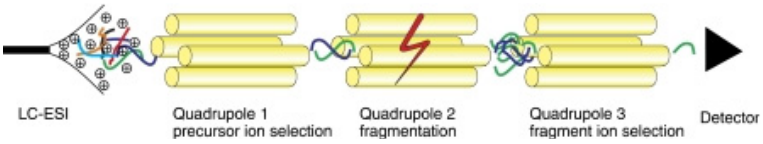

Figure 9. Triple quadropole experiment linked to LC-ESI in a SRM setup. The first quadropole selects peptides as a filter for further analyses, the second quadropole fragments the selected peptides, and the third select fragments for analysis. Figure adapted from Lange [109] under Creative Commons licence.

Time of Flight (TOF) mass spectrometry is a more specific mass analyzer than quadropoles. Ions enter and the m/z is measured on basis of their flight time through a charged vaakum tube. This is a versatile and stable mass analyzer and is the common mass analyzer for MALDI.

Detectors of TOF and trap-instruments are of electron multiplier types, and they typically add up information with a trap to detect the m/z and relative intensity of a molecule. Such detectors work by principle of an emissive material, such as if a charged particle hits the detector, several electrons might be emitted and each lead to new emissions, causing the generation of a detectable current on the end plate [111].

A Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FT-ICR-MS) applies another principle of detecting masses. Ions are gathered into an ionization chamber and are exited in a magnetic field cyclotron. Detectors measure the movement or orbit of ions in the cyclotron. The signal decay from excitations of ions, known as free inductance decay (FID), is detected and transformed to resonance frequencies by Fourier transform and these frequencies are proportional to mass/charge. These mass analyzers have high resolution and can be used for a precise measurement of masses. Orbitrap MS applies the same principle as FT-ICR-MS, using an oscillating electric field instead of an expensive superconducting magnet [90]. The principle is to inject ions to the Orbitrap observing the stabilizing of an orbit based on electrostatic attraction and centripetal acceleration, depending on m/z. The m/z of trapped ions is thus easily deductible by Fourier transform detected ion orbits, where resolution is increased on every ion "passing" the detector in its orbit. Orbitrap MS instruments are well described by Hu et al [112] and Olsen et al [113].

2.10.1 Challenges for mass spectrometry in proteomics

The difference in concentration between the most abundant proteins and proteins of possible interest that exist in lower concentration is a large challenge in proteomics. For any instrumental set-up, the difference between the most abundant protein in concentration and the one with lowest concentration detectable is termed the dynamic range. The dynamic range is often measured in orders of magnitude, where most instrument set-ups can handle 10^4-10^6, while the biological systems subject for

methodology relevant for this thesis, cerebrospinal fluid and plasma is up to 10^{10}-10^{11}. This can be exemplified further. Using plasma as an example, the concentration of one single protein – albumin, makes up for about 2/3 of the total protein content of about 60 g/litre. Added up, immunoglobulins, blood coagulation factors fibrinogen and lipoproteins together with albumin make up for 99 % of the protein content in plasma. Therefore, a strategy is needed to overcome that problem.

Spreading the proteins entering the mass spectrometer over time by chromatography is a strategy much employed to allow the instrument to focus at a few polypeptides at a time. Still, the abundant proteins tend to dominate any sample due to their sheer numbers. Several approaches have been applied in proteomics; unspecific depletion of abundant proteins, specific immunoassay targeting of selected proteins to selectively remove abundant proteins, and extensive fractionation prior, to analysis.

Removal of high abundant proteins from body fluids can be conducted in several different ways, but the most recognised approach is to use columns with antibodies agains the most abundant proteins. Examples of such columns are MARS (multiple removal affinity system) from Agilent, Seppro IgY from Genway Biotech, and Proteoprep from Sigma. Each vendor has several different and more complex columns absorbing more high-abundant proteins from a sample. For the future, this might be expected to further increase the feasibility of analysis of low-abundant proteins.

2.10.2 Alternatives to mass spectrometry in proteomics

Antibodies can also target directly the specific protein in question, by methods such as Enzyme-linked immunosorbent assay (ELISA) and western blot. The principle of ELISA is to create an assay where the substance in question is attached to a surface or solid support by antibodies; a new “detection” antibody is then attached with a reporter, such as an enzyme so that, by measuring enzyme activity, the amount of a protein is reported by indirect measures. This is a useful method for control procedures

and routines, but not in the discovery phases. Western blot is more useful in proteomics, especially in hypothesis-driven proteomics. A western blot consist of a separation on a gel, transfer to a membrane, incubation with primary antibody against the protein in question, and later incubation with secondary antibody against the first, attached to a reporter enzyme. Gel electrophoresis, as previously mentioned, has been the traditional alternative to mass spectrometers, but is on a decline due to sensitivity and reproducibility issues.

2.11 Quantitative proteomics

Quantitative proteomics is defined as methods able to quantify absolute or relative differences in protein concentrations between a sample and a control or reference. Kroksveen et al wrote a concise review article concerning quantitative proteomics and neurological diseases [70]. They discuss several methods for quantitative proteomics, chemical isotope labelling, label free and MS/MS- based methods.

In label-free quantitative proteomics, proteins are quantified either by spectral counting or by intensity measures. In spectral counting, the number of spectra containing fragments selected for identification represents the relative quantity of a protein [70, 114]. Label-free quantitative proteomics by peak intensity utilise the configuration of the peak between two samples as a measure of quantity, either measuring the highest intensity or the integral of one or more peaks to represent the relative intensity between two samples [70, 115, 116].

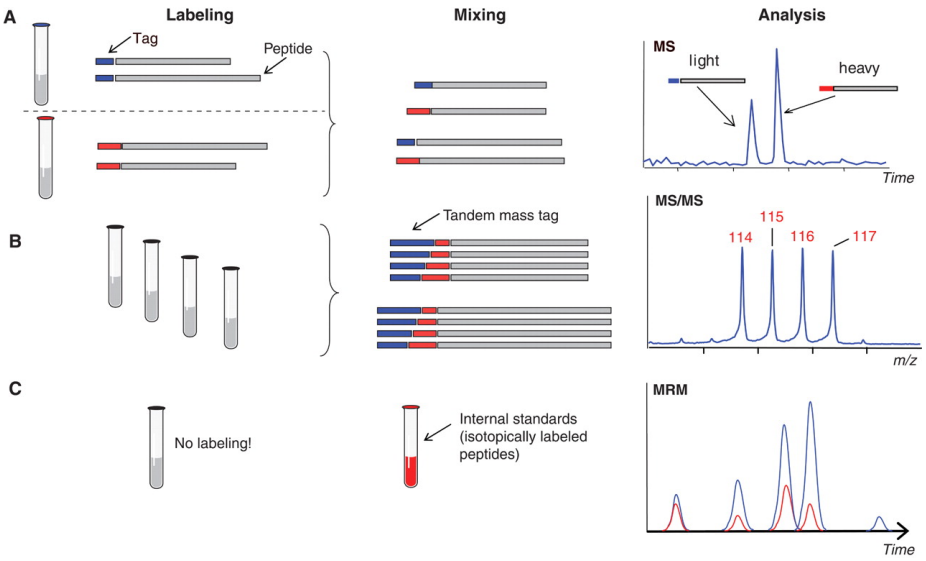

Figure 10. Strategies for peptide quantitation.

A: Stable isotope labelling, with a heavy and a light tag.

B: Isobaric tandem mass tag, where the tag separate in MS/MS and the same peptides are separated by 1Da. Quantification is by relative quantities to a standard, one of the tags.

C: Selective (SRM) or multiple reactions monitoring (MRM) where quantification is based on relative abundance to internal or external standard.

Adapted from Domon [90], reprinted with permission from AAAS

A method for performing quantitative proteomics is iTRAQ – isobaric tag for relative and absolute quantitation, first published by Ross et al in 2004 [117]. This is a commercial kit from Applied Biosystems (Life Technologies, Carlsbad CA, USA). This is one of several methods utilizing reporter ions attached to peptides from different samples or sample fractions, which is then mixed and analyzed together by MS/MS and the relative quantities of proteins in mixture appear in the spectra. The

method works by attaching reporter ions separated only by 1 Da MW to free amines in the peptides. The term “isobaric” means that the reporter before fragmentation is attached to a weighted molecule, so that the different labelled groups will have equal mass. The reporter ion will follow through MS fragmentation and disintegrate and separate in MS/MS-fragmentation, giving peak heights representative of relative quantity of each protein The typical workflow of such a procedure is to have a known and equal amount of protein in each sample, deplete the more abundant proteins, digest the proteins to peptides with trypsin, add the different reporter ions to the different samples and mix, fractionate the sample by off-line HPLC, and analyze each fraction by LC-MS/MS. The reporter released after MS/MS, termed by mass 114, 115, 116 and 117 will be detected as four separate peaks, where signal intensity is a relative measure of concentration of the same peptide between samples.

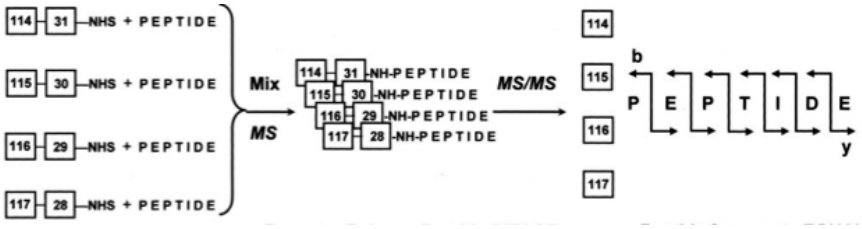

Figure 11: Structure of reporter ions attached to peptides. Adapted from Ross et al [117]

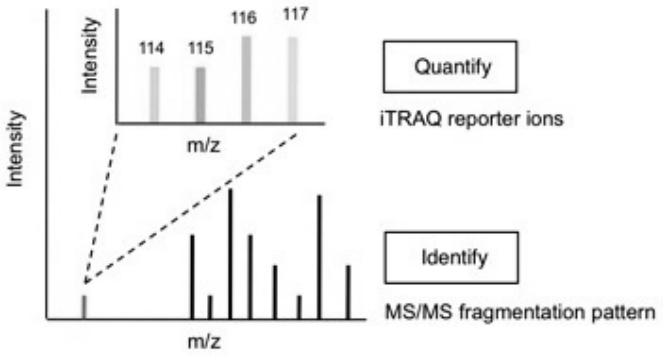

Figure 12. Mass spectrum of iTRAQ reporter fragmentation pattern. Adapted from Tweedie-Cullen et al [118].

2.12 Clinical plausibility

The objective of proteomic studies is in general to identify specific changes at a molecular level representing the disease or phenomenon in question. Given that a change, either by the presence or changed concentration of a specific analyte, is present in a large number of "diseased" samples but absent in controls, then it might be used to discriminate an unknown sample (or patient) to have or have not the specific condition in question. An analyte or a set of analytes in combination is termed a biomarker. The principle specified for proteomics will be as proteins perform a multitude of cellular functions, a change in function will be found in a molecular scale and thus a change in protein content will mirror a change in function.

For proteomics, there are yet not any large-scale biomarkers which have been approved for clinical use, as is emphasised by Ioannidis [119] who identified four major causes for this failure. Clinical reversal, exemplified by the prostate specific antigen, does not show as promising results in clinical practise as was expected upon

its introduction. Validation failure is when initial promising analytes fail to provide analytical validity in an external sample set. Non-optimised clinical translation is when the analytes selected for further development is not the most promising but the more available for a research group, as well as a smaller sample size gives a skewed presentation of reality. Promotion despite nonpromising evidence is when biomarkers are taken further from discovery phase even though results from research should indicate further discovery research – either for commercial or non-science causes. A roadmap for implementation of clinical proteomic biomarkers is well evaluated by Mischak et al [120].

3. The Thesis

3.1 Aim of the Thesis

The main objectives of the thesis were to characterise AC fluid by clinical chemistry and proteomics and compare it with CSF from the same individuals. This information would be used to gain knowledge of where AC fluid comes from, as well as the mechanisms of filling and sustaining of such cysts.

Secondary objectives were

1. Compare the clinical chemistry between AC fluid and CSF
2. Evaluate the proteome of AC by qualitative proteomics by comparison to published databases of CSF and plasma proteomes
3. Compare AC fluid and CSF proteomes by quantitative proteomics
4. Evaluate the results of analyses versus previous studies on mRNA and DNA of AC

3.2 Materials and Methods

Patients were recruited by the responsible surgeon prior to elective surgery for fenestration of symptomatic AC in the temporal fossa. AC fluid and CSF was collected with written informed consent from 19 patients, age 22-78, nine females and ten males in the period January 2008 to September 2010.

Patient	Age (yrs), sex	Side	Galassi-stage[47]	Remarks
1	26, f	Left	2	
2	43, m	Left	2	
3	58, f	Left	3	Old hematoma
4	34, f	Left	2	Reoperation
5	22, f	Right	1	
6	36, f	Right	2	Slight hemolysis CSF
7	35, f	Right	2	
8	77, f	Left	1	
9	42, f	Left	1	
10	60, m	Left	2	Slight hemolysis CSF
11	56, m	Right	2	
12	25, m	Left	1	
13	30, f	Left	1	
14	37, m	Left	2	
15	63, m	Left	2	
16	58, m	Right (Bilateral)	1	Bilateral, opposite already operated
17	62, m	Right	1-2	Chronic SDH. Thin membrane between AC and SDH. Slight blood contamination.
18	63 m	Right	3	Reoperated. Shunt. Previous meningitis x2, multiple previous intracranial operations.
19	50, m	Left	1	

Table 1: Patients included in the study.

3.3 Ethics

This project was approved by The Regional Committee for Medical and Health Research Ethics (REK) of Western Norway (approvals REK 70.03, NSD 9634 and REK 2009/1885).

3.4 Surgical method and sample collection

All patients were operated with a craniotomy under general anaesthesia, given as total intravenous anaesthesia (TIVA) with propofol and remifentanyl. Vecuronium bromide (Norcuron®) was used as the neuromuscular blocking agent. A burr hole was made with a high-speed drill immediately posterior to the sphenoid wing in order to gain access to the anterior and most basal aspects of the middle cranial fossa. The dura and the underlying cyst membrane were punctured through the burr-hole with a 23 G, 25 mm long syringe connected to an Optidynamic® spinal fluid manometer (Mediplast AB, Malmo, Sweden). After pressure equilibration and registration, a cyst fluid sample (3 - 5 ml) was collected using the manometer tube as a siphon. The sample was immediately transferred to a sterile centrifuge tube for centrifuging and further analytic processing as described below (sample handling). After this procedure, a standard craniotomy with a microsurgical resection and fenestration of the cyst membranes was performed. Before opening the medial cyst wall and thus communicating the cyst interior to the basal arachnoid space/CSF, all cyst fluid was aspirated from the cyst cavity to avoid cyst fluid contamination of the CSF.

After opening the medial cyst membrane that covered the basal structures (the tentorial slit, the oculomotor nerve, the carotid artery, and the optic nerve), thus creating communication to the basal cisterns and the posterior fossa, a CSF-sample was collected with a pre- cut baby-feeding catheter #6, connected to a 10 ml syringe. The catheter was placed below the tentorium via the tentorial slit and fluid was aspirated

gently from the posterior fossa. The collected CSF was transferred to centrifuge tubes and processed in an identical manner to the cyst fluid.

3.5 Sample handling

The samples were transferred to polypropylene tubes (Nunc CryoTube, Thermo-Fischer Scientific, Roskilde, Denmark) and centrifuged for five minutes at 450 x g to remove cells and cell debris. The supernatant was transferred to new polypropylene tubes and immediately stored on dry ice prior to long term storage at -80°C. Such sample handling has previously been demonstrated to reduce degradation of components and lysis of cells in centrifugation precipitate, which may change the composition of the sample [68].

3.6 Chemical analysis

Analysis of the AC fluid and CSF for Paper I were performed at the Laboratory for Clinical Biochemistry, Haukeland University Hospital. The laboratory is accredited by Norwegian Accreditation (accreditation number "TEST 231") as a testing laboratory and complies with the requirements of NS-EN ISO 15189. Clinical chemistry analysis was performed on a Modular Analytics System by Roche Diagnostics (Roche Diagnostics GmbH, Mannheim, Germany), except for osmolarity, which was measured by freeze point depression on a Fiske Micro-Osmometer (Fiske Associates, Massachusetts, USA) and immunoglobulins, which were measured by nephelometry with system specific N antisera to Human Immunoglobulins (Dade Behring Marburg GmbH, Marburg, Germany) on a BN Pro Spec System (Siemens Healthcare Diagnostics, Illinois, USA).

3.7 Qualitative proteomics

The qualitative proteomics for Paper II was performed as two experiments of several sequential steps. The quantitative data in paper III provides information on qualitative identities on identified proteins. In paper II, the first part is an evaluation of the similarity in the qualitative proteome between the individual patients included. The second part is an evaluation of a pool of a selection of the same patients.

