UNIVERSITÀ DEL PIEMONTE ORIENTALE

SCHOOL OF MEDICINE

Department of Health Science

Master's degree in Medical Biotechnology

Experimental thesis:

Untargeted Analysis of Protein Complexes and Interactions in CSF from Alzheimer Disease Patients

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Academic Year 2023/204

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1. Title

Untargeted analysis of protein complexes and interactions in CSF from Alzheimer Disease patients

2. Summary

RATIONAL OF THE STUDY: The assembly of proteins into protein complexes and their interactions with different molecular partners significantly influence their biological function. However, the characterization of protein complexes and protein interactions in their native form is still challenging. In this field, all the studies reported in literature were carried-out using stabilized cell-cultures, whereas researches on biological fluids are still lacking. The study of protein-protein interactions, and protein complexes is both intriguing and challenging for the biomedical research. Furthermore, this kind of analysis could provide new biochemical information for better understanding disease pathogenesis and progress, to identifying potential therapeutic targets and new biomarkers.

PLANNING OF THE STUDY: We developed a new workflow that uses size exclusion chromatography, mass spectrometry, bioinformatic analysis and bibliographic research to find protein complex and protein biomarker in the CSF of Alzheimer patients as an untargeted approach to obtained therapeutic targets.

RESULTS: The present work reports a new untargeted workflow that could be a remarkable option to detect and characterize protein complexes, and protein-protein interactions in the CSF samples of Alzheimer patients Finally, we mapped for the first time in four CSF samples of Alzheimer patients and three non-neurodegeneration patients the protein complexes, and protein-protein interactions as therapeutic targets .To underline its potential biomedical application, we reported some of the most significant results.

CONCLUSIONS: The possibility to detect, characterize and monitor *in vivo* these molecular interactions can open new perspectives for the study of the pathogenesis and the progression of specific diseases but also to identifying new biomarkers for the early diagnosis. The method will be particularly useful to study all the diseases where the degradation of proteins is particularly important such as neurodegenerative diseases and pathologies associated to aging.

3.Introduction

3.1. Neurodegenerative diseases and protein folding

The human body contains a variety of organs, each playing a unique role, but the nervous system stands out as one of the most significant and vast. It is highly susceptible to various stimuli. Each of these stimuli can be dangerous and affects the neurons in the nervous system, causing acute or chronic damage. Many neurological diseases are the consequences of these effects. Neurodegenerative disorders are one of the most significant groups of diseases that are the results of the degeneration or destruction of neurons in the nervous system or disruption of their functions for many reasons. Interestingly, people in developed countries face many factors that can lead to these types of diseases. Elevated stress levels, less exercise, and an unsuitable diet, in addition to the industrialized lifestyle, are relevant factors. However, the natural aging process and genetic factors play a key role in the process of neurodegeneration.

In the neurodegenerative process, various parts of the nervous system—not only the central but also peripheral parts—are involved. Impairments occur in neurons, synapses, glial cells, and even microenvironment components. These changes can impact the physical and chemical structure of different proteins, thereby deteriorating the structure and function of the nervous system. To classify different types of neurodegenerative diseases, clinical symptoms are very important. Moreover, we can distinguish different types of neurodegenerative disorders by other criteria such as accumulation of synaptic proteins, extracellular protein aggregation, damaged anatomical areas, and affected cell types. (Kovacs, 2016, 2019)

Various parts of the nervous system network, including the ubiquitin-proteasome system and the autophagy-lysosome process, significantly contribute to protein damage or buildup in neurodegenerative disorders. Elderly patients experience a decrease and disruption in these two pathways. It was also shown that when proteasomes don't work properly, different proteins like beta-amyloid, tau, SOD1, and others are increased, especially in neurological diseases like Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis. (Schmidt et al., 2021)

In the neurodegenerative process, misfolded and aggregated proteins like beta-amyloid are recognized as the cause of brain tissue damage, in addition to disrupting the function of the neural cells. (Cornejo & Hetz, 2013)

According to numerous studies, various neurodegenerative disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD), involve multiple pathogenic misfolded or

accumulated proteins, such as tau and the synuclein alpha (SNCA) protein. This evidence reveals that overlapping pathogenic proteins cause dysfunction of the nervous system in numerous neurodegenerative diseases. (Gratuze et al., 2016; Jellinger, 2012; Ling et al., 2013) Nevertheless, significant diversity of these proteins in terms of several features like size, structure, sequences functional capacity, and expression is recognized in various neurodegenerative diseases. Furthermore, misfolding of these proteins results in the formation of large fibrillar structures and beta-sheet oligomers, which facilitate protein aggregation within neuronal cells. (Ross & Poirier, 2004; Soto, 2003)

When the neural cells undergo structural destruction and malfunction due to pathological protein aggregation, a variety of clinical symptoms manifest. These symptoms display similarities across various stages of different neurodegenerative disorders. On the other hand, additional strategies such as brain imaging and laboratory tests serve as alternative tools for diagnosing these disorders, complementing clinical indications. Moreover, in evaluating the pathological process and diagnosing or differentiating these diseases, biological biomarkers are reliable indicators. (Atkinson et al., 2001; Wagner et al., 2007)

3.2. Native proteomics

To gain a thorough understanding of the structure of protein complexes, protein-protein interaction (PPI) can offer valuable insights into the mechanisms of complex aggregation and the accumulation of misfolded proteins in neurodegenerative diseases. These protein-protein interactions, along with their characteristics, provide evidence for the function and interplay effects of the proteins. The rate of protein aggregation in the nervous system—the decrease or increase rate—is affected by these PPIs. These protein accumulations have a detrimental impact. (Mogk & Bukau, 2017; Morley et al., n.d.; Pechmann et al., 2009)

A top-down characterization of protein complexes can be conducted through native proteomics. In this context, the primary method to detect complexes by using mass spectrometry is known as “proteomics.”. (Korecka & Shaw, 2021a)

Native proteomics is a significant technology that provides a good understanding of the structure of protein complexes in their native form and PPI.

(Skinner OS, Haverland NA, Fornelli L, Melani RD, Do Vale LH, Seckler HS, Doubleday PF, Schachner LF, Srzentić K, Kelleher NL, Compton PD.)

Proteins are highly desirable biomarkers for biological processes and pathological conditions in the human body. Biological fluids and tissues contain concentrated amounts of protein. In diagnosing neurodegenerative diseases, proteomics technology is a significant tool to find out the protein biomarkers needed to detect changes in protein levels and abundance and understand which proteins are involved in these diseases (Fountoulakis & Kossida, 2006)

Research on neurodegenerative disorders showed that Alzheimer's disease and Parkinson's disease are the most prevalent ones in neurodegenerative disorders, which can be considered proper candidates for proteomics analysis to understand more proteins as diagnostic or therapeutic biomarkers. (Wei & Li, 2009)

Biological fluids such as cerebrospinal fluid (CSF) and plasma are the most suitable options for identifying various markers. CSF is in contact with brain tissue; as a result, the protein content is similar to that of brain tissue. As before mentioned, mass spectrometry-based approaches are reliable for detecting protein biomarkers in different diseases, specifically neurodegenerative ones. By accurately quantifying pathological protein biomarkers through mass spectrometry technology, the diagnosis and treatment of these diseases can be made more reliable. (Korecka & Shaw, 2021b).

3.3. Alzheimer disease

There are numerous age-related disorders, including neurological conditions, which can cause significant difficulties for individuals. Among these neurological diseases, Alzheimer is the most common type of dementia, with a prevalence of 60–70%. Around 50 million people worldwide are affected, and the mean age is 65 and more, and they are recognized as elderly people (Korecka & Shaw, 2021a) (https://www.alzheimersnewstoday.com/).

In the occurrence of Alzheimer disease, different factors, like age, diet, environment, stress level, genetics, bacterial and viral infections, and the quality and quantity of sleep, have strong effects on the pathogenesis of this neurodegenerative disease. (Guo et al., 2020)

In the development of neurodegenerative diseases, there are various mechanisms that are involved, but two important mechanisms are more significant: the amyloid beta cascade and

the tau hyperphosphorylation that lead to the production of intracellular neurofibrillary tangles (NFTs). (Fan et al., 2020)

As a result of NFT production in the cells and its aggregation, different side products and various side effects can be dangerous for the cells and they can cause neurodegenerative processes. Oxidative stress and dysfunction of the mitochondria, along with impairment of the cytoskeleton structure and synapse's function, are the results of the NFT aggregation. These consequences disrupt neural cells and create conditions for tau protein accumulation, which is associated with neurodegenerative diseases, particularly Alzheimer's disease. (Gao et al., 2018)

In Alzheimer disease, the most important events are aggregated amyloid beta and TNF production, which play a role in the occurrence of Alzheimer disease as the main chemical aetiology. (Cras et al., 1991)

It should be noted that in the process of this disease, the presence of aggregated amyloid beta precedes other processes such as NFT production, loss of synapses, and the appearance of clinical symptoms that can be illustrated in Fig. 3 (Sperling et al., 2011).

As a result of all of these mechanisms that speed up the process of neurodegeneration, the function and structure of the neural cells will be disrupted, and the synaptic connection, which is one of the most crucial parts of transferring messages, will be lost.

3.4. Protein aggregates in Alzheimer diseases

Proteins are involved in almost all of the cellular functions in our body, and a change in the level or structure of these biological elements in many cases may cause different pathological situations. As a result, the comparison between healthy and diseased protein coverage in both controls and patients can lead to the discovery of different therapeutic targets. (Aluise et al., 2008; Wang et al., 2010).

Because biomarkers change even before the onset of the early clinical symptoms of neurodegenerative diseases in biological fluids like plasma and blood or cerebrospinal fluid (CSF), detecting these biomarkers that are related to the neurological diseases could lead to the provision of suitable therapeutic drugs to treat these patients or at least control the rapid progression of these diseases. (Keeney et al., 2013a)

The research on the pathology of Alzheimer indicates that there are many approved protein complexes that play a significant role in causing this disease. As previously mentioned, b-amyloid peptide-containing plaques and protein tau, composed of hyperphosphorylated microtubules that create neuropathological lesions in the nervous system, are the two most important aggregated proteins (Bourdenx et al., 2017).

Furthermore, many other high-molecular-weight protein complexes are believed to cause Alzheimer disease, such as Presenilin, which is a 52,668-Da protein in the form of a homodimer complex located in the transmembrane proteins localized in the nuclear envelope, the endoplasmic reticulum, and the Golgi apparatus (Sherr et al., 1995). The central nervous system and other types of tissues express it. (*47.Presenilin*, n.d.; Lee et al., 1996). In terms of what presenilin does in the body, it takes part in the Notch and Wnt signalling pathways and controls protein processing further down the line. For example, CTNNB1 encodes the protein beta-catenin, which is essential for cell adhesion and signalling. (Ray et al., 1999)

Presenelin-1 has two aspartate residues that are required for the cleavage of both Notch and beta-amyloid precursor protein (APP). (*50.Presenilin*, n.d.) Mutations in these residues inhibit gamma-secretase processing of APP and cause abnormal aggregation of b-amyloid plaque and apolipoprotein E4 in familial AD (FAD) and sporadic AD (SAD), respectively, so that these types of mutations in Presenilin enhance b-amyloid aggregation, which is the result of impaired g-secretase activity (Islam et al., 2022).

Apolipoprotein A-I (ApoA-I) is an important protein whose level changes or goes through oxidation in several neurodegenerative diseases or in cancer patients after chemotherapy. This is because chemotherapeutic agents produce ROS. (Hansson et al., 2004) APOA1 is a 30,778 Da homodimer protein that plays a role in the metabolism of lipoproteins, which are in the plasma membrane structure. APOA1 is present as a cofactor alongside the enzymes that are involved in lipid metabolism or maintain lipoprotein particles (Mahley et al., n.d.).
The liver clears these cholesterol-fortified chylomicron-related particles, but hyperlipoproteinemia's plasma membrane contains them.

Furthermore, another function of APOA is in inflammatory situations, where it acts as an anti-inflammatory or antioxidant agent, whose level decreases in inflammation and increases in CSF in damaged brains. (Keeney et al., 2013b; Saito et al., 1997)

On the other hand, in the pathogenesis of neurodegenerative diseases like Alzheimer disease, proinflammatory molecules and oxidative stress responses have important roles, so that many oxidised proteins like activated microglia are present in the brains of people who are affected by AD, which can give the prospective of different pathways and molecules that are related to AD. (Butterfield et al., 2007; Itagaki et al., 1989; Sultana et al., 2006).

3.5. Liquid chromatography mass spectrometry analysis in different diseases

One of the most significant steps in proteome studies and the detection of protein and peptide biomarkers, particularly in blood samples such as plasma and CSF, is the separation of protein complexes prior to any analysis. In analytical chemistry, High Performance Liquid Chromatography (HPLC) is one of the branches of column chromatography that plays an important role in order to separate, detect, and measure the soluble components in a liquid, like proteins, in their complex form for future analysis. (Kumar Bhardwaj, 2015)

After the separation the protein complexes in HPLC, the content of the proteome is now ready to go for analysis. In disease diagnosis through biomarker identification, molecular mass spectrometry (MS) is one of the most significant tools in this regard, which analyses proteins based on their mass-to-charge ratio. (Geyer et al., 2017; Hanash et al., 2008). In various neurodegenerative disorders, HPLC coupled with liquid chromatography-mass spectrometry are crucial tools in biomarker discovery.

Based on the mass spectrometry mechanism, the mass-to-charge (m/z) ratio of ionized molecules is measured, and in this way different types of molecules such as protein complexes and protein biomarkers can be identified (Awad et al., 2015)

In different types of diseases, MS is a significant tool in the diagnosis of biomarkers as therapeutic markers, like cancer and neurodegenerative disorders.

In cancer research, biomarkers let us diagnose cancer in its early stages. Also, protein complex biomarkers can lead us to differentiate various subtypes of a specific cancer, utilise them as prognostic factors, introduce new therapeutic targets to get the medications, and also follow the alteration of the structure and the microenvironment of the cancer cells in response to the

therapy (Bacher et al., 2008; Draisma et al., 2009; Fine & Amler, 2009; J. S. Ross et al., 2008; Sauter et al., 2009).

Two types of protein biomarkers are useful in cancer research and important to developing diagnosis and therapy. Not only can we measure them in tissues, but they are also present in blood products such as plasma and CSF. Blood products are more advantageous in cancer research than tissues because we can analyze tissue biomarkers only after diagnosing potential cancer and obtaining a biopsy. (Fine & Amler, 2009)

On the other hand, from an oncological perspective, alteration in proteome coverage is necessary for the discovery and validation of protein biomarkers, enabling patients to monitor their treatment needs and benefit from blood products. Mass spectrometry is a suitable tool for obtaining strong, comprehensive insights into protein biomarkers and observing proteome variation. (Macklin et al., 2020)

In different age-related disorders that are common in elderly people, there is a very significant group that is related to damaged neurons. This is known as neurodegenerative diseases, which contain various types of diseases such as Alzheimer’s disease, Parkinson's disease, frontotemporal dementia, and amyotrophic lateral sclerosis.

CSF is one of the important blood products used to study these diseases in patients. Through the CSF samples, many significant biomarkers can be obtained.

Protein complexes are a huge group of CSF biomarkers that can be identified to diagnose diseases or find useful therapeutic biomarkers for treatment. Moreover, these complexes that are identified through the liquid chromatography-mass spectrometry analysis of proteomics are useful for acquiring significant data related to the structure of the neurons, their function, the content and structure of the microenvironment, and the behaviour of the neural cells in a pathological condition in comparison with a healthy situation. (Li et al., 2019)

4.Objective of thesis

The thesis aims to identify protein complexes in the CSF, a crucial biological fluid, of patients suffering from Alzheimer's, a common neurodegenerative disease. Proteins have crucial roles in the human body, and their structures, amounts, and functions in cells cause biological effects, as many studies have indicated. Any disruption in these features of the proteins can result in malfunction and, consequently, the development of various disorders. Specifically, in the human nervous system and neurons, malfunction or aggregation of proteins leads to disturbances in the function of these cells. In Alzheimer's disease, identifying these proteins creates an opportunity to use these protein complexes as either therapeutic or diagnostic targets. To reach this goal, using the CSF of these patients to find specific protein complexes using HPLC along with LC/MS can show how to find a therapeutic target in living organisms for one of the most common and important neurodegenerative diseases in humans and how to make drugs that target these protein complexes.

