TESE DE DOUTORAMENTO

DEVELOPMENTS ON METALLOMIC AND PROTEOMIC STRATEGIES FOR EARLY DIAGNOSIS OF ALZHEIRMER'S DISEASE

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DEVELOPMENTS ON METALLOMIC AND PROTEOMIC STRATEGIES FOR EARLY DIAGNOSIS OF ALZHEIRMER'S DISEASE

Dna. María del Pilar Chantada Vázquez

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DEVELOPMENTS ON METALLOMIC AND PROTEOMIC STRATEGIES FOR EARLY DIAGNOSIS OF ALZHEIRMER'S DISEASE

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ABREVATIONS

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1D-PAGE:	one-dimensional gel electrophoresis
2D-NMR:	two-dimensional NMR
2D-PAGE:	two-dimensional polyacrilamide gel electrophoresis
3D-NMR:	three-dimensional NMR
A1AT:	alpha-1-antritrypsin
A2M:	alpha-2-macroglobulin
AChEI:	acetylcholinesterase inhibitors
ACTB:	actin cytoplasmatic 1
AD:	Alzheimer´s disease
ADH:	alcohol dehydrogenase
AEC:	anion exchange chromatography
AFM:	atomic force microscopy
AHSG:	alpha-2-HS-glycoprotein
ALBU:	serum albumin
ANS:	amino-2-naphthol-4-sulfonic
APDC:	ammonium pyrrolidinedithiocarbamate
Apo A-II:	apoliprotein A-II
APP:	amyloid precursor protein
Asp:	aspartic acid
ATH:	avian thymic hormone
Aß:	ß amyloid protein
BMIMBr:	1-butyl-3-methylimidazoliumm bromide
BMIMCl:	1-butyl-3-methylimidazoliumm chloride
BSA:	bovine serum albumin
C9JKR2:	CRA_k isoform
CA:	carbonic anhydrase
CaM:	calmodulin
Cap:	capillary
CCDs:	charge-coupled devices
CD:	circular dichroism
CD5L:	CD5 antigen-like
CE:	collision energy

CF1:	complement factor 1
CHMP1A:	charged multivesicular body protein 1A
CP:	ceruloplasmin
CRM:	certified Reference Material
CSF:	cerebral spinal fluid
CTR:	control
CUR:	curtain gas
CX3CL1:	fractalkine
CXCL10:	interferon gamma-induced protein 10
Cys:	cysteine
Da:	Dalton
DBS:	dried blood spot
DC:	direct current
DDA:	shotgun data-dependent acquisition
DIA:	Data Independent Acquisition
DLS:	dynamic light scattering
DMS:	dried matrix spot
DNMT1:	DNA (cytosine-5)-methyltransferase 1
DTT:	dithiotreitol
EcMetAP:	*Escherichia coli* methionine aminopeptidase
EDS:	energy dispersive spectroscopy
EDTA:	ethylenediaminetetraacetic acid
EfCP:	*E. fetida* coelomic proteins
EPR:	electron paramagnetic resonance
EPR:	electron paramagnetic resonance
ESI:	electrospray ionization
ESR:	electron spin resonance
ETAAS:	electrothermal atomic absorption spectrometry
EXAFS:	X-ray absorption fine structure
FAAS:	flame atomic absorption spectrometry
FBS:	fetal bovine serum
FC:	force-clamp

FDA:	Food and Drug Administration
FDR:	false discovery rate
FNC3:	ficolin-3
FT:	Fourier transforms
FTIR:	Fourier-transform infrared spectrometry
FT-IRC:	Fourier transform ion cyclotron resonance
FX:	force-extension
GDP:	gross domestic product
GMCSF:	granulocyte-macrophage colony-stimulating factor
GS:	ion source gas
HD:	Huntington's disease
His:	histidine
HP:	haptoglobin
HPP:	Human Proteome Project
HSQC:	Heteronuclear Single Quantum Coherence
HT-XAS:	high-throughput techniques based on X-ray absorption spectrometry
ICP-MS:	inductively coupled plasma mass spectrometry
ICP-OES:	inductively coupled plasma - optical emission spectrometry
IDA:	isotopic dilution analysis
IEC:	ion exchange chromatography
IEF:	isoelectric focusing
IEF:	Isoelectric focusing
IFN-γ:	interferon-gamma
IgG:	immunoglobulin G
IGKC:	immunoglobulin kappa constant
IGLC7:	immunoglobulin lambda constant 7
IL-1β:	interleukin-1 beta
IL-8:	interleukin-8
ISVF:	ion spray voltage floating
ITC:	isothermal titration calorimetry
K2C1:	keratin, type II cytoskeletal 1
KED:	kinetic energy discrimination

KNG1:	kininogen-1
KNG1:	kininogen-1
LA:	laser ablation
LA-ICP-MS:	laser ablation – inductively coupled plasma mass spectrometry
LC:	liquid chromatography
LOD:	limit of detection
LOQ:	limit of quantification
MAD:	multi-wavelength anomalous diffraction
MALDI:	matrix-assisted laser desorption/ionization
MALDI-TOF/TOF:	matrix-assisted laser desorption ionization time of flight
MCI:	mild cognitive imparment
MD:	molecular dynamics
MMAPA:	(4-monomethylaminophenyl)-acrylonitrile
MMSE:	mini mental state examination
MoCA:	Montreal cognitive assesment
MRI:	magnetic resonance imaging
MRM:	multiple reaction monitoring
SM:	multiple sclerosis
MS:	mass spectrometry
MS/MS:	tandem mass spectrometry
MYO:	myoglobin
n-ESI:	nanospray ionization
NFT:	neurofibrillary tangles
NMDAR:	N-methyl-D-aspartate antagonists receptor
NMR:	nuclear magnetic resonance spectrometry
NOESY:	nuclear overhauser effect spectroscopy
NPs:	nanoparticles
PAGE:	polyacrylamide gel electrophoresis
Parvs:	parvalbumins
PCA:	principal components analysis
PD:	Pick's disease
Pdi:	Parkinson's disease

PEDF:	pigment epithelium-derived factor
PET:	positron emission tomography
Phe:	phenylalanine
PMF:	peptide mass fingerprinting
PrP:	prion protein
PSP:	Progressive Supranuclear Palsy
p-tau:	phosphorylated Tau
QDs:	quantum dots
QQQ:	triple-quadrupole
Rc:	rusticyanin
RF:	radio frequency
RNS:	reactive nitrogen species
ROS:	reactive oxygen species
SAD:	single-wavelength anomalous dispersion
sCaM4:	soybean calmodulin isoform 4
sCD40L:	soluble CD40 ligand
SDS:	sodium dodecyl sulfate
SDSL:	site-directed spin labeling
SEC:	size exclusion chromatography
SEM:	scanning electron microscopy
SEPINC1:	antithrombin-3
SERPINA1:	alpha-1-antitrypsin
SERS:	surface enhanced Raman spectroscopy
SMFS:	single molecule force spectroscopy
SOD:	superoxide dismutase
SP:	senile plaques
SRM:	selected reaction monitoring
SWATH:	sequential window acquisition of all theoretical spectra
Syt1:	synaptotagmin
TEM:	transmission electron microscopy
TMAH:	tetramethyl ammonium hydroxide
TnC:	Akazara scallop troponin C

TNF-**α:**	tumor necrosis factor-alpha
TOCSY:	total correlation spectroscopy
TOF:	time-of-flight
TRFE:	serotransferrin
Trp:	tryptophan
t-tau:	total Tau
Tyr:	tyrosine
UV:	ultraviolet
VascD:	vascular dementia
VEGF:	vascular endothelial growth factor
VTNC:	vitronectin
XANES:	X-ray absorption near edge structure
XAS:	X-ray absorption spectroscopy

ABSTRACT

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Abstract

Alzheimer's disease (AD) is considered the new epidemic of the 21st century because of the increase on the year's life expectancy which generates a remarkable increase of patients suffering this pathology. Etiology of AD is still unknown, but it is well-known that there are several factors related to AD. Regarding treatments, there is no medication that recovers patients, although there are available several drugs that diminish patient deterioration which are applied at advanced stages of the disease.

Therefore, the knowledge of the behavior of this disease in the early stages is a goal in biomedical research. There are several studies that compare healthy people with patients who suffer AD but currently these studies are focused on knowing the evolution of the disease, that is, in studying the differences between an early stage such as mild cognitive impairment (MCI) and AD.

The objective of this thesis has been focused on optimizing several techniques (multi-element analysis and proteomics) for searching potential biomarkers for an early AD diagnosis. The two first chapters have been devoted to the optimization of methods for the determination of several elements, which led to successful results (good precision and accuracy). The developed techniques differ in the sample introduction mode in ICP-MS, but both share short analysis time for assessing simultaneously several elements, and the use of very small sample volume. The first technique consists of using paper as a support for serum samples (*dried blood spot*, DBS) followed to introduction in ICP-MS after laser ablation (LA-ICP-MS). The second technique is based on discrete sampling for ICP-MS. Both techniques require very small sample volumes, short analysis times, as well as minimum sample handling and sample conservation.