The protein concentration in AC fluid was measured using a QubitTM, a step necessary to control the amount of protein in each sample. This is a method using dye to bind protein and measure the concentration on basis of ultraviolet spectrometry versus a calibration curve. AC fluid was concentrated and desalted using molecular weight cut-off filters and the solvent was removed in a vacuum concentrator. The amount of protein from each patient was standardised to avoid the introduction of variance in the data set.

The proteins were digested into peptides using laboratory trypsin protocols [121]. For the second experiment, the peptides were fractionated by SCX chromatography to 28 fractions and vacuum concentrated to dryness after thorough preparation through desalting.

In the first part of Paper II, the AC protein comparison experiment, the peptides were prepared for and analyzed by a nano-HPLC coupled to an ion trap mass spectrometer. Data were collected in automatic MS-MS-mode. The raw data was processed with the Spectrum Mill search engine. Peptide and protein identifications were accepted when the probability of correct identification was greater than 95 %, with a minimum of two identified peptides per protein.

The statistical analysis for evaluating similarity was performed by principal component analysis [122]. Models for this analysis were made both for all proteins and for the 50 most prominent proteins in order to reduce the probability of proteins near the detection limit to introduce noise to the dataset. The intent of this approach was to evaluate statistical outliers. Furthermore, a sub-sampling scheme was performed where 50 proteins were drawn on random to build a model, evaluating the stability of the dataset – a design made to reveal if any particular sample would cause divergence in data. [123, 124]. Statistics was performed in the statistical software package R.

For the second part of Paper II, the AC proteome evaluation experiment, the separate fractions from the SCX fractionation of peptides were prepared and analyzed by a nano-HPLC system coupled to an ESI-Q-TOF mass spectrometer.

To reduce false positive proteins reported specifically for AC fluid, a supplementary experiment was performed, where supposedly selective proteins for AC were searched for by targeted proteomics. We used the MIDAS workflow (Multiple Reaction Monitoring (MRM) initiated detection and sequence analysis) [125] as hypothesis driven proteomics increase the sensitivity for specific proteins. MIDAS is a method selecting the mass spectrometer to look only for specific predicted masses from peptides, thereby increasing sensitivity in the instrument. These analyses were performed on a nano-HPLC system coupled to a Q-TRAP 5500 mass spectrometer. Data was extracted with Masslynx, merged utilizing PklFileMerger [126]. MS/MS data were searched using the MASCOT software platform against the IPI-human database [127]. Protein organization and redundancy reduction was done using the Scaffold software [128]. Peptide and protein identifications were accepted when the

probability of correct identification was greater than 95 %, with a minimum of two identified peptides per protein.

The protein list resulting from these experiments was compared with published proteome libraries of CSF [129] and plasma [130]. Database comparison, as well as information about protein functional characteristics, was performed using ProteinCenter with mappings from the Gene Ontology Consortium website [131].

3.8 Quantitative proteomics.

In Paper III we performed two semi-quantiative experiments, one using a label-free global shotgun approach on individual patient samples, and one approach based on a pool of several samples quantified using an iTRAQ approach combined with extensive fractionation.

The individual label-free quantitation experiments were performed by LC-MS on samples subjected to in solution digestion [121]. The peptide mixture was separated by reverse phase C18 chromatography, then transferred by electrospray and analyzed by the LTQ-Orbitrap Velos Pro. The resulting data was searched against the SwissProt database using SearchGUI with OMSSA and XTandem as search engines and PeptideShaker [132] for combining the results. Progenesis LC-MS (Nonlinear Dynamics Ltd. Newcastle upon Tyne, UK) was used in the data analysis.

The iTRAQ-labelled quantification experiment was performed on a sample-mix depleted of high abundant proteins by a Multiple Affinity Removal System (MARS hu-14) (Agilent Technologies), removing the 14 most abundant proteins in the samples by immuno depletion to increase the dynamic range of the experiment and the sensitivity for detection of low-abundant proteins. The samples were digested by trypsin [121], and then labelled by iTRAQ chemical labelling according to manufacturers protocol with a mixture of the two samples as control. The mixture of

labelled AC fluid, the CSF and the 50:50 control sample were combined as iTRAQ-labelled peptides. To increase the proteome coverage, the samples were fractionated into 28 fractions using mix-phase (RP-WAX) chromatograpy. This is a method combining the chemical properties in reverse phase chromatography and ion exchange chromatograpy. The samples were analyzed by LTQ-orbitrap Velos Pro mass spectrometry coupled to an on-line nano-HPLC in the similar manner as the label-free experiment, using HCD instead of CID as method for collision cell. The resulting data was searched against the SwissProtKB database using using the Spectrum Mill software package (Agilent Technologies, Santa Clara, CA).

3.9 Statistical methods

Principal component analysis (PCA) is a workhorse in complex statistical analysis with more than one variable. The principle of PCA is to create arbitrary multidimensional vectors through the dataset, where the first vector explains as much as possible of the variance in the dataset. The second vector orthogonal to the first vector and explains the remaining variance in the dataset after the first vector is explained. The data are projected down on the new vectors as arbitrary axes, supplementing the original axes. This is a way to handle variation across multiple axes without knowing the degree of independence in the dataset.

False Discovery rate (FDR) is a statistical method to handle multiple testing problems [133, 134]. FDR determines the amount of false discoveries in a dataset, such as the erroneous reporting of a protein being present. Most instrument packages for the analysis of mass spectrometry reporting data have a cut-off at around 1.0 %, implying that not more than 1.0 % of the reported proteins are erroneously identified.

Bonferroni correction [135] is a way to handle the problems of multiple testing, correcting for false positives only. The Bonferroni correction divides the p-value

limitation on the number of tests, thus increasing the significance needed when testing for multiple tests. As a method, this is considered extremely conservative**.**

3.10 Databases

The International Protein Index (IPI) [136] has been common database for searching mass spectrometry data using search algoritms like Spectrum Mill and Mascot. The IPI database of protein identities is currently on the verge of being discontinued, and UniProtKB is currently the most used database. This is a more specific and stringent database. The UniProtKB/Swissprot database is a high-quality register of protein, manually reviewed by experts and is the current consensus database for reporting of protein identities. The UniProtKB is developed by a consortium consisting of the European Bioinformatics Institute, the Swiss Bioinformatics Institute and the Protein Information Resource, supported by US and EU funding. We used IPI human in Paper II and UniProtKB in Paper III.

Proteins with increased or reduced abundance between CSF and AC fluid in both label free and iTRAQ labelled experiment from paper III were compared to the mRNA microarray results previously published using J-Express Pro (MolMine AS, Bergen, Norway).

Gene ontology data for the identified proteins in the mapping experiment in Paper II and the iTRAQ experiment in paper III was obtained using ProteinCenter (Thermo-Fischer scientific, Odense, Denmark).

3.11 Synopsis of results:

In Paper I, we found that AC fluid and CSF had the same osmolarity. We did not find any significant differences in the concentrations of sodium, potassium, chloride,

calcium, magnesium or glucose. The concentration of phosphate was significantly elevated in AC fluid (0.39 versus 0.35 mmol/L in CSF; p = 0.02), whereas concentrations of total protein (0.30 versus 0.41 g/L; p = 0.004), ferritin (7.8 versus 25.5 ug/L; p = 0.001) and lactate dehydrogenase (17.9 versus 35.6 U/L; p = 0.002) were significantly reduced in AC fluid relative to CSF. Based on these observations we concluded that AC fluid is not identical to CSF. The differential composition of AC fluid relative to CSF supports secretion or active transport as the most probable mechanism underlying cyst filling. Oncotic pressure gradients or slit-valves as mechanisms for generating fluid in temporal ACs are not supported by these results.

In Paper II, we did not identify systematic trends or grouping of data, implying low variability between individual proteomic profiles of AC.
In a pool from 11 patients, we identified 199 proteins. The identified proteins, as well as supplementary MIDAS experiment, show a high similarity in protein profiles (192/199) between AC and CSF, as well as a considerably lower similarity towards plasma. The high qualitative overlap gives an indication that CSF should be somewhat involved in the mechanisms for filling and sustaining of such cysts. This was the first evaluation of arachnoid cysts by proteomics.

In Paper III, we quantified 348 proteins to obtain an abundance ratio between AC fluid and CSF proteins in these samples collected from five individual patients. In a pool from the same patients in depleted and fractionated samples, we quantified 1425 proteins between AC fluid and CSF. 296 proteins were identified in common in both individual and fractionated depleted pooled samples.

In the individual samples, based on strict identification criteria (2 peptides, 95 % peptide and protein confidence, FDR <1.0 %), 150 protein groups (43 %) (of which 133 also were identified from the iTRAQ experiment) were identified as significantly different between the two groups by paired two-sided t-test on 95 % confidence. From

the differentially abundant proteins, 22 proteins had a fold change of >1,5 (log2 = 0,58) in both individual label-free average as well as in iTRAQ-labelled samples, while 24 proteins had a fold change of <0,67 (log2 = -0,58) in both the label-free experiment on individual patients and in iTRAQ experiment performed with pooled samples. This finding supports our previous claim that AC fluid is different from CSF.

We did not quantify any of the proteins associated to genes previously mentioned as regulated in relation to AC [43, 46]. Searching mRNA microarray data [46] against our proteins with change in abundance do not show a strong correlation – an observation we attribute to membrane proteins not necessarily present in liquid phase due to the hydrophobic nature of membrane proteins. Our data from quantified proteins did not support the previously suggested gradient in molecular weight over the AC membrane.

3.12 Discussion

The scope of this thesis has been to gain further insight in the mechanisms for filling and sustaining of AC. This is a topic where basal research is scarce. Rengachary et al [27, 28] analysed membranes of AC by histology and electron microscopy. Interesting findings in these studies is of a single-layer covering of AC by mesotelial cells, the absence of inflammatory cells and the unusual presence of clear arachnoid cells resembling fetal arachnoid cells. Schachenmayr and Fride [31] point at a layer of extremely flattened cells lining the arachnoid membrane. Sandberg et al [42] performed chemical analyses in AC fluid, reporting similar values as expected for reference CSF. Helland et al [43] and Aarhus et al [46] identified slight differences in DNA microsatellites and mRNA microarray of membranes of AC relative to control arachnoid membrane. The nature and mechanisms of the splitting and change of structure of AC membrane, as well as the content and composition of AC fluid, is still unclear. This was the basis on which the studies were undertaken.

In our studies, we find that AC fluid is different from CSF both evaluated by clinical chemistry and by quantitative proteomics. Our observation that the protein concentration is lower in AC fluid relative to CSF is not consistent with the suggested mechanism of oncotic pressure. The hypothesis in oncotic pressure mechanism is that proteins bind salts, increasing osmotic pressure binding water. The most known mechanism of oncotic pressure is the role of albumin in the blood system. A reduced protein concentration is thus not consistent with such findings. We find a reproducible difference in concentration between the individual patients, as well as low blood signatures. We interpret this as a stable difference and relatively safe as a conclusion in the current studies.

The qualitative proteomics data show a large degree of similarity of AC fluid relative to CSF, while larger differences to that of plasma. This is a relatively simple comparison, but it clarifies both that the similarity to CSF is present, judging by the difference to plasma. The comparison between the individual samples, using our known deviating samples as internal controls demonstrate consistent results in the content of individual cysts. Paper II was the first proteomics analysis of AC while Paper III is the largest.

The quantitative proteomics performed by both global shotgun label-free proteomics and iTRAQ chemical labelling after extensive fractionation is relatively sophisticated as methodology. The proteins reported as altered in abundance in these studies are only the ones being consistently different in both label-free and iTRAQ-labelled samples. In the methodology applied, we tried to correct for the possibility of single outliers in individiual experiment using the t-test. The significance criteria are probably too stringent. With large resources, reducing the significance criteria and

introducing verification steps, this experiment could yield reports with higher confidence.

3.12.1 Paper I:

The scope of this paper [137] is relatively simple; to characterise AC fluid and CSF by clinical chemistry. The samples were collected and handled in accordance with a standardised protocol, and stored in an ultra freezer at -80^{o}C until analysis, and analysed at the same time in an accredited hospital laboratory.

The rationale for this paper was that there are three prevailing theories for the filling of AC. Go et al [38] suggested in 1984 that the presence of transport ATPase in the luminal cell membrane of AC could be interpreted as evidence of an active transport mechanism. Dyck and Gruskin [39] suggested in 1977 that filling of cysts could happen by osmotic gradients such as xantochromia after a previous hemorrhage. Smith and Smith [40] suggested a ball-valve mechanism for trapping of fluid, causing the cyst to expand. Santamarta et al [41] claim to have observed a slit valve in preoperative MRI for such a cyst. Such valves have however only been observed intraoperatively in suprasellar AC and not in temporal cysts.

These different mechanisms of filling of AC would be expected to cause different clinical chemical profiles. Osmotic gradients would necessarily demand a higher osmolarity inside the cyst, perhaps also equal, as the cyst may have reached equilibrium. To create this osmotic gradient, elevated protein content would be needed. Slit valves would probably cause some sort of skewed distribution of ions, as one would expect some sort of general up-concentration while some water and ions might be resorbed or leak. A mechanism of active transport might cause a skewed distribution of some, but not all ions, depending of the type of transporter.

The results in this paper did not show significant changes in most electrolytes except for phosphate, which is 1,11 times higher in AC. The distribution of larger proteins as measured by clinical chemistry was interesting as a scientific finding. The possibility of a decreasing gradient of proteins dependant on protein molecular weight in AC relative to CSF is a finding that cannot be attributed to possible blood contamination in CSF. This might imply some kind of unknown protein selectivity. However an interesting hypothesis, we later refuted it in Paper III. The finding that protein concentration is lower in AC did not support an oncotic pressure mechanism, where proteins bind up electrolytes creating an osmotic pressure – the same mechanism as that of albumin in plasma.

Concerning the technical set-up for this experiment and future improvements, performing sample collection with a small aliquot set aside and analyzed by cell-counter, would give information on the cells and debris now precipitated away before freezing. In a new set-up like this, more analytes could probably be included. The possibility of including more patients might as well increase the significance of this study, perhaps also with a more thorough control with factors such as previous operations, bleeding in close proximity or other cases useful for evaluating and differentiating the primary from the secondary arachnoid cysts [13].

3.12.2 Paper II:

The scope of this paper [138] was to explore the nature of the content of AC by proteomics, evaluated by qualitative proteomics. The sample material for these studies origins from the same patients as for the clinical chemistry study. This study was exploratory in nature; to our knowledge there are no similar studies on AC.

The qualitative proteome of AC was relatively similar to that of normal CSF, but not to that of plasma. As a simple observation, if the mechanism is due to active secretion, either the mechanisms have to be like that of CSF-production in the choroid plexus as is not observed in microscopy studies, or there is a mechanism transporting some components from CSF to AC fluid. The qualitative profiles of individual AC show a high similarity to each other, a finding supportive of but not conclusive, that primary AC is a unique condition.

Concerning the selection criteria for the second part of article II, we did not have a good prior scientific foundation on which patient's samples to include or exclude in the studies. We excluded samples with detected content of blood proteins, as well as the two patients previously either operated or with a prior bleeding in close proximity to the AC.

From the analysis of AC fluid versus CSF, we also found the similarity so high that we suggested quantitative strategy as more appropriate for evaluating AC by proteomics, as were performed in Paper III.

3.12.3 Paper III.

The scope of Paper III [submitted] was to compare AC fluid and CSF, with high qualitative similarity as observed in Paper II, by quantitative measures. The concept is that even though the same proteins are identified, quantitative differences in proteins may represent a change in function as proteins perform a multitude of cellular and biologic functions. Furthermore, we wanted to link the results from this experiment against prior research on AC, in particular the mRNA microarray data in Aarhus et al [46].

For proteomic data, this is a study where the number of patients is low (n=5). Furthermore, the labelled quantitative proteomic data is performed as a single experiment in a pool of the same patients. We did not perform external validation, such as selective reaction monitoring (SRM). Proteomic results are constructed as layered probabilistic statistics, where mass spectrum data are annotated to peptides and peptides to protein groups. Annotation as protein groups instead of single proteins is an effect of shared peptides between different versions of the same protein, where the post-translational modification (PTM) is not a part of the peptide identified [139].

Comparing quantitative data of a multitude of proteins with probable partial co-variation calls for sober use of significance criteria. In the experiment of label-free individual samples, 150 of 348 protein groups (43 %) were significant as different in abundance in a two-sided paired t-test, treated individually. A problem to be handled is the fact that this is in principle multiple testing. In a normally distributed population, one would expect 5 % of the samples to be reported as significant, implicit in the p=0,05. Bonferroni correction of results is in general considered too stringent for such a purpose [140]. False Discovery Rate [133, 134] is currently used to determine a cut-off on the number of protein groups reported, to reduce the probability of reporting proteins findings not present. Reproduction of results by different modalities, in instrumental and biologic replicates, as well as external validation, are good strategies to confirm that a differential abundance of a protein group is in fact explained by biology, rather than being an analytical error.

In the CNS, and in particular CSF, blood contamination is a great risk factor for false reporting of findings. This is due to the fact that the protein concentration in CSF is 0,5-1 % of that of blood/plasma. In the CSF, 80 % of the amount proteins are blood-derived and blood contamination will skew the distribution of proteins in CSF relative to any other liquid medium in the CNS, such as AC fluid. The AC fluid collected in

this experimental set-up was relatively safe from blood contamination. The CSF will, no matter the rigidity in collection, contain a minute amount of blood.