5.Materials and Methods

Overview of t the method

In order to find protein complexes as biomarkers for Alzheimer's disease, the human CSF of the patients and controls was fractionated through high-performance liquid chromatography (HPLC) and then analysed using the high-resolution mass spectrometry method.

Figure 1 demonstrates the first phase of the workflow briefly. The non-denatured CSF samples were prepared in order to maintain the native condition of the protein complexes and preserve the protein-protein interactions. These CSF samples were fractionated and analysed through HPLC in combination with the spectrophotometer. The size-exclusion chromatography column in the HPLC instrument was used to separate the samples. Additionally, we analyzed the albumin standard to assess the chromatographic profile and its retention time in the size exclusion chromatographic analysis process.

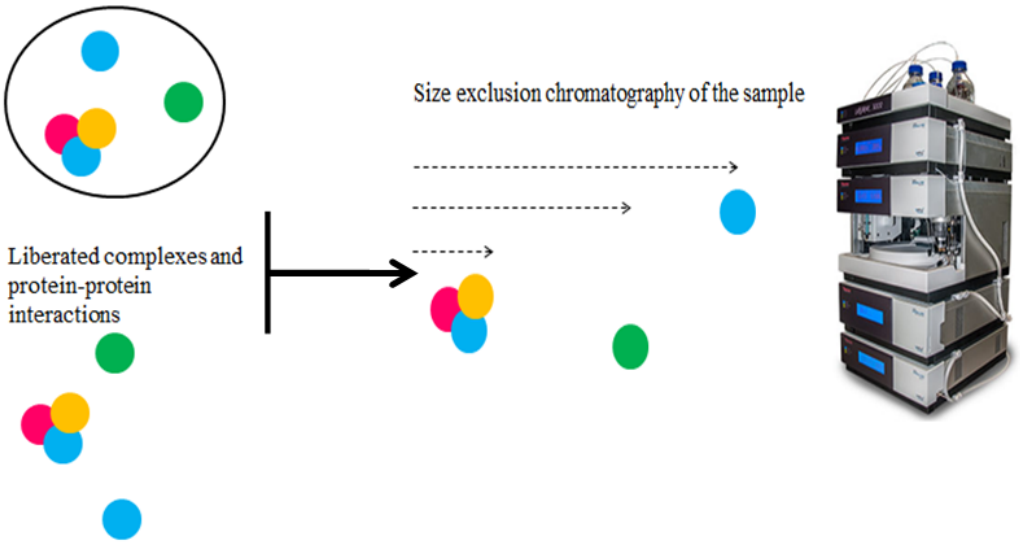

Figure 1: Size exclusion chromatography of plasma samples

Figure 2 shows the size exclusion chromatography elution profile obtained through spectrophotometry. We collected the fractions from minute 18 to minute 50 of the SEC-elution; the total was 30 fractions. We performed the size exclusion chromatography at a wavelength of 280 nm.

After collecting the 30 fractions, we started to prepare them and then continued the process with proteomics analysis.

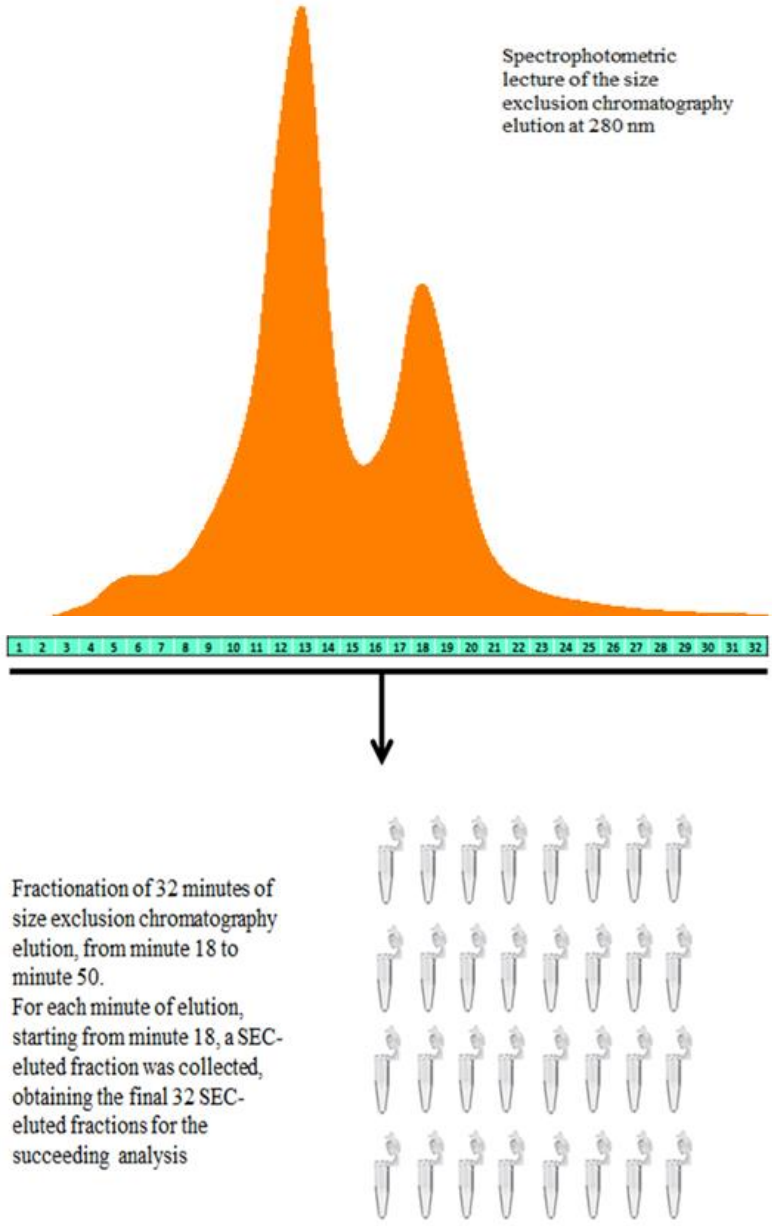

Figure 2: Spectrophotometric lecture and fractionation of the sample.

In order to achieve this goal, we precipitated the 32 SEC-eluted fractions that had undergone denature and disrupted protein folding overnight using cold acetone at -20 degrees C. Next, we discarded the supernatant and resuspended the pellet for quantification using the BCA colorimetric assay. The TECAN Spark spectrophotometer quantified all 30 SEC-eluted fractions at 562 nm wavelength in a spectrophotometric lecture.

The trypsin enzyme then digested the fractions to extract the peptides from each fraction.

Then we purified and desalted the fractions through solid-phase extraction (SPE) to remove contaminants in the samples and enrich interested proteins to increase the accuracy and sensitivity of the analysis.

In the last step, the 30 digested and purified SEC-eluted fractions were collected from the SPE and dried, then we resuspended them with Mobile Phase to go to analysis through liquid chromatography coupled with mass spectrometry (LC/MS) to identify and quantify the proteome coverage of each SEC-eluted fraction.

5.1.Sample preparation

5.1.1.CSF preparation

We extracted the native sample from the CSF vials at -80 degrees and refrigerated it at room temperature to slowly thaw without compromising the protein content. Then we took 600 µl of the human CSF from the sample. Then we put two 10 kDa Amicon filters in two Eppendorf tubes and loaded these filters with 300 µl of sample. After that, we centrifuged these two eppendorfs at 14000 g for 10 min. We then turned the Amicon filters loaded with the sample upside down, placed them in a new Eppendorf, and discarded the liquid from the first Eppendorf. Again, we started to centrifuge the new ones at 1000 g for 2 minutes. Then, we repeated this cycle once more, loading two new Amicon filters with the liquid from the current Eppendorf and centrifuging them at 14000 g for 10 minutes. After that, we again used two new tubes turned upside down, loaded filters in them, and centrifuged at 1000 g for 2 minutes. In the end, we collected the samples and quantified the amount of each to understand how much mobile phase we should add in the vials for the HPLC size-exclusion chromatography. The volume of each vial was 310 µl. It should contain the amount of the sample, and the best part is the amount of the mobile phase.

5.1.2. Size-exclusion chromatography

The size-exclusion chromatography of the samples was carried out with an UltiMate 3000 HPLC system (Thermo Scientific). BioSepSEC3000, a size exclusion column that is 300 mm long, 7.8 mm wide, has silica particles that are 5 µm thick, and pores that are 290 A big, was used to separate the proteins in the sample by size. The column performed the separation at a constant flow rate of 150 µl/min and at a constant temperature of 20 degrees. We carried out the analysis using an isocratic modality, using a mobile phase of water containing 150 mM NaCl and 50 mM HEPES. The chromatographic run took 60 minutes in total. We carried out the fractionation of the SEC-eluted sample from min 18 to min 50. We measured the absorbance using a spectrometer with a wavelength of 280 nm. We performed 5 chromatographic runs for the non-denatured plasma, followed by 5 fractionations, and pooled 3 chromatographic SEC-eluted fractions for each minute of fractionations.

We organized the size-exclusion chromatography runs and fractionation for the proteomic analysis as follows: Five runs and fractionations were performed for the mildly denatured plasma.Upon completion of the fractionation, we pooled each SEC-eluted fraction with its corresponding SEC-eluted fraction from the other runs.

5.2. Preparation of the SEC-eluted fractions

5.2 .1. Denaturation and precipitation

In this step we added 150 µl of lysis buffer (50mm Tris HCl, 0.05%SDS, Ph7) to the SEC-eluted fraction in order to be lysed. Then we sonicated the sample with Ultrasonication instrument(minichiller300) for 5 runs, each run 20 sec. Then we put the Eppendorf in the rotator for 15 min. All of these processes were carried out with the presence of ice to keep the suitable temperature for the proteins to avoid damaging them. Then the lysed proteins were precipitated overnight by adding the cold acetone (-20 degree). The day after, the sample was centrifuged at 14000g at 4 degrees for 10 min. We discarded the supernatant and resuspended the pellet with 20 µl of 8mM urea buffer and 100 µl of 100mM ammonium bicarbonate solution.

5.2.2. Protein quantification

For protein quantification we used BCA colorimetric assay to measure the concentration of each SEC-eluted fraction. In this way, a 96 well plate with a BCA assay kit was used.

In a Falcone we prepared the reagent that is contained 4800 µl of BCA solution and 96 µl cupric sulfate 4%. Besides, the buffer of urea 8mM and ammonium bicarbonate 100mM was prepared in an Eppendorf. Then we take 128 µl of the reagent for each well of the well plate for 32 SEC-eluted fraction and the blank. Then 16 µl of each fraction was added to the wells of the well plate and 16 µl of the buffer was added to the blank.

The sample and the BCA reagent were added in 1:8 ratio. After that the 96 well plate was incubated at 37 degrees for 30 min in order to catalyze the reaction of the BCA.

Then we put the 96 well plate in TECAN spark spectrometer in order to measure the absorbance in 562nm wavelength. The absorbance of all the fractions were exported in an excel file. The absorbance is useful to understand the protein concentration with the help of a formula to obtain a standard curve.

5.3. Trypsin digestion and SPE desalting

Each fraction were reduced with DTT 200mM , alkylated with IAM 200mM and we used Trypsin (Sigma-Aldrich Inc., St. Louis , MO, United states) to digest the proteins. The mixture solution was desalted and purified through the DSC-18 solid phase extraction (SPE). The preconditioning of the SPE columns was performed by 1ml of acetonitrile to open the carbon chain of the columns, and 2ml of water. Then we loaded the columns with SEC-eluted samples and then washed with 1ml of water. In the next step we put the tubes under the SPE to collect the adsorbed proteins due to adding 800 µl of acetonitrile 80%. After that, the samples were put in the SpeedVac to be evaporated. Then the samples were ready to be resuspended with 50 µl of acetonitrile with formic acid 0.1% for LC/MS analysis.

5.4. LC-MS/MS -analysis with Orbitrap mass spectrometer

5.4.1. Proteomic analysis

For the analysis of the proteins in the samples we used a UHPLC Vanquish system (Thermo Scientific, Rodano, Italy) in combination with Orbitrap Q-Exactive Plus (Thermo Scientific, Rodano, Italy). Proteins were separated with the reverse phase column (Accucore RP-MS100 x2.1mm, particle size 2.6µm)

5.5. Data processing

Digested peptides were analyzed on an Ultimate 3000 RSLC nano coupled directly to an Orbitrap Exploris 480 with a High-Field Asymmetric Waveform Ion Mobility Spectrometry System (FAIMS) (all Thermo Fisher Scientific). Samples were injected onto a reversed-phase C18 column (15 cm × 75 µm i.d., Thermo Fisher Scientific) and eluted with a gradient of 6% to 95% mobile phase B over 41 min by applying a flow rate of 500 nL/min, followed by an equilibration with 6% mobile phase B for 1 min. The acquisition time of one sample was 41 min and the total recording of the MS spectra was carried out in positive resolution with a high voltage of 2500 V and the FAIMS interface in standard resolution, with a CV of -45V. The acquisition was performed in data-independent mode (DIA): precursor mass range was set between 400 and 900, isolation window of 8 m/z, window overlap of 1 m/z, HCD collision energy of 27%, orbitrap resolution of 30000 and RF Lens at 50%. The normalized AGC target was set to 1000, the maximum injection time was 25 ms, and microscan was 1. For DIA data processing, DIA-NN (version 1.8.1) was used: the identification was performed with “library-free search” and “deep learning-based spectra, RTs and IMs prediction” enabled. Enzyme was set to Trypsin/P, precursors of charge state 1–4, peptide lengths 7–30 and precursor m/z 400–900 were considered with maximum two missed cleavages. Carbamidomethylation on C was set as fixed modification and Oxidation on M was set as variable modification, using a maximum of two variable modifications per peptide. FDR was set to 1%.

6) Results

6.1) Experimental Workflow

The objective of the present research is to map the proteomic landscape of the cerebrospinal fluid (CSF) of four patients who are affected by Alzheimer's disease (ALZ patients) and three patients with non-neurodegenerative pathologies (NN patients). In particular, we performed a native proteomics analysis, focusing on the differences in the proteins' structure between the two groups and then investigated the presence and the abundance of protein complexes and interactions detected in the CSF. To this aim, we separated CSF's proteins through size exclusion chromatography, under native conditions, then we collected the 30 obtained SEC-fractions and subjected them to lysis, protein precipitation, tryptic digestion, and protein analysis with LC-MS. After data acquisition, mass spectra were searched against the human protein database and we obtained a list of proteins, with their abundances, for each fraction of each sample. Then, we applied a bioinformatic workflow to infer protein complexes' presence and abundance and to compare them between ALZ patients and NN patients.

6.1.1) Mass spectrometry analysis of native cerebrospinal fluid

1. **Fractionation of proteins in native conditions:**

   We fractionated CSF proteins in native conditions using size exclusion chromatography (SEC). In **figure 3** Is reported the chromatogram of a non-denatured CSF sample obtained through the separation by the size exclusion column: the x-axis reports retention time (in minutes), while the y-axis reported the absorbance at 280 nm, expressed in mUA, measured through the LC-coupled diode array detector. Two main chromatographic peaks were detected: a first one characterized by a maximum intensity of 260 mUA at 33 minutes, and a second one, a little bit smaller, with a maximum intensity of 140 mUA at 41 minutes. Five chromatographic runs were performed for each CSF sample.

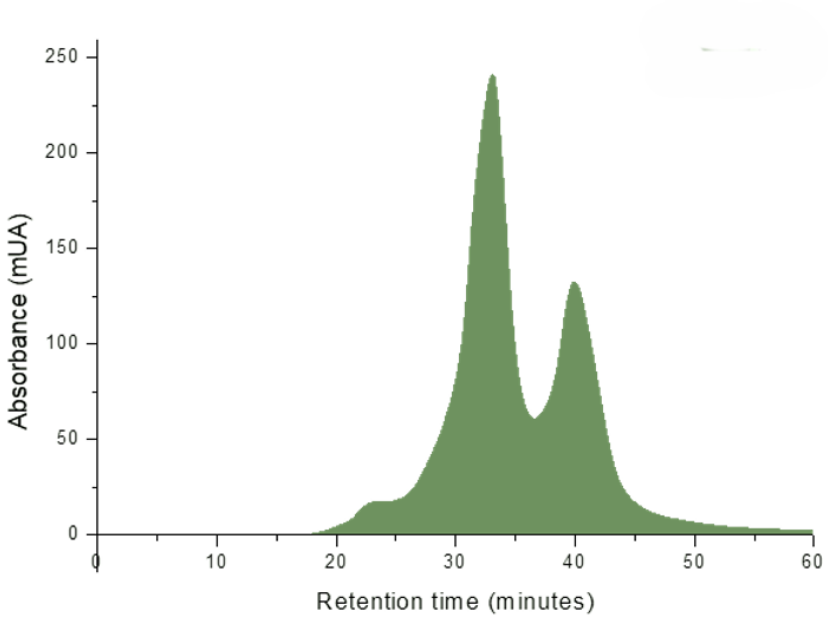

Fig 3 Chromatogram of one example of CSF sample fractionated by size exclusion

In figure 4 the workflow that from one CSF sample gives origin to 30 SEC-eluted fractions is summarized.