In the second part of this thesis regarding proteomics, we have been able to find several proteins for differentiating three groups of population (healthy people, AD patients, and MCI patients). The study

has been addressed qualitatively and quantitatively, and these preliminary results are first step for designing a panel of biomarkers.

In a final chapter, LA conditions have been optimized for the determination of metals in the isoforms of two proteins [Serotransferrin (TRFE) and Keratin type II cytoskeletal 1 (K2C1)] after 2D electrophoresis.

The current research is therefore a first step to study the differences between groups of patients suffering AD using elements and proteins as discriminating features, but also the levels of metals bound to proteins.

I. INTRODUCTION

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1. DEMENTIA, PROTEOMICS AND METALLOMICS

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1.1 DEMENTIA

Dementia is defined as the acquired and sustained impairment of the cognitive capacities in a patient, which hinders the satisfactory completion of daily activities. In this sense, this illness must be distinguished from mental retardation and delirium. Dementias can be so severe that they significantly affect patients social sphere, family and work [1, 2]. A differential diagnosis must be made with mild cognitive impairment (MCI), a state which could increase the risk of dementia and can manifest itself through normal memory disorders and cortical function disorders, without meeting the diagnostic criteria for dementia. In some cases, MCI is associated with 12-15% increased risk of dementia per year, in comparison with 2% community risk [3]. A major problem with dementias is that they have multiple diagnosis. Thus, the establishment of clinical and paraclinical criteria is needed. Dementias could be degenerative, as Alzheimer's Disease (AD), Pick's Disease (PD), Parkinson's Disease (PDi), Huntington's Disease (HD) and the Progressive Supranuclear Palsy (PSP), among many others; acquired, such as Vascular Dementia (VascD), Multiple Sclerosis (MS), intracranial neoplasia, traumatism, hydrocephalus, and prions, among others; and potentially reversible, such as toxic/metabolic disorders (hypothyroidism, kidney dialysis, vitamin B12 deficiency, alcoholism, malnutrition, etc.), infectious diseases (HIV/AIDS, neurosyphilis, tuberculosis, cryptococcosis, viral encephalitis, etc.) and major depression [2].

The main cause of degenerative dementia (60%) is AD, followed by VascD, which accounts for 12.5-27% of cases. Other frequent degenerative dementias are frontotemporal dementia, which is present in young individuals, and Lewy Body Dementia, present in individuals over 65 [4].

It is crucial to understand that, currently, very few people are familiar with the care and treatment of a person with dementia. As yet there is no cure for this disease and therefore this generates severe

disabled patients with a high dependence on their caregivers, whose quality of life is also affected [5].

The worldwide cost of dementia has increased from 604 billion dollars in 2010 to 800 billion dollars at present, which means an increase of 35.4%. This amount represents about 1.09% of global gross domestic product (GDP). Even these expenses may seem unbearable, it is calculated that the total costs will exceed a trillion dollars in 2030. In Western Europe, 50.8 million dollars are invested in direct health care (about 19.6% of total health expenditure), 113 millions in direct costs of care (about 43% of the total), and 98.9 millions in costs of informal care (about 37.6% of the total) [6]. Diagnostic services are still insufficient and become a barrier for the adequate provision of care to dementia patients. Although currently there are no treatments that modify the disease, the correct and timely diagnosis is a precondition to have access to supporting services (e.g., subsidized home upgrades) and to symptomatic treatment. Only 20 to 50% of dementia patients are estimated to have a documented diagnosis at primary care, this ratio being substantially lower in developing countries [7].

1.1.1 Epidemiology of Dementia

In 2011 there were 36.5 million people with dementia, figure which is increasing. The prevalence is 2-3% between the ages of 70 to 75, and it dramatically increases to 20-25% when people are 85 or older [8]. It is estimated that there will be 42 million patients with dementia in 2020, and 81 million patients in 2040 [9, 10]. Several epidemiologic studies mention that about 2 billion people worldwide will be over 60 years old in 2050 [5]; that is why a drastic increase in the number of patients with dementia is expected (*Figure 1*).

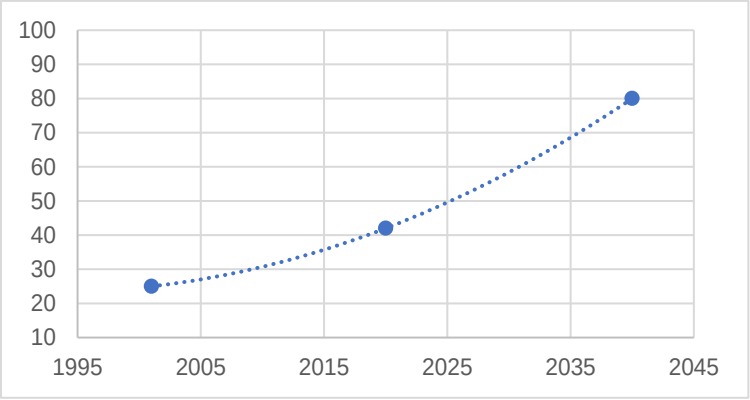

Figure 1. Estimated number of people living with dementia worldwide. The data is shown in millions (Figure taken from reference 9).

1.1.2 Classification of Dementias

Dementias must be regarded as a progressive multifactorial syndrome, produced by several diseases rather than just one. The traditional concept of dementia has to main categories: a) neurodegenerative dementias considered irreversible and b) non-neurodegenerative or potentially reversible dementias (*Table 1*) [11].

Table 1. Traditional concept in the classification of dementias.

Neurodegenerative	No Neurodegenerative
Alzheimer´s Diseasse	Vascular dementia (multi-infarct dementia, small vessel disease, subacute / chronic subdural hematomas, hypoxic / ischemic encephalopathy)
Dementia with Lewy bodies, Parkinson´s disease/Dementia	Normal pressure hydrocephalus
Frontotemporal degeneration	Metabolic causes (limbic encephalitis, Hashimoto encephalopathy, encephalopathy by voltage-dependent potassium channels)
Non-parkinosis movement disorders (Huntington's disease, Wilson's disease, Dentadorubral-palidoluisian atrophy)	Depression, bipolar disorder
Cognitive disorder / Alcohol dementia	Neoplastic / paraneoplastic causes (anti-NMDA and CRMP-5 encephalopathy, brain tumor)
Chronic traumatic encephalopathy	Infectious causes (syphilis, neurocognitive disorder associated with VIH)
Prion disease (Creutzfeldt-Jakob disease, fatal familial insomnia)	Toxic causes (lead, arsenic, organophosphorus pesticides)
Dementia related to Multiple Sclerosis	Vasculitis (primary vasculitis of the central nervous system, Behcet's disease, related to Systemic Lupus Erythematosus)
Motor neuron disease (Amyotrophic Lateral Sclerosis, Primary Lateral Sclerosis)	Vitamin deficiency (B12, thiamine, folic acid)

1.1.3 Diagnosis of Dementias

The accurate diagnosis of dementia is complex, and it must include four elements for being a proper one: a) clinical record, b) physical exploration, with an emphasis on the state of mind, c) laboratory analysis aimed at discarding systematic or metabolic problems (blood chemistry, vitamin B12 levels, thyroid profile, vitamin levels, among

others), and d) structural or functional imaging studies such as tomography or magnetic resonance [12]. The clinical assessment must include a mental function test comprising an analysis of multiple domains, such us mental alertness, attention, concentration, memory, language, visuo-spatial skills, executive functions, social behavior aptitudes, personality, and judgement, among others. The assessment of mental functions is difficult to accomplish, and their quantification is even more complex. Therefore, several scales have been developed to facilitate this task. The most widely used is the "Mini Mental State Examination" (MMSE) [13]. Its main problem being that it was not created to detect MCI, so the "Montreal Cognitive Assesment" (MoCA) is used for this purpose [14].

1.2 ALZHEIMER

AD includes a series of neurological disorders characterized by memory loss and cognitive impairment. The most common early symptom of dementia is the difficulty to remember recent events. As the disorder is developing, a wide range of other symptoms may appear, such as disorientation, mood changes, confusion, severe memory loss, behavioral changes, difficulty speaking and swallowing and walking problems. The progressive accumulation of disability, with deterioration of multiple cognitive domains, interferes with patients daily functioning. Thus, dementia substantially affects not only patients themselves, but also their families and society as a whole [15].

This progressive degenerative process has been divided into several clinical phases according to the symptoms and the evolution of the different disease indicators:

There exists a **pre-symptomatic or pre-clinic phase**, corresponding to stage 1 (*Table 2*), in which, as its name suggests, there are no symptoms, but a risk of developing the pathology. The individuals studied in this phase show family history, and so autosomal dominant mutations. A lower cognitive level has also been detected, but it is not sufficiently differentiated to be used in a diagnostic [16].

In **MCI**, corresponding to stages 2 and 3 (*Table 2*), there is clear evidence of a decrease in the cognition levels, but patients autonomy and their ability to perform daily tasks is barely reduced [17]. Risen levels of oxidative stress are also observed. However, this is the only typical marker of Alzheimer found to be increased at this stage [18]. It must be noticed that only 15% of patients with mild cognitive impairment end up developing Alzheimer.