Comparing the mRNA microarray data against our proteins with change in abundance did not show a strong correlation – an observation we attribute to membrane proteins not necessarily present in liquid phase due to the hydrophobic nature of membrane proteins. As a research hypothesis, this is still an interesting question. Performing proteomics on AC membranes could be a strategy for evaluating active transport by membrane proteins. A challenge in this particular set-up will be the same as in this paper, the need for adequate controls. Presumably arachnoid membranes from the same individual outside the cyst could be used with the patient as his own control, but no method with acceptable and feasible sampling has been devised.

Our data did not support a gradient in molecular weight from quantified proteins previously suggested. The observation on clinical chemistry in AC fluid compared to CSF in Paper I was tested by plotting the fold change between proteins in AC fluid and CSF against the molecular weight of the proteins. There was no correlation in this plot, a finding that supports rejection of the molecular weight gradient hypothesis.

3.12.4 Methodological considerations

In the process of science, an increased insight into the potential for better research is quite common, as it has been in this project as well. A first observation is that the collection of high-quality samples is a challenge in a clinical and intraoperative setting. Firstly, since the surgeon or clinician collecting the samples and the person handling the sample are not the same person, the information transfer on a synopsis of previous medical history could be improved before making the samples anonymous.

Second, during the collection of CSF in the basal cisterns the risk of minute amounts of blood mixing with the CSF is present. During sample collection, blood contamination of samples is a problem since it is difficult to control. This was not the case for the AC fluid, collection methods has been relatively controlled. Puncture of the AC with a needle, using the pressure measurement device (a CSF manometer) as siphon, the collection of AC fluid is not very prone for blood contamination. CSF was collected from the bottom of the operative field after complete hemostasis before field closure. CSF was aspired through a small plastic tube, a baby feeding tube modified by cutting the end for having a flat hole, held by operative forceps under the microscope and carefully aspired manually by a syringe. Although observably clean, this fresh collected CSF might contain blood and the blood content will not necessarily be identical between individuals. As CSF contains 0,3-1 % of the protein content of serum, a miniscule contamination of blood will introduce a change in the proteome composition. Currently, visual inspection of the pellet after centrifugation has been the easiest way to estimate the presence of blood contamination, even if the sample appears clear before centrifugation. For future sample collections, saving a small aliquot for cell differential counting and perhaps a serum/plasma sample as well for future reference could increase the sample quality.

Third, the development and improvement in methodology for proteomics is clear for such a project as ours, where in Paper II, we identify 199 proteins by qualitative measures after fractionation, while in Paper III, we identify 1425 in one experiment while utilizing a more stringent databases and search criteria. This is in part the availability of MARS-14 abundant protein depletion, the improved fractionation of mix-phase columns and especially the use of a modern Orbitrap instrument replacing an elderly qTOF instrument.

Fourth, patient selection could in our material include more information on past medical history before anonymisation. As the responsible surgeon and the persons

handling the samples is not the same, there might be better information transfer possible. Still, this is a balance on the definition of what is anonymous, and was not specified in the informed consent. Exclusion criteria were therefore relatively simple.

Fifth, the comparison of mRNA from membranes of the same patients as were operated, as well as possible control from the same patients, could reduce the biological variation in our comparison.

Sixth, the statistics applied is not ideal, seen from a statistician's perspective. This is a consistent problem in proteomics, where methods are developing along with the technical and methodological progress of the field in general. Currently, there are consensus criteria on what constitute a real difference in datasets such as the ones applied, but neither the limitations nor the results are cut in stone.

3.12.5 Future perspectives

In the perspective for future research on AC, the arachnoid cells lining the cavity of the AC would be very interesting for targeted proteomics approaches. There have been some studies in this direction, Schachenmayr and Friede did not identify pinocytotic vesicles in the membranes, using this as argument against transcellular active transport [31]. Later, with the recognition of other transport mechanisms, some attempts to demonstrate aquaporins in the AC membrane have failed [141]. The salt pump NKCC1 [43] is currently the most interesting mechanism suggested, but needs verification in larger datasets. Targeted searches against molecular machinery by proteomics may identify the presence of active pumps in the inner membranes. The possibility of analysing AC membrane and normal arachnoid membrane from the same patients would probably be an alternative strategy if we disregard current resources and availability of sample material.

4. Conclusions

4.1 Main objectives:

We have characterised AC fluid by clinical chemistry and proteomics. The AC fluid and CSF is very similar, but not identical. The reduced protein concentration in AC fluid relative to CSF does not support oncotic pressure as a mechanism of filling and sustaining of arachnoid cysts. Differences in both total and individual protein abundance do not support a valve as a filling mechanism. From our data, we believe some kind of active transport to be the most plausible of the three mentioned mechanisms for filling of AC.

Paper II was the first proteomics evaluation of AC fluid, while Paper III is the first quantitative proteomics paper on AC fluid versus CSF and the largest qualitative characterization of AC fluid.

4.2 Secondary objectives:

1. The clinical chemistry between AC fluid and CSF show high similarity between the two fluids, but protein concentration to be reduced in AC fluid relative to CSF, while the phosphate is elevated.
2. The evaluation of the qualitative proteome of AC fluid show a high similarity between individual patients, this as an indication of AC being the same condition. There is a high similarity to reference CSF databases and a reduced similarity relative to plasma reference databases. This is indicative for CSF to have some involvement in the filling mechanism of AC.
3. The quantitative proteomics evaluation was performed as two different experiments; a labelfree shotgun approach on individual patient samples quantifying 348 proteins between AC fluid and CSF, and an iTRAQ chemically labelled approach in a pool of patients combined with extensive fractionation,

quantifying 1425 proteins between AC fluid and CSF. With strict criteria, we identified 22 proteins with significant increased abundance 24 with significant decreased abundance in AC fluid relative to CSF. We did not observe any molecular weight gradient over the arachnoid cyst membrane.

4. We did not observe any pattern in change in the proteins with altered abundance between AC fluid and CSF in the mRNA microarray data between AC membranes and control arachnoid membrane from a previous study. We did not identify the correspondingproteins mentioned as regulated in previous studies on DNA and mRNA of AC membranes. We cannot draw conclusions from these comparisons due to the nature of the experiment.

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Appendix

Consent form in Norwegian.

HELSE BERGEN - Alt handlar om menneske
Haukeland Universitetssjukehus

Nevroklinikken

Nevrokirurgisk avdeling

Forespørsel om å få oppbevare vev i biobank for forskning

Til pasienten

Du er nå innlagt for å bli operert, og vi ønsker ditt samtykke til at noe av vevet som fjernes kan oppbevares i en biobank og brukes til forskning.

Du mottar denne forespørselen fordi du har fått diagnostisert en cyste i hjernens spindelvevshinne (araknoidal cyste). Din identitet blir ikke lagt inn i forskningsregisteret før du eventuelt samtykker ved å signere denne forespørselen.

Hvorfor oppbevare og bruke vev til forskning?

Det cystevevet som ikke trengs for å stille diagnosen blir vanligvis kastet. I stedet for å kaste dette vevet vil vi gjerne bruke noe av det til forskning. Cystevevet og en blodprøve er frosset ned. Det vil bli undersøkt på forskjellige proteiner og på arvestoffet (DNA) i både cysteveggen og i cystevæsken. Resultatene får ingen konsekvenser for deg, men kan på sikt gi oss nye opplysninger om hvordan slike cyster oppstår og skal behandles. Slik kan du bidra til å hjelpe pasienter i fremtiden.

Hvilke opplysninger innhentes om pasienten og hvordan blir opplysningene brukt?

Vevet i biobanken merkes med et kodenummer for pasienten, slik at data om pasientens kjønn, alder, diagnose og behandlingsforløp kan skaffes fra sykehusets pasientjournal og røntgenarkiv. Disse blir sammenholdt med vevet i biobanken.

Resultater blir publisert i medisinske tidsskrifter, men ikke presentert slik at pasienter kan gjenkjennes. Opplysningene lagres i et elektronisk register sammen med et kodenummer. Registeret er lagt på en egen forskningsserver på Haukeland Universitetssykehus, i.h.t. Helse Bergens rutiner for datasikkerhet. Alle med tilgang har taushetsplikt og behandler informasjonen konfidensielt. Registeret er tidsavgrenset, og alt vev destrueres og analyseresultater slettes når prosjektet er ferdig, senest i 2100. Prøver kan bli sendt til analyse hos forskningspartnere i utlandet, men personopplysninger blir ikke utlevert.

Hvilke rettigheter har du som pasient?

Samtykket er helt frivillig, og du tar den betenkningstid du trenger. Har du spørsmål som er ubesvart i dette skrivet kan du få snakke med lege eller sykepleier tilknyttet prosjektet.

Hvorvidt du samtykker har ingen konsekvenser for behandlingen eller forholdet til behandlere og andre. Du kan når som helst trekke samtykket tilbake, uten å måtte begrunne dette. Vevsprøven blir da destruert, og opplysningene blir slettet fra registeret. Informasjonen som inngår i større vitenskapelige arbeider vil ikke kunne trekkes tilbake i ettertid. Prosjektet er meldt til Personvernombudet for forskning, Norsk samfunnsvitenskapelige datatjeneste og opprettelse av biobank er gjort etter tilråding fra Regional Etisk Komite (REK) for Vestlandet og godkjennelse fra sosial- og helsedirektoratet.

Registeret er finansiert av Den Norske kreftforeningen og Universitet i Bergen, og mottar ingen støtte fra kommersielle aktører. Ansvarlig for biobanken vil være per Øyvind Enger, overlege ved Nevrokirurgisk avdeling på Haukeland Universitetssykehus og forsker ved Universitetet i Bergen.

HELSE BERGEN - Alt handlar om menneske
Haukeland Universitetssjukehus

Nevroklinikken

Nevrokirurgisk avdeling

Jeg har mottatt skriftlig og muntlig informasjon, og er villig til å delta i studien og tillater at vev oppbevares i biobanken med opplysninger slik det er angitt ovenfor.

_____ _____

Sign. Dato

(Dersom pasient er under 18 år signerer foresatte under for samtykke sammen med pasient)

Navn .....

Bruk blokkbokstaver

Sted.....Dato.....

.....

Foresattes underskrift

Papers

I

Berle et al. Cerebrospinal Fluid Research 2010, 7:8
http://www.cerebrospinalfluidresearch.com/content/7/1/8

CEREBROSPINAL FLUID RESEARCH

RESEARCH Open Access

Arachnoid cysts do not contain cerebrospinal fluid: A comparative chemical analysis of arachnoid cyst fluid and cerebrospinal fluid in adults

Magnus Berle[1*], Knut G Wester[2,3], Rune J Ulvik[1,4], Ann C Kroksveen[1], Øystein A Haaland[5], Mahmood Amiry-Moghaddam[6], Frode S Berven[7], Christian A Helland[3,2]

Abstract

Background: Arachnoid cyst (AC) fluid has not previously been compared with cerebrospinal fluid (CSF) from the same patient. ACs are commonly referred to as containing "CSF-like fluid". The objective of this study was to characterize AC fluid by clinical chemistry and to compare AC fluid to CSF drawn from the same patient. Such comparative analysis can shed further light on the mechanisms for filling and sustaining of ACs.

Methods: Cyst fluid from 15 adult patients with unilateral temporal AC (9 female, 6 male, age 22-77y) was compared with CSF from the same patients by clinical chemical analysis.

Results: AC fluid and CSF had the same osmolarity. There were no significant differences in the concentrations of sodium, potassium, chloride, calcium, magnesium or glucose. We found significant elevated concentration of phosphate in AC fluid (0.39 versus 0.35 mmol/L in CSF; $p = 0.02$), and significantly reduced concentrations of total protein (0.30 versus 0.41 g/L; $p = 0.004$), of ferritin (7.8 versus 25.5 ug/L; $p = 0.001$) and of lactate dehydrogenase (17.9 versus 35.6 U/L; $p = 0.002$) in AC fluid relative to CSF.

Conclusions: AC fluid is not identical to CSF. The differential composition of AC fluid relative to CSF supports secretion or active transport as the mechanism underlying cyst filling. Oncotic pressure gradients or slit-valves as mechanisms for generating fluid in temporal ACs are not supported by these results.

Background

Arachnoid cysts (AC) are relatively common benign lesions of the arachnoid, with a reported prevalence as high as 1.1% in the adult population [1]. Clinical presentations of AC include headache, dizziness, seizures [2] and dyscognition [3]. They can be found all along the cranio-spinal axis, but have a marked predisposition for the temporal fossa [4]. The mechanisms underlying the formation and filling of arachnoid cysts are not well understood, but clinical, epidemiological, and laboratory data indicate that genetic mechanisms are involved in the *formation* of arachnoid cysts [5,6]. Three prevailing theories exist for the *filling* of the cyst: 1) active secretion of fluid by cells in the cyst wall [7,8], 2) fluid influx due to an oncotic pressure gradient [9], and 3) trapping of fluid by a valve mechanism [10]. It is conceivable that the chemical composition of the AC fluid relative to the cerebrospinal fluid CSF reflects the mechanism by which the fluid enters the cyst. If the composition is identical to CSF a valve mechanism appears likely, whereas if the filling is caused by oncotic pressure, a higher concentration of proteins in the cyst fluid compared with CSF would be expected. Likewise, cyst fluid composition could reflect the mechanism of transport across the cyst wall, if such a mechanism is involved.

In their study of clinical chemical analysis of cyst fluid in pediatric patients, Sandberg *et al*. [11] described a similar chemical composition to that of reference CSF in the majority of patients investigated, but in 14 of 41 (34%) the protein concentrations were elevated above

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0.50 g/L in the cyst fluid. Based on these findings, the authors hypothesized that higher protein content could contribute to the expansion of the cysts by an oncotic pressure gradient. We have recently described the up-regulation of the CSF-secreting cation chloride co-transporter NKCC1 in AC membranes compared with normal arachnoid [8]. This finding supports fluid secretion as the main mechanism of fluid accumulation in AC. The objective of the present study was to analyze the chemical parameters of AC fluid and compare with CSF from the same patient, to gain further knowledge of AC concerning the mechanisms of filling and sustaining of such cysts.

Methods

Patients

A total of 15 patients (9 female, 6 male, age 22-77) with unilateral, temporal AC were included. Cyst type and sidedness are summarized in table 1. Patient 3 had previously had a chronic subdural hematoma, most probably caused by the cyst [12]. Patient 4 had undergone previous surgery for the cyst. The other patients had no previous history of intracranial hematomas or surgery. Patients were recruited by written informed consent by the responsible surgeon. This project was approved by The Regional Committee for Medical and Health Research Ethics (REK) of Western Norway (approvals REK 70.03, NSD 9634 and REK 2009/1885).

Operative technique and fluid sampling

Details for the surgical procedure have previously been given elsewhere [12-14] but a short description is given here. All patients were operated with a craniotomy under general anaesthesia, given as total intravenous anaesthesia (TIVA) with propofol and remifentanyl. Vecuronium bromide (Norcuron®) was used as the neuromuscular blocking agent. A burr hole was made with a high-speed drill immediately posterior to the sphenoid wing in order to gain access to the anterior and most basal aspects of the middle cranial fossa. The dura and the underlying cyst membrane were punctured through the burr-hole with a 23 G, 25 mm long syringe connected to an Optidynamic® spinal fluid manometer (Mediplast AB, Malmo, Sweden). After pressure equilibration and registration, a cyst fluid sample (3 - 5 ml) was collected using the manometer tube as a siphon. The sample was immediately transferred to a sterile centrifuge tube for centrifuging and further analytic processing as described below (sample handling). After this procedure, a standard craniotomy with a microsurgical resection and fenestration of the cyst membranes was performed. Before opening the medial cyst wall and thus communicating the cyst interior to the basal arachnoid space/CSF, all cyst fluid was aspirated to avoid cyst fluid contamination of the CSF.

After opening the medial cyst membrane that covered the basal structures (the tentorial slit, the oculomotor nerve, the carotid artery, and the optic nerve), thus creating communication to the basal cisterns and the posterior fossa, a CSF-sample was collected with a pre-cut baby-feeding catheter #6, connected to a 10 ml syringe. The catheter was placed below the tentorium via the tentorial slit and fluid was aspirated gently from the posterior fossa. The collected CSF was transferred to centrifuge tubes and processed in an identical manner to the cyst fluid.

Table 1 Characteristics of patients in study with age, gender, Gallasi-stage [20] and remarks.