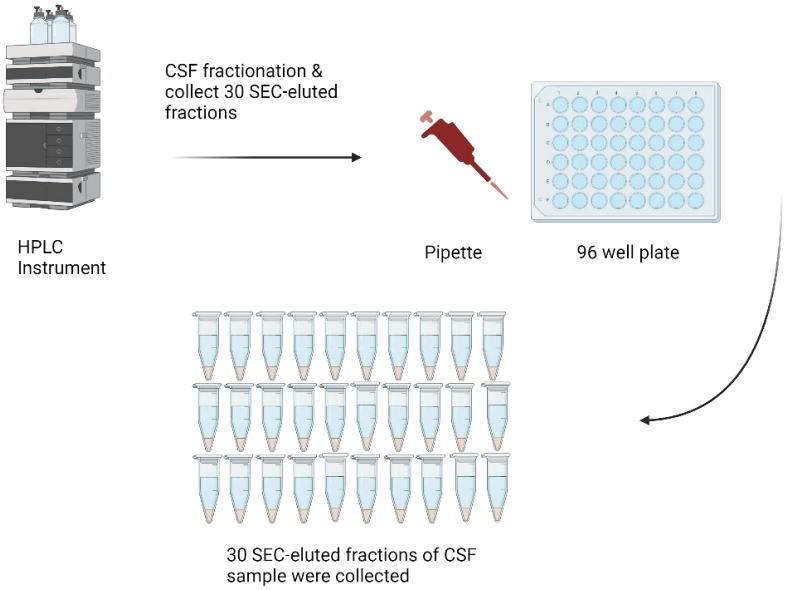

Fig 4 CSF sample was fractionated through a size exclusion column and 30 SEC-eluted fractions derived from one CSF sample were collected in the 96 well plate and then stored in Eppendorf for the subsequent analysis.

2. Quantification of total protein content:

After the lysis of SEC-eluted CSF fractions, the protein content was precipitated and after resuspension it was quantified through BCA assay. In figure 5 is report the protein absorbance, measured at 562nm, of the 30 fractions. We can notice an higher absorbance for fraction 27, 28, 29 and 30.

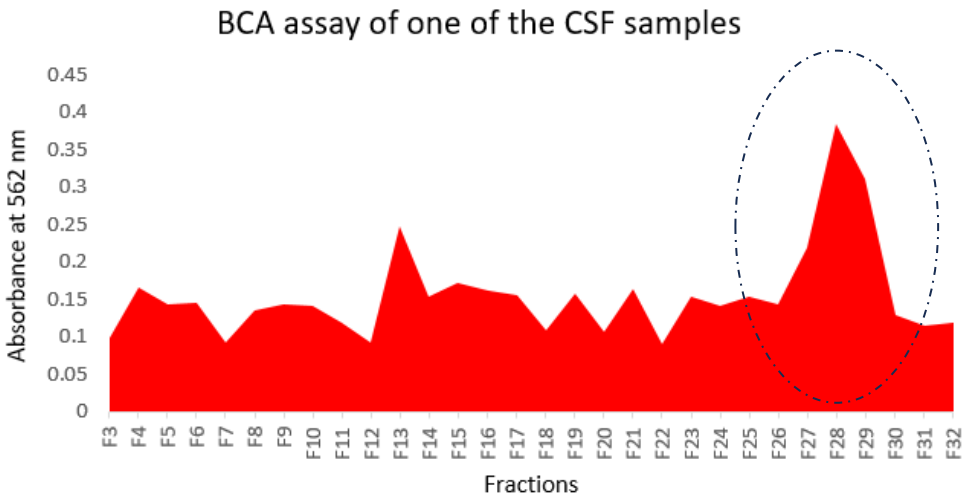

Fig 5 Measure at 562 nm of the absorbance of the protein BCA assay of the 30 SEC-eluted fractions of one of the CSF samples

3. Mass spectrometry analysis of the proteomic content

After tryptic digestion, we reconstituted the peptide mixture for each fraction using mobile phase A (water acidified with 0,1% formic acid) for the liquid chromatography-coupled mass spectrometry analysis.

LC-MS/MS data were search against human protein database through DIA-NN software to identify and quantify proteins in the 30 SEC-eluted fractions of each CSF sample. Totally, 1511 unique proteins were detected. However, the distribution of these 1511 proteins and their intensity is not uniform across these 30 fractions.

Indeed, the most intense fractions are fraction 28, 29 and 30, reflecting the absorbance among fractions reported in Figure 6

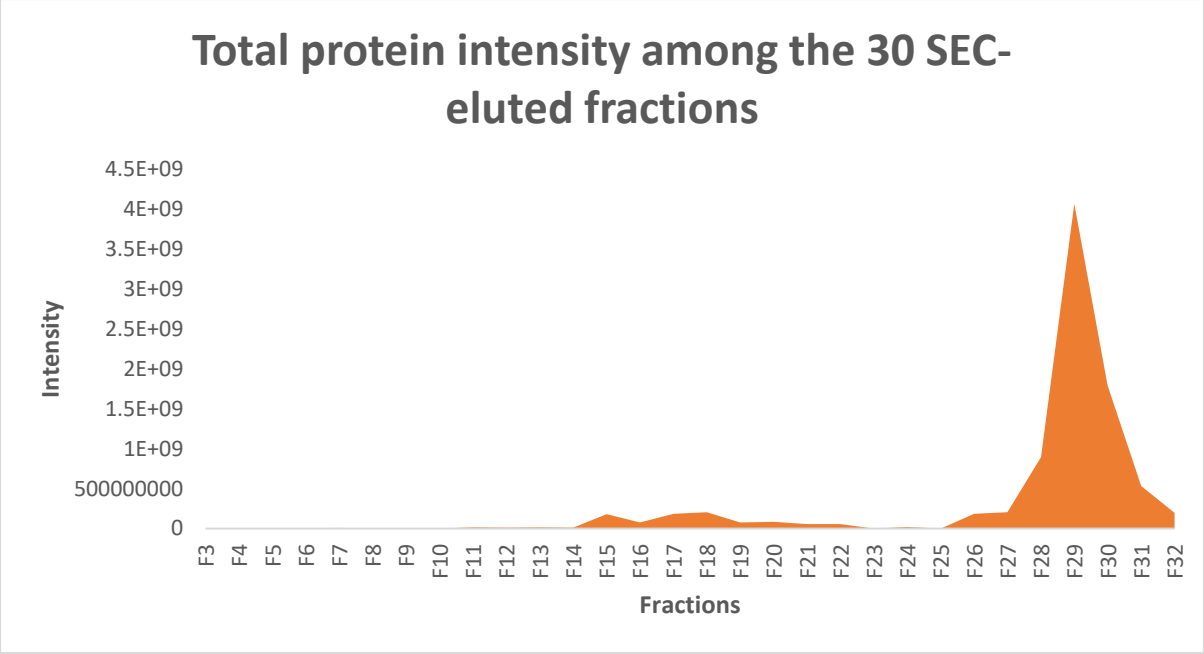

Fig 6 Total protein intensity among the 30 SEC-eluted Fractions of all the seven samples

We analyzed also the intensity of Albumin among the 30 SEC-eluted fractions of all samples, as it is reported in the Figure 7. As we can see, the most intense fractions are the 28, 29 and 30. This behavior is similar to the absorbance chart reported in Figure 5 and the total protein intensity of Figure 6.

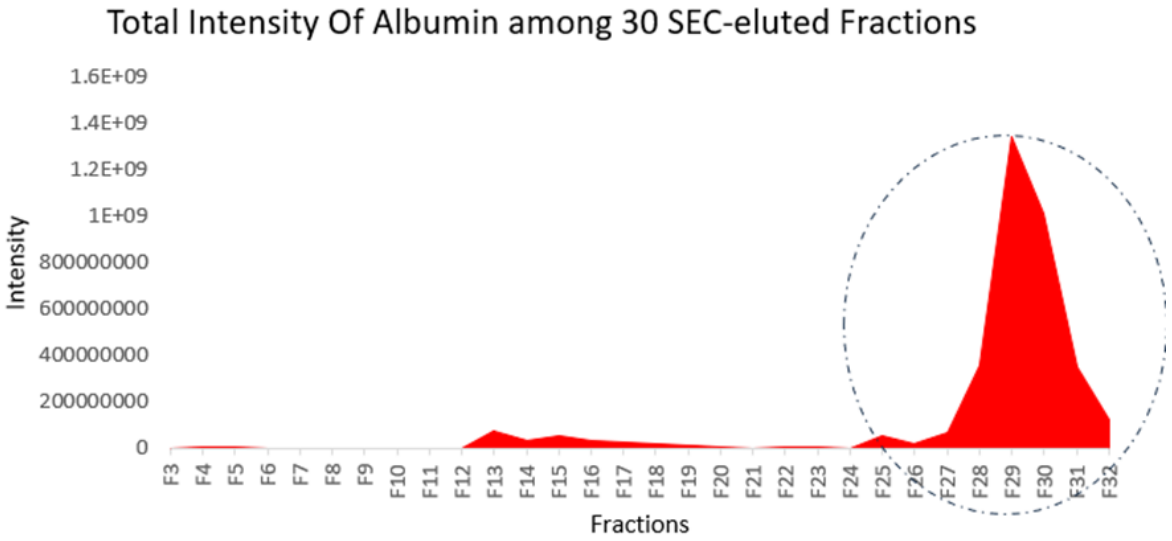

Fig 7 Total intensity of Albumin in the 30 SEC-eluted Fractions

6.1.2) Gene ontology of the identified proteins

1. **Biological process analysis**

The 1511 unique proteins detected in the fractions of the seven CSF samples are involved in different biological processes. In order to investigate the biological role of the identified proteins, we performed an enrichment analysis through STRING software. We investigated Biological processes, Molecular functions and Tissue expression: in order to select the most relevant processes, functions and expression, we used a cut-off of 0.6 strength, which measures how impacting the enrichment effect is, then we excluded redundant terms and we reported the significance of each detected process, function and expression using the -log(FDR) (the FDR refers to the p-values corrected for multiple testing within each category using the Benjamini–Hochberg procedure. Concerning the analysis of the biological processes, it emerged that the most significantly represented processes are the Intermediate filament organization(-log(FDR)= 20,327), the complement activation (-log(FDR)= 15,123) and the acute inflammatory response (-log(FDR)= 7,627). In addition, several biological processes

involved in brain homeostasis resulted significantly enriched, such as Negative regulation of axon regeneration (-log(FDR)= 7,57), Neuron recognition (-log(FDR)= 6,153), Synaptic membrane adhesion (-log(FDR)= 4,06), Axonal fasciculation(-log(FDR)= 3), Regulation of presynapse assembly (-log(FDR)= 2,79), Protein refolding(-log(FDR)= 1,987), Neuron maturation (-log(FDR)= 1,91) and Axon regeneration (-log(FDR)= 1,46).(Fig 8)

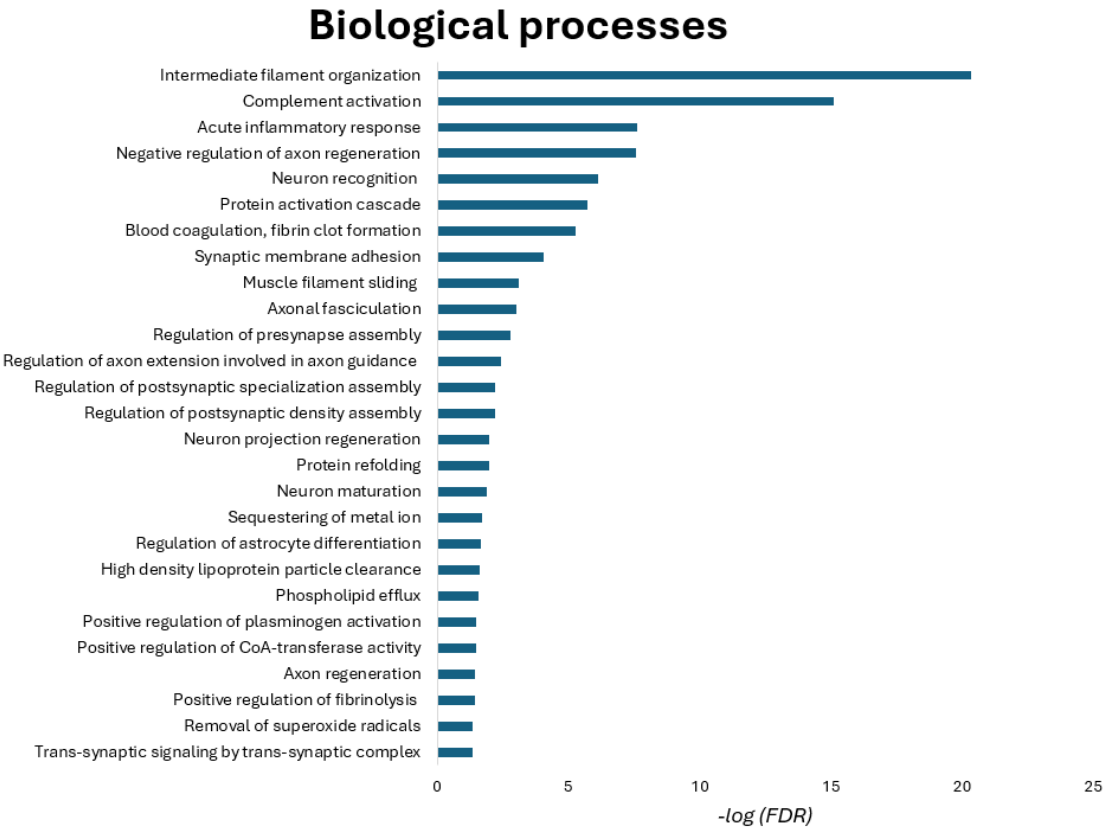

Fig 8 Most significant Biological processes of proteins identified in CSF samples

2. Molecular Function

As we can see in Fig 9 our detected proteins in the CSF samples have appropriate molecular functions. Peptidase inhibitor activity (-log (FDR)= 16,05), Glycose aminoglycan binding (-log (FDR)= 15,45), Serin-type endopeptidase inhibitor activity (-log(FDR)= 13,52), Heparin Binding (-log(FDR)= 12,18), and Growth factor binding (-log(FDR)= 7,25) are the most significant molecular function of the obtained proteins in the CSF samples. Moreover, Cell-cell adhesion mediator activity, Transmembrane receptor kinase activity, Extracellular matrix binding, Collagen binding, complement binding, Structural constituent of muscles, Proteoglycan binding, Carboxypeptidase

activity, Laminin & Opsonin binding, immunoglobulin receptor binding, and platelet - derived growth factor binding are the other molecular functions that our proteins are involved. On the other hand, our proteins play key roles in misfolded protein binding activity (-log (FDR)= 1,385) and Ciliary neurotrophic factor receptor binding (-log (FDR)= 1,369) in the cells.