Once **AD** is clinically diagnosed, it can be divided into three stages taking into account the progression of symptoms. In the mild stage the patient presents recent memory loss, but the memory of past events is minimally affected. Furthermore, they suffer from cognitive impairment, behavioral changes and difficulties in daily live activities. As the disease progresses, its effects increase, and in the medium-stage the patients depend on other people to perform their activities of daily life (such as having a bath or getting dressed) done, and they experience hallucinations, paranoia and irritability. Finally, in the severe stage of the disease patients require 24-hour care. They have a complete memory loss, and they are not able to recognize their family members and friends [19] (*Table 2*). In any of these three stages, patients can suffer from depression, anxiety fear and even aggressiveness due to confusional states [20].

This division into three categories is the basis for the cognitive categorization in many current studies [21-24].

Table 2. Clinical classification of stages of AD.

Stage 1
Performance within the expected range in objective cognitive tests. The performance of the cognitive test can be compared with the normative data chosen by the researchers, with or without adjustment (the choice of the researchers) by age, sex, education, etc. - No report a recent deterioration in cognition or a new onset of symptoms of neurobehavioral concern. - There is no evidence of recent cognitive impairment or new neurobehavioral symptoms in the observer's report (for example, study partner) or by longitudinal cognitive tests, if available.
Stage 2
Normal performance within the expected range in objective cognitive tests - Cognitive impairment of transition: decrease in the previous level of cognitive function, which can involve any cognitive domain (not exclusively of memory). - Although cognition is the main characteristic, mild neurobehavioral changes, for example, changes in mood, anxiety or motivation, can coexist. In some individuals, primary compliance may be neurobehavioral rather than cognitive. The neurobehavioral symptoms must have a recent onset defined in an educational way, which persists and cannot be explained by life events. - No functional impact on daily life activities.
Stage 3
Altered or abnormal performance in objective cognitive tests - Evidence of decrease from the beginning, documented by the individual's report or by the report of the observed (for example, study partner) or by changes in longitudinal cognitive tests or neurobehavioral behavior evaluations. - It can be characterized by cognitive presentations that are not primarily amnesic.

Table 2. Clinical classification of stages of AD (Continued)

Stage 3
- Performs activities of daily life independently, but the cognitive difficulty can be a slight but detectable functional impact in the most complex activities of daily life, that is, it can take more time or be less efficient, but even so it can be completed, either self-employed or corroborated by fellow student.
Stage 4
Mild dementia - Substantial progressive cognitive impairment that affects several domains and / or neurobehavioral disorders. Documented by the individual's report or by observer's report (for example, study partner) or by change in longitudinal cognitive tests. - Functional impact clearly evident in daily life, which mainly affects instrumental activities. It is no longer totally independent / requires occasional assistance with activities of daily living.
Stage 5
Moderate dementia - Extensive functional impact in daily life with deterioration in basic activities. It is no longer independent and requires frequent assistance with activities of daily living.
Stage 6
Severe dementia - The clinical interview may not be possible. - Complete dependence due to the severe functional impact in daily life with deterioration in basic activities, including basic self-care.

AD is the most common form of dementia and represents from 50% to 70% of cases. Ageing is the most important risk factor for AD, and as the average age of the population is rising, the amount of people with dementia is expected to rise. It was estimated that almost 50 million people worldwide were affected by dementia in 2018, and the figures are expected to reach 75 million in 2030 and 131 million in 2050, with a higher increase in low-and-middle-income countries [25].

AD is a neurodegenerative disease described in 1906 by the medical psychiatrist and pathologist Alois Alzheimer, who presented the case of Auguste Deter, now a well-known 51-year-old patient whose symptom picture included memory loss, hallucination and dementia and resulted in her early death at the age of 55. In the anatomopathological study of her brain, it was discovered a marked atrophy of her brain cortex, with two pathologies which would become the main histopathologic alterations of AD: a huge neuronal loss, and the presence of amyloid plaques and neurofibrillary tangles [26].

Due to the high worldwide prevalence as well as the high social and economic brunt of AD, this disease is regarded as an important public health issue. In fact, figures point out that it will be the "pandemic of the 21st century", making it a priority for medical research [25].

The prevalence and incidence of AD have been widely studied due to the deep concern about this disease. One of the most recent studies puts numbers on these terms. Thus, in Europe, the prevalence of AD was 5.05% and, disaggregated by sex, 1.74% of men and 3.31% of women. As for the incidence of AD, it was 11.08 out of 1000 people/year in the case of men, and 7.02 out of 1000 people/year in the case of women. Moreover, both the prevalence and the incidence show an increasing trend with ageing [13].

This disease can be classified according to the age of onset into: AD of early onset and AD of late onset. Approximately between 1% and 6% of all AD cases have an early onset; that is, the first symptoms

appear at the age of 30 to 60 or 65, whereas in the AD of late onset, which is the most common form of AD, the symptoms appear at the age of 60 or 65 at the earliest. Both can happen in people whose families are affected by Alzheimer. In spite of the fact that first-degree relatives of AD of late onset patients double the risk of developing the disease, the transmission pattern is rarely consistent with the Mendelian inheritance. In contrast, approximately 60% of cases of AD of early onset have multiple cases of AD in their families, and 13% of these cases are inherited in an autosomal dominant manner, with at least three generations affected [27].

1.2.1 Etiology of AD

1.2.1.1 Neuropathological characteristics

Many neurodegenerative disorders are characterized by the formation of insoluble deposits and oligomers composed by individual amyloidogenic proteins as diverse as β amyloid protein (Aβ), tau, prion protein (PrP), α-synuclein and huntingtin [28].

At a neuropathological level, Alzheimer's disease is characterized by the appearance of two characteristic structures: I) Aβ extracellular plaques or deposits and II) neurofibrillary tangles (*Figure 2*) [29].

The brain tissue shows "neurofibrillary tangles" (twisted fragments of protein within neurons that clog them up), "neuritic plaques" (abnormal clusters of dead and dying neurons, other brain cells and proteins) and "senile plaques" (areas where products of dying nerve cells have accumulated around proteins). Although these changes happen to a certain extent in all brains as a result of ageing, they are much more common in the brains of people with Alzheimer's disease [30].

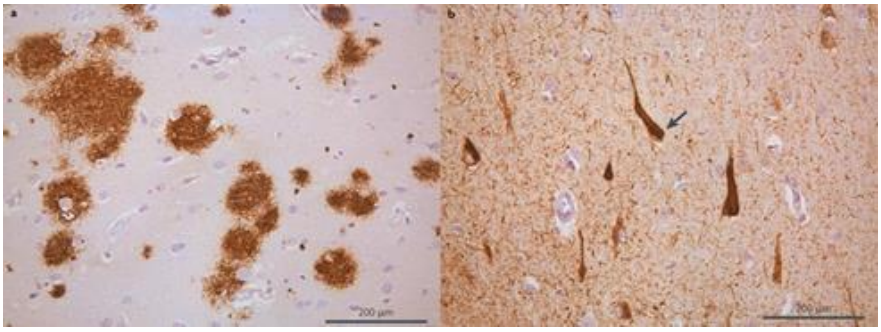

Figure 2. Cortical sections of the brain from a patient affected by AD. (a) Senile **plaques are labeled with a specific antibody for Aß and (b) neurofibrillary** tangles were stained with a specific antibody for phosphorylated tau (Figure taken from reference 31).

1.2.1.1.1 Aβ extracellular plaques or deposits

Aβ extracellular plaques or deposits are complex structures consisting of accumulated Aβ peptide, specifically Aβ 40 and Aβ 42, which is characterized by the presence of 39 and 43 amino acids and molecular weight of 4 kDa in its central part [32]. Aβ peptide originates from an abnormal proteolytic rupture of the amyloid precursor protein (APP), a transmembrane glycoprotein with a membrane domain and a short cytosolic domain [33] (*Figure 3*).

The amyloid cascade hypothesis considers that the processing of APP can occur by two metabolic pathways: a non-amyloidogenic pathway, or an amyloidogenic pathway. The former is the most frequent and is produced by the action of α-secretase, which cleaves the APP in the amino acid 83 from the C-terminal, generating two fragments: N-terminus and C-terminus. The former is secreted in the extracellular medium (sAPPα), and the latter, a C-terminal fragment of 83 amino acids (C83), is retained in the membrane and subsequently cleaved by a γ-secretase, producing a small soluble peptide (p3). However, if the processing takes place by an amyloidogenic pathway, the first proteolytic cleavage is mediated by β-secretase and located 99 amino acids away from the C-terminus. Thus, there is a release of sAPPβ and a membrane-bound fragment containing 99 amino acids (C99) is

generated. Then, C99 is processed by γ-secretase. This process may lead to a proteolytic cleavage in different positions. As a result, there is a release of Aβ peptide, which may contain between 37 to 49 amino acids (Aβ37- Aβ49), being Aβ40 the most frequent fragment. In any case, these aggregated Aβ peptides in small oligomeric structures give rise to the typical senile plaques observed in AD [34-36]. Different mutations related to the disease were found in APP proteolytic cleavage areas. They were identified as α-, β- and γ-secretases [37].