Patient	Age (yrs), sex	Side	Galassi-stage [20]	Remarks
1	26, f	Left	2	
2	43, m	Left	2	
3	58, f	Left	3	Old hematoma
4	34, f	Left	2	Reoperation
5	22, f	Right	1	
6	36, f	Right	2	Slight hemolysis CSF
7	35, f	Right	2	
8	77, f	Left	1	
9	42, f	Left	1	
10	60, m	Left	2	Slight hemolysis CSF
11	56, m	Right	2	
12	25, m	Left	1	
13	30, f	Left	1	
14	37, m	Left	2	
15	63, m	Left	2	

Sample handling

The samples were transferred to polypropylene tubes (Nunc CryoTube, Thermo-Fischer Scientific, Roskilde, Denmark) and centrifuged for five min at 450 x g to remove cells and cell debris. The supernatant was transferred to new polypropylene tubes and immediately stored on dry ice prior to long term storage at -80°C. Such sample handling has previously been demonstrated to reduce degradation of components and cell lysis, which may change the composition of the sample [15].

Samples were thawed for analysis at room temperature and transferred to pre-marked analysis tubes for laboratory analysis.

Chemical analysis

The samples were analyzed at Laboratory for Clinical Biochemistry, Haukeland University Hospital, 5021, Bergen, Norway. The laboratory is accredited by Norwegian Accreditation (accreditation number "TEST 231") as a

testing laboratory and complies with the requirements of NS-EN ISO 15189. Clinical chemistry analysis was performed on a Modular Analytics System by Roche Diagnostics (Roche Diagnostics GmbH, Mannheim, Germany). The analytical coefficient of variation (CV) is noted in parenthesis for each analyte; Sodium (CV 1%), potassium (CV 2%) and chloride (CV 2%), were measured by ion-selective electrode on an ISE 1800 module. Magnesium (CV 2.5%), phosphate (CV 3%), calcium (CV 2%), bilirubin (CV 6%) lactate dehydrogenase (CV 2.5%), protein (CV 1.8%) glucose (CV 2.5%), triglycerides (CV 3%) and iron (CV 2%) were measured by photometic assays on a P 800-module. Ferritin (CV 5%) was measured by an ECLIA (electrochemiluminescence) immunoassay on an E 170-module. Osmolarity (CV 1.5%) was measured by freeze point depression on a Fiske Micro-Osmometer (Fiske Associates, Massachusetts, USA). Immunoglobulins (CV 2-5%) were measured by nephelometry with system specific N antisera to Human Immunoglobulins (Dade Behring Marburg GmbH, Marburg, Germany) on a BN Pro Spec System (Siemens Healthcare Diagnostics, Illinois, USA)

Statistical analysis

For each of the thirteen patients without previous operations or known injuries to the AC, the differences in concentration between cyst fluid and corresponding CSF were calculated. A paired T-test was then utilized to check if the mean ratio was equal to one. Due to slight hemolysis of two CSF samples (patients 6 and 10), data from these patients were omitted from the statistical analyses for lactate dehydrogenase, ferritin and protein. Two patients (patient 3 and 4) were described clinically as different from the others, and were compared separately with the thirteen native patients using a two-sample t-test assuming equal variances. Correlation between lactate dehydrogenase, ferritin and protein was determined by correlation analysis as a control against a possible contamination of the samples with blood. *P*-values were calculated utilizing Pearson's product moment correlation test. Statistical analysis was performed using the statistics software package R version 2.10.1 (The R foundation for Statistical Computing, Vienna, Austria).

Results

Chemical analysis of AC fluid and CSF, obtained during elective surgery for arachnoid cysts from 15 patients, was performed in a routine hospital laboratory. The results from the measurements are presented in table 2. There was no significance difference in osmolarity or concentrations of sodium, potassium, chloride, calcium, magnesium or glucose between AC fluid and CSF. The concentration of phosphate was higher in AC fluid relative to CSF (0.39 versus 0.35 mmol/L; $p = 0.02$), while the concentration of total protein (0.30 versus 0.41 g/L; $p = 0.004$), lactate dehydrogenase (17.9 versus 35.6 U/L; $p = 0.002$) and ferritin (7.8 versus 25.5 ug/L; $p = 0.001$) was significantly lower in AC relative to CSF. Bilirubin, iron, triglycerides and immunoglobulins were below

Table 2 Results of chemical analysis of AC fluid and CSF in the same patients, with units, number of samples in calculation (n), mean results for AC fluid and CSF with standard error of mean (SEM) and means of AC fluid/CSF ratio.

	Unit	n	Mean AC fluid +/- SEM	Mean CSF +/- SEM	Mean cyst/CSF
Sodium	mmol/L	13	142.23 +/- 2.14	142.08 +/- 2.71	1.00
Potassium	mmol/L	13	2.47 +/- 0.04	2.35 +/- 0.07	1.05
Chloride	mmol/L	13	121.23 +/- 1.94	120.15 +/- 2.54	1.01
Calcium	mmol/L	13	1.07 +/- 0.01	1.03 +/- 0.03	1.04
Magnesium	mmol/L	13	1.20 +/-0.01	1.14 +/- 0.03	1.05
Phosphate	mmol/L	13	0.39 +/-0.01	0.35 +/- 0.01	1.11*
Glucose	mmol/L	13	2.85 +/- 0.09	3.13 +/- 0.11	0.92
Protein	g/L	10	0.30 +/-0.03	0.41 +/- 0.04	0.71**
Lactate dehydrogenase	U/L	11	17.91 +/- 2.93	35.55 +/- 4.61	0.57**
Ferritin	ug/L	11	7.82 +/- 1.00	25.55 +/- 6.20	0.31**
Osmolarity	mosmol/L	13	290.15 +/-1.07	290.08 +/- 0.96	1.00
IgG	g/L		< 1.50	< 1.50	
IgA	g/L		< 0.25	< 0.25	
IgM	g/L		< 0.18	< 0.18	
Iron	umol/L		low	low	
Triglycerides	mmol/L		low	low	

Significance levels are denoted as $p < 0.05$ (*) and $p < 0.01$ (**). Data from patients #3 and #4 are excluded as clinical outliers from this table. Data from patients #6 and #10 were excluded for protein, lactate dehydrogenase and ferritin due to slight hemolysis in the CSF. Patient #12 was excluded from protein concentration measurement as an extreme statistical outlier.

quantification limit for analysis setup. Patient 12 was identified as an extreme outlier for protein measurement and was excluded from this analysis.

Correlations were calculated between possible blood contamination parameters ferritin, lactate dehydrogenase and protein on the CSF samples without observed blood contamination (n = 11). The correlation between ferritin and LD was 0.77 (p = 0.006), ferritin and protein was 0.36 (p = 0.312) and lactate dehydrogenase and protein was 0.34 (p = 0.34). The protein concentrations in the cyst fluid from the two clinically different patients (patient 3 and 4, respectively 6.16 g/L (hematoma) and 3.54 g/L (previous operation)) were significantly elevated relative to the others ($p < 10^{-16}$).

Discussion

The aim of the present study was to collect information that may contribute to the understanding of the mechanism for arachnoid cyst filling. If the cyst were filled by a simple valve mechanism, which has been observed in suprasellar cysts, it would be expected that the composition of the cyst fluid would be identical to that of CSF. On the other hand, if the filling was caused by oncotic pressure, one would expect a difference in osmolarity between the AC fluid and CSF as well as a higher protein content in the AC. If the underlying mechanism were active secretion or transport, this would probably cause a different concentration of certain molecules or ions depending on the underlying transport mechanisms. We found an isotonic AC fluid with a lower protein concentration than in CSF; this is not consistent with an oncotic pressure filling mechanism.

Macromolecules such as albumin (67 kDa), ferritin (24 subunits between 19 and 21 kDa, total weight around 450 kDa) and lactate dehydrogenase (tetramer of about 37 kDa, total weight around 140 kDa) would be expected to pass freely through a slit in the cyst membrane. However, the ratio between cyst fluid and CSF for these three protein complexes was reduced (dependent on size), from 0.73 for protein (of which 2/3 albumin) to 0.31 for ferritin. Our findings are therefore contradictive of a slit valve mechanism underlying the filling of temporal AC.

The skewed distribution of phosphate could imply a selective or active transport of fluid and solutes over the AC membranes. This is consistent with previous findings of morphological and enzyme ultracytochemical structures in the wall of arachnoid cysts assumed to be capable of fluid secretion, as reported by Go *et al* [7]. Furthermore, our group has recently published evidence that there are differences between arachnoid cysts and normal arachnoid tissue in that the Na-K-Cl cotransporter NKCC1 is up-regulated in arachnoid cysts compared with normal arachnoid [8], and a small subset genes are differentially expressed in arachnoid cysts compared with normal arachnoid tissue [5]. The phosphate level in the CSF is kept lower than in the blood [16], due to active transport mechanisms in the choroid plexus epithelia. Higher phosphate concentration in the cyst fluid could imply that the cyst epithelium is either not, or is differentially equipped with transport mechanisms relative to the choroid plexus. There are several lines of evidence suggesting that co-transporters such as GLUT1, MCT1 and NKCC1 have the ability to transport water along with their respective substrates, regardless of the osmotic gradients [17].

As we have only studied temporal cysts, we cannot generalize the assumption of an active transport mechanism to all ACs. For other locations, other fillings mechanisms may well exist, such as a slit-valve in supracellar cysts [18,19]. In our study, two patients differed from the others - one with a previous hematoma in close proximity to the cyst and one being a reoperation. The protein levels in the cyst fluid from these patients were significantly higher than in the rest of the study group. In their study Sandberg *et al* [11] found that 14 of the 41 patients had markedly higher protein concentrations (above 0.5 g/L) in the cysts; four of those had extreme values such as the ones we observed. The cause for some cysts to have a marked elevated protein level is not well understood, but products from previous bleeding in the cyst may be one explanation. Correlation analysis between protein, ferritin and lactate dehydrogenase was performed as a statistical control to rule out blood contamination as a possible explanation of the protein content in the CSF. The protein concentration of CSF relative to plasma is about 0.5-1%; a leak of plasma to the CSF would introduce a large source of error to the analysis. If the difference in concentration of protein, lactate dehydrogenase and ferritin was caused by blood contamination, the correlation should be expected to be significant and close to 100%. These correlation results do not show a strong association between assumed blood contaminants and the findings, thus not supporting blood contamination as explaining variable.

A limitation for this study is the small number of samples. Applied on such a dataset, the T-test is vulnerable to outliers such as the excluded patient 12 in protein concentration (outlier plots not shown). Nonetheless, our results can support reflections around the filling mechanisms for arachnoid cysts. As far as the authors know, this is the first publication of AC fluid analysis matched with CSF from the same patient.

Conclusions

The chemical composition of AC fluid found in this study does not support an oncotic pressure or valve

mechanism as responsible for filling an AC. Due to the pattern of differences, we postulate that the filling mechanism for temporal AC is by either a selective or active transport mechanism or a secretion from the cyst-lining cells.

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Authors' contributions
MB applied for ethical approval, collected the samples, processed the samples for analysis, processed the data and drafted and edited the manuscript. KGW is the senior neurosurgeon and operated on the patients, devised sampling technique and drafted and edited parts of the manuscript. RJU applied for ethical approval, organized clinical chemical analysis and participated in editing of the manuscript. ACK worked on the biobanking design of the study, discussion around and editing of the manuscript. OAH did the statistical analysis and statistics interpretation. MAM contributed on writing the discussion part and the intellectual interpretation of the data. FSB worked on biobanking design and the editing of the manuscript. CAH conceived of this study, operated on the patients and helped draft and edit the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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FLUIDS AND BARRIERS OF THE CNS

RESEARCH Open Access

Protein profiling reveals inter-individual protein homogeneity of arachnoid cyst fluid and high qualitative similarity to cerebrospinal fluid

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Abstract

Background: The mechanisms behind formation and filling of intracranial arachnoid cysts (AC) are poorly understood. The aim of this study was to evaluate AC fluid by proteomics to gain further knowledge about ACs. Two goals were set: 1) Comparison of AC fluid from individual patients to determine whether or not temporal AC is a homogenous condition; and 2) Evaluate the protein content of a pool of AC fluid from several patients and qualitatively compare this with published protein lists of cerebrospinal fluid (CSF) and plasma.

Methods: AC fluid from 15 patients with temporal AC was included in this study. In the *AC protein comparison experiment*, AC fluid from 14 patients was digested, analyzed by LC-MS/MS using a semi-quantitative label-free approach and the data were compared by principal component analysis (PCA) to gain knowledge of protein homogeneity of AC. In the *AC proteome evaluation experiment*, AC fluid from 11 patients was pooled, digested, and fractionated by SCX chromatography prior to analysis by LC-MS/MS. Proteins identified were compared to published databases of proteins identified from CSF and plasma. AC fluid proteins not found in these two databases were experimentally searched for in lumbar CSF taken from neurologically-normal patients, by a targeted protein identification approach called MIDAS (Multiple Reaction Monitoring (*MRM*) initiated detection and sequence analysis).

Results: We did not identify systematic trends or grouping of data in the *AC protein comparison experiment*, implying low variability between individual proteomic profiles of AC.
In the *AC proteome evaluation experiment*, we identified 199 proteins. When compared to previously published lists of proteins identified from CSF and plasma, 15 of the AC proteins had not been reported in either of these datasets. By a targeted protein identification approach, we identified 11 of these 15 proteins in pooled CSF from neurologically-normal patients, demonstrating that the majority of abundant proteins in AC fluid also can be found in CSF. Compared to plasma, as many as 104 proteins in AC were not found in the list of 3017 plasma proteins.

Conclusions: Based on the protein content of AC fluid, our data indicate that temporal AC is a homogenous condition, pointing towards a similar AC filling mechanism for the 14 patients examined. Most of the proteins identified in AC fluid have been identified in CSF, indicating high similarity in the qualitative protein content of AC to CSF, whereas this was not the case between AC and plasma. This indicates that AC is filled with a liquid similar to CSF. As far as we know, this is the first proteomics study that explores the AC fluid proteome.

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Background

Arachnoid cysts (AC) are benign intracranial lesions with a reported prevalence in the adult population of up to 1% [1,2]. AC can be found all along the cranio-spinal axis, but have a marked predilection for the temporal fossa [3]. Anatomically, ACs are formed by a splitting of the arachnoid mater (AM) creating a potential space that when filled with fluid appears as a cyst [4,5]. Temporal ACs are classified according to Galassi *et al.* [6]. Briefly, a type I cyst is small, biconvex, and located at the anterior temporal pole. A type II cyst involves the proximal and intermediate segments of the Sylvian fissure, and a type III cyst involves the entire Sylvian fissure and has often a marked radiological mass effect. The cyst wall is composed of non-neoplastic arachnoid cells with a capacity to secrete fluid [7,8] that slightly differs in chemical composition from that of cerebrospinal fluid (CSF) [9]. The genetic profile of temporal AC membranes [10] indicates that these cysts represent a homogenous condition, but the underlying cause of AC formation is unknown. Further analyses of the cyst fluid with proteomics, the large-scale studies of proteins, might give indications of the aetiology of AC and thus shed further light on the mechanisms underlying fluid secretion and transport.

One aim of this study was to compare the protein content of cyst fluid from temporal AC of different individuals using proteomics (*AC protein comparison experiment*). Our hypothesis was that AC is a homogenous condition, and that we could identify a similar protein profile from AC from different patients. Homogenous protein content between AC fluid from different patients would point towards similar filling mechanisms for the examined patients. Large differences in some patients would indicate different filling mechanisms in these patients. Such a difference, if present, would be revealed by a label-free proteomics comparison approach. Mass spectrometry-based label-free approaches are commonly used for semi-quantitative comparison of complex protein samples [11].

Another aim of the study was to identify the major proteins present in AC fluid and examine if these proteins also appeared in CSF and plasma (*AC proteome evaluation experiment*). For the proteins that did not appear in the CSF and plasma protein databases, we used a targeted mass spectrometry protein identification approach referred to as MIDAS in an attempt to identify these AC fluid proteins in CSF. The protein content of AC fluid is largely unknown, but it has been shown to have reduced protein content relative to that of CSF from the same patient, as evaluated by clinical chemistry [9]. In a similar study, Sandberg *et al.* [12] studied the clinical chemistry of AC fluid in pediatric patients. We did not have specific hypotheses on the degree of similarity of AC fluid to CSF or plasma, as this has not been thoroughly demonstrated in literature. This AC fluid protein identification part of our study and comparison with CSF and plasma might give information about the origin of the AC fluid and the mechanisms of filling and sustaining of AC.

Methods

Participants and collection of AC fluid and CSF

15 patients (six male, nine female, age 22-77 y) with unilateral, temporal AC were included in the study. AC fluid was collected during surgery for AC at Haukeland University Hospital (Bergen, Norway). The patients' characteristics are summarized in Table 1. One patient (No. 3) had previously been operated for a chronic subdural hematoma, most probably caused by the cyst [13], and another patient (No. 4) had undergone previous surgery for the AC. The other patients had no previous history of intracranial hematomas or surgery. The sample collection and handling protocol used in this study have been described in detail elsewhere [9,14]. Briefly, AC fluid was collected during elective surgery for AC (craniotomy with fenestration and extirpation of the cyst) by puncturing the dura with a 23G, 25 mm long syringe needle using an Optidynamic® spinal fluid manometer (Mediplast AB, Malmo, Sweden) by siphoning through a burr hole before the craniotomy/opening of the dura. This procedure ensures that CSF does not contaminate the collected AC fluid: The remaining fluid in the cyst was removed by suction during opening of the cyst wall. The collected AC fluid was centrifuged at 450 × *g* for 5 min to remove cells and cell debris, and the supernatant was aliquoted and frozen at -80°C.