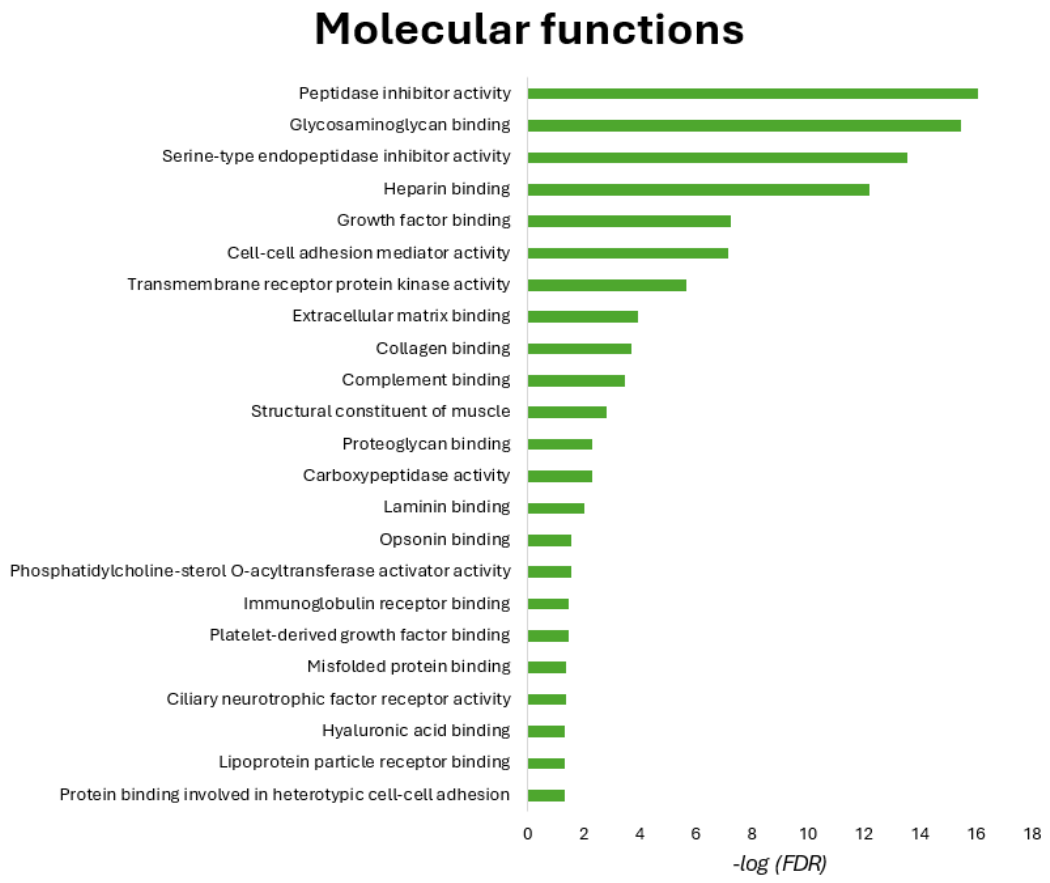

Fig 9 Most significant Molecular Function of proteins which are present in CSF samples

3. Tissue expression of the CSF proteins

As shown in Fig 10 , it is clear that most of the available proteins in the samples originate from bone marrow cells (-log(FDR)= 63,059). However, the other most relevant tissues that indicate the original expression of these proteins are plasma cells

(-log(FDR)= 57,857) , subarachnoid space (-log(FDR)= 22,762), cerebral spinal fluid (-log(FDR)= 22,429) and serum(-log(FDR)= 19,987).

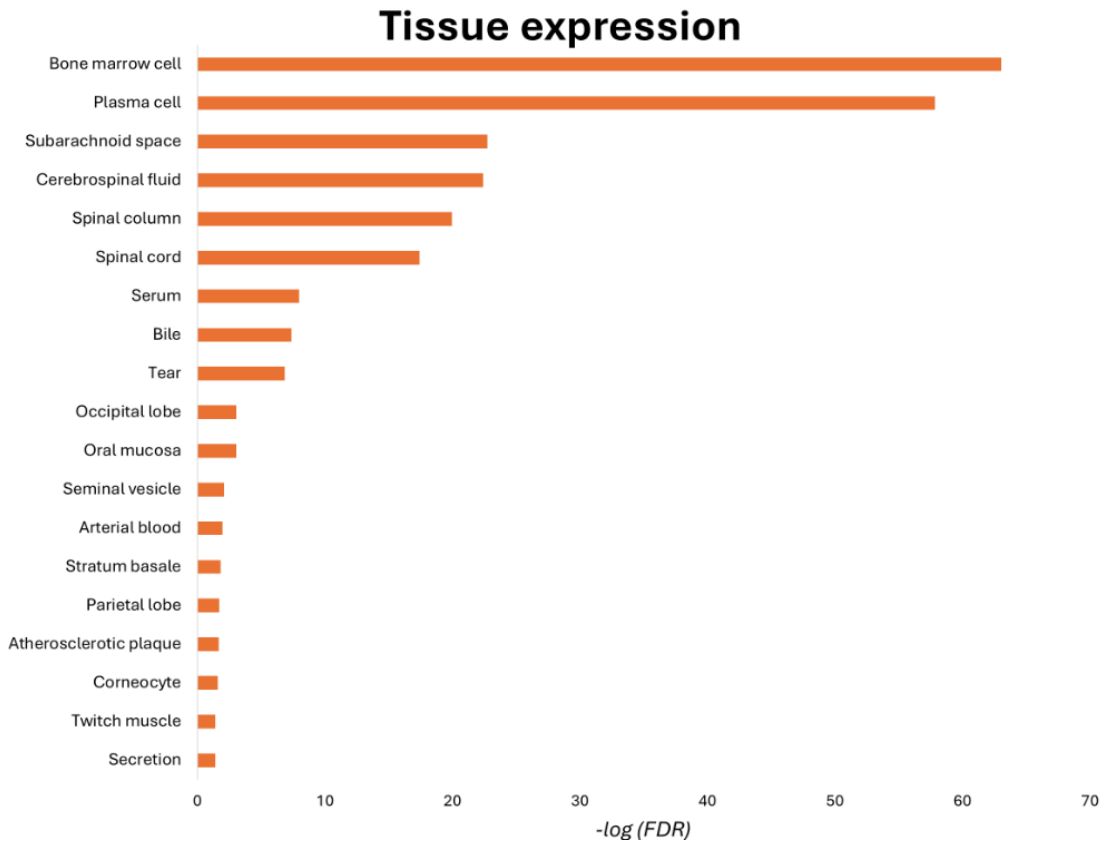

Fig 10 Most significant Tissue expression of the identified proteins

4. Cellular component analysis

We also investigated the subcellular location of the identified proteins, exploiting the Cellular Components enrichment of FunRich software. The most significant cellular components are reported in fig 11.

34% (-log10 (P-Value) =100) of the proteins in the cells are significantly originated from exosomes. Moreover, 33% of gene expression in the cells related to the extracellular matrix (-log10 (P-Value) =98). Plasma membrane is responsible of 32.8% (-log10 (P-Value) =96) of protein expression in the cells.

On the other hand, nucleosome as one of the cellular components is just responsible of 1.6% of protein expression in the cells, which is the lowest amount among other cellular components. (-log10 (P-Value) < 5)

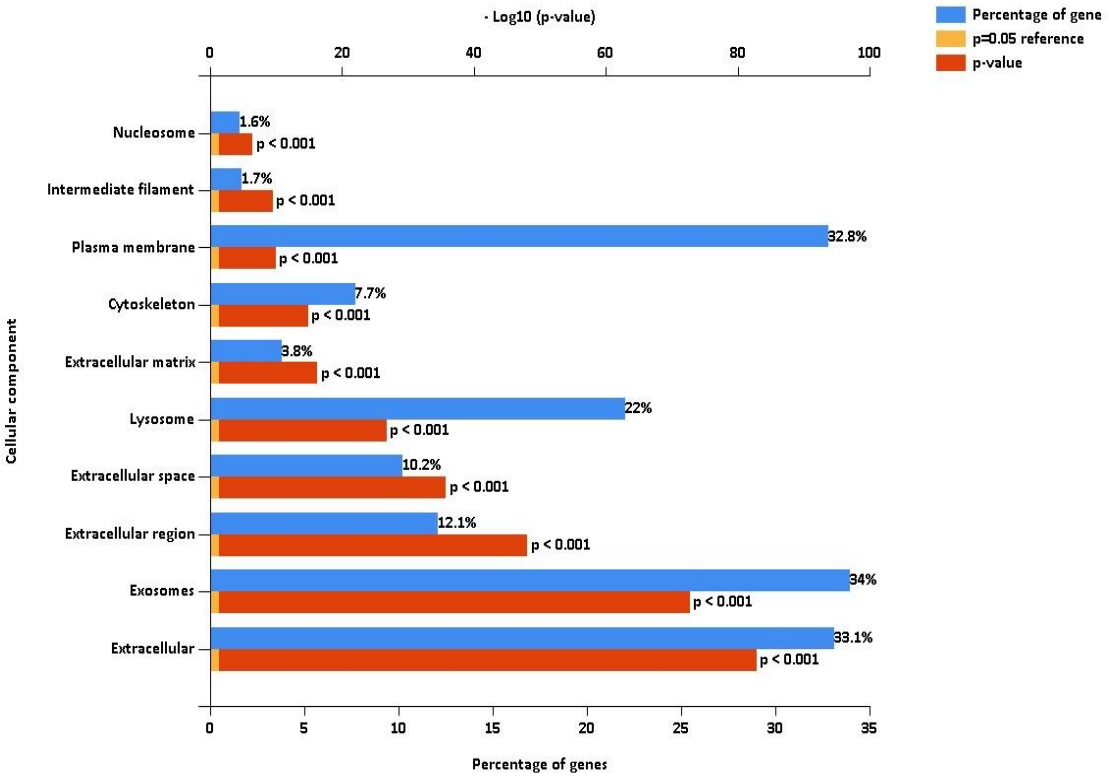

Fig 11 Cellular component analysis of CSF identified proteins

6.1.3) Protein biomarkers among the identified proteins of CSF

1. CSF protein biomarkers

Here we report the most important and relevant protein biomarkers that we have detected in the CSF samples of AD and NN patients. In particular, **Table 1** illustrates a list of biomarkers relevant for neurodegenerative diseases, detected in the analyzed CSF samples.

Name of protein biomarker	Biological Function	Role in neurodegeneration
Brevican core protein	It plays role in nervous system differentiating	It is cleaved during neurodegeneration, and their fragments were indicated as potential blood biomarkers of neurodegenerative diseases, like dementia
Semaphorin-7A	It Plays an important role in inflammation, axon growth,integrin-mediated signalling	Aberrant Semaphorin expression may result in altered neuronal connectivity or synaptic function and inflammation associated with a number of degenerative neuronal disorders like Alzheimer
Neurofascin	It is involved in Neural cells growth	It was reported its involvement in Neurodevelopmental disorder with central and peripheral motor dysfunction (NEDCPMD)
Calsyntenin-1	It Promotes synapse development by acting as a cell adhesion molecule	It Stabilizes Amyloid-beta precursor protein (APP) metabolism and enhances APBA2-mediated suppression of beta-APP40 secretion

Superoxide dismutase(SOD1)	It protects against damage mediated by free radicals of oxygen.	Mutant SOD1 protein form aggregates that damage motor neurons and it is frequently mutated in Amyothrophic lateral sclerosis
Apolipoprotein E (Apo-E)	It Associates with lipid particles, to transport lipids between organs via plasma and interstitial fluids	It has a known role in Alzheimer disease, where regulatesamyloid-β aggregations and protein clearancein the brain
Lactotransferrin	It binds and transports iron and aluminium	Iron and Aluminium ions' impairment are involved in the pathogenesis of many dementia-like diseases.
Amyloid-beta precursor protein (APP)	Serves as cell surface receptor, neurite growth, neuronal adhesion and axonogenesis	They aggregate, forming plaques and cerebrovascular residues which causes neurotoxic effects which characterized Alzheimer patients
Calgranulin-B	It has a role in the regulation of inflammatory processes and immune response	It is reported as increased in Alzheimer
Clusterin	It is an extracellular chaperone that prevents aggregation	It delays Tau fibril formation but enhanced the activity of Tau oligomers to seed aggregation of endogenous Tau in vitro. It

	of non-correctly folded proteins	also inhibits the propagation of α-Synuclein aggregates associated with Parkinson's disease.
Insulin-like growth factor-binding protein 3	It is elated to inflammation and cell-cell adhesion.	In AD brains, the expression of IGFBP-3 was found to be increased

Table 1: Most relevant protein biomarkers identified in CSF from MS patients. Protein names, biological roles and references are also reported

Referring to **Table 1,** we have detected some interesting markers: I) Semaphorin 7A**,** which is a protein that plays a role in the immune system and the central nervous system. and it is reported also to be involved in the pathogenesis of Multiple sclerosis (https://doi.org/10.1038/nature01790); II) Neurofascin, that is expressed in both immature and mature neurons. It is an important player in neurite outgrowth and post-synaptic structure in immature neural cells. In mature neural cells, it stabilizes the synaptic structure.(https://doi.org/10.1016/j.neuron.2011.02.021) (https://doi.org/10.1016/j.biocel.2012.01.012). III) Calsyntenin-1 is a protein known as the Alzheimer-related cadherin-like protein. Calsyntenin-1 stabilizes APP metabolism and improves the way APBA2 stops beta-APP40 from being released by slowing down the maturation of APP inside cells. IV) Superoxide dismutase 1 has a great tendency to aggregate in neural cells. The aggregated form of this protein is the result of the mutant SOD1 gene, which causes cytotoxic effects in the nervous system and it is the key feature of amyotrophic lateral sclerosis (https://doi.org/10.1016/j.bbrc.2006.06.092). V) Amyloid-beta precursor protein is responsible for forming plaques in Alzheimer patients' brain, causing the damage of neural cells **(**https://doi.org/10.1073/pnas.88.22.10302**).**

6.1.4) CSF protein complexes

To study the SEC-elution behavior of the identified and quantified proteins, the intensity of each detected protein was considered across each of the 30 SEC-eluted fractions. Subsequently the obtained profile was analyzed. We considered the molecular weight of each protein that was found based on both literature and the one that correspond to the fraction, which was previously determined fractionating a standard molecular weight mix with the same LC-run used for the CSF samples. For example, if we found a protein in one SEC-eluted fraction, we searched its molecular weight in literature and complex database and we compared this molecular weight with the one suggested from the SEC-fraction where it was detected. In this way, we can understand the conformational structure of this complex in these eluted fractions. Moreover, we calculated the p-Value and Fold Change for this putative complex. In this way we can illustrate the behavior of that proteins in the groups of ALZ and NN patients. We reported the most significant examples. The list of the most relevant complexes, for neurodegeneration and neuroinflammation, that we identified in CSF is reported in Table 2.

Complex name	Biological role	Reference
ATXN1-CIC complex	Neurodegeneration	"Disruption of the ATXN1–CIC complex causes a spectrum of neurobehavioral phenotypes in mice and humans"
STI1-Hsp70-Hsp90	Cellular stress response, Neurodegeneration	"The Hsp70/Hsp90 Chaperone Machinery in Neurodegenerative Diseases"
HSP70-MBP complex	Cellular stress response, Neurodegeneration	"Hsp70 and Its Molecular Role in Nervous System Diseases"
TTR-RBP complex	Retinol transport, Neurodegeneration	"Transthyretin and familial amyloidotic polyneuropathy, Recent progress in understanding the molecular mechanism of neurodegeneration"

Calprotectin	Immune response, Neuroinflammation	"Calprotectin levels in the cerebrospinal fluid reflect disease activity in multiple sclerosis"
CP-MPO complex	Immune response, Inflammation	"Interaction of ceruloplasmin, lactoferrin, and myeloperoxidase"
PRNP homo-oligomer-complex	Protein aggregation, Neurodegeneration	"Importance of dimerization in aggregation and neurotoxicity of Prion and a-Synuclein in prion and Parkinson's diseases"
C1q complex	Immune response, Neuroinflammation	"Emerging Roles of Complement Protein C1q in Neurodegeneration"
Reelin complex	Neural development, Neurodegeneration	"Reelin in Alzheimer's Disease, Increased Levels but Impaired Signaling: When More is Less"
Amyloid beta precursor complex	Protein aggregation, Neurodegeneration	"Amyloid β protein toxicity mediated by the formation of amyloid-β protein precursor complexes"
S100A10-annexin A2-AHNAK	Neurodegeneration	"Modulation of Ion Channels and Receptors by p11 (S100A10)"
Apolipoprotein A-II Complex	May stabilize HDL (high density lipoprotein) structure by its association with lipids	lowered apolipoprotein A (ApoA) levels are observed in patients with schizophrenia and deficit schizophrenia
Brevican Complex	Plays role in neural growth	Brevican is a central nervous system (CNS)-specific

		chondroitin sulphate proteoglycan (CSPG) primarily expressed in astrocytes and neurons Elderly Cognitively Impaired by this protein
Ceruloplasmin complex	It is involved in iron transport across the cell membrane	Clinical features consist of the triad of retinal degeneration, diabetes mellitus and neurological disturbances
Homodimer N-acetylglucosamine-1-phosphotransferase subunit gamma Complex	It is an enzyme that catalyzes the formation of mannose 6-phosphate (M6P) markers	The decreased level of this complex is related to increase rate of neural cell degeneration

Table 2: Most relevant protein complexes identified in CSF from AD patients. Complex names, biological roles and references are also reported.