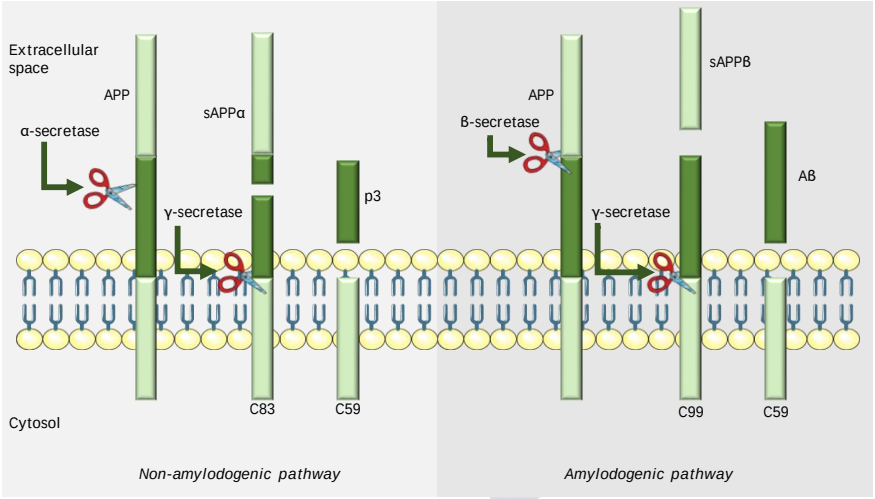

Figure 3. Scheme of the processing mechanisms of amyloid precursor protein (APP): non-amyloidogenic pathway and amyloidogenic pathway. The latter **allows the generation of β-amyloid peptide (Aβ) and the formation of senile** plaques (Figure taken from reference 38).

1.2.1.1.2 Neurofibrillary tangles

Neurofibrillary tangles are intracellular deposits formed by residues of hyperphosphorylated tau protein. In AD neurofibrillary tangles are more common in the areas of higher neuronal destruction, such as the hippocampus and the area of the temporal lobe [39]. Tau is a cytosolic protein which is part of the structure of microtubules present in the neurons, and it is widely present in the central nervous system. A characteristic of this protein is that it presents many areas of

phosphorylation, some of which can modify the union of the protein to the microtubules. Tau hyperphosphorylation produces the accumulation of fibrils forming tangles, which do not allow microtubule stabilization. This creates disruptions in the nutrient transport, and nerve signals, axon degeneration, neurotoxicity and, finally, cognitive impairment, typical of this pathology [39, 40] (*Figure 4*).

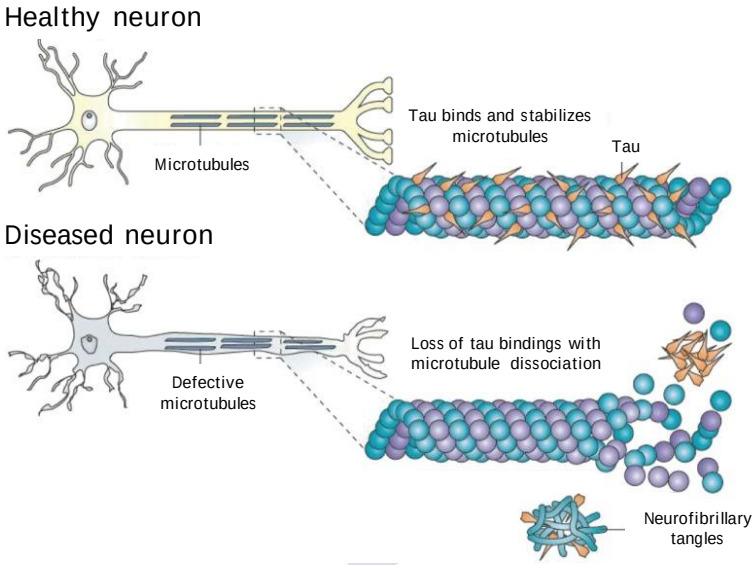

Figure 4. Neuronal death due to the destabilization of microtubules by tau hyperphosphorylation (Figure taken from reference 41).

In this context, currently three cerebral spinal fluid (CSF) markers of AD are included in research guides, and their use as inclusion criteria and/or outcome measures in clinical trials is increasing. These are Aβ - 42, total Tau (t-tau) and Tau phosphorylated at threonine 181 (p-tau) [42- 45]

1.2.1.2 Complementary hypotheses

Even so, the etiology of AD is unknown. There is growing evidence of the neuropathological processes explained above being the main cause of this disease, although it is well-known that genetic,

environmental and ageing factors may have an influence on this pathology. Traditionally, it has been considered that AD specifically affects the central nervous system, but recent studies are starting to consider that it may also affect the peripheral system. So, in the last years there has been a growing concern and interest in studies using biological fluids, such as serum or blood plasma, CSF, peripheral cells (e.g. lymphocytes, skin fibroblasts) and non-brain tissue [46].

There are many studies that relate the progression of AD, not only with pathologies such as plaques or extracellular Aβ deposits or neurofibrillary tangles, but also with other mechanisms [47]. Thus, other complementary hypotheses that try to explain the complex etiology of AD have been proposed. Some of these hypotheses are the cholinergic hypothesis, oxidative stress, inflammation, or the involvement of the metabolism of different metals. These hypotheses give a global view of the multiple pathological mechanisms behind the disease (*Figure 5*).

1.2.1.2.1 Cholinergic hypothesis

This is one of the most ancient pathologies, and it is related to the decrease of the neurotransmitter acetylcholine, as a result of the loss of cholinergic neurons in the hippocampus and brain cortex. Acetylcholine plays an important role in the functioning of memory and learning, which decreases in AD patients. This is one of the main hypotheses, as it has been a basis for the development of the most widely used drugs in palliative care of AD [48].

1.2.1.2.2 Inflammation

Neuroinflammatory processes were first related to AD many years ago, but there is great controversy over whether these processes cause the neuronal damage observed in this disease or they are just a natural protection response against other pathological processes (e.g. deposition of Aβ, formation of neurofibrillary tangles). These inflammatory mechanisms seem to be caused by overactive glial cells,

which produce a huge amount of pro-inflammatory molecules such as cytokines, the complement system and eicosanoids. Thus, the levels of interleukins, tumor necrosis factor and other cytokines increase in brain tissue of patients with AD. However, these disruptions have not only been found in the central nervous system, but also in the peripheral one [49,50].

1.2.1.2.3 Oxidative stress

It is a molecular mechanism present in many diseases. Oxidative stress which is consider an imbalance between free radicals and antioxidants mainly, reactive oxygen species (ROS) and reactive nitrogen species (RNS), caused a default in organisms protection. It is present in many diseases including AD. Thus, the brain has proved to be highly susceptible to oxidative stress, due to its high metabolic capacity, high concentration of easily oxidizable substrate and scarcity of antioxidant compounds, in comparison with other tissues.

So, numerous oxidative stress markers have been proposed as potential evidence for AD diagnosis. These markers can be simultaneously identified in both the central and peripheral nervous systems. These include the production of isoprostanes, protean carbonyls, 3-nitrotyrosine, thiobarbituric acid reactive substances, oxidized bases and decreased levels of antioxidants, among others [51-53].

1.2.1.2.4 Metal homeostasis

Metal elements play a crucial role in the development of the AD [54], involving both essential elements and toxic species. The involvement of the homeostasis of iron, copper and zinc in the pathogenesis of AD has been widely documented, as these essential metals take part in the proteinopathies which characterize this neurodegenerative disorder. These metals develped in the amyloid deposits in the brain, causing Aβ peptide aggregation and senile plaque deposition [55].

Moreover, have also been established relations between AD and different metals with a protective function. In this sense, it has been shown that there exists a negative correlation between cognitive impairment and levels of selenium and the activity of different selenoproteins in AD patients [56]. Furthermore, zinc has been reported to play a neuroprotective role –due to its antioxidant capacity- against Aβ-induced cytotoxicity in spite of its neurotoxic properties described above. This paradox proves the high complexity of the pathological mechanisms associated to AD [57, 58].

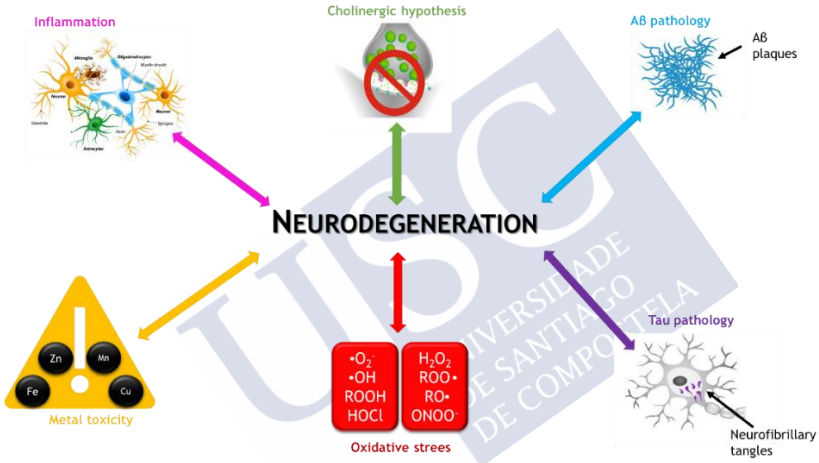

Figure 5. Pathological mechanisms implicated in AD.