Table 1 Table of arachnoid cyst patients in study, with age/sex, sidedness, Galassi-stage [6] and remarks.

Patient	Age (yrs), sex	Side	Galassi-stage [6]	Remarks
1	26, f	Left	2	
2	43, m	Left	2	
3	58, f	Left	3	Old haematoma
4	34, f	Left	2	Reoperation
5	22, f	Right	1	
6	36, f	Right	2	
7	35, f	Right	2	
8	77, f	Left	1	
9	42, f	Left	1	
10	60, m	Left	2	
11	56, m	Right	2	
12	25, m	Left	1	Slight observed haemolysis
13	30, f	Left	1	
14	37, m	Left	2	
15	63, m	Left	2	

Deviations and observations on individual sample material were noted on sampling. During this sampling, slight hemolysis was observed in one patient sample (No 12). The CSF used for the *targeted AC protein identification experiment* was collected under informed consent as lumbar CSF before spinal anaesthesia in patients undergoing lower extremity orthopedic surgery from neurologically healthy individuals. The CSF was handled by the same protocol as for the AC fluid, and the CSF used in this experiment was pooled from 11 individuals.

Ethics

Patients were recruited by the responsible surgeon and signed a written informed consent. This project was approved by the Regional Committee for Medical and Health Research Ethics (REK) of Western Norway (approvals REK 70.03, NSD 9634, REK 151.06 and REK 2009/1885).

Chemicals

Trypsin was purchased from Promega (Fitchburg WI, USA). Urea, acetonitrile (ACN), formic acid (FA), calcium chloride ($CaCl_2$), iodoacetamide (IAA) and dithiothreitol (DTT), potassium phosphate monobasic (KH_2PO_4), potassium chloride (KCl), water and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis MO, USA). Water and ACN were of HPLC quality.

Sample preparation and protein digestion

The protein concentration in AC fluid was measured using a Qubit™ fluorometer (Life Technologies, Carlsbad CA, USA). AC fluid was concentrated and desalted using Amicon 3 kDa molecular weight cut-off filters (Millipore, Billerica, MA, USA) and dried in a vacuum concentrator (Eppendorf, Hamburg, Germany). The proteins were digested into peptides using in-solution digestion, as follows: The dried protein pellet was dissolved in 6 M Urea and 100 mM DTT and incubated for 1 h at 37°C. Cysteins were alkylated using 200 mM iodoacetamide and the samples were incubated for 1 h at 37°C. Chymotrypsin activity was inhibited by adding 2 mM $CaCl_2$ the proteins were digested to peptides over night using a protein:trypsin ratio of 1:50. Each sample was acidified using 5% TFA to quench the digestion activity, followed by drying the sample completely in a vacuum concentrator.

Strong cation exchange (SCX) chromatography

The samples were dissolved in 120 μL of SCX loading buffer (5 mM KH_2PO_4, 25% ACN, 0.05% FA, pH = 3) and fractionated by SCX chromatography using an Ultimate 3000 LC system (Dionex, Ultimate, Sunnyvale, CA, USA) equipped with a BioBasic SCX column (150 mm × 2.1 mm, 5 μm, Thermo Scientific, Ontario, Canada). The peptides were eluted in SCX elution buffer (500 mM KCl, 5 mM KH_2PO_4, 25% ACN, 0.05% FA, pH 3.0) over 55 min with a flow rate of 0.2 mL/min. A total of 28 SCX fractions were collected. The first two fractions (SCX fraction 1 and 2) were collected with 5.5 min intervals (first 11 min) and the last fraction (fraction number 28) was collected over the last 5 min. Fractions number 3-27 was collected with 1.5 min intervals. After collection, each SCX fraction was vacuum concentrated to dryness.

Sample clean-up

The samples were desalted using a 96 well reverse phase Oasis® HLB μElution Plate 30 μm (Waters, Wilford, MA, USA). The wells in the μElution plate were conditioned with solvent B (80% ACN, 0.1% FA) and thereafter washed twice with solvent A (0.1% FA). The peptides were re-suspended in solvent A, added to the μElution plate, and washed thrice with solvent A before the peptides were eluted twice using solvent B. One-minute centrifugation at 200 × *g* was used for all centrifugation steps except for addition of sample where 3 min at 150 × *g* was used. The samples were concentrated to dryness under vacuum and frozen at -80°C prior to mass spectrometry (MS) analysis.

Mass spectrometry

In the *AC protein comparison experiment*, the peptides were dissolved in 0.1% FA, and 4 μL (1.6 μg) of the sample was injected onto a 40nL enrichment column (Zorbax 300SB C18 5 μm, Agilent Technologies, Santa Clara, CA, USA) at a flow rate of 3 μL/min using 3% ACN, 0.1% TFA. The separation column (0.0075 × 43 mm Zorbax 300SB C18 5 μm, Agilent Technologies) was used with the following gradient and a flow rate of 300 nL/min using solvent A (0.1% FA) and solvent B (90% ACN, 0.1% FA): 3-15% solvent B for 3 min, 15-45% solvent B for 42 min, 45-90% solvent B for 5 min and back to 3% solvent B after 5 min. Both columns were integrated in a CHIP (Agilent Technologies, Santa Clara, CA, USA), and an 1100 cap/nano HPLC coupled to a chip-cube-LC/MSD XCT Plus ion trap mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) was used for separation and analysis, respectively. MS data was acquired using the AutoMS2 mode of the three precursors with highest intensity active exclusion for 1 min.

For the *AC proteome evaluation experiment*, the peptides were dissolved in 0.1% FA and 5 μL of the sample was injected to the analytical fused-silica capillary column (15 cm long, 75 μm i.d.) packed with Reprosil-Pur 3 μm C18 resin (Dr. Maisch, Ammerbuch-Entringen, Germany). The settings for LC were: Trap column: 2%

ACN, 0.1% FA with a flow rate of 25 μL/min. Analytical column: solvent A was 0.1% FA and solvent B was 90% ACN, 0.1% FA. The flow rate was 0.288 μL/min with the following gradient: 5-12% solvent B for 2 min, 12-30% solvent B for 48 min, 30-50% solvent B for 20 min, 50-95% solvent B for 1 min and 95% solvent B was kept constant for 5 min before regeneration of the column for 24 min. The nano-HPLC system (Dionex, Ultimate, Sunnyvale, CA, USA) was coupled to an Ultima Global ESI-Q-TOF mass spectrometer (Waters, Wilford, MA, USA). The scan area for the MS survey scan was *m/z* 300-1500 with automatic fragmentation of the three ions with highest intensity. All the data was acquired in data dependent mode.

For the *targeted AC protein identification experiment*, the tryptic peptides of CSF from neurologically normal patients were dissolved in 0.1% FA and 1 μL was injected into the Q-TRAP 5500 (AB Sciex, Foster City, CA, USA) coupled to a nano-HPLC system (Dionex, Ultimate, Sunnyvale, CA, USA). The targeted mass spectrometry analysis was done using the MIDAS (Multiple Reaction Monitoring (*MRM*) initiated detection and sequence analysis) workflow [15] selecting a minimum of 3 peptides per protein, and three transitions per peptide based on information from *in silico* digestion. The instrument settings were 15 ms dwell time with approximately 3.5 s cycle time for 100 transitions per method in four analyses. MRM was then used as a survey scan in information dependent acquisition (IDA) to detect specific peptide peaks, and each resulting MRM peak was examined by two full MS/MS-scans to obtain sequence verification of the hypothesized peptide.

Mass spectrometry data analysis

For the *AC protein comparison experiment*, the raw data was processed with the Spectrum Mill search engine (Rev A.03.03.084) (Agilent Technologies, Santa Clara, CA, USA) using Carbamidomethylation (C) as fixed modification. The precursor mass tolerance was set to 2.5 Da with a product mass tolerance of 0.7 Da, and two trypsin miss-cleavages were allowed. The default autovalidation settings were applied for both protein and peptide level validation. Briefly, peptides were accepted at charge +2 if score >11 and%SPI >60 and at charge +3 if score >13 and%SPI >70. Proteins with a score >20 were accepted. A threshold of 2 was set both for peptides and proteins for the forward-reverse score and the rank 1-2 score, except for charge +2 with score >6 and%SPI > 90 where the threshold was 1.

For the *AC proteome evaluation experiment*, the mass list was extracted into PKL files with Masslynx (Waters, Wilford, MA, USA) and the PKL files were merged into a single MGF file using PklFileMerger [16]. All MS/MS data were searched using the MASCOT (version 2.2.2) software platform (Matrix Science, London, UK) against the IPI-human database (v3.69, 174784 entries). Missed cleavages were set to one using trypsin as the enzyme. Carbamidomethyl (C) was set as fixed modification whereas Oxidation (M) was set as variable modifications. The peptide tolerance was set to 40 ppm and the MS/MS tolerance was set to 0.6 Da. Protein organization and redundancy reduction was done using the Scaffold software (v3.00.02 [17]). Peptide and protein identifications were accepted when the probability of correct identification was greater than 95%, with a minimum of two identified peptides per protein.

To verify peptide specificity in designated protein targets in the *targeted AC protein identification experiment*, MS/MS spectra were extracted using Analyst software (version 1.5) (Life Technologies, Carlsbad CA, USA). The selected spectra were searched using the MASCOT (version 2.3.0) software platform (Matrix Science) against the IPI-human database (version 3.78, 302626 entries) using precursor mass and MS/MS tolerance at 0.2 Da. Carbamidomethyl (C) was set as fixed modification and 0 miss cleavages were chosen.

The protein list from the AC fluid pool created in the *AC proteome evaluation experiment* was compared to published proteome libraries of CSF [18] and plasma [19]. Database comparison was performed using ProteinCenter, v3.2.0.9 (Proxeon, Odense, Denmark). The grouping of proteins and analysis of molecular function and biological processes was performed in ProteinCenter, where gene ontology terms linked to specific proteins are obtained using mappings from the Gene Ontology Consortium website [20].

Statistical methods

Principal component analysis (PCA) is a mathematical method that transforms a number of possibly correlated variables into a smaller number of uncorrelated variables called principal components, with the objective of improved understanding of the data [21]. The algorithm of PCA gives the weight of each principal component, meaning the percentage of variation in the dataset explained. In the *AC protein comparison experiment*, the protein content between individual samples was compared based on spectral intensities. The spectral intensities in each sample were normalized based on the total sum of all intensities in that sample relative to a chosen reference samples. The protein hits from the *AC protein comparison experiment* were sorted according to protein score. To check for possible outliers, we first did a PCA including all identified proteins in the analysis, and plotted the patients according to the two first principal components. Next, we compared the samples based on the 50 proteins with highest protein score to avoid large influence from proteins identified in a low number of

samples. Finally, we used a sub-sampling scheme [22,23] consisting of two steps:

1 - select 50 proteins at random and measure the distance normalized to standard deviation from the origin of each patient and 2 - repeat the first step 1000 times, and calculate the average distance from the origin over the 1000 replications.

This analysis was designed to reveal the degree to which any particular sample is divergent from the rest. Statistics was performed in the statistical software package R, version 2.11.1. (The R Foundation for Statistical Computing, Vienna, Austria).

Results

A flow chart of the two main experiments conducted in this study, the *AC protein comparison experiment* and the *AC proteome evaluation experiment* is given in Figure 1.

The AC protein comparison experiment

In the *AC protein comparison experiment* the protein contents of AC fluid from 14 patients (patients 1-14) were semi-quantitatively compared using a label-free proteomics approach to determine the protein homogeneity across the different patients. The peptides from each of the 14 patients were analyzed on an ion-trap mass spectrometer, and the protein extracted ion chromatographic (XIC) intensity values, extracted from the Spectrum Mill searched data, were compared between the different patients using PCA. In total, 139 proteins were identified from the 14 different patients. When all 139 proteins were compared using PCA, the two first principal components (PC) explained 33% of the total variation in the dataset (Figure 2). As low scoring proteins with few associated spectra can lead to more inaccurate semi-quantitative measurements, we also compared the 50 proteins with highest protein scores. For this dataset, the two first PCs explained 48% of the total variation in the dataset (Figure 3). This was a larger degree of explained variation than the model based on all 139 proteins. A comparison based on the 50 highest scoring proteins gave a clearer separation in absolute distance from the origin for samples from patients 3 (old hematoma), 4 (previous operation) and 12 (slight observed hemolysis), as well as possibly patient 9, than for the other patients. To check whether or not this separation was due to a single or a few proteins, we performed a sub-sampling scheme. Selecting 50 of the 139 proteins at random for 1000 iterations and evaluating absolute distance in standard deviations from the origin showed that patients 3, 4 and 12, the samples presumed to be different, are consistently further away from the sample mean than the rest of the patients (Table 2), while this was not the case for patient 9. Disregarding patients 3, 4 and 12, the dataset of individual patient's AC fluid samples presents little systematic variation. We

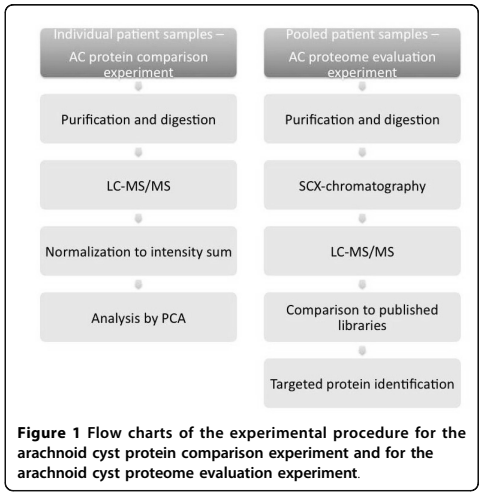

Figure 1 Flow charts of the experimental procedure for the arachnoid cyst protein comparison experiment and for the arachnoid cyst proteome evaluation experiment.

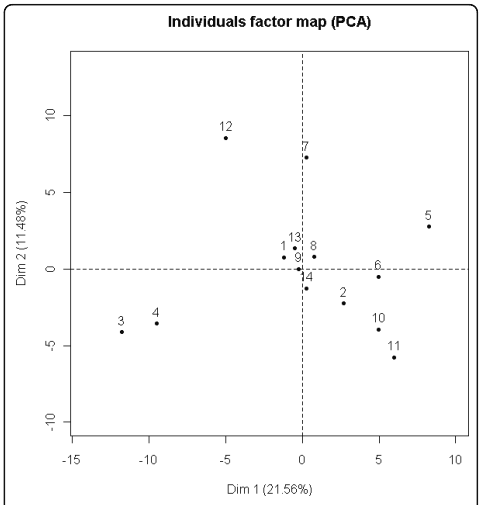

Figure 2 Arachnoid cyst protein comparison experiment (all proteins). Principal component analysis (PCA) of individual AC fluid LC-MS/MS results, normalized to sample sum. The figure is based on evaluation of all 139 proteins. Axis labels are weights of axes and the amount of total variance explained by the PC. The percentage of variance explained by the first two PCs was 33%. Observe that patients 3 and 4, as well as patient 12 were apart from the other patients.

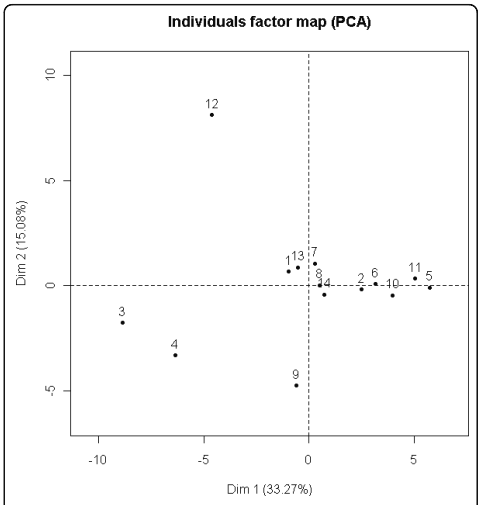

Figure 3 Arachnoid cyst protein comparison experiment (top 50 proteins). Principal component analysis (PCA) of individual AC fluid LC-MS/MS results, normalized to sample sum. The figure is based on evaluation of top 50 proteins on protein score. Axis labels are weights of axes, the amount of total variance explained by the PC. The percentage of variance explained by the first two PCs is 48%. Observe that patients 3 and 4, as well as 12 and possibly 9 were apart from the other patients.