We also analyzed the regulation of these proteoforms between ALZ and NN patients, identifying several significant up-regulated and down-regulated protein complexes and structures. Here we report some of the most relevant.

1. **Apolipoprotein A-II homodimeric complex**

   Apolipoprotein A-II (APOA2) is a homodimer protein complex, with a molecular weight of 36 kDa. (Uniprot). This apolipoprotein can also create heterodimeric complexes with APOD. In our data, the SEC-elution profile of APOA2, displays two different most intense areas in correspondence of fractions 7–21 and 22–32. In particular, in the area of the elution profile between fraction 22 and fraction 23, which corresponds to a mean molecular weight of almost 90 kDa, a significant intensity modulation between ALZ patients and NN patients is present.(p-Value: 0.02)(Fig 12)

The molecular weight of nearly 90kDa corresponds to an Homotetrameric form of APOA2 and it is significantly decreased in ALZ patients as respect to NN patients. (FC: 0.18)(Fig 13)

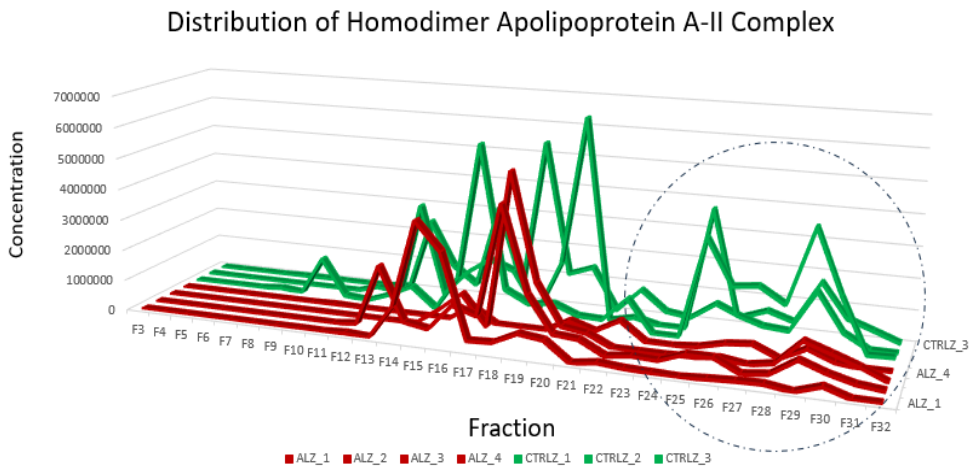

Fig 12 Distribution of Homodimer APOA2 Complex in 30 SEC-eluted fractions.

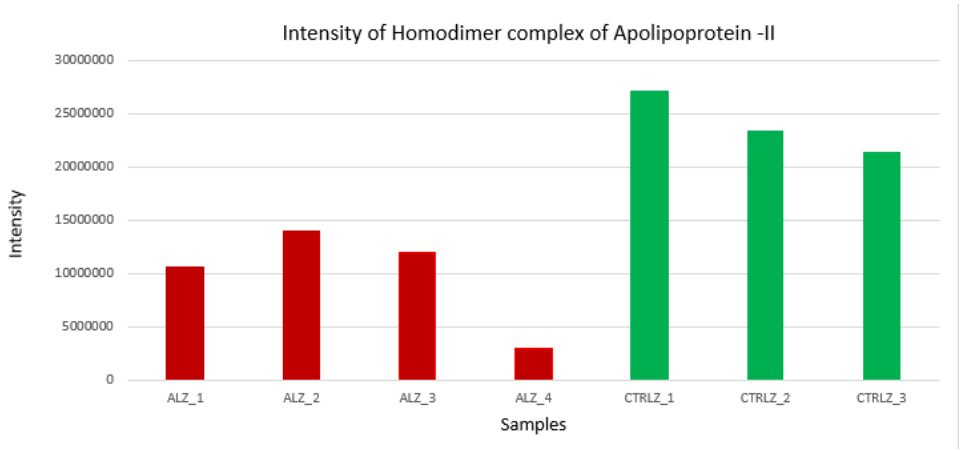

Fig 13 Intensity of Homodimer APOA2 in the AD and NN patients.

2. Truncated form of Brevican core protein

Brevican is a 99 kDa protein that is usually cleaved during neurodegeneration and, in our experiment, we obtained this protein between fraction 26 and 32 which

corresponded to the molecular weight of 67kDa and this form is significantly increased in Alzheimer samples as respect to NN patients (p-value :0.006; FC: 2.78) (Fig14). So, we can assume that we have detected this increased cleaved form of Brevican complex in our Alzheimer samples. (Fig15)

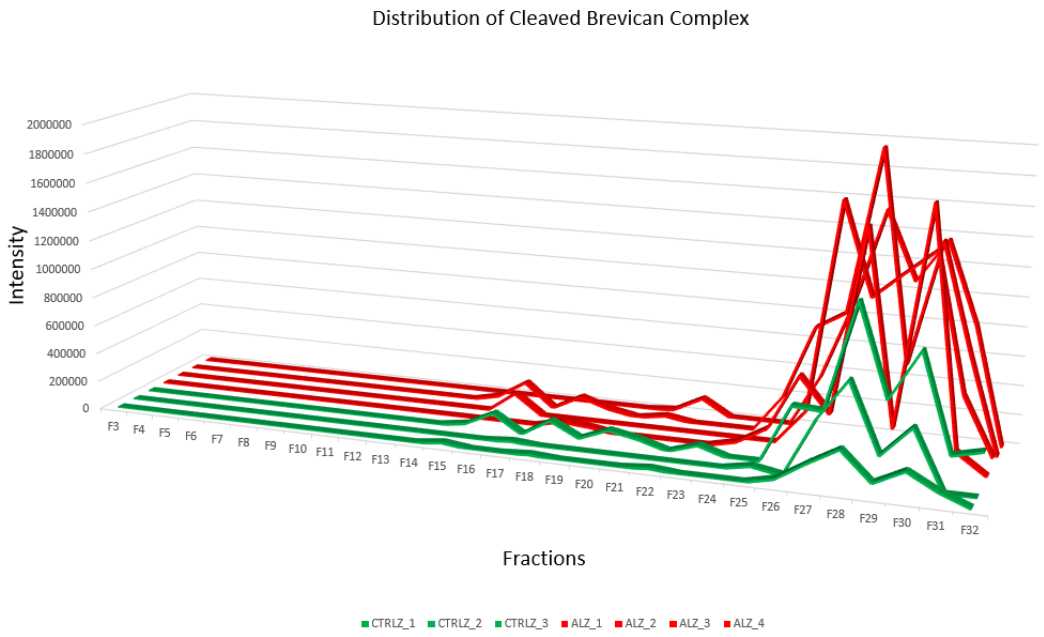

Fig 14 Distribution of Cleaved Brevican Complex in 30 eluted fractions.

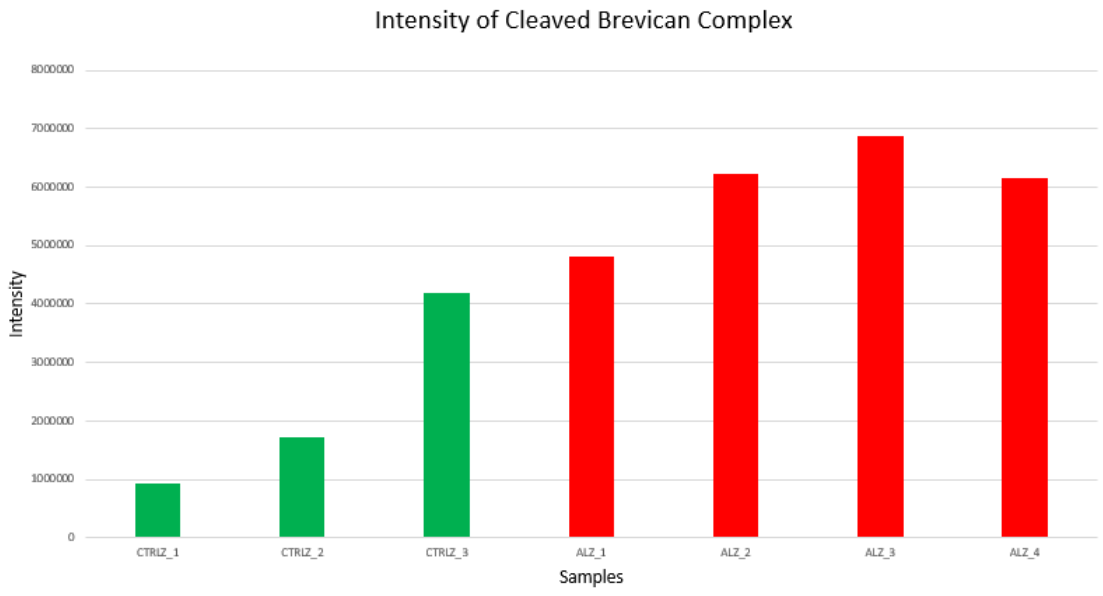

Fig 15 Intensity of Cleaved Brevican Complex in Samples

3. Homotrimeric Ceruloplasmin

Ceruloplasmin is a 122 kDa protein that can be found in complex with Lactotransferrin. However, in our data we detected this protein between fraction 13 and 16 which is related to the molecular weight of 366 kDa which corresponds to Homotrimeric form and we did not detect in Heterocomplex with LTF. (Fig 16)

This protein complex is significantly increased in CSF of NN patients' samples with respect to Alzheimer Samples (p-value :0.02; FC: 0.324) (Fig 17)

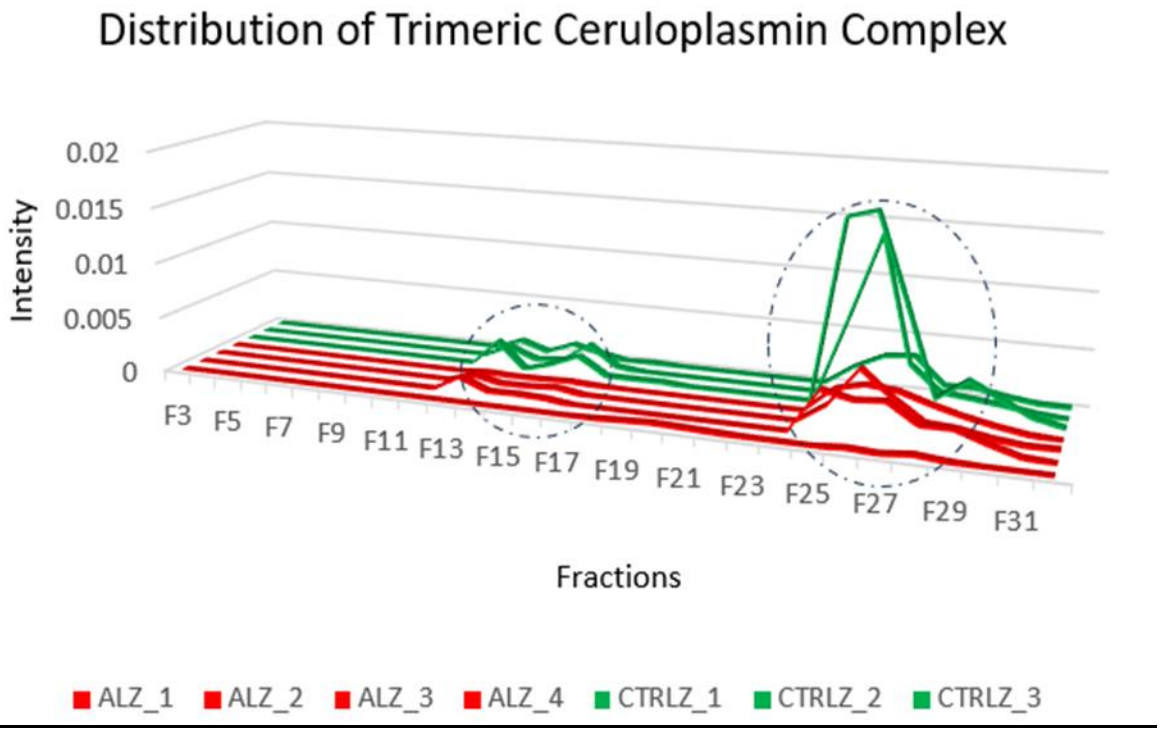

Fig 16 Distribution of Trimeric Ceruloplasmin Complex in 30 SEC-eluted fractions

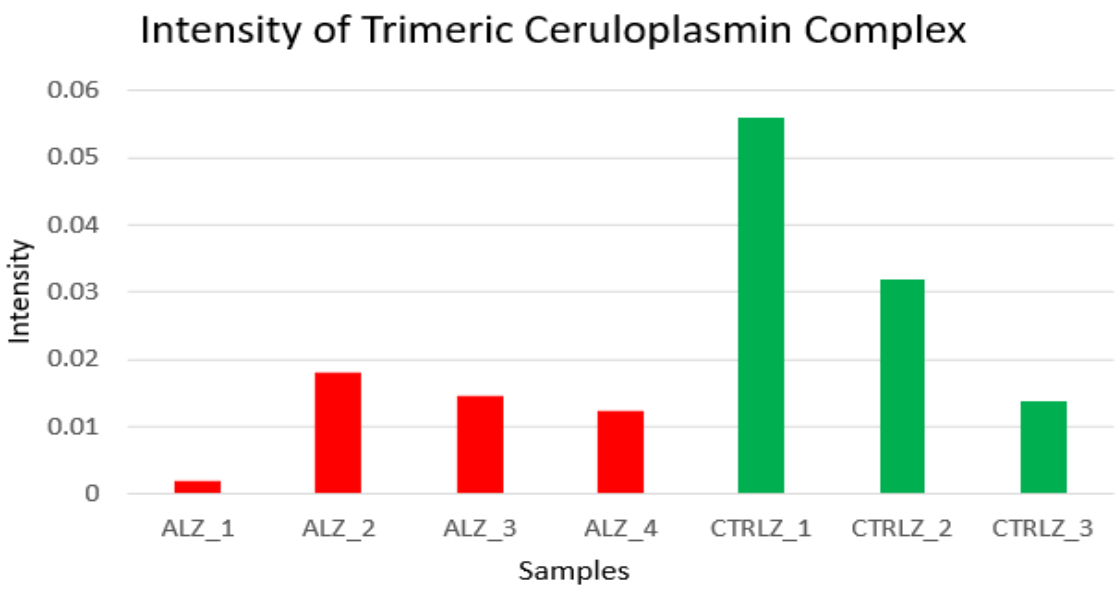

Fig 17 Intensity of Trimeric Ceruloplasmin Complex in Samples

4. **N-acetylglucosamine-1-phosphotransferase subunit gamma homodimeric complex**

The N-acetylglucosamine-1-phosphotransferase subunit gamma complex is a 67-kDa homodimer protein complex. We can see from our data that this complex is much more common in the fractions 28–30 of SEC-eluted fractions of control CSF samples than in Alzheimer's CSF samples (p-value :0.018, FC: 0.084). (Fig18). As anticipated, the average molecular weight of fractions 28, 29, and 30 is 66 kDa, aligning with the molecular weight of this homodimer complex (Fig19).