1.2.2 Therapeutic approaches

Despite the scientific and clinical advances in the study of AD in the last 30 years, the available treatments are symptomatic; that is, they just alleviate the symptoms of the disease [59]. During the last decade, from 1998 to 2011, about 100 compounds, tested with the aim of altering the progression of the disease, have failed when they were in phase of clinical development [60, 61]. The reason for their failure could be explained by the complexity of the disease, due to its multifactorial etiology and its fisiopathological complexity. Finding a suitable drug, effective in all the trial population, is a challenging task.

Although certain key aspects of the pathogenesis of AD remain unsolved, the scientific advances in the last 25 years have made it possible to establish several strategies for the development of treatments with potential to alter the progression of AD [62]. Thus, among all the different therapeutic approaches in progress, those aimed to decrease the formation of Aβ42, and tau protein phosphorylation are the most promising [45]. These two types of disruptions are the best studied in this field, and may be the key for AD treatment in the near future.

Currently, there are only four drugs approved for the AD treatment. These belong to two groups: acetylcholinesterase inhibitors (AChEI) and N-methyl-D-aspartate receptor (NMDAR) antagonists [61, 63].

However, new treatments and therapeutic approaches are being investigated with the aim of stopping the disease progression, focusing on different targets and the drug administration in the early stages of AD. To enable effective treatments to be developed, new diagnostic techniques need to emerge. They must allow early diagnosis of AD in a preclinic phase (before symptoms begin to manifest) or even predict AD development. AD prevention is a realistic challenge for researchers. However, to make it possible, it is necessary a better understanding of its etiology and of the extent to which environmental influences and life style have an influence on the risk of developing this disease [64].

Nowadays, in addition to those mentioned above there exist many therapeutic approaches under consideration in order to address AD (*Figure 6*). There are as many approaches as hypothesis that try to explain this complex disease because, so far, researches have neither been able to elucidate the mechanisms involved in this pathology nor its origin.

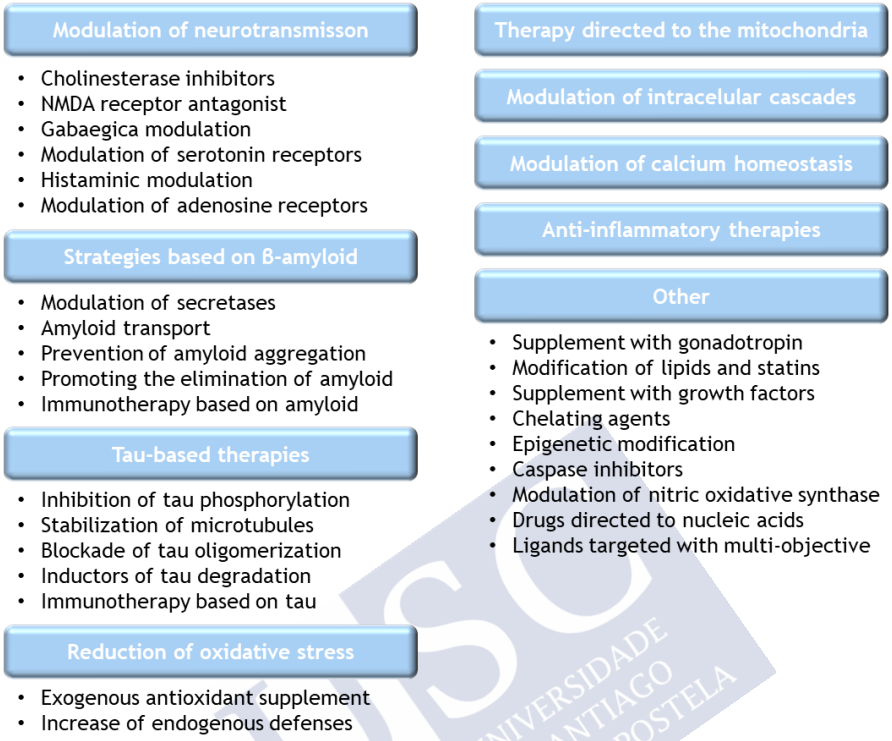

Figure 6. Current therapeutic strategies in AD.

1.3 BIOMARKERS

In the field of human health, the development, validation and use of biomarkers as information tools for evaluating risk factors associated to environmental exposures increase every day, due to the need of knowing the adverse effects generated by the different work environments and life styles.

Nowadays it is known that many diseases related to life styles; that is, in many occasions environmental exposure, food, physical activity or even geographic location are key factors in the manifestation and development of certain diseases such as several carcinogenesis, teratogenesis, genotoxicity, nephrotoxicity, neurotoxicity or

immunotoxicity, among others [65]. The risk of suffering health deterioration can be evaluated thanks to the use of biomarkers and it is expressed as the probability of an undesirable effect happening as a result of an exposure [66].

There are many and very diverse definitions of "biomarker". A wide and inclusive definition can be: "characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic pathways, or a biological response to a therapeutic intervention", which takes place at a cell or molecular level, and it is also associated with the probability of developing a disease [67]. Biomarkers are used for the understanding of different diseases in various aspects such as their treatment, prevention, diagnosis and progression, responses to therapy, experimental toxicological assessment of drugs and pesticides, measurement of environmental and epidemiologic risk, and assessment of therapeutic intervention, among others [68].

It is essential for a biological marker to be validated before its use in human health studies. Thus, its selection and validation require careful consideration. Its specificity, sensitivity and reliability as a risk measure need to be considered, establishing the correctness, accuracy and quality assurance of the analytical process and the interpretation of the measured data [69-73].

The factors that need to be considered in the selection and validation of a biomarker are [70]:

i. Identification and definition of the relevant biological process;

ii. Previous studies regarding the exposure agent, the biomarker and the effect to be assessed (in vitro studies in humans and other organisms);

iii. Identification of the variable to be quantified, to assess marker sensitivity and specificity in relation to the exposure;

iv. Selection of available tests for the analysis, reliability of the sample integrity between its collection and analysis;

v. Revision of the available analytic procedures for the marker quantification and its limitations with regard to detection, sensitivity, precision and accuracy;

vi. Standardization of a protocol which ensures adequate quality and control levels;

vii. Assessment of the intra and interindividual variation of unexposed population;

viii. Data analysis to establish the dose-response relationship and its variation, taking into account individual susceptibility;

ix. Prediction of health hazard for the general population or for the subgroup; and

x. Review of ethical and social considerations.

The use of biomarkers as elements of health risk assessment has growing importance, as they provide much information on the different types of diseases, the identification of health risk factors for individuals and populations, the selection of drugs, the evaluation of the progressions of diseases and their treatments, and the development of workplace and environmental health policies. Technology progress enables that implemented biomarkers are more and more specific, and they are essential in the development of the different biomedical disciplines that elaborate the strategies and policies to improve the living conditions of AD patients and people in general. This is so because they give information that allows for making correctives to decrease mortality and morbidity of individuals [74-85].

1.3.1 AD biomarkers

Early detection of AD in presymptomatic stages would offer a great opportunity to establish early therapeutic interventions with a higher chance of success, as they would take place before both the synaptic damage and the neuronal loss would be widespread. In this scenario, the incorporation of new more accessible and more affordable biomarkers in clinical practice would be very useful [86].

The physiological changes in AD pathology, such as neuritic plaques, neurofibrillary tangles, synaptic and neuronal loss, are widely analysed. These changes are also accompanied by disorders in the levels of some molecules in samples from the SCF, blood, serum, or even saliva [87, 88].

Biomarkers are promising tools to enable the development of more effective drugs for AD, and to establish a more personalized medicine [89]. Fluid biomarkers have the potential of being easy to implement in clinical trials, and several biomarkers affecting different pathophysiological mechanisms can be analysed in a single sample. Furthermore, SCF or blood can provide an informational window for the detection of some biomarkers which cannot be identified in brain images [90].

Currently, the distinctive features of AD can be assessed in vivo by biomarker analysis in two main categories: i) fluid biomarkers, including CSF and promising developments in blood; and ii) image biomarkers, such as positron emission tomography (PET) and magnetic resonance imaging (MRI) [91]. These biomarkers are currently providing reliable quantitative measures which can support early and accurate diagnosis of AD and have the potential to exceed the levels of precision by using clinical measures [92].

Thus, the biomarkers map related to AD increases and all of them are complementary (*Table 3*).

Table 3. Summary of selected candidate AD fluid biomarkers (Table taken from reference 48).