Table 2 Arachnoid cyst fluid protein comparison experiment: distance in dataset standard deviation from the origin for each patient

Patient	All protein scores - figure 2	50 highest protein scores - figure 3	1000 iterations of 50 randomly-selected protein scores
1	0.3	0.3	0.5
2	0.7	0.6	0.7
3	2.3	2.2	2.1
4	1.9	1.9	1.8
5	1.6	1.4	1.7
6	0.9	0.7	1.0
7	1.8	0.4	1.3
8	0.2	0.1	0.8
9	0.0	1.7	0.7
10	1.3	1.0	1.2
11	1.8	1.2	1.5
12	2.3	3.0	1.8
13	0.3	0.3	0.5
14	0.3	0.2	0.4

Evaluation of all 139 proteins (Column 2), the top 50 proteins on protein score (Column 3) and an average over 1000 reiterations of PCA performed on pulling 50 of the 139 proteins in a random sub-sampling scheme (Column 4). The table show that the standard deviations of patient 3, 4 and 12 were higher than for the others. This was not the case for patient 9. The values represent the distance from each individual patient to the mean of all patients, using the first two principal components from the PCA. The results were normalized with respect to sample standard deviations.

did not find that a few specific proteins create the separation of patients 3, 4 and 12 from the others. There was no apparent sub-grouping or trends in the data. The lack of systematic trends is an indication of homogenous sample material, thus suggesting that AC is a homogenous condition as evaluated by proteomics.

The AC proteome evaluation experiment

In the *AC proteome evaluation experiment*, the aim was to identify the major protein components of AC fluid, and to qualitatively compare these proteins to the proteins previously identified in CSF and plasma and thereby learn more about the composition and origin of AC fluid. Based on the results in the *AC protein comparison experiment*, AC fluid from patients with identified blood protein haemoglobin in the cyst fluid was excluded from this analysis (patients 7, 12, 13 and 14). In this experiment, 10 μg AC fluid from the remaining 11 patients was pooled, digested, fractionated using SCX chromatography, purified and analyzed on a Q-ToF mass spectrometer. From these analyses, 199 proteins were identified in the AC fluid (Additional File 1: List of 199 proteins detected in a pool of AC fluid from 11 patients, with number of peptides and sequence coverage). The identified proteins spanned a large range of MWs, with apolipoprotein C-I being the smallest with a MW of 9.3 kDa, and protocadherin fat 2 being the largest with a molecular weight of 479.3 kDa. The isoelectric point of the proteins ranged from 4.35 to 9.96, represented by cell growth regulator with EF hand domain protein 1 and NANUC-1 heavy chain protein, respectively. The MW and pI calculations were performed using the Compute pI/Mw tool [24]. The 199 identified proteins were compared based on IPI accession numbers to published libraries of CSF [18] and plasma [19] (Figure 4). The database comparison identified 15 proteins that were not reported in CSF or plasma and we identified 11 of these by targeted protein identification in lumbar CSF samples using the MIDAS workflow on a Q-TRAP 5500 mass spectrometer (Table 3). Hence, most proteins identified in AC fluid (195 out of 199) were proven to also be present in CSF or plasma. The four proteins that were not found in the databases were; Isoform 3 of seizure 6-like protein 2, full-length cDNA clone CS0DD006YL02 of neuroblastoma of *Homo sapiens*, isoform 2 of neuroendocrine protein 7B2, and cell adhesion molecule 1 (Table 3). The overlap between AC fluid and plasma only contained 3 supplementary proteins not identified in CSF, whereas CSF and AC fluid had 89 proteins in common but not found in plasma (Figure 4). The 199 identified

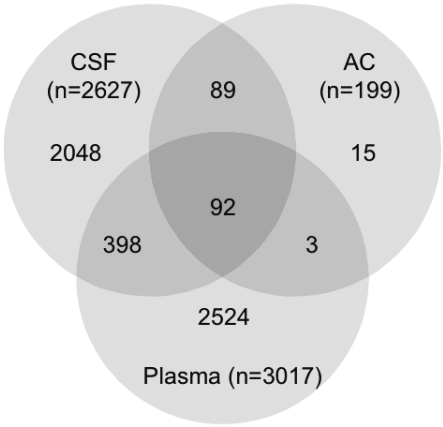

Figure 4 Venn-diagram showing the protein IPI-identity overlaps between arachnoid cyst fluid, previously recorded CSF [18] and plasma [19]. Each large circle defines the content of the three individual dataset. The numbers indicate proteins in each group, the overlap between larger circles indicate the number of protein identities in common.

proteins identified in AC fluid were annotated to a diverse range of biological processes, molecular functions, and sub cellular compartments using the ProteinCenter, v3.2.0.9 software (Proxeon, Odense, Denmark) (Figures 5 and 6 and 7). Of the 199 proteins we identified, 39.6% are involved in transport as a cellular activity (Figure 5), 20.6% are involved in transport activity on molecular function (Figure 6), 81.9% are annotated as extracellular proteins, while 66.8% are membranous proteins (Figure 7). A comparison of molecular functions of the proteins identified in AC and CSF demonstrated a similar distribution (Figure 8). Ten genes have previously been found to be differentially expressed in AC membranes compared to normal arachnoid; NKCC1 [7] and ASGR1, DPEP2, SOX9, SHROOM3, A2BP1, ATP10D, TRIML1, BEND5 and NMU [10]. We did not find the corresponding protein products among the 199 identified proteins in our study.

Discussion

As far as we know, this is the first study of proteomic evaluation of AC fluid. In the *AC protein comparison experiment* we looked for systematic differences or distributions between AC from different patients. The hypothesis underlying this experiment was that the protein profile in AC fluid would be similar between the examined patients, and this was supported by our data. This indicates a common filling mechanism and source for AC fluid in these patients. Previous observations of left side domination in males as well as a predilection for the middle cranial fossa do imply a common origin of AC fluid between patients. One possible method of formation is a defect during the embryological development of the Sylvian fissure [25]. AC could potentially be two or more conditions or subgroups with different filling mechanisms but our proteomics data from the different samples of AC fluid did not support this hypothesis, as no systematic variation in PCA-plots was

Table 3 Proteins in arachnoid cyst fluid not identified in published libraries of CSF [18] or plasma [19], and peptides identified from targeted identification in samples of normal CSF

IPI number	Protein name	Molecular weight (KDa)	# peptides identified in CSF
IPI00178854	Contactin-4	113.454	1
IPI00332887	cDNA, FLJ92887, Homo sapiens protein tyrosine phosphatase, non-receptor typesubstrate 1 (PTPNS1), mRNA	54.967	4
IPI00218803	Isoform B of Fibulin-1	77.214	7
IPI00855821	NRXN1-alpha	169.913	4
IPI00018276	Isoform 3 of Seizure 6-like protein 2	97.501	ND
IPI00216250	Cell recognition protein CASPR4	145.660	1
IPI00479708	Full-length cDNA clone CS0DD006YL02 of Neuroblastoma of Homo sapiens (human)	41.273	ND
IPI00022418	Fibronectin	262.625	12
IPI00645363	Putative uncharacterized protein DKFZp686P15220	51.725	7
IPI00470716	Isoform 2 of Neuroendocrine protein 7B2	23.730	ND
IPI00003813	Cell adhesion molecule 1	48.509	ND
IPI00435020	Isoform 2 of Neural cell adhesion molecule 1	94.574	6
IPI00384998	Isoform 7 of Neurofascin	150.027	4
IPI00451624	Cartilage acidic protein 1	71.421	3
IPI00219664	Isoform 2 of Myelin-oligodendrocyte glycoprotein	28.179	1

Proteins that were not found in any of the databases or in the targeted identification experiment is indicated by not detected (ND).

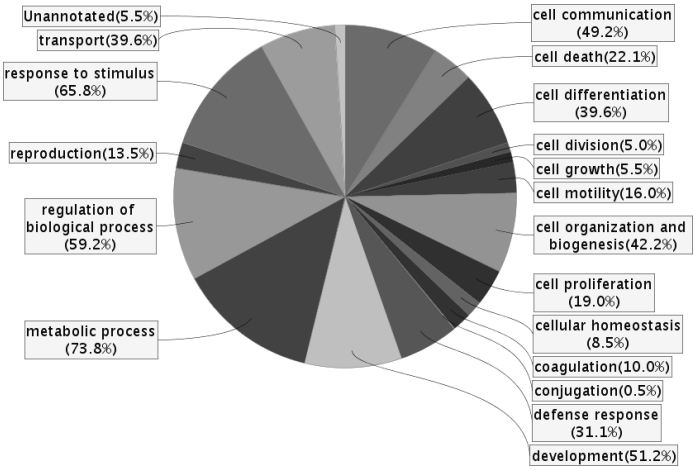

Figure 5 Biological process involvement of the 199 arachnoid cyst fluid proteins identified, denoted in percentage of proteins. Note that any one protein may be involved in several processes. Observe that 39.6% of the proteins are involved in transport processes. The figure was made using ProteinCenter, v3.2.0.9.

observed (Figures 2, 3). This finding supports previous experiments performed by clinical chemistry [9] and mRNA [10]. We observed some variation for patients 3, 4 and 12, which can be explained by patient 3 having an old hematoma in close proximity and patient 4 previously operated for the same condition. Patient 12 had a slight observed haemolysis of the AC fluid. We had anticipated a change in the proteome as a consequence of a local trauma and consecutive repair mechanisms.

There was an increase in the percentage variation explained by the first two PCA components when looking at only the 50 highest scoring proteins compared to all 139 proteins. This can be explained by the fact that low scoring proteins often are represented by few

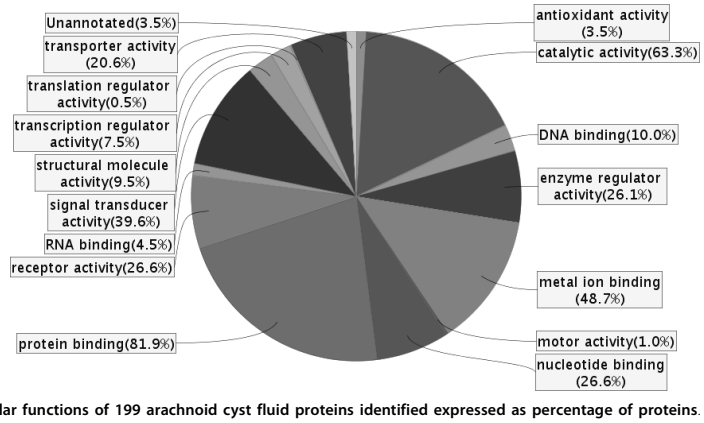

Figure 6 Molecular functions of 199 arachnoid cyst fluid proteins identified expressed as percentage of proteins. Note that any one protein may have several functions. Observe that 20.6% of the proteins are involved in transporter activity. The figure was made using ProteinCenter, v3.2.0.9.

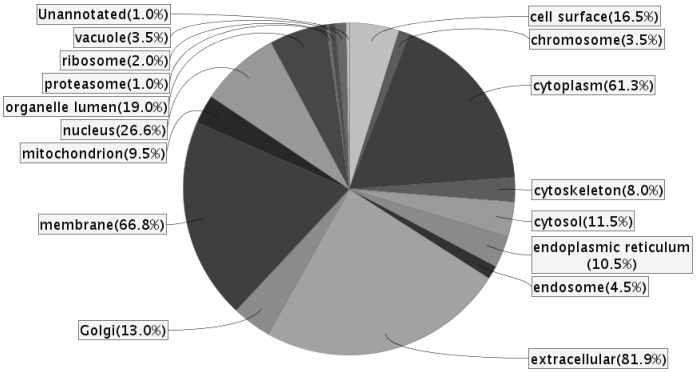

Figure 7 Cellular component localization of 199 arachnoid cyst fluid proteins identified, expressed as percentage of proteins. Note that each protein may be identified in several localizations. 81.9% of the proteins are annotated as extracellular proteins, while 66.8% are annotated as membranous proteins. The figure was made using ProteinCenter, v3.2.0.9.

peptide observations, which will lead to few values for semi-quantitative measurements, and the uncertainty in the measurements is increased. Hence, focusing only on the proteins with the highest protein scores will give a more accurate picture of the comparison. The method of sub-sampling was useful for evaluation of the stability of a result, such as for the evaluation on whether patient 9 was an actual outlier relative to the remaining patient samples.

The *AC proteome evaluation experiment* resulted in the identification of 199 proteins from pooled AC fluid from 11 patients. In order to identify more AC proteins than the 199 we found in our study, more extensive fractionation of the sample material could have been done, although this is limited by amount of sample material, as well as use of mass spectrometers with higher sequencing capacity and sensitivity. Using lumbar CSF as the basis for comparison with AC fluid is possible when doing a qualitative protein comparison, as proteins are not expected to disappear during migration towards the lumbar area. In a quantitative study between AC fluid and CSF on the other hand, it would be important to use CSF collected in the temporal fossa to avoid effects of the rostro-caudal protein gradients. Of the 199 proteins we identified 15 were not found among the reported 2627 proteins in the CSF database [18] or the 3017 in the plasma database [19]. This does, however, not imply that these proteins are not present in CSF or plasma, as they may not have been previously identified and therefore not added to the database or they could be in the database but under a different accession number, leading to a mismatching. This may explain why we identified 11 of the 15 AC fluid proteins not found in the CSF or plasma databases, in the collected CSF samples, using targeted identification with the MIDAS workflow. Hence the number of proteins that potentially are unique to AC fluid was further decreased to four proteins after this experiment.

The large overlap between AC fluid proteins and CSF (192 of 199 proteins in common) indicates that CSF is important in the filling of the AC. There was much less overlap between identified plasma proteins and AC fluid proteins, with 104 proteins only identified in AC fluid. As the identified protein content of AC almost completely overlapped with the proteins previously identified in CSF, it was not surprising that the biological function annotation of the identified AC fluid also corresponded well with the annotations of the CSF proteins (Figure 8). We did not observe that any particular protein group was not present in AC fluid compared to CSF. In addition small (9.3 kDa), large (479.3 kDa), basic (pI 9.96), and acidic (pI 4.35) proteins were observed in AC fluid, indicating that there were no absolute exclusion of proteins with these different characteristics even though the most extreme basic and acidic proteins were not observed. Certain protein groups could be expected to not be present in AC if there was a selective transport mechanism of fluid across the AC membrane. There could, however still be quantitative differences between certain protein groups in CSF and AC fluid, which would then point towards properties in the filling mechanism. Given the high qualitative similarity we found between AC fluid proteins and CSF proteins, a relevant future study would be to do a quantitative protein comparison of the temporal fossa CSF and AC fluid from the same patient collected at the same time. This

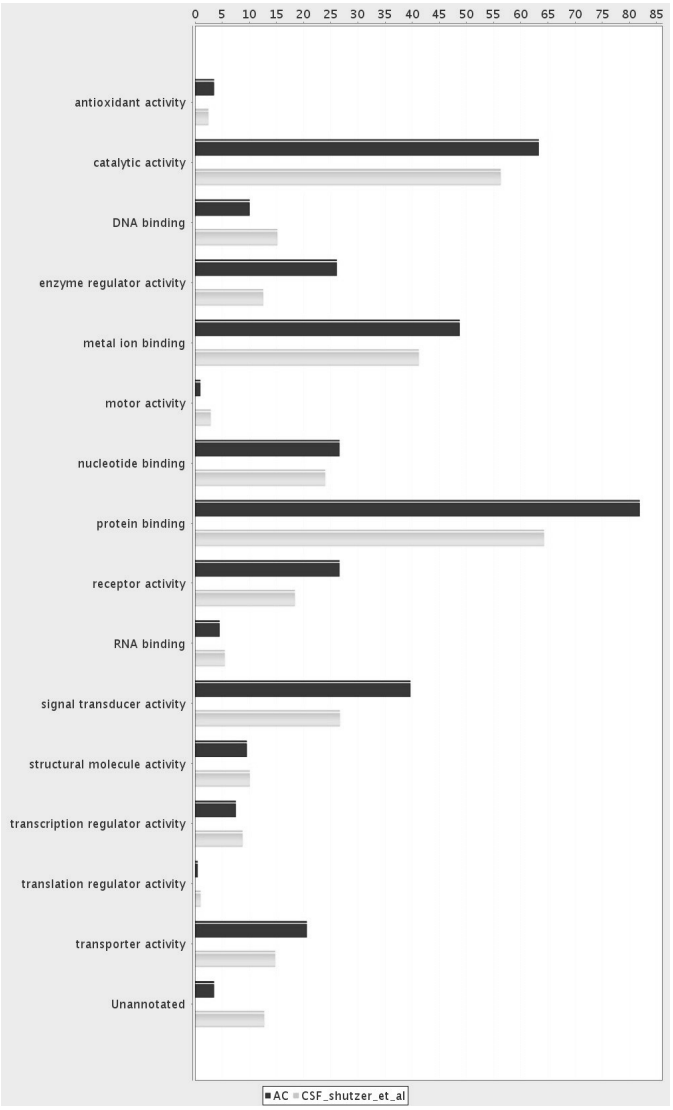

Figure 8 Comparison of proteins in arachnoid cyst fluid and CSF [18]**based on molecular function, expressed as percentage of proteins**. Note a large difference in number of proteins detected and used as a basis for creating the figure: 199 proteins in AC and 2627 in the CSF database. The figure was made using ProteinCenter, v3.2.0.9.

would give information about which proteins or protein classes that are differentially expressed between AC fluid and CSF in proximity to the AC, possibly giving further indications of the filling mechanism of AC fluid and the origin of the AC fluid.