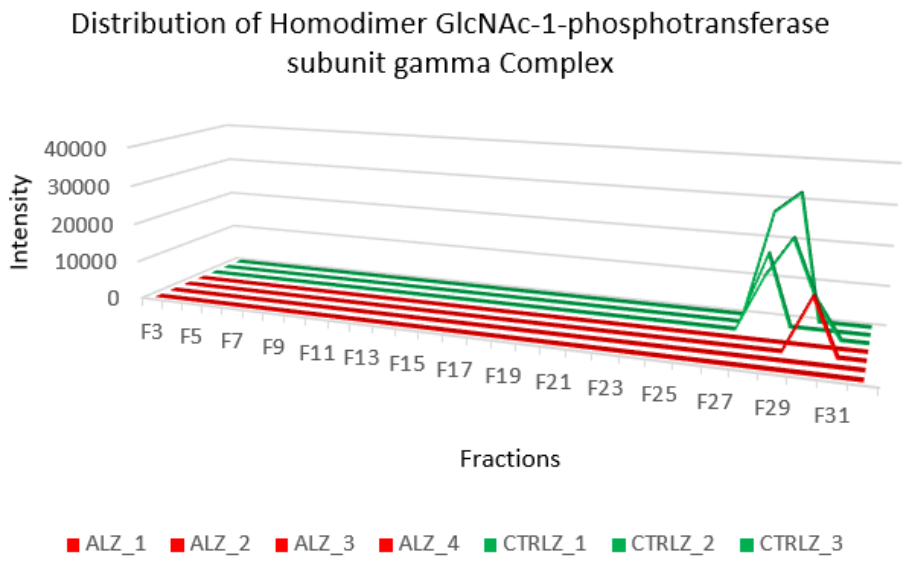

Fig 18 Distribution of Homodimer GlcNAc-1phosphotransferase subunit gamma Complex in 30SEC-eluted fractions

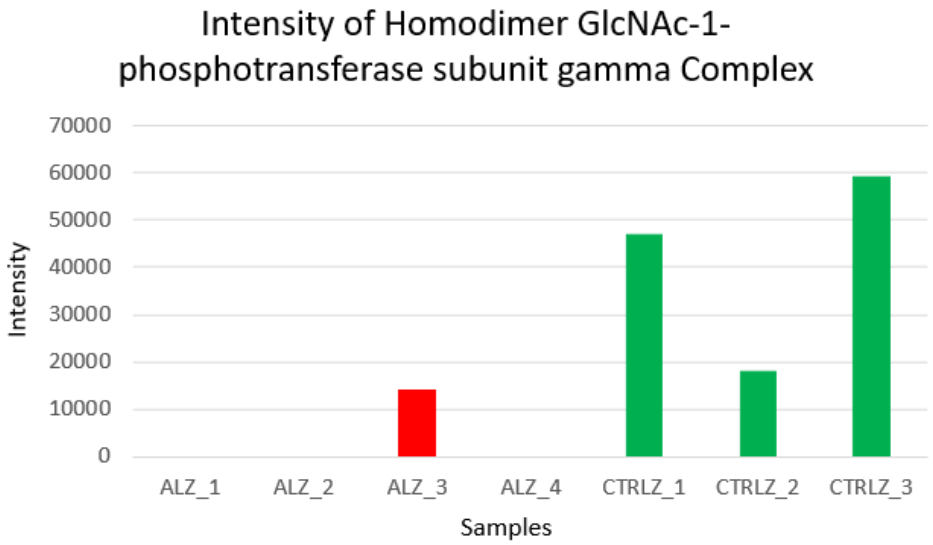

Fig 19 Intensity of Homodimer GlcNAc-1phosphotransferase subunit gamma Complex in the Samples

7) Discussion

In the present research we have performed untargeted analysis of the CSF samples of Alzheimer patients (ALZ patients) and patient with non-neurodegenerative conditions (NN patients) characterizing the biological pathways and functions of the detected proteins, finding a list of putative CSF protein biomarkers and, protein Complexes modulations among the two groups, through size-exclusion chromatography under native conditions, LC-MS analysis and bioinformatic research. Here we discuss about the most significant results that validate our method for obtaining protein biomarkers and protein complexes in cerebrospinal fluid.

7.1) Method assessment

The initial part of the results that we obtained is related to the chromatographic profile of the 30 SEC-eluted CSF fractions which were collected from the five runs of CSF sample through size-exclusion chromatography. In Fig 7, which is refers to the intensity of Albumin (one of the most abundant and stable proteins in the CSF), among the 30 SEC-eluted fractions obtained from the fractionation the CSF samples. As we can see in this graph, the most intense part of the chromatograph of Albumin is located in the last 10 SEC-eluted fractions, especially in fraction 28, 29 an 30, which correspond to a medium molecular weight of 66 kDa, which rightly corresponds to Albumin molecular weight reported in literature (66,5 kDa). Correctly, from the figure of the Absorbance of protein BCA assay among the fractions (Fig5) it is clear that the absorbance of the protein in BCA assay and the intensity of the Albumin have a significant association, since they have peaks in the same fractions. This indicates that the most abundant protein concentration among CSF SEC-eluted fractions are the ones in which Albumin elutes. After fractionation, the proteins were extracted, digested and prepared to be analyzed through LC-MS and the mass spectra are searched against human protein database in order to obtain a list of proteins, with their quantification, among fractions for all samples.

7.2) CSF biomarkers

Before moving to the structural proteomic analysis, we have focused on the identities of the proteins found in the cerebrospinal fluid of ALZ and NN patients, with the aim of finding out putative CSF biomarkers. We detected several proteins with a known role in neurodegeneration. Here we discuss some examples of particular interest. For instance, we detected Semaphorin-7A, which exerts its function in the immune system and the central nervous system. In particular, in the brain it has an important role in axon growth (Jeroen Pasterkamp et al., 2003) and it is reported also to be involved in the pathogenesis of Multiple sclerosis (Semaphorin 7A as a Potential Therapeutic Target for Multiple Sclerosis). In addition, Semaphorins family is related to the axon-guidance, whose impairment is involved in the onset and progression of Alzheimer disease. These proteins, indeed, plays crucial functions in the brain: at hippocampal level, they prompt the synaptic signalling and mediate the migration of neural cells. They also have an effect on memory pathways and enhance dendritic growth (Roles and Mechanisms of Axon-Guidance Molecules in Alzheimer's Disease)**.** Another example is Neurofascin, which is an important player in neurite outgrowth and post-synaptic structure formation in immature neural cells, while in mature neural cells, it stabilises the synaptic structure. (Zonta et al., 2011); (Kriebel et al., 2012) The decrease of this protein concentration is also significantly related to neurodegenerative disorders, comprising Alzheimer (Brinkmalm et al., 2018) Also Calsyntenin-1 has a relevant role in brain during neurodegeneration: indeed, this protein is known as the Alzheimer-related cadherin-like protein, since it stabilises APP metabolism and improves the way APBA2 stops beta-APP40 from being released by slowing down the maturation of APP inside cells (PubMed:12972431). Researchers have demonstrated a decrease in calsyntenin-1 in Alzheimer's disease' brains. The lower amount of this protein is linked to changes in the processing of APP, which causes more amyloid-beta aggregation (Vagnoni et al., 2012). We also found Superoxide dismutase 1, which has already a well-known role in Amyotrophic Lateral Sclerosis: SOD1 has a great tendency to aggregate in neural cells. The aggregated form of this protein is the result of the mutant SOD1 gene, which causes cytotoxic effects in the nervous system, characterizing the molecular pathological pattern of ALS (Fei et al., 2006) The alteration of SOD1 is also present in AD. In particular, the pathogenic effect of this protein arises when the capacity of the protein of binding zinc ions is affected (Structural effects of stabilization and complexation of a zinc-deficient

superoxide dismutase). Another widely described protein that we have also detected is Amyloid-beta precursor protein, which is responsible for the formation of plaques in Alzheimer patients' brain, causing the damage and the consequent death of neural cells (Van Nostrand et al., 1991) The identification of these proteins in our SEC-eluted fractions from CSF, underlines that with our workflow we are able to detect and monitor key proteins for neurodegeneration in general and specifically for Alzheimer's disease, opening possible perspectives for the use of these proteins in the context of CSF-based liquid biopsy.

7.3) Gene ontology of the identified proteins

To characterize the biological features of the identified proteins, we investigated biological processes, Molecular functions and Tissue expression through STRING software. Concerning the analysis of the biological processes, it emerged that the most significantly represented processes are the Intermediate filament organization, the Complement and the Acute inflammatory response. In addition, several biological processes involved in brain homeostasis resulted significantly enriched (Negative regulation of axon regeneration, Neuron recognition, Synaptic membrane adhesion, Axonal fasciculation, Regulation of presynapse assembly, Regulation of axon extension in axon guidance, Regulation of postsynaptic specialization assembly, Regulation of postsynaptic density assembly, Neuron projection regeneration, Neuron maturation, Regulation of astrocyte differentiation, Axon regeneration, Trans-synaptic signalling by trans-synaptic complex). The most significant biological process is Intermediate filaments: this kind of filaments are the major components of neurons' cytoskeleton and, dysregulation and aggregation of these components are reported to be a typical sign during neurodegeneration. However, the mechanism that provoke the formation of these aggregates is actually unclear, and in literature there are also some evidence of a beneficial effect of aggregation of some specific types of intermediate filaments in motor neuron disease (Perrot & Eyer, 2009) Then, Complement is reported as one of the most significant processes and its dysregulation is a frequent event in NDD. In addition, a crescent number of works indicate its up regulation as a key player in the onset and progression of these kind of diseases (Targeting complement in neurodegeneration: challenges, risks, and strategies). Indeed, an increase of the

complement-promoted synaptic pruning can be a toxic event in neurodegeneration and the presence of an augment of proteins involved in complement activation is clearly a detrimental signature. For these reasons, there are already several research that indicate complement cascade as a possible source of target for NDD (The good, the bad, and the opportunities of the complement system in neurodegenerative disease). Even Acute inflammatory response, which is the third most significant process, is already well described in NDD, and it represents both a key aspect of neurodegeneration and an actor of its onset: the aggregates which are typical in NDD lead to neuroinflammation, that further induce aggregation, in a continue pathological cycle. Since evidence reported that inflammation is an earlier event as respect to protein aggregation, it might be crucial to target its mediators, in order to exert a therapeutic action during a very early stage of NDD (Role of neuroinflammation in neurodegeneration development). Then, several biological processes related to the regulation and maintenance of neural environment are reported. For example, there are processes involved in synapsis assembly and signalling (Synaptic membrane adhesion, Regulation of presynapse assembly, Regulation of postsynaptic specialization assembly, Regulation of postsynaptic density assembly and Trans-synaptic signalling by trans-synaptic complex): synaptic dysfunction is a key aspect of NDD, specifically, it is reported to be involved in the pathogenesis of Alzheimer's disease and Parkinson's disease. In fact, it concurs to detrimental protein accumulation, which leads to neural loss. Also axonal regeneration and neural cells maturation and differentiation are processes involved in NDD. In fact, axonal regeneration has been indicated as a possible strategy for therapeutic options both in AD and PD, however, many limitations are present for the developing of these therapeutic options (From cradle to grave: neurogenesis, neuroregeneration and neurodegeneration in Alzheimer's and Parkinson's diseases). Astrocytes impairment is relevant too in neurodegeneration, since it is caused by neurodegenerative environment and can result in further detrimental alterations: astrocytes differentiation driven by a maladaptive transition can impair some brain areas, however further research on the role of these cells is required (Astrocytes in selective vulnerability to neurodegenerative disease). As regard to molecular functions, the most significant ones are Peptidase inhibitor activity, Glycosaminoglycan binding and Serine-type endopeptidase inhibitor activity. Peptidase inhibitor activity comprises pathways whose proteins are enriched in tau-transgenic mice and have a role in tau-induced neurodegeneration (Peptidomic Approaches and Observations in Neurodegenerative Diseases). Even Glycosaminoglycan binding can be connected to neurodegenerative environment: intriguingly, inside protein aggregation plaques, like amyloid beta aggregates and neurofibrillary tangles, which are both

found in Alzheimer disease, sulphated glycosaminoglycans were also detected (Glycosaminoglycans and β-amyloid, prion and tau peptides in neurodegenerative diseases). Moreover, serine endopeptidase like Prolyl oligopeptidase, is a key actor in brain homeostasis, since it is involved in cognitive function, it controls the level of brain inositol 1,4,5-triphosphate and neuronal signal transduction. According to recent research its impairment might be involved in molecular patterns of Alzheimer disease. (Issues About the Physiological Functions of Prolyl Oligopeptidase Based on Its Discordant Spatial Association With Substrates and Inconsistencies Among mRNA, Protein Levels, and Enzymatic Activity). Other interesting and significant reported molecular functions are Misfolded protein binding and Ciliary neurotrophic factor receptor activity. The former is a function clearly associated to Neurodegeneration since decades: indeed, unfolded proteins become insoluble and lead to the formation of amyloid aggregates , which are a key feature of the wide majority of ND, obviously comprising Alzheimer disease (Quantifying misfolded protein oligomers as drug targets and biomarkers in Alzheimer and Parkinson diseases). The Ciliary neurotrophic factor receptor activity instead, is a function related to the effect of Ciliary neurotrophic factor (CNTF). CNTF acts as a transcription factor and ensures neuronal survival and differentiation at motor neuron level. Given its important roles in brain homeostasis' maintenance, it has been indicated as a possible preventing agent in neurodegeneration , especially in amyotrophic lateral sclerosis (Ciliary neurotrophic factor (CNTF): New facets of an old molecule for treating neurodegenerative and metabolic syndrome pathologies). These findings emerged from the Gene ontology analysis are fascinating, since highlight the presence, in our analysed CSF, of proteins that belongs to key biological pathways in neurodegenerative diseases, and so they have the potential to be tested as potential therapeutic targets. Finally, the tissue expression analysis revealed that the most significantly represented tissue, based on the expression of the identified proteins, is Bone marrow, followed by Plasma, subarachnoid space, cerebrospinal fluid, Spinal column and spinal cord. While it is obvious that a great number of the identified proteins derive from cerebrospinal fluid and anatomical structures in contact with cerebrospinal fluid (Spinal cord and column and different brain's areas), the heavy presence of proteins derived from bone marrow might be explainable considering the presence of dura-skull channels, which connect cerebrospinal fluid flux and bone marrow niches in the skull. It is also reported that this connection via cerebrospinal fluid can mediate immune response at central nervous system level (Cerebrospinal fluid regulates skull bone marrow niches via direct access through dural channels). Concerning plasma tissue expression, it is known that an exchange between plasma and cerebrospinal fluid proteins can occur at the level of choroid plexus. This

structure forms the barrier between cerebrospinal fluid and plasma and its integrity is crucial for avoiding brain damages. In fact, its perturbations have been already described in the pathology of several diseases, comprising NDD like Alzheimer disease. More precisely, β-amyloid in choroid plexus is related to an augment of immunoglobulins that can provoke damages at capillaries' level and can also boost the secretion of pro-inflammatory cytokines, in turns provoking damage in the barrier. (Choroid plexus and the blood–cerebrospinal fluid barrier in disease). As regard to Cellular component analysis, the most represented cellular compartments are Extracellular region, space and matrix, as we can expect, since we used an extracellular fluid as protein source. Other represented components are lysosomes, which are organelles whose function is frequently impaired in neurodegeneration. Specifically, there is a crescent number of evidence indicating a connection between the corruption of autophagy-lysosomal pathways and AD development (Impairment of the autophagy–lysosomal pathway in Alzheimer's diseases: Pathogenic mechanisms and therapeutic potential).