Biomarker	Stage of clinical validation	Levels in AD vs. healthy controls	Stage of assay development	
			CSF	Plasma/ Serum
$A\beta_{42}$	CSF $A\beta_{42}$ is accepted as part of research criteria IWG-2 criteria recommend using CSF Aβ42 in combination with CSF t-tau or p-tau Many studies on plasma but inconsistent results	- Consistently decreased in CSF. - Inconsistent results in plasma, although recent studies have shown a decrease.	Commercially available assays, including fully automated (IVDs in Europe)	Commercially available assays
$A\beta_{40}$	Many studies on CSF and plasma Inconsistent results for Aβ40 alone Consistent results for ratio of CSF Aβ42/Aβ40 Consistent results for ratio of plasma Aβ42/Aβ40	- Aβ40 alone: inconsistent results in CSF and plasma. - Ratio of Aβ42/Aβ40: consistently decreased in CSF and plasma.	Commercially available assays (IVDs in Europe)	Commercially available assays
$A\beta_{38}$	Several studies on CSF Aβ38 alone Inconsistent results for Aβ38 alone Very few studies on CSF Aβ42/Aβ38 One study on plasma Aβ38	- Inconsistent results in CSF but most studies showed increased levels/activity. - Increased activity in plasma but data are limited.	Commercially available assays	Commercially available assays
BACE1	Several studies on CSF Inconsistent results Very few studies on plasma	- Inconsistent results in CSF but most studies showed increased levels/activity. - Increased activity in plasma but data are limited.	Commercially available assays	Commercially available assays

Table 3. Summary of selected candidate AD fluid biomarkers (Table taken from reference 48) (Continued).

Biomarker	Stage of clinical validation	Levels in AD vs. healthy controls	Stage of assay development	
			CSF	Plasma/ Serum
T-tau	CSF t-tau is accepted as part of research criteria IWG-2 criteria recommend using CSF t-tau in combination with CSF Aβ42 Several studies on plasma Consistent results	- Consistently increased in CSF. - Consistently increased in plasma.	Commercially available assays, including fully automated (IVDs in Europe)	Commercially available assays
P-tau	CSF p-tau is accepted as part of research criteria IWG-2 criteria recommend using CSF p-tau in combination with CSF Aβ42 Few studies on plasma or serum Consistent results	- Consistently increased in CSF. - Increased in plasma and serum but data are limited.	Commercially available assays, including fully automated (IVDs in Europe)	In-house assays
hFABP	Several studies on CSF Consistent results Very few studies on plasma or serum	- Consistently increased in CSF. - No change in plasma or serum but data are limited.	Commercially available assays	Commercially available assay
TREM2	Few studies on CSF Inconsistent results Few studies on blood	- Inconsistent results in CSF but most studies showed an increase. - No change in plasma levels but data are limited; increased mRNA and protein levels in blood cells but data are limited.	Commercially available assays	Commercially available assays

Table 3. Summary of selected candidate AD fluid biomarkers (Table taken from reference 48) (Continued).

Biomarker	Stage of clinical validation	Levels in AD vs. healthy controls	Stage of assay development	
			CSF	Plasma/ Serum
IP-10	Few studies on CSF Inconsistent results Very few studies on plasma or serum	- Inconsistent results in CSF. - Inconsistent results in plasma or serum.	Commercially available assays	Commercially available assays
YKL-40	Several studies on CSF Inconsistent result Very few studies on plasma	- Inconsistent results in CSF but most studies showed an increase. - Increased in plasma but data are limited.	Commercially available assays	Commercially available assays
Neurogranin	Many studies on CSF Inconsistent results Few studies on plasma	- Inconsistent results in CSF but most studies showed an increase. - No change in plasma but studies are limited; decreased in plasma neuronally derived exosomes but data are limited.	Commercially available assays	Commercially available assays
SNAP-25	Two studies on CSF No studies on plasma	- Increased in CSF but data are limited. - Unknown for plasma.	Commercially available assays	Commercially available assays
TDP-43	No studies on CSF	- Unknown for CSF. - Increased in plasma but data are limited.	Commercially available assays	Commercially available assays

Table 3. Summary of selected candidate AD fluid biomarkers (Table taken from reference 48) (Continued).

Biomarker	Stage of clinical validation	Levels in AD vs. healthy controls	Stage of assay development	
			CSF	Plasma/ Serum
Synaptotagmin	One study on CSF One study on plasma	- Increased in CSF but data are limited. - Decreased in plasma neuronally derived exosomes but data are limited.	Commercially available assays	Commercially available assays
α-Synuclein	Few studies on CSF Inconsistent results Very few studies on plasma	- Inconsistent results in CSF but most studies showed an increase.	Commercially available assays	Commercially available assays
	Very few studies on plasma	- No change in plasma but data are limited.		
Ferritin	Very few studies on CSF Very few studies on plasma	- No change in CSF but data are limited; increased CSF levels are associated with cognitive decline, but data are limited. - No change in plasma but data are limited; plasma levels are associated with PET Aß but data are limited.	Commercially available assays	Commercially available assays

Table 3. Summary of selected candidate AD fluid biomarkers (Table taken from reference 48) (Continued).

Biomarker	Stage of clinical validation	Levels in AD vs. healthy controls	Stage of assay development	
			CSF	Plasma/ Serum
VILIP-1	Several studies on CSF Inconsistent results	- Inconsistent results in CSF but most studies showed an increase. - Increased in plasma but data are limited.	Commercially available assays	Commercially available assays
NF-L	Several studies on CSF Consistent results Few studies on plasma or serum	- Consistently increased in CSF. - Increased in plasma but data are limited.	Commercially available assays (IVDs in Europe)	Commercially available assays

Aß$_{38}$ amyloid beta 38, *Aß$_{40}$* amyloid beta 40, *Aß$_{42}$* amyloid beta 42, *AD* Alzheimer's disease, *BACE1* ß-site amyloid precursor protein cleaving enzyme 1, *CSF* cerebrospinal fluid, *hFABP* heart-type fatty acid-binding protein, *IP-10* interferon-ɣ-induced protein 10, *IVD* in vitro diagnostic, *IWG-2* International Working Group 2, *NF-L* neurofilament light, *P-tau*phosphorylated tau, *SNAP-25* synaptosome-associated protein 25, *TDP-43* transactive response DNA-binding protein 43, *TREM2* triggering receptor expressed on myeloid cells 2, *T-tau* total tau, *VILIP-1* visinin-like protein 1.

1.4 PROTEOMICS

The Human Genome Project revealed that the human genome is composed by approximately $3x10^9$ base pairs or nucleotides far, they have sequenced 25,000 genes that codify proteins, which represents 1.5% of the genome, whereas the rest are repeated DNA sequences which do not codify or codify regulatory sequences and introns for non-coding RNA [93, 94].

The completion of this project led to the current post-genomic era, when new research fields such as functional genomics, comparative genomics, transcriptomics, metallomics and proteomics, started to grow by leaps and bounds.

In 1994, Wilkins introduced the term proteome as a linguistic equivalent of genome [95]. The proteome defines the set of proteins that can be expressed by a genome. Unlike the genome, which is always the same in each cell throughout life, the proteome is an extremely dynamic element. It undergoes variations within a single organism, tissue, cell or subcellular compartment, in response to environmental and physiological factors such as age, stress conditions, toxic agents, drugs or hormones [96, 97].

The analysis of the proteome is a difficult task, due to the high number of existing proteins. As a single gen may codify multiple proteins, the proteome is estimated to have a more complex order of magnitude than the genome [98, 99]. Thus, a typical proteome may contain tens of thousands of unique proteins, which are present in a wide range of concentrations [100].

Once the proteins are synthesized, they may undergo modifications in their structure or in their basic sequence by proteolytic processes, as well as post-translational modifications, including methylations, phosphorylations and acetylations. All the above helps us to understand the high complexity of the proteome in a cell [92].

Proteomics is a tool endowed with a set of techniques and instruments with a high resolving power. For a robust proteomic analysis, it is necessary to optimize the current techniques. Moreover, their development and assessment are also needed in order to apply them in routine analysis, especially in the identification of biomarkers [101].

The strategies used or applied to the biological samples are vital, due to their great complexity. Thus, the analysis and characterization of the proteome will also be influenced by the sensitivity of the equipment used. In most cases, it is necessary or even obligatory the prefractionate of the sample, due to its complexity and the need for high resolution, as well as good results [102].

The application of proteomic techniques in the medicine field is called clinic proteomics, and its main aim is the identification of new biomarkers [103]. Proteomics provide information on the changes in the specific protein expression profiles and on the mechanisms associated to them in different cell stages (healthy and diseased), with the long term aim of developing better diagnosis methods and treatment of diseases [104]. This is not an easy task as the whole sequence of the complete human proteome is not still available (we only know a small part of the proteins present in the human body). Currently, the "Human Proteome Project" (HPP), which is focused in the characterization of the known 21,000 gens of the human genome, is being developed. The target of this project is to generate protein maps based on the molecular structure of the human body. The HPP will allow us to explain the biological function of proteins and how their modifications are affected under different cellular conditions [105].

The study areas of proteomics are wide and diverse. Among them, protein function, protein-protein interaction analysis, and their role in the metabolic and signaling pathways are worth-mentioning. All these have a vital importance to understand the role of proteins in biological processes [105-108].

There is a wide range of strategies or workflows to carry out a proteomic analysis. Exists such robust proteomic techniques that they comprise most of the range in protein concentration in a complex biofluid, from ultrasensitive (~0.05 pg / mL) to extremely abundant (~ 50 mg / mL). Conducting an experimental selection and a careful design has great importance in order to maximize the probability of quantifying precisely an object of interest (*Figure 7*).