Conclusions

The results of this study indicate that AC fluid is homogenous between patients when evaluated by protein content using a label-free semi-quantitative proteomics approach, a finding supporting results from previous experiments regarding clinical chemistry and mRNA. This points towards a similar filling mechanism of the AC for the examined patients. We found that most proteins identified in AC fluid also could be identified in CSF, while plasma had fewer proteins in common with AC fluid. This indicates that CSF has similar properties to AC fluid. We did not find specific groups of proteins with given properties absent from AC fluid, but there could still be different quantitative trends between CSF and AC fluid. A future quantitative proteomics comparison between CSF and AC collected from the same patients at the same time would reveal this information.

Additional material

Additional file 1: List of 199 proteins detected in a pool of AC fluid from 11 patients, with number of peptides and sequence coverage. Proteins are denoted by lead protein IPI accession number.

Abbreviations

AC: arachnoid cyst; ACN: acetonitrile; AM: arachnoid mater; CSF: cerebrospinal fluid; DTT: dithiothreitol; FA: formic acid; HPLC: high pressure liquid chromatography; IAA: iodoacetamide; IDA: information dependent acquisition; LC: liquid chromatography; MIDAS: multiple reaction monitoring initiated detection and sequence analysis; MRM: multiple reaction monitoring; MS: mass spectrometer; ND: not detected; PC: principal component; PCA: principal component analysis; PPM: parts per million; SCX: strong cation exchange; TFA: trichloro formic acid; qTOF: quadropole time-of-flight; XIC: extracted ion chromatographic intensity value.

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Authors' contributions

MB, ACK, KW, RJU, CAH, FSB conceived and designed the experiments. ACK, TTA, JAO, EO performed the experiments. MB, ACK, ØAH, TTA analyzed the data. KW, CAH operated on the patients. MB performed sample collection and handling. MB, ACK, ØAH, TTA, JAO, EO, KW, RJU, CAH, FSB wrote the paper. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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III

Quantitative proteomics comparison of arachnoid cyst fluid and cerebrospinal fluid collected perioperatively from arachnoid cyst patients

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Abstract

Background: There is little knowledge concerning the content and the mechanisms of filling of arachnoid cysts. The aim of this study was to compare the protein content of arachnoid cysts and cerebrospinal fluid by quantitative proteomics to increase the understanding of arachnoid cysts.

Methods: Arachnoid cyst fluid and cerebrospinal fluid from five patients were analyzed by quantitative proteomics in two separate experiments.
In a label-free experiment arachnoid cyst fluid and cerebrospinal fluid samples from individual patients were trypsin digested and analyzed by Orbitrap mass spectrometry in a label-free manner followed by data analysis using the Progenesis software.
In the second proteomics experiment, a patient sample pooling strategy was followed by MARS-14 immunodepletion of high abundant proteins, trypsin digestion, iTRAQ labelling, and peptide separation by mix-phase chromatography followed by Orbitrap mass spectrometry analysis. The results from these analyzes were compared to previously published mRNA microarray data obtained from arachnoidal membranes.

Results: We quantified 348 proteins by the label-free individual patient approach and 1425 proteins in the iTRAQ experiment using a pool from five patients of arachnoid cyst fluid and cerebrospinal fluid. This is by far the largest number of arachnoid cyst fluid proteins ever identified, and the first large-scale quantitative comparison between the protein content of arachnoid cyst fluid and cerebrospinal fluid for the same patients at the same time. Consistently in both experiment, we found 22 proteins with significantly increased abundance in arachnoid cysts compared to cerebrospinal fluid and 24 proteins with significantly decreased abundance. We did not observe any molecular weight gradient over the arachnoid cyst membrane. Of the 46 proteins we identified as differentially abundant in our study, 45 were also detected from the mRNA expression level study. None of them were previously reported as differentially expressed. We did not quantify any of the proteins corresponding to gene products from the ten genes previously reported as differentially abundant between arachnoid cysts and control arachnoid membranes.

Conclusions: From our experiments, the protein content of arachnoid cyst fluid and cerebrospinal fluid appears to be similar. There was however proteins significantly differentially abundant between arachnoid cyst fluid and cerebrospinal fluid. This could reflect that these proteins are affected by the filling mechanism of arachnoid cysts or are shed from the membranes into arachnoid cyst fluid. Our results do not support the proposed filling mechanisms of oncotic pressure or valves.

Introduction

Arachnoid cysts (AC) are congenital malformations of the arachnoid; a benign malformation with reported prevalence of up to 1.1 % [1, 2]. The mechanism of formation of such cysts is not known, although several studies have tried to investigate and understand the biological basis of AC [3-11]. Anatomically, AC originate from splitting of the arachnoid mater (AM), thus AC are truly intra-arachnoid in nature [9, 10]. True AC are considered to be developmental or congenital mistakes of the arachnoid architecture [12]. By electron microscopy, Rengachary et al [10] observed that the inner membrane of AC is covered by hyperplastic arachnoid cells, as well as cells in the cyst membrane – resembling foetal human arachnoid cells.

Using chemical analyses on AC fluid, Sandberg et al [7] observed composition comparable with cerebrospinal fluid (CSF). In addition, they found that some cysts had elevated protein levels in their fluid, relative to reference values. In a previous study on AC fluid and CSF from the same patient population as the current study, Berle et al [4] found AC fluid and CSF to be similar in electrolyte content, except for an increased phosphate content in cyst fluid. The following components were reduced in AC fluid compared with CSF: 1) the total protein amount 2) lactate dehydrogenase and ferritin. Based on the decrease in protein concentration, we would suspect a molecule weight gradient, although the number of measured proteins was low.

Helland et al [5] and Aarhus et al [6] found differentially expressed mRNA and DNA copy number in AC membrane relative to normal arachnoid membrane for the genes NKCC1 [5] and ASGR1, DPEP2, SOX9, SHROOM3, A2BP1, ATP10D, TRIML1, BEND5 and NMU [6]. The NKCC1 is an active salt pump, that conceptually could contribute to the filling of AC. Zeuthen [13] discussed water transport in tissues against osmotic barriers and suggested that

this transport is energised by ion transport, thus opening for active or selective transport as a filling mechanism. For active pumps such as the sodium – potassium – chloride transporter NKCC1, the amount of water co-transported is interesting - a single load of 1 Na^+, 1 K^+, 2 Cl^- may co-transport as much as 590 H_20-molecules.

In the same patient population as the current study, Berle et al [11] performed a qualitative proteomics study of AC fluid, where the 199 identified proteins in a pool from 11 individual patients did show a similar protein expression as in normal CSF (195/199 proteins), dissimilar from that of plasma. A qualitative protein comparison study between 14 patients did indicate AC fluid protein profiles to be relatively homogenous between patients.

Label-free quantitative proteomics by measured precursor intensity is a well-established method for obtaining relative quantitative measurements of a large number of proteins between samples [14]. Quantitative proteomics by Isobaric tag for relative and absolute quantification (iTRAQ) [15] allows for extensive fractionation at the peptide level of samples pooled after labeling without loosing analytical reproducibility. The possibility for extensive fractionation makes it possible to identify and quantify a larger portion of the proteome. This provides in part qualitative information of the proteome of AC. The quantitative proteomics methods applied here is thoroughly revised elsewhere [16].

In this study we used proteomics to quantitatively compare the protein content of AC fluid and CSF from the same patients, in order to identify possible differences in the proteomes between these two fluids**.** Two different approaches were undertaken; one where samples from individual patients were analysed using a label-free approach, and one where individual patient samples were pooled, iTRAQ-labelled and extensively fractionated to allow for a more in-depth quantitative analysis of the proteomes. The results form the two complementary proteomics approaches were expected to give us a better insight in the content of AC fluid and potentially the mechanisms of fluid filling. Furthermore, we wanted to use the quantitative data to evaluate previously published results on AC, both to test the hypothesis of a molecular weight gradient over the AC membrane, and the comparison to previous published DNA and mRNA-results.

Materials and Methods

Participants

Five participants, two males, three females, age 26-60 years, with unilateral temporal AC were included in this study (Table 1). AC fluid was collected during decompressive cyst surgery at Haukeland University Hospital (Bergen, Norway). The samples were selected from our biobank, that contains samples of AC fluid and CSF from the same patients. All the patients fulfilled the following criteria: no previous intracraial surgery, no intracranial bleeding or trauma, and relatively low intraoperative blood contamination in both AC fluid and CSF as estimated from visual inspection and measured by mass spectrometry.

The methods for sample collection and handling protocol as well as the laboratory work-up used in this study have previously been described in detail [4, 17]. Briefly, AC fluid was collected during elective surgery for AC (craniotomy with fenestration and extirpation of the cyst) by puncturing the dura with a 23G, 25mm long syringe needle using an Optidynamic® spinal fluid manometer (Mediplast AB, Malmo, Sweden) as siphon through a burr hole before the craniotomy and opening of the dura. The fluid was centrifuged at 450 *x g* for 5 minutes to remove cells and cell debris, and the supernatant was aliquoted and frozen at -80 °C. Deviations and observations on individual sample material were noted on sampling.

After opening the medial cyst membrane that covered the basal structures (the tentorial slit, the oculomotor nerve, the carotid artery, and the optic nerve), thus creating communication to the basal cisterns and the posterior fossa, a CSF-sample was collected with a pre- cut baby-feeding catheter #6, connected to a 10 ml syringe. The catheter was placed below the tentorium via the tentorial slit and fluid was aspirated gently from the posterior fossa. The collected CSF was processed in the same manner as the cyst fluid.

An overview of the procedures is given in Figure 1.

Chemicals

Trypsin was purchased from Promega (Fitchburg WI, USA). N-octyl-β-D-glycopyranoside (NOG) was purchased from Anatrace (Maumee, OH, USA). Urea, acetonitrile (ACN), formic acid (FA), calcium chloride (CaCl2), iodoacetamide (IAA) and dithiothreitol (DTT), potassium phosphate monobasic (KH2PO4), potassium chloride (KCl), water and

trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis MO, USA). Water and ACN were of HPLC quality.

Lable free sample preparation

The protein concentration in AC fluid was measured using a QubitTM fluorometer (Life Technologies, Carlsbad CA, USA). The individual AC fluid and CSF samples were concentrated and desalted using Amicon 3 kDa molecular weight cut-off filters (Millipore, Billerica, MA, USA) and dried in a vacuum concentrator (Eppendorf, Hamburg, Germany). The proteins were digested into peptides by trypsin as described [18], a brief summary follows: the dried protein pellet was dissolved in 6M Urea and 100mM DTT and incubated for 1h at room temperature (RT). Cysteins were alkylated using 200 mM iodoacetamide during one hour incubation at RT. Chymotrypsin activity was inhibited by adding 2 mM CaCl2 and the proteins were digested to peptides over night at 37°C using trypsin (Sequencing Grade Modified Trypsin, Promega) at a protein:trypsin ratio of 1:50. Each sample was acidified using 10 % FA to quench the digestion activity, desalted and concentrated on an Oasis HLB µElution Plate (Waters) as previously described [14] followed by drying the sample completely in a vacuum concentrator.

Sample preparation prior to iTRAQ-labelling

150 µl AC fluid from each of the five patients was used to generate a pool of 750 µl, and a pool of CSF was made in the same way for the same patients. 250 µl of each of these two pools were combined into a mix-pool. 500 µl from each of the AC fluid pool, CSF pool and mix-pool was concentrated using 3 kDa ultracentrifugation filters (Amicon Ultra-4, Millipore, Bedford, MA), which were pre-rinsed with 0.1 % NOG. The samples were then depleted from high abundant proteins using a human Multiple Affinity Removal System (MARS Hu-14) 4.6 mm × 50 mm LC column (Agilent Technologies) according to the protocol provided by the supplier, using a Dionex 3000-series LC system. This column depletes albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, alpha-2-macroglobulin, alpha-1-acid glycoprotein, IgM, apolipoprotein AI, apolipoprotein AII, complement C3, and transthyretin. The high abundant protein depleted samples were concentrated using 3 kDa ultracentrifugation filters which were pre-rinsed with 0.1 % NOG. Each sample was freeze-dried prior to protein digestion and iTRAQ labelling.

Protein digestion and iTRAQ-labelling

For the pooled patient samples, the entire amount of protein in each depleted sample was reduced, alkylated, digested with trypsin, and iTRAQ-labeled according to the manufacturer's protocol using the reagents provided (Applied Biosystems, Foster City, CA). The reduced and S-methylmethanethiosulfonate (MMTS) treated proteins were digested to peptides over night at 37 °C using 2.5 µg trypsin as protease. The peptides were iTRAQ-labeled (4plex) where the mix-pool 50:50 AC fluid and CSF with the 114 label, the AC fluid with 115, and CSF with 116. All samples were combined after the labeling was conduced.

Mix-phase chromatograpy

iTRAQ labelled peptides were fractionated in 28 fractions using mix-phase chromatography utilizing a Sielc Promix column (MP-10.250.0530, 1.0 x 250 mm, 5 µm, 300Å, Sielc Technologies, Prospect Heights, Illinois), using an Agilent 1260 series LC system (Agilent Technologies, Palo Alto, CA). The peptides were reconstituted in buffer A (20 mM ammuniumformate, 3 % ACN) and loaded on the Mix phase column using 85 % A for 10 minutes at a flowrate of 50 µl/min. The peptides were eluted using a gradient of 15 % - 60 % buffer B (2mM ammonium formate, 80 % ACN, pH 3.0) over 35 minutes, 60 %-100 % B over 10 minutes and hold constant for 5 minutes. The fractions were collected every 2 minutes until 60 minutes, the last 10 minutes of the LC run was collected in 2 fractions of 5 minutes. The fractions from the 8 first minutes of the gradient were discarded.

Orbitrap mass spectrometry

Injection and LC

About 0.5µg of each peptide sample, dissolved in 1 % aqueous formic acid, were injected into an Ultimate 3000 RSLC system (Thermo Scientific, Sunnyvale, California, USA) connected online to a linear quadrupole ion trap-Orbitrap (LTQ-Orbitrap Velos Pro) mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nanospray Flex ion source (Thermo Scientific).

The sample was loaded and desalted on a pre-column (Acclaim PepMap 100, 2cm x 75µm i.d. nanoViper column, packed with 3µm C18 beads) at a flow rate of 5µl/min for 6 min using an isocratic flow of 0.1 % FA (vol/vol) with 2 % ACN (vol/vol).

Peptides were separated during a biphasic ACN gradient from two nanoflow UPLC pumps with flow rate of 280 nl /min on the analytical column (Acclaim PepMap 100, 15 cm x 75µm i.d. nanoViper column, packed with 2µm C18 beads). Solvent A was 0.1 % FA (vol/vol) with 2 % ACN (vol/vol). Solvent B was 0.1 % FA (vol/vol) with 90 % ACN (vol/vol). The

gradient composition was 5-38 % B from LC starts to 67 minutes, then 38-90 % B from 67-70 minutes. 90 % B was held constant for 5 minutes, followed by column conditioning for 12 minutes with 5 % B.

Individual patient samples

The eluting peptides were ionised in the electrospray and analyzed by the LTQ-Orbitrap Velos Pro. The mass spectrometer was operated in the DDA-mode (data-dependent-acquisition) to automatically switch between full scan MS and MS/MS acquisition. Survey full scan MS spectra (from m/z 300 to 2000) were acquired in the Orbitrap with resolution R = 60000 at m/z 400 (after accumulation to a target value of 1e6 in the linear ion trap with maximum allowed ion accumulation time of 500ms). The seven most intense eluting peptides above an ion threshold value of 1000 counts, and charge states 2 or higher, were sequentially isolated to a target value of 1e4 and fragmented in the high-pressure linear ion trap by low-energy CID (collision-induced-dissociation) with normalised collision energy of 40 % and wideband-activation enabled. The maximum allowed accumulation time for CID was 200ms, the isolation width maintained at 2Da, activation q = 0.25, and activation time of 10ms. The resulting fragment ions were scanned out in the low-pressure ion trap at normal scan rate, and recorded with the secondary electron multipliers. One MS/MS spectrum of a precursor mass was allowed before dynamic exclusion for 30s. Lock-mass internal calibration was not enabled.

Pooled patient samples

The settings were identical to those mentioned above for CID fragmentation, with the exception that the seven most intense eluting peptides were sequentially isolated in the high-pressure linear ion trap by low-energy CID and in the octopole HCD collision cell by HCD (Higher Energy Collision Dissociation) fragmentation. Both fragmentation forms used normalised collision energy of 40 %. For HCD fragmentation the isolation with was 3Da and the activation time 0.10ms. After fragmentation in the HCD cell the fragments are transferred via the C-trap to the Orbitrap and scanned out with resolution R = 7500. One MS/MS spectrum of a precursor mass was allowed before dynamic exclusion for 20s.

Ethics

Patients were recruited by the responsible surgeon and had signed a written informed consent. This project was approved by the Regional Committee for Medical and Health Research Ethics (REK) of Western Norway (approvals REK 70.03, NSD 9634 and REK 2009/1885).

Data analysis

The handling of multiple testing and validation of peptide and protein hits were assessed by false discovery rate (FDR) [19]. The data from the individual patients label-free experiment were compared by paired 2-sided t-test with $p < 0.05$.