7.4) CSF protein complexes

Among the CSF protein complexes detected, several of them have known roles in neurodegeneration, neuroinflammation but also in immune defense/response, retinol transport, cellular stress response and neuronal development. For example, the amyloid-β protein precursor is the protein from which the amyloid β-protein derives, which is the toxic peptide at the basis of Alzheimer's disease pathogenesis. We detected amyloid-β protein precursor, which is a protein of 87kDa, at a molecular weight of nearly 350kDa, corresponding to the tetrameric complex. Lu et al. have reported that two amyloid-β protein precursor proteins can be complexed with each other, and it seems that soluble amyloid β protein accelerates the formation of this complex. The formation of this complex leads to an increase of neuronal cell death. So, the amyloid β peptide might exert its toxic function also by favoring the formation of this complex. Amyloid β expends its harmful activities in Alzheimer's disease, however, in the last past years it has emerged a role also in other neurodegenerative diseases including multiple sclerosis: indeed, amyloid β levels are increased in the CSF and brain tissue of MS patients. For these reasons it might be useful to detect and monitor the presence and abundance of amyloid-β protein precursor complexes, which are a toxic product of amyloid-β, in the CSF of MS patients. Major prion protein is a small protein of nearly 27kDa with a not well elucidated physiological function. Instead, it has been well described in a considerable number

of papers that the dimerization of major prion protein could be a crucial event in the formation of toxic aggregates during neurodegenerative conditions. The dimerization of prion protein has been reported in prion and Parkinson disease and spongiform encephalopathies. Other studies suggested that the homodimerization of this protein is a consequence of the stress response and it is more susceptible to the α-cleavage, which is a key event to avoid the toxic aggregate formation, offering a therapeutic option for the treatment of prion disease. Thus, the homodimerization might be both beneficial and detrimental, reasonably depending on the context. Our data suggest the presence of the homodimeric form of prion protein with a molecular weight of nearly 50kDa. Calprotectin is a protein complex composed by the protein S100-A8 and the protein S100-A9. It is released by monocytes during inflammatory response and its presence in cerebrospinal fluid could be a signal of neuroinflammation. In particular, a high concentration in CSF might be positively associated to the phase of relapse of MS. Calprotectin is secreted as a heterodimer, and it associates into an heterotetramer as a consequence of the increase of calcium levels. It has been reported that neuronal calcium overburden leads to axonal damage , and the augment of CSF calcium concentration has been correlated with neurodegeration . For this reason, also the calcium-dependent heterotetramerization of calprotectin might be associated with neurodegenerative processes and future comparisons between CSF heterotetramer levels of MS patients with different severity or during the relapse and the remission phases could be highlighting to elucidate the correlation that links inflammation and neurodegeneration. Another interesting complex related to inflammation is the Ceruloplasmin-Myeloperoxidase complex: during an inflammatory state, ceruloplasmin (CP) associates to myeloperoxidase (MPO). Ceruloplasmin is a protein of a molecular weight of 132 kDa and Myeloperoxidase is a homodimer of 140 kDa: these two proteins form a complex in a 2:1 stoichiometric ratio, for a total molecular weight of nearly 400kDa . Ceruloplasmin abnormalities have been associated with neurodegenerative diseases and Myeloperoxidase has been related to tissue injury in several diseases comprising multiple sclerosis , however, the role of their complex in this kind of disease is still undefined. The association of the two proteins into the complex results in a modification of both their activity: ceruloplasmin is able to oxidate different substrates such as copper and iron, serotonin and epinephrine and the lack of its activity leads to oxidative stress, and it seems to provoke neurodegeneration. On the other hand, myeloperoxidase catalyzes the formation of reactive species to act against microbial pathogens during infection, and it potentially have cytotoxic effects. It has been suggested that the complex formed by CP and MPO decreases the oxidative stress during inflammation, by blocking the action of MPO. However, the massive

ceruloplasmin binding to myeloperoxidase would avoid also the exertion of its oxidant activities on other fundamental substrates, maybe resulting in another damage. To elucidate its real role in neuroinflammation and neurodegeneration, it could be interesting to compare the levels of CP-MPO complex and the free form of CP and MPO between MS patients with different severity or during relapse and remission phases. Finally, Reelin is a large protein of nearly 386 kDa which is fundamental for brain development, synaptic formation, dendritic growth, and memory. Reelin can homodimerize and both the monomer and the homodimer can bind to the ApoER2 receptor, nevertheless only the dimeric form can activate the signaling pathway . Moreover, the truncated form of Reelin leads to the formation of huge complexes, larger than a homodimer, which are unable to trigger the signaling. The increase of these larger complexes has been associated to Alzheimer's disease . In our analysis, we have detected the reelin complex with a molecular weight of nearly 750kDa.

Regarding protein-protein interactions, we identified several interactions. Among them we found an interaction between Cystatin C and Complement C4A that was already found in literature ,even if the biological effect is still unknown. Both Cystatin C and Complement C4A are related to neurodegenerative diseases: in fact, Cystatin C has been correlated to several neurodegenerative diseases such Alzheimer's, Parkinson diseases and Amyotrophic lateral sclerosis, since its expression is modulated during these pathologies, and it seems to have a protective role. Instead, an up-regulation of Complement C4A in CSF and plasma is directly involved in multiple sclerosis, and it underlines the pathological role of complement hyper-activation in this disease. Another interesting interaction is the one from neuronal pentraxin 1 and its receptor. The neuronal pentraxins are a group of proteins that could be a marker of synaptic defects in different neurodegenerative diseases. Moreover, neuronal pentraxins are structurally like immune pentraxins, and this could suggest a role in neuroinflammation. Limbic System Associated Membrane Protein and neuronal growth regulator 1 interaction represents another important target. but its exact function is unknown. Limbic System Associated Membrane Protein has a role in serotoninergic pathway, whereas neuronal growth regulator 1 supports axon connections and they are both involved in major depressive disorder. These findings underline the presence of complexes involved in neurodegeneration, neuroinflammation, immune defense/response retinol transport, cellular stress response and neuronal development. The study of complexes and protein-protein interactions and their presence and abundance in the CSF could put in evidence new insights for the elucidation of the pathogenesis but also for the identification of new therapeutic targets. Moreover, the present

approach could also allow the identification of new markers that can help in discriminating different subtypes at onset and to predict evolution and treatment response of NDD.

Several protein complexes are differently modulated between ALZ patients and NN patients. We reported some examples which have a role in neurodegeneration in general and in Alzheimer disease in particular. For instance, we found a significant decrease of a tetrameric form of Apolipoprotein A II, (APOA2) which corresponds to nearly 90kDA, in ALZ patients as respect to NN patients. APOA2 is an apolipoprotein member of the high-density lipoproteins (HDL) family. In literature the decrease of this protein has already been associated with dementia occurrence. In addition, lower plasma levels of APOA2 combined with a parallel augment of APOB/APOA1 increase the cognitive decline during Alzheimer disease (Song et al., 2012) Apolipoproteins are often dysregulated at brain levels in several neurological disorders. Apolipoproteins like APOA-I and APOA-II can enter the CSF through the choroid plexus and then affect the central nervous system environment: it is already well known that an alteration of the cholesterol pathways could play a role in neurological and psychiatric pathologies, however the role of APOA-II dysregulation in neurological diseases has not been clarified yet (Elliott et al., 2010) APOA-II usually circulates as a homodimer, however also tetrameric structures are reported in literature (Structures of Apolipoprotein A-II and a Lipid−Surrogate Complex Provide Insights into Apolipoprotein−Lipid Interactions). Since we have found a significant decrease of the tetrameric form of APOA-II in the CSF of ALZ patients, we can speculate that this quaternary structure might be crucial to guarantee a correct cholesterol metabolism in brain, thus avoiding lipid impairment which can contribute to the formation of a neurodegenerative environment. We also detected N-acetylglucosamine-1-phosphotransferase subunit gamma complex which is a 67-kDa homodimer protein complex. N-acetylglucosamine-1-phosphotransferase subunit gamma is a monomeric protein of nearly 33kDa that can associate to form homodimers (Qian et al., 2010) and can also associate with homodimers of the subunits alpha and beta, forming the hexameric lysosomal enzyme N-acetylglucosamine-1-phosphotransferase (Functions of the alpha, beta, and gamma subunits of UDP-GlcNAc:lysosomal enzyme N-acetylglucosamine-1-phosphotransferase). Mutations in one of the genes for this enzyme result in, which is an autosomal disorder that affect lysosomal storage(Identification and molecular characterization of six novel mutations in the UDP-N-acetylglucosamine-1-phosphotransferase gamma subunit (*GNPTG*) gene in patients with mucolipidosis III gamma[‡]). Little is known about this homodimeric protein's role in nervous system, however, the lysosomal impairment is a key aspect in neurodegeneration, since they

regulate proteostasis and aging related pathways. In addition, lysosomal enzymes are reported to be reduced in Alzheimer disease (Chapter Eleven - Endosomal-lysosomal dysfunction in metabolic diseases and Alzheimer's disease). Since we found the homodimer significantly down-regulated in ALZ patients, we can assume that maybe the homodimer formation is crucial to exert its correct function in brain, and their lack can be a signature of the neurotoxic environment during Alzheimer disease.

In addition, we found an anomalous structure of Brevican core protein significantly up-regulated in ALZ patients compared to NN patients. Brevican core protein is a nearly 100 kDa protein, however we have detected a modulated form with a lower molecular weight of nearly 67kDa. Interestingly, there are already evidences highlighting the presence of truncated forms of this protein in neurodegeneration. Brevican is a brain specific chondroitin sulfate proteoglycan that is secreted by neurons and astrocytes and play an important role in the organization of extracellular structures named perineuronal nets. Since these structures exert a protective action for neurons, it is clear that a cleaved, incorrect form of Brevican core protein can result in neurotoxic effects and thus, it can result increased in the CSF of ALZ patients. (Jonesco et al., 2020). Lastly, we reported an augmented amount of a high molecular weight form of Ceruloplasmin in NN-patients as respect to ALZ patients. Ceruloplasmin is an enzyme that is fundamental for the iron efflux from cells, indeed, its absence causes neurodegeneration driven by iron accumulation (Cheli et al., 2023). Interestingly, we found both the monomeric form (nearly 120kDa) and the homotrimeric form (nearly 370 kDa) in our dataset, however, only the tetrameric form was modulated, specifically it is much lower in ALZ patients, suggesting a putative physiological role of this form and the relation between its loss and neurodegenerative environment.

8) Bibliography

1. Aluise, C. D., Sowell, R. A., & Butterfield, D. A. (2008). Peptides and proteins in plasma and cerebrospinal fluid as biomarkers for the prediction, diagnosis, and monitoring of therapeutic efficacy of Alzheimer's disease. In *Biochimica et Biophysica Acta - Molecular Basis of Disease* (Vol. 1782, Issue 10, pp. 549–558). https://doi.org/10.1016/j.bbadis.2008.07.008

2. Atkinson, A. J., Colburn, W. A., DeGruttola, V. G., DeMets, D. L., Downing, G. J., Hoth, D. F., Oates, J. A., Peck, C. C., Schooley, R. T., Spilker, B. A., Woodcock, J., & Zeger, S. L. (2001). Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework. In *Clinical Pharmacology and Therapeutics* (Vol. 69, Issue 3, pp. 89–95). https://doi.org/10.1067/mcp.2001.113989
3. Awad, H., Khamis, M. M., & El-Aneed, A. (2015). Mass spectrometry, review of the basics: Ionization. In *Applied Spectroscopy Reviews* (Vol. 50, Issue 2, pp. 158–175). Bellwether Publishing, Ltd. https://doi.org/10.1080/05704928.2014.954046
4. Bacher, U., Zander, A. R., Haferlach, T., Schnittger, S., Fehse, B., & Kröger, N. (2008). Minimal residual disease diagnostics in myeloid malignancies in the post transplant period. In *Bone Marrow Transplantation* (Vol. 42, Issue 3, pp. 145–157). https://doi.org/10.1038/bmt.2008.185
5. Bourdenx, M., Koulakiotis, N. S., Sanoudou, D., Bezard, E., Dehay, B., & Tsarbopoulos, A. (2017a). Protein aggregation and neurodegeneration in prototypical neurodegenerative diseases: Examples of amyloidopathies, tauopathies and synucleinopathies. In *Progress in Neurobiology* (Vol. 155, pp. 171–193). Elsevier Ltd. https://doi.org/10.1016/j.pneurobio.2015.07.003
6. Bourdenx, M., Koulakiotis, N. S., Sanoudou, D., Bezard, E., Dehay, B., & Tsarbopoulos, A. (2017b). Protein aggregation and neurodegeneration in prototypical neurodegenerative diseases: Examples of amyloidopathies, tauopathies and synucleinopathies. In *Progress in Neurobiology* (Vol. 155, pp. 171–193). Elsevier Ltd. https://doi.org/10.1016/j.pneurobio.2015.07.003
7. Brinkmalm, G., Sjödin, S., Simonsen, A. H., Hasselbalch, S. G., Zetterberg, H., Brinkmalm, A., & Blennow, K. (2018). A parallel reaction monitoring mass spectrometric method for analysis of potential CSF biomarkers for Alzheimer's disease. *PROTEOMICS–Clinical Applications*, *12*(1), 1700131.
8. Butterfield, D. A., Reed, T., Newman, S. F., & Sultana, R. (2007). Roles of amyloid β-peptide-associated oxidative stress and brain protein modifications in the pathogenesis of Alzheimer's disease and mild cognitive impairment. In *Free Radical Biology and Medicine* (Vol. 43, Issue 5, pp. 658–677). https://doi.org/10.1016/j.freeradbiomed.2007.05.037
9. Cheli, V. T., Sekhar, M., Santiago González, D. A., Angeliu, C. G., Denaroso, G. E., Smith, Z., Wang, C., & Paez, P. M. (2023). The expression of ceruloplasmin in astrocytes is essential for postnatal myelination and myelin maintenance in the adult brain. *Glia*, *71*(10), 2323–2342.
10. Cornejo, V. H., & Hetz, C. (2013). The unfolded protein response in Alzheimer's disease. In *Seminars in Immunopathology* (Vol. 35, Issue 3, pp. 277–292). https://doi.org/10.1007/s00281-013-0373-9
11. Cras, P., Kawai, M., Loweryf, D., Gonzalez-Dewhit-T, P., Greenbergt, B., & Perry, G. (1991). Senile plaque neurites in Alzheimer disease accumulate amyloid precursor protein (P-amyloid protein/T/ubiquitin/neurofflaments/dystrophic neurites). In *Proc. Nadl. Acad. Sci. USA* (Vol. 88). https://www.pnas.org
12. Draisma, G., Etzioni, R., Tsodikov, A., Mariotto, A., Wever, E., Gulati, R., Feuer, E., & de Koning, H. (2009). Lead time and overdiagnosis in prostate-specific antigen screening: Importance of methods and context. *Journal of the National Cancer Institute*, *101*(6), 374–383. https://doi.org/10.1093/jnci/djp001
13. Elliott, D. A., Weickert, C. S., & Garner, B. (2010). Apolipoproteins in the brain: implications for neurological and psychiatric disorders. *Clinical Lipidology*, *5*(4), 555–573. https://doi.org/10.2217/clp.10.37

14. Fainaru, M., Mahley, R. W., Hamilton, R. L., & Innerarity', T. L. (n.d.). *Structural and metabolic heterogeneity of @-very low density lipoproteins from cholesterol-fed dogs and f rom humans with Type 111 hyperlipoproteinemia*.
15. Fan, L., Mao, C., Hu, X., Zhang, S., Yang, Z., Hu, Z., Sun, H., Fan, Y., Dong, Y., Yang, J., Shi, C., & Xu, Y. (2020). New Insights Into the Pathogenesis of Alzheimer's Disease. In *Frontiers in Neurology* (Vol. 10). Frontiers Media S.A. https://doi.org/10.3389/fneur.2019.01312
16. Fei, E., Jia, N., Yan, M., Ying, Z., Sun, Q., Wang, H., Zhang, T., Ma, X., Ding, H., Yao, X., Shi, Y., & Wang, G. (2006). SUMO-1 modification increases human SOD1 stability and aggregation. *Biochemical and Biophysical Research Communications*, *347*(2), 406–412. https://doi.org/https://doi.org/10.1016/j.bbrc.2006.06.092
17. Fine, B. M., & Amler, L. (2009). Predictive biomarkers in the development of oncology drugs: A therapeutic industry perspective. In *Clinical Pharmacology and Therapeutics* (Vol. 85, Issue 5, pp. 535–538). https://doi.org/10.1038/clpt.2009.9
18. Fountoulakis, M., & Kossida, S. (2006). Proteomics-driven progress in neurodegeneration research. In *Electrophoresis* (Vol. 27, Issue 8, pp. 1556–1573). Wiley-VCH Verlag. https://doi.org/10.1002/elps.200500738
19. Gao, Y., Tan, L., Yu, J.-T., & Tan, L. (2018). Tau in Alzheimer's Disease: Mechanisms and Therapeutic Strategies. *Current Alzheimer Research*, *15*(3), 283–300. https://doi.org/10.2174/1567205014666170417111859
20. Geyer, P. E., Holdt, L. M., Teupser, D., & Mann, M. (2017). Revisiting biomarker discovery by plasma proteomics. *Molecular Systems Biology*, *13*(9). https://doi.org/10.15252/msb.20156297
21. Gratuze, M., Cisbani, G., Cicchetti, F., & Planel, E. (2016). Is Huntington's disease a tauopathy? *Brain*, *139*(4), 1014–1025. https://doi.org/10.1093/brain/aww021
22. Guo, T., Zhang, D., Zeng, Y., Huang, T. Y., Xu, H., & Zhao, Y. (2020). Molecular and cellular mechanisms underlying the pathogenesis of Alzheimer's disease. In *Molecular Neurodegeneration* (Vol. 15, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s13024-020-00391-7
23. Hampel, H., Hardy, J., Blennow, K., Chen, C., Perry, G., Kim, S. H., Villemagne, V. L., Aisen, P., Vendruscolo, M., Iwatsubo, T., Masters, C. L., Cho, M., Lannfelt, L., Cummings, J. L., & Vergallo, A. (2021). The Amyloid-β Pathway in Alzheimer's Disease. In *Molecular Psychiatry* (Vol. 26, Issue 10, pp. 5481–5503). Springer Nature. https://doi.org/10.1038/s41380-021-01249-0
24. Hanash, S. M., Pitteri, S. J., & Faca, V. M. (2008). Mining the plasma proteome for cancer biomarkers. In *Nature* (Vol. 452, Issue 7187, pp. 571–579). Nature Publishing Group. https://doi.org/10.1038/nature06916
25. Hansson, S. F., Puchades, M., Blennow, K., Sjögren, M., & Davidsson, P. (2004). Validation of a prefractionation method followed by two-dimensional electrophoresis - Applied to cerebrospinal fluid proteins from frontotemporal dementia patients. *Proteome Science*, *2*. https://doi.org/10.1186/1477-5956-2-7
26. Hölscher, C. (1998). Possible Causes of Alzheimer's Disease: Amyloid Fragments, Free Radicals, and Calcium Homeostasis. *Neurobiology of Disease*, *5*(3), 129–141. https://doi.org/https://doi.org/10.1006/nbdi.1998.0193
27. Islam, S., Sun, Y., Gao, Y., Nakamura, T., Noorani, A. A., Li, T., Wong, P. C., Kimura, N., Matsubara, E., Kasuga, K., Ikeuchi, T., Tomita, T., Zou, K., & Michikawa, M. (2022). Presenilin Is Essential for ApoE Secretion, a Novel Role of Presenilin Involved in Alzheimer's Disease Pathogenesis. *Journal of Neuroscience*, *42*(8), 1574–1586. https://doi.org/10.1523/JNEUROSCI.2039-21.2021