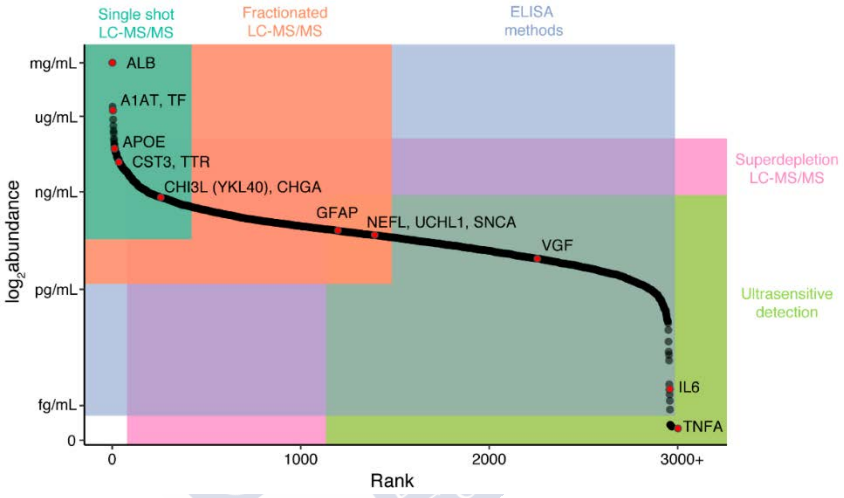

Figure 7. Chart of the main proteomics and molecular biology techniques according to their suitability to detect different concentration ranges in biofluids analytes (Figure taken from reference 94).

Usually, the experimental design of a proteomic analysis has the following stages: preparation and splitting of the sample, an analysis by mass spectrometry (MS) and computer analysis of the data (identification of proteins) [106].

1.4.1 Preparation and prefractionate of the sample

Sample preparation before its analysis is one of the most critical points of the work in a proteomic laboratory. Pre-analytical factors are those which take place before the sample analysis, including collection methods and materials, hemolytic contamination of the samples, sample

handling, storage temperature, thaw conditions and sample stability before processing. All these factors may affect accuracy and precision of the measured analytes [110-111]. The proteins integrity varies a lot with the frost/thaw cycle, which is specific of each proteomic platform, depending on the detection sensitivity. Ideally, sample collection methods and timing have to be strictly controlled to minimize diurnal effects, as well as taking into account the differences in the protein concentration between fasting and non-fasting, which may affect hormone, triglyceride and other marker levels. Certain protein levels may vary a lot from day to day, for this reason, it is important to examine the bitemporal stability of proteins before validating the results [113].

Most of the biological samples are very complex protein mixtures which cannot be directly analysed. Consequently, prefractionation is a crucial step in proteomics. The main problem of this type of samples is the masking of low-abundance proteins by high-abundance ones when, with the aim of finding a biomarker, the best expected results are found in the minority protein fraction. There are a number of techniques applied for this purpose, of which the recent use of nanotechnology (use of bare or functionalized nanoparticles), the one/two-dimensional gel electrophoresis and the high-performance liquid chromatography (HPLC) must be highlighted [114].

1.4.2 Analysis by mass spectrometry

MS is an analytic technique that emerges in the 60's, but it is widely known in the 70's. In the field of biological sciences, this technique has no relevance until the 90's since it is in this decade when the ionization techniques allow for the protein and peptide analysis. It is a widely used technique in many and different scientific areas, such as biomedicine, environment, pharmaceutical companies, food sector, toxicological analysis and even in the world of cosmetics [115].

The main feature of MS is that it not only provides the accurate molecular weight of the compound, but also its structural data, and in

many occasions, it is used for the structural analysis of certain substances or species [116]. That is, it is an analytic technique that provides quantitative and qualitative information of the analysed molecules.

A mass spectrometer is composed by three basic functional units: the ionization source, the mass analyser and the detector (*Figure 8*).

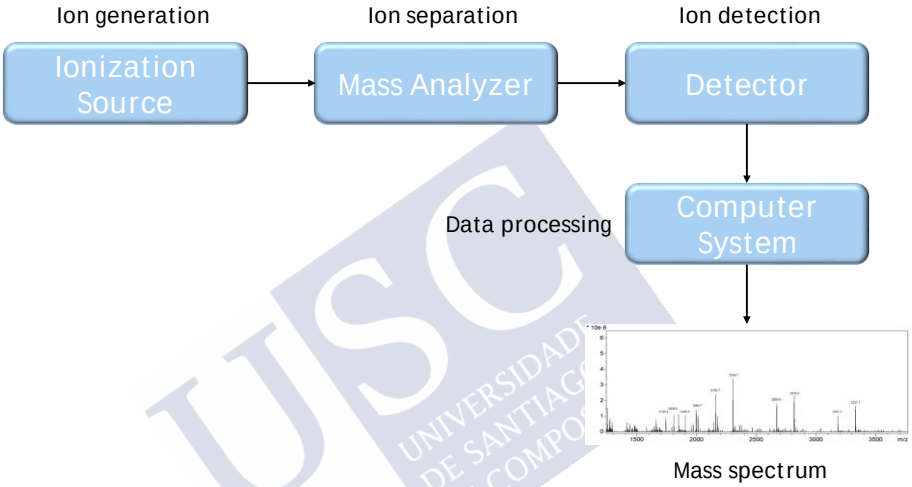

Figure 8. Parts of the mass spectrometry equipment.

The **ionization source** is the part that turns molecules into ions in the gaseous phase by means of gain or loss of charge (for example, loss of electrons, desprotonation or protonation). There are multiple ionization methods. They depend on the nature of the sample and the molecules to be detected in the analysis. In the case of biomolecular analysis in liquid and solid samples the matrix-assisted laser desorption/ionization (MALDI) and the electrospray ionization (ESI) are the most commonly used [117].

- MALDI. It was developed by Karas and Hillenkamp in the late 80's when observing the alanine co-adsorption. It was only expected to

visualize tryptophan at a wavelength of 266 nm, but it gave rise to soft ionization [118].

This technique consists on mixing analyte with an organic compound -such as sinapinic acid or α-cyano-4-hydroxycinnamic acid called matrix in the presence of an organic solvent, and the deposit of the mixture is a metal plate. When the organic solvent evaporates, the matrix co-crystallizes with the analyte. The plate is introduced in the ionization chamber of the mass spectrometer, which is under hard-vacuum conditions, and an ultraviolet (UV) laser is applied in it. The crystalized mixture absorbs the laser energy, producing analyte ions in the gaseous phase, most of them having just one positive charge. This results in an ion beam which is oriented and redirected to the ion analyzer (*Figure 9*) [117].

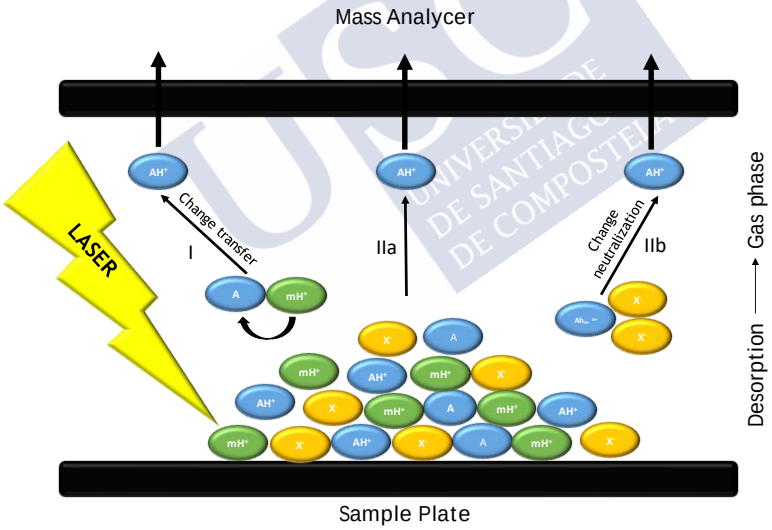

Figure 9. Major proposed models for MALDI ionization; [I] Gas-phase protonation, showing the charge transfer from the ionized matrix (mH+) to the analyte (A). [IIa] Direct desorption of the preformed singly charged analyte (AH+). [IIb] The multiply charged analyte (AHn+), where incomplete **neutralization by the counterions (X−) or electrons occurs in the gas phase, producing the singly charged analyte ions (AH+). (A = analyte, m= matrix, x− =** counterion).

- ESI. This technique emerged in the late 60's, with the experiments made by Dole and his collaborators [119]. However, it is not until 2002 when John Fenn publishes an interesting article on the personal and historical memories of the pros and cons of ESI [120], being awarded the Nobel Prize in Chemistry together with Koichi Tanaka.

It is an ionization technique in which analytes are in dissolution. This dissolution is drawn through a thin stainless-steel capillary to which a high electric potential -around 5.000 watts- is applied. The result is the nebulization of the dissolution. Thus, small charged drops are formed in the form of spray and subjected to a high-temperature gas stream, which removes the solvent and leaves protoned analytes in gaseous phase. The generated ions are later accelerated in an electric field towards the mass analyzer (*Figure 10*) [117].