The individual patient data obtained from the label-free quantitative analysis was compared using Progenesis LC-MS (Nonlinear Dynamics Ltd. Newcastle upon Tyne, UK) (v4.0.4573.30654). The data was searched against the SwissProtKB database (release 2011_10) using SearchGUI (1.8.3) with OMSSA and XTandem as search engines and PeptideShaker (0.16.2) (http://code.google.com/p/peptide-shaker/) for combining the results [20]. The following search criteria were used: fixed modifications carbamidomethylation, oxidated methionin as variable modification, a maximum of two missed cleavages, precursor mass tolerance 15 ppm, and product mass tolerance 0.7 Da. The peptides were auto-validated with maximum FDR of 1.0 %. The quantitative data obtained from the individual patients in the label-free experiment was compared by a paired 2-sided t-test. Proteins with a p-value of less than 0.05 combined with an average fold change of more than +/- log2 (0.58) (Fold change >1.5 or <0.67) were considered as differentially abundant between CSF and AC fluid.

The pooled patient iTRAQ-data was searched using the Spectrum Mill software package v4.0 (Agilent Technologies, Santa Clara, CA) using the same settings as previously described [14]. MS/MS data was searched against the SwissProtKB database (release 2011_10) with a precursor mass tolerance of 15 ppm, and a product mass tolerance 0.7 Da. The peptides were validated by auto-determined score by delta R1-R2 threshold with max FDR of <1.2 %. Proteins were validated by minimum protein score of 20. The quantitative data were recentered to obtain an average ratio of 1 for each iTRAQ-channel relative to the mix-pool reference sample. The reference/CSF value was divided by the reference/AC fluid value for every protein to obtain a CSF-to-AC fluid ratio. All proteins with a fold change more than +/- log2 (0.58) (Fold change >1.5 or <0.67) were considered as differentially abundant between CSF and AC fluid.

Using the bioinformatic software J-Express Pro 2.7 (MolMine AS, Bergen, Norway) proteins with increased or reduced abundance between CSF and AC fluid in both label-free and iTRAQ labelled experiments were compared with the corresponding gene expresson profiles of a previously published study [6]. The objective of this examination was to evaluate whether AC fluid protein expression could be linked to membrane mRNA expression. The proteins were searched in PubMed for official gene symbol, and then searched against the microarray data to obtain the corresponding mRNA expression profile.

Gene ontology data for the identified proteins in the iTRAQ experiment were obtained using ProteinCenter version 3.9.10025 (Thermo-Fischer scientific, Odense, Denmark).

Results

In the quantitative individual label-free comparison, we quantified 348 proteins, of which 150 were differentially expressed between AC and CSF ($p < 0.05$ in a paired two-sided t-test, minimum 2 peptides, FDR < 1.0 %). In the iTRAQ-labeled quantitative experiment, 1425 proteins were identified (minimum 2 peptides, FDR <1.2 %). 296 protein groups were identified both in the label-free and the iTRAQ-labelled experiment. The list of proteins identified from the quantitative iTRAQ-labelled experiment, ranked by fold change AC/CSF is presented in Supplementary table 1. Proteins identified in the label-free experiment with individual and average fold change, p-value and corresponding iTRAQ fold change value (when applicable) are shown in Supplementary table 2. This iTRAQ quantification experiment is also the single largest qualitative characterization of AC fluid proteins. The 1425 proteins quantified in the iTRAQ-experiment were annotated to a multitude of cellular localizations, of which the principal classes are presented in Figure 2. A high proportion of the proteins were annotated to extracellular or membrane space. Note that one protein may be allocated to several localizations and may therefore be counted more than once.

Examining the proteins with significantly differential abundance by t-test between individual samples, as well as fold change +/- log2 (0.58) (Fold change >1.5 or <0.67) in both individual label-free samples as well as pooled iTRAQ-labelled samples. We found 22 proteins with significantly higher abundance in the AC fluid relative to CSF and 24 proteins with significantly lower abundance (Table 2). Concerning the proteins with changed abundance in AC fluid relative to CSF, we observed no specific pattern in the type of

proteins. Some, but not all, of the proteins with reduced abundance in AC relative to CSF are typical blood proteins. Examples of such proteins were carbonic anhydrase 1, fibrinogen, alpha-1-antitrypsin and haemoglobin. Carbonic anhydrase 1 has previously been reported to be present in CSF due to contamination of blood introduced during sample collection [21].

Among the proteins being more abundant in AC fluid was ribonuclease T2 (Gene name: RNASET2). Defects in RNASET2 are proposed to be the cause of leukoencephalopathy cystic without megalencephaly (LCWM), and the brain of such affected indivuduals shows anterior temporal lobe subcortical cysts.

We were not able to link the molecular functions of the 46 differentially abundant proteins to the cyst fluid biology based on the examined GO functional terms and Uniprot functional annotation (data not shown). The cellular location of the differentially abundant proteins between AC fluid and CSF were mainly membrane and secreted proteins as seen from the GO analysis (data not shown).

In the iTRAQ quantification, we identified 1129 proteins that were not present in the list from the label-free experiment. The depletion of the most abundant proteins as well as more extensive fractionation caused this increase in the number of proteins quantified. We observed that 480 of the proteins quantified in the iTRAQ experiment were outside the selected boundaries of significant fold change (+/- log2 (0.58)), but we cannot from this experiment conclude if any of these proteins represent a true biological change in abundance without additional verification. We observe that these 480 proteins to a lesser degree seem to represent membrane or extracellular proteins, relative to the proteins reported as differential abundant in both experiments (data not shown).

From the 46 proteins we identified as differentially abundant in our study, 45 were also detected from the mRNA expression level study. None of them were reported as differentially expressed [6]. We identified no specific patterns of altered abundance between the membrane mRNA and the 46 differentially abundant cyst fluid proteins (Supplementary table 3). We did not quantify any of the proteins associated with the ten genes previously reported as differentially abundant between AC membrane and AM [5, 6] (data not shown).

To test the hypothesis of molecular weight gradients over the AC membrane, we created a scatter plot for all the iTRAQ protein ratios sorted based on protein molecular weight. We could not see any correlation between increased molecular weight and decreased abundance in AC compared to CSF from this plot (results not shown).

Discussion

In this study, we identified by far the largest number of AC fluid proteins ever reported, and this is also the first large-scale quantitative comparison between the protein content of AC fluid CSF collected from the same patients. At present, there are three dominating hypotheses on the mechanisms of filling and sustaining of AC: secretion or selective transport, oncotic filling, and a slit-valve mechanism. Previous reports of reduced protein content in AC fluid relative to CSF [4, 7] do weaken the hypothesis of filling by oncotic pressure.

From our two quantitative proteomics experiments, we identified 46 proteins with altered abundance between AC fluid and CSF. Some of the protein groups with lower abundance in AC fluid seem to have a high representation of blood proteins. Blood contamination is an obvious problem when sampling is not identical between the sample types to be compared, in this case AC fluid and CSF. Because we sample AC fluid through a syringe needle after a direct puncture of the cyst, the risk for blood contamination is low. CSF is collected in the basal cistern after the operation is finished and haemostasis is ensured. This might lead to might be a small and variable contamination of blood in the CSF samples. Hence, abundance changes in these typical blood proteins are probably not representing AC biology, but are rather introduced to CSF during sample collection. Currently, there is no consensus on how to handle skewed blood contamination. Most of proteins with reduced abundance in AC relative to CSF are however not termed “blood specific” proteins.

A challenge in the evaluation of quantitative difference is the defining criteria on what is differentially abundant protein. In our case, we choose to include proteins that were observed with fold change above +/- log2 (0.58) in both experiments, as well as a p-value below 0.05 for the label-free experiment. This result might be somewhat conservative for exploratory analyses, hence we also evaluated the abundance changes for the 1129 proteins only quantified in the iTRAQ study.
The 480 proteins from the iTRAQ study with fold change above +/- log2 (0.58) in this analysis does however need further verification in different sample sets, by different methods

or specific validation by for example selective reaction monitoring to increase the certainty of these findings. Further verification is also necessary to confirm the differential abundance of the 46 proteins found as differentially abundant in both experiments in a larger number of patients. Our current results does however support to the previous claim [4] that AC fluid is different from CSF. As observed from our Qubit protein measurements, the general trend of reduced total protein concentration in AC fluid relative to CSF does not support oncotic pressure gradients. Observing differential abundance of proteins between AC fluid and CSF, as well as the lack of observed slit valves in general in the literature, do in principle not support the theory of valve mechanism but is no definitive evidence. Differential abundance of proteins between AC fluid and CSF is supportive of some kind of secretion or selective transport, but we are not able to elute which.

Previous reports have identified an up-regulation of mRNA for several ion transporters [5] and other genes [6] in AC membrane when compared with normal arachnoidea membrane. In our proteomics study, we hypothesised that some of the proteins in the AC membrane corresponding to these mRNA transcripts also could be found in the AC fluid, but not to the same extend in CSF, and that such proteins possibly could indicate the mechanism of transport over the membrane. However, we were not able to draw such lines based on our obtained data. Concerning the proteins that make up active pumps, such as NKCC1, they would be a part of the AC wall and thus probably not detectable in AC fluid due to the hydrophobic nature of such membrane proteins. A lack of confirmation in our data do therefore not exclude that such pumps can be found in the AC membrane, in particular since hydrophobic membrane proteins might not at all be detectable in the AC fluid due to solubility issues.

In a previous study, Berle et al [4] suggested a MW gradient from the reported reduced concentration of macromolecules ferritin and lactate dehydrogenase. The extended examinations of the data presented in the current work, contradict such a hypothesis of MW gradients over the AC membrane.

Conclusions:

From our experiments, the protein content of AC fluid and CSF appears to be very similar. Some proteins were, however, significantly differentially abundant between AC fluid and CSF. This could reflect that these proteins are affected by the filling mechanism of arachnoid

cysts or are shed from the membranes into arachnoid cyst fluid. Our results do not support the mechanisms of oncotic pressure or valves. Based on these results we suggest that AC filling is caused by secretion or selective transport across the membrane.

List of abbreviations

AC – Arachnoid cyst

ACN – Acetonitrile

CID – Collision-induced dissosiation

CSF – Cerebrospinal fluid

DTT – dithiothreitol

FA – Formic acid

FDR – False discovery rate

HCD – Higher Energy Collision Dissociation

HPLC – High pressure liquid chromatography

IAA – Iodoacetamide

iTRAQ – Isobaric tag for relative and absolute quantification

LTQ – Linear trap quadropole

MW – Molecular weight

NOG – N-octyl-β-D-glycopyranoside

NSE – Gamma-enolase

PPM – Parts per million

REK – Regional Committee for Medical and Health Research Ethics

TFA – Trifluoroacetic acid

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MB, ACK, HG, MA, OAH, KW, RJU, CAH, FSB conceived and designed the experiments. ACK, HG, performed the experiments. MB, ACK, HG, MA, OAH, FSB analyzed the data. KW, CAH operated on the patients. MB performed sample collection and handling. MB, ACK, HG, MA, KW, CAH, FSB wrote the paper. All authors read and approved the final manuscript.

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Figure Legends

Figure 1: Overview of procedures undertaken

Figure 2: Genetic Ontology of cellular localization of the 1425 quantified protein groups in the iTRAQ-labeled pooled patient samples. Figure made utilizing ProteinCenter version 3.9.10025.

Tables

Table 1 - Age, gender and protein concentration in AC fluid and CSF for the included patients.

Table 2: Proteins with different abundance between AC fluid and CSF, determined by significant ($p < 0.05$) differential abundance in individual label-free samples, as well as fold change +/- log2 (0.58) (Fold change >1.5 or <0.67) in both label-free and iTRAQ experiment

Supplementary table 1: Proteins identified in the iTRAQ-labelled experiment, ranked by fold change between AC fluid and CSF.

Supplementary table 2: Proteins identified in the label-free experiment with individual and average fold change, p-value and corresponding iTRAQ fold change value (when applicable).

Supplementary table 3: Proteins with increased or decreased abundance in AC fluid relative to CSF, plotted against corresponding membrane mRNA microarray data in order of significance in membrane data.

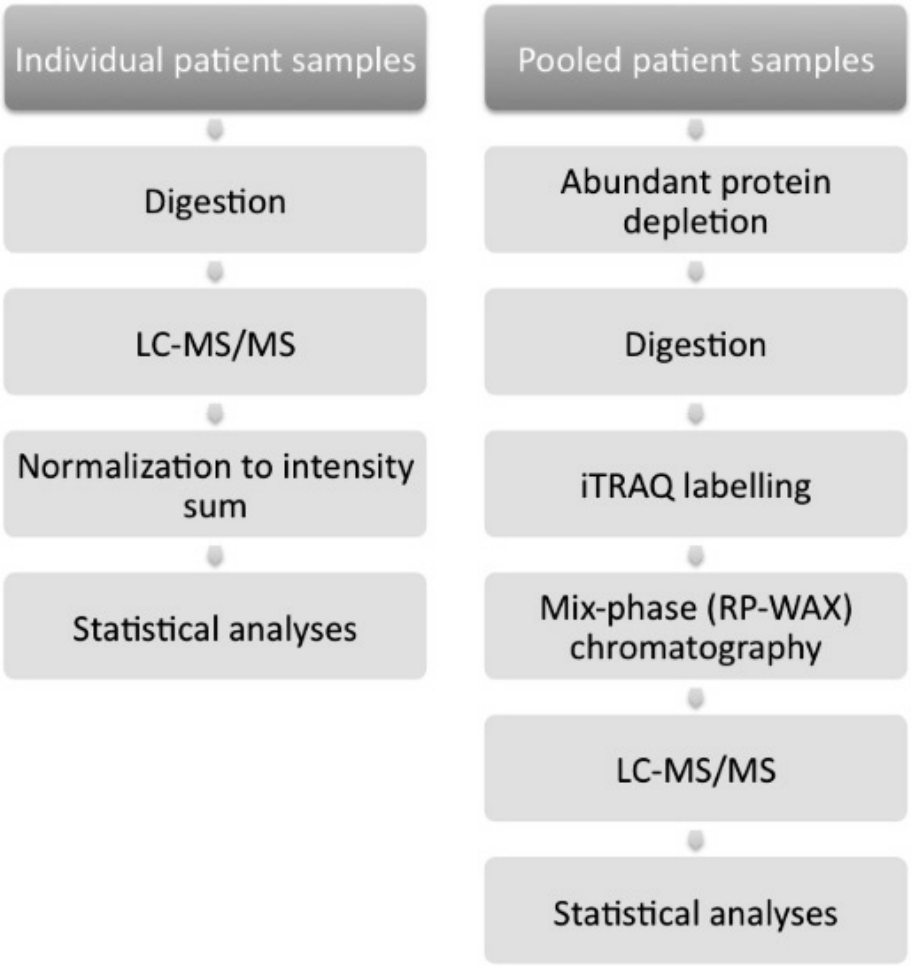
Individual patient samples
Digestion
LC-MS/MS
Normalization to intensity sum
Statistical analyses
Pooled patient samples
Abundant protein depletion
Digestion
iTRAQ labelling
Mix-phase (RP-WAX) chromatography
LC-MS/MS
Statistical analyses

Figure 1

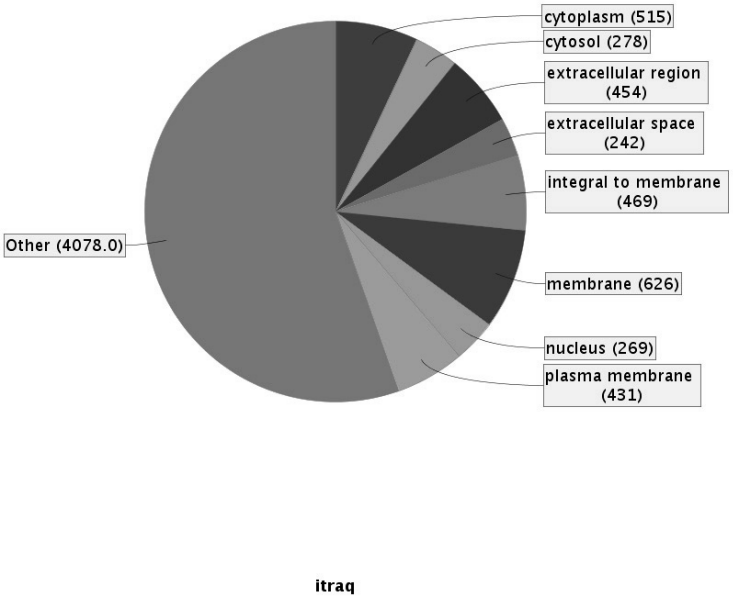
cytoplasm (515)
cytosol (278)
extracellular region (454)
extracellular space (242)
integral to membrane (469)
Other (4078.0)
membrane (626)
nucleus (269)
plasma membrane (431)
itraq

Figure 2

Patient number	**Age**	**Gender**	**protein content AC fluid µg/µl**	**protein content CSF µg/µl**
1	27	f	0,44	0,57
2	44	m	0,38	0,62
3	23	f	0,31	0,59
4	42	f	0,29	0,59
5	61	m	0,50	0,59
Average			0,38	0,59
protein content ratio AC fluid / CSF				0,65

Table 1