28. Itagaki, S., Mcgeer, P. L., Akiyama, H., Zhu, S., & Selkoe, D. (1989). Relationship of microglia and astrocytes to amyloid deposits of Alzheimer disease. In *Journal of Neuroimmunology* (Vol. 24).
29. Jellinger, K. A. (2012). Interaction between pathogenic proteins in neurodegenerative disorders. *Journal of Cellular and Molecular Medicine*, *16*(6), 1166–1183. https://doi.org/10.1111/j.1582-4934.2011.01507.x
30. Jeroen Pasterkamp, R., Peschon, J. J., Spriggs, M. K., & Kolodkin, A. L. (2003). Semaphorin 7A promotes axon outgrowth through integrins and MAPKs. *Nature*, *424*(6947), 398–405. https://doi.org/10.1038/nature01790
31. Jerrett, J. T., & Lansbury, P. T. (1993). Seeding uOne-Dimensional Minireview Crystallization" of Amyloid: A Pathogenic Mechanism in Alzheimer's Disease and Scrapie? In *Cell* (Vol. 73).
32. Jonesco, D. S., Karsdal, M. A., & Henriksen, K. (2020). The CNS-specific proteoglycan, brevican, and its ADAMTS4-cleaved fragment show differential serological levels in Alzheimer's disease, other types of dementia and non-demented controls: A cross-sectional study. *PLOS ONE*, *15*(6), e0234632-. https://doi.org/10.1371/journal.pone.0234632
33. Kanekiyo, T., Xu, H., & Bu, G. (2014). ApoE and Aβ in Alzheimer's disease: Accidental encounters or partners? In *Neuron* (Vol. 81, Issue 4, pp. 740–754). https://doi.org/10.1016/j.neuron.2014.01.045
34. Keeney, J. T. R., Swomley, A. M., Förster, S., Harris, J. L., Sultana, R., & Butterfield, D. A. (2013a). Apolipoprotein A-I: Insights from redox proteomics for its role in neurodegeneration. In *Proteomics - Clinical Applications* (Vol. 7, Issues 1–2, pp. 109–122). https://doi.org/10.1002/prca.201200087
35. Keeney, J. T. R., Swomley, A. M., Förster, S., Harris, J. L., Sultana, R., & Butterfield, D. A. (2013b). Apolipoprotein A-I: Insights from redox proteomics for its role in neurodegeneration. In *Proteomics - Clinical Applications* (Vol. 7, Issues 1–2, pp. 109–122). https://doi.org/10.1002/prca.201200087
36. Korecka, M., & Shaw, L. M. (2021a). Mass spectrometry-based methods for robust measurement of Alzheimer's disease biomarkers in biological fluids. In *Journal of Neurochemistry* (Vol. 159, Issue 2, pp. 211–233). John Wiley and Sons Inc. https://doi.org/10.1111/jnc.15465
37. Korecka, M., & Shaw, L. M. (2021b). Mass spectrometry-based methods for robust measurement of Alzheimer's disease biomarkers in biological fluids. In *Journal of Neurochemistry* (Vol. 159, Issue 2, pp. 211–233). John Wiley and Sons Inc. https://doi.org/10.1111/jnc.15465
38. Kovacs, G. G. (2016). Molecular pathological classification of neurodegenerative diseases: Turning towards precision medicine. In *International Journal of Molecular Sciences* (Vol. 17, Issue 2). MDPI AG. https://doi.org/10.3390/ijms17020189
39. Kovacs, G. G. (2019). Molecular pathology of neurodegenerative diseases: Principles and practice. In *Journal of Clinical Pathology* (Vol. 72, Issue 11, pp. 725–735). BMJ Publishing Group. https://doi.org/10.1136/jclinpath-2019-205952
40. Kriebel, M., Wuchter, J., Trinks, S., & Volkmer, H. (2012). Neurofascin: A switch between neuronal plasticity and stability. *The International Journal of Biochemistry & Cell Biology*, *44*(5), 694–697. https://doi.org/https://doi.org/10.1016/j.biocel.2012.01.012
41. Kumar Bhardwaj, S. (2015). *A Review: HPLC Method Development and Validation*. http://www.urpjournals.com

42. Lee, M. K., Slunt, H. H., Martin, L. J., Thinakaran, G., Kim, G., Gandy, S. E., Seeger, M., Koo, E., Price, D. L., & Sisodia, S. S. (1996). *Expression of Presenilin 1 and 2 (PS1 and PS2) in Human and Murine Tissues*.
43. Li, K. W., Ganz, A. B., & Smit, A. B. (2019). Proteomics of neurodegenerative diseases: analysis of human post-mortem brain. In *Journal of Neurochemistry* (Vol. 151, Issue 4, pp. 435–445). Blackwell Publishing Ltd. https://doi.org/10.1111/jnc.14603
44. Ling, S. C., Polymenidou, M., & Cleveland, D. W. (2013). Converging mechanisms in als and FTD: Disrupted RNA and protein homeostasis. In *Neuron* (Vol. 79, Issue 3, pp. 416–438). https://doi.org/10.1016/j.neuron.2013.07.033
45. Macklin, A., Khan, S., & Kislinger, T. (2020). Recent advances in mass spectrometry based clinical proteomics: Applications to cancer research. In *Clinical Proteomics* (Vol. 17, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s12014-020-09283-w
46. Mahley, R. W., Innerarity, T. L., Rall, S. C., & Weisgraber, K. H. (n.d.). *I I revzezo Plasma lipoproteins: apolipoprotein structure and function*.
47. Mogk, A., & Bukau, B. (2017). Role of sHsps in organizing cytosolic protein aggregation and disaggregation. In *Cell Stress and Chaperones* (Vol. 22, Issue 4, pp. 493–502). Cell Stress and Chaperones. https://doi.org/10.1007/s12192-017-0762-4
48. Morley, J. F., Brignull, H. R., Weyers, J. J., & Morimoto, R. I. (n.d.). *The threshold for polyglutamine-expansion protein aggregation and cellular toxicity is dynamic and influenced by aging in Caenorhabditis elegans*. www.pnas.orgcgidoi10.1073pnas.152161099
49. Pechmann, S., Levy, E. D., Gaetano Tartaglia, G., & Vendruscolo, M. (2009). Physicochemical principles that regulate the competition between functional and dysfunctional association of proteins. In *PNAS* (Vol. 106, Issue 25).
50. Perrot, R., & Eyer, J. (2009). Neuronal intermediate filaments and neurodegenerative disorders. *Brain Research Bulletin*, *80*(4), 282–295. https://doi.org/https://doi.org/10.1016/j.brainresbull.2009.06.004
51. Qian, Y., Lee, I., Lee, W.-S., Qian, M., Kudo, M., Canfield, W. M., Lobel, P., & Kornfeld, S. (2010). Functions of the α, β, and γ Subunits of UDP-GlcNAc:Lysosomal Enzyme N-Acetylglucosamine-1-phosphotransferase*. *Journal of Biological Chemistry*, *285*(5), 3360–3370. https://doi.org/https://doi.org/10.1074/jbc.M109.068650
52. Ray, W. J., Yao, M., Mumm, J., Schroeter, E. H., Saftig, P., Wolfe, M., Selkoe, D. J., Kopan, R., & Goate, A. M. (1999). *Cell Surface Presenilin-1 Participates in the-Secretase-like Proteolysis of Notch**. http://www.jbc.org
53. Ross, C. A., & Poirier, M. A. (2004). Protein aggregation and neurodegenerative disease. *Nature Medicine*, *10*(7), S10. https://doi.org/10.1038/nm1066
54. Ross, J. S., Hatzis, C., Symmans, W. F., Pusztai, L., & Hortobágyi, G. N. (2008). Commercialized Multigene Predictors of Clinical Outcome for Breast Cancer; Commercialized Multigene Predictors of Clinical Outcome for Breast Cancer. *The Oncologist*, *13*, 477–493. https://doi.org/10.1634/theoncolo
55. Saito, K., Seishima, M., Heyes, M. P., Song, H., Fujigaki, S., Maeda, S., Vickers, J. H., & Noma, A. (1997). Marked increases in concentrations of apolipoprotein in the cerebrospinal fluid of poliovirus-infected macaques : relations between apolipoprotein concentrations and severity of brain injury. In *Biochem. J* (Vol. 321).
56. Sauter, G., Lee, J., Bartlett, J. M. S., Slamon, D. J., & Press, M. F. (2009). Guidelines for human epidermal growth factor receptor 2 testing: Biologic and methodologic considerations. *Journal of Clinical Oncology*, *27*(8), 1323–1333. https://doi.org/10.1200/JCO.2007.14.8197

57. Schmidt, M. F., Gan, Z. Y., Komander, D., & Dewson, G. (2021). Ubiquitin signalling in neurodegeneration: mechanisms and therapeutic opportunities. In *Cell Death and Differentiation* (Vol. 28, Issue 2, pp. 570–590). Springer Nature. https://doi.org/10.1038/s41418-020-00706-7
58. Sherr, R., Leresque, G., Ikeda, M., Ch, H., Lin, C., Holman, K., Tsuda, T., Mar, L., Foncin, J., Bruni, A. C., Montesi, M. P., Sorbi, S., Rainero, I., Pinessi, L., Nee, L., Chumakov, I., Pollen, D., Brookes, A., Sanseau, P., … Rommenst Be P H St George-Hyslop, J. M. (1995). *FAD4 (ref. 18), TORl.l (ref. 19) and 603 (ref. 20)), each of which provided an individual log NATURE ·* (Vol. 375).
59. Song, F., Poljak, A., Crawford, J., Kochan, N. A., Wen, W., Cameron, B., Lux, O., Brodaty, H., Mather, K., & Smythe, G. A. (2012). Plasma apolipoprotein levels are associated with cognitive status and decline in a community cohort of older individuals. *PloS One*, *7*(6), e34078.
60. Soto, C. (2003). Unfolding the role of protein misfolding in neurodegenerative diseases. *Nature Reviews Neuroscience*, *4*(1), 49–60. https://doi.org/10.1038/nrn1007
61. Soto, C., Estrada, L., & Castilla, J. (2006). Amyloids, prions and the inherent infectious nature of misfolded protein aggregates. In *Trends in Biochemical Sciences* (Vol. 31, Issue 3, pp. 150–155). https://doi.org/10.1016/j.tibs.2006.01.002
62. Sperling, R. A., Aisen, P. S., Beckett, L. A., Bennett, D. A., Craft, S., Fagan, A. M., Iwatsubo, T., Jack, C. R., Kaye, J., Montine, T. J., Park, D. C., Reiman, E. M., Rowe, C. C., Siemers, E., Stern, Y., Yaffe, K., Carrillo, M. C., Thies, B., Morrison-Bogorad, M., … Phelps, C. H. (2011). Toward defining the preclinical stages of Alzheimer's disease: Recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's and Dementia*, *7*(3), 280–292. https://doi.org/10.1016/j.jalz.2011.03.003
63. Sultana, R., Boyd-Kimball, D., Poon, H. F., Cai, J., Pierce, W. M., Klein, J. B., Merchant, M., Markesbery, W. R., & Butterfield, D. A. (2006). Redox proteomics identification of oxidized proteins in Alzheimer's disease hippocampus and cerebellum: An approach to understand pathological and biochemical alterations in AD. *Neurobiology of Aging*, *27*(11), 1564–1576. https://doi.org/10.1016/j.neurobiolaging.2005.09.021
64. Vagnoni, A., Perkinton, M. S., Gray, E. H., Francis, P. T., Noble, W., & Miller, C. C. J. (2012). Calsyntenin-1 mediates axonal transport of the amyloid precursor protein and regulates Aβ production. *Human Molecular Genetics*, *21*(13), 2845–2854. https://doi.org/10.1093/hmg/dds109
65. van Nostrand, W. E., Farrow, J. S., Wagner, S. L., Bhasin, R., Goldgaber, D., Cotman, C. W., & Cunningham, D. D. (1991). The predominant form of the amyloid beta-protein precursor in human brain is protease nexin 2. *Proceedings of the National Academy of Sciences*, *88*(22), 10302–10306. https://doi.org/10.1073/pnas.88.22.10302
66. Wagner, J. A., Williams, S. A., & Webster, C. J. (2007). Biomarkers and surrogate end points for fit-for-purpose development and regulatory evaluation of new drugs. In *Clinical Pharmacology and Therapeutics* (Vol. 81, Issue 1, pp. 104–107). https://doi.org/10.1038/sj.clpt.6100017
67. Wang, E. S., Sun, Y., Guo, J. G., Gao, X., Hu, J. W., Zhou, L., Hu, J., & Jiang, C. C. (2010a). Tetranectin and apolipoprotein A-I in cerebrospinal fluid as potential biomarkers for Parkinson's disease. *Acta Neurologica Scandinavica*, *122*(5), 350–359. https://doi.org/10.1111/j.1600-0404.2009.01318.x
68. Wang, E. S., Sun, Y., Guo, J. G., Gao, X., Hu, J. W., Zhou, L., Hu, J., & Jiang, C. C. (2010b). Tetranectin and apolipoprotein A-I in cerebrospinal fluid as potential

biomarkers for Parkinson's disease. *Acta Neurologica Scandinavica*, *122*(5), 350–359. https://doi.org/10.1111/j.1600-0404.2009.01318.x
69. Wang, P., Whiteaker, J. R., & Paulovich, A. G. (2009). The evolving role of mass spectrometry in cancer biomarker discovery. In *Cancer Biology and Therapy* (Vol. 8, Issue 12, pp. 1083–1094). Landes Bioscience. https://doi.org/10.4161/cbt.8.12.8634
70. Wei, X., & Li, L. (2009). Mass Spectrometry-Based Proteomics and Peptidomics for Biomarker Discovery in Neurodegenerative Diseases. In *Int J Clin Exp Pathol* (Vol. 2). www.ijcep.com/IJCEP806008
71. Zonta, B., Desmazieres, A., Rinaldi, A., Tait, S., Sherman, D. L., Nolan, M. F., & Brophy, P. J. (2011). A Critical Role for Neurofascin in Regulating Action Potential Initiation through Maintenance of the Axon Initial Segment. *Neuron*, *69*(5), 945–956. https://doi.org/https://doi.org/10.1016/j.neuron.2011.02.021