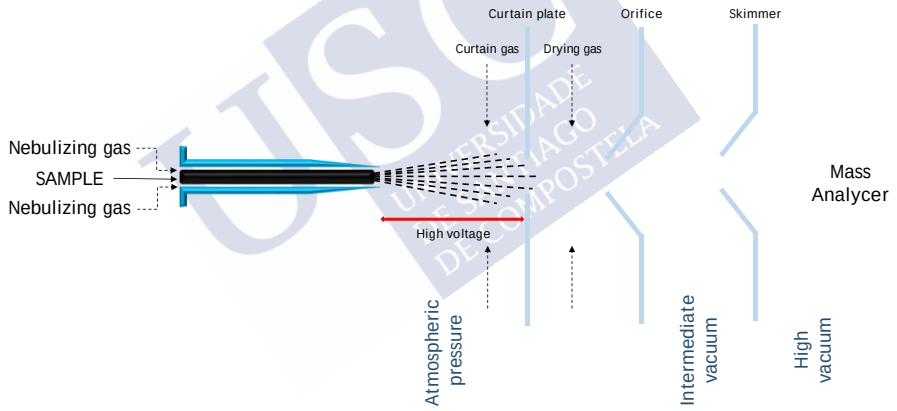

Figure 10. Schematic diagram of an ESI source.

ESI ionization allows direct connections with the mass spectrometer such as the capillary electrophoresis and the liquid chromatography, which facilitate and make it possible the massive identification of analytes in very complex samples. Nowadays, the MALDI technique is still being used, but the number of proteins which is able to identify is much lower. Thus, sample simplification by pre-fractionation techniques such as 2-DE or liquid chromatography in this case is needed [121].

In the **mass analyzer**, ions are separated according to their mass-to-charge-ratio (m/z) by means of magnetic and/or electric fields. This part is considered the heart of the equipment and it is currently available in a wide range of analyzers, each of them with advantages and disadvantages according to the required results. Among them, the use of the cuadrupole, the ion trap and the time-of-flight (TOF) must be highlighted [122].

- CUADRUPOLE. This type of analyzers contains four equidistant metallic cylinders which are applied a direct current (DC) potential, and another of radio frequency (RF). When applying variable RF voltage, ions of a determined m/z ratio go through the cylinders, keeping the trajectory, while others are diverted. In this way, ions of different m/z ratio can be sent to the detector successively, whereas the rest are discarded (*Figure 11*). This type of analyzers is the most widely used in tandem mass spectrometry. Among its advantages, it has to be emphasized the fact that they are extremely fast, the narrower the scanning range, the higher the speed [123, 124].

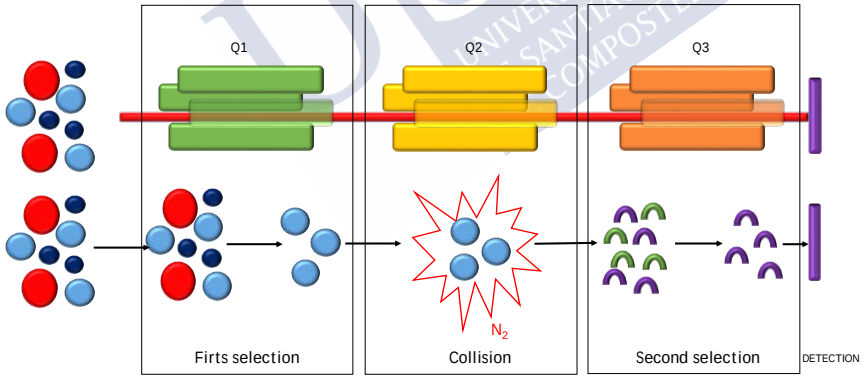

Figure 11. Scheme of a triple quadrupole mass analyzer.

- IONS TRAP. It is an analyzer able to isolate ions within a circular electrode, which has two pierced hemispherical electrodes on top and below. The ions go in and out through these pierced electrodes. Thus, the ions of a certain m/z ratio are trapped according to the electrode voltage setting. After changing this setting, ions go towards the detector

(*Figure 12*). The analyzers may be one of these two types: Orbitrap, if the applied field is electric, or ion cyclone resonance using Fourier (FT-IRC), if the field is magnetic [124].

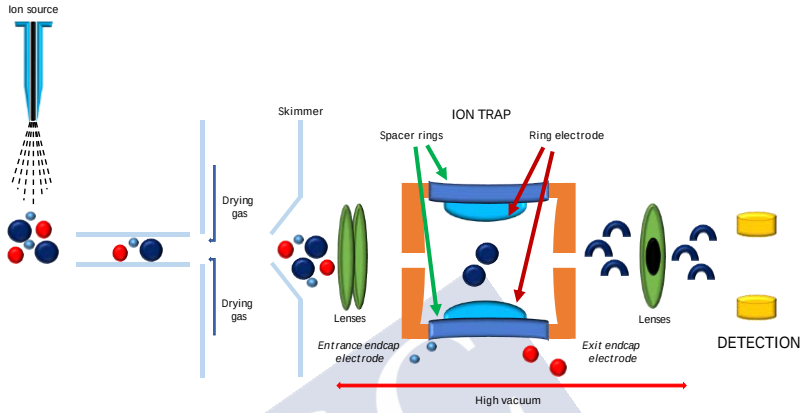

Figure 12. Scheme of an ion trap mass analyzer.

- TOF. It is one of the simplest and most widely used analyzers in laboratories. Ions are accelerated with a determined voltage towards the flight tube. Ion separation is based on the relation between mass and speed of ions; that is, in the amount of time needed to cover the length of the tube. These analyzers may include a reflectron, which allows to increase the resolution and improve ion separation [124, 125].

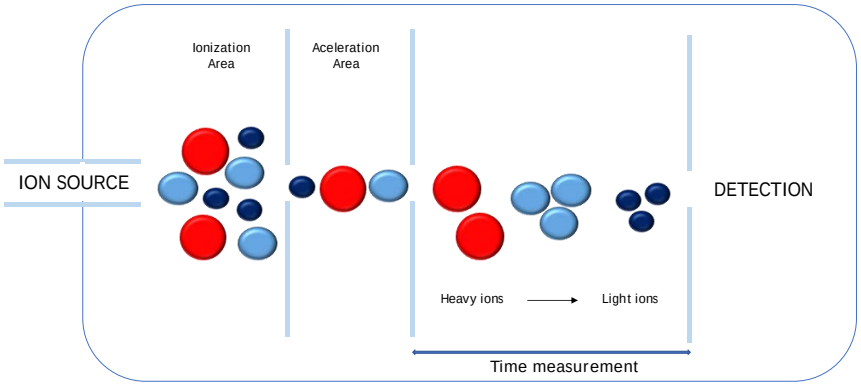

Figure 13. Scheme of a TOF mass analyzer.

The TOF analyzer analyzes ion packages coming from the ionization source with the same features, which makes it a perfect combination with MALDI ionization. Thus, MALDI-TOF mass spectrometers are the most widely used in peptide and protein analysis. Among the different advantages of this combination, it must be emphasized the wide range of ion masses that can be analyzed, from peptides of few amino acids to proteins with a high molecular weight [126].

Finally, the **ion detector** generates signals which are proportional to the abundance of each ion, by recording the induced charge or the current produced when an ion passes by or hits a surface. There are different types of detectors, such as Faraday cup, electron multiplier and the photographic plate [117].

1.4.3 Protein identification

The most widely used methods of protein identification are peptide mass fingerprinting (PMF) and the peptide sequencing by tandem mass spectrometry (MS/MS).

PMF corresponds to the peptide fragments resulting from the digestion of a protein with a specific enzyme, usually trypsin, which specifically cleaves before the residues of lysine and arginine. In this way, small fragments with a specific mass are generated and compared with the calculated values of the virtual digestion of numerous known proteins collected in databases such as NCBInr, MSDB or Swissprot.

The problem with this type of identification is that only pure proteins or proteins from not very complex protein mixtures. That is why currently this type of identification is used for structural purposes in the characterization of proteins, and MALDI-TOF is the technique used [127].

MS/MS not only provides identification of proteins and their structural information, but it also eliminates interferences in the case of

quantitative studies. Currently, these quantitative analyses are vital due to an upturn in biomarker studies in the field of clinic proteomics [117].

In this technique, several analyzers are combined as a tandem. In the first one, an ion of a determined m/z, which is called precursor ion, is isolated. It is fragmented by an inert gas and multiple collisions between the ions, and the resulting fragments go to the next mass analyzer. Hence, the protein is not only identified by its peptide fingerprint, but also by the fragments of its peptides. It must be taken into account that the peptide fragmentation spectrum is characteristic of each peptide as the amino acid sequence is unique. All this information is compared with databases and the protein identified accurately.

1.5 METALLOMICS

The term metallome was initially defined as the distribution of an element, the distribution of free metal ions, or the free content of an element in a cellular compartment, cell or organism. Currently, the term metallome refers to all the species of a metal or metalloids present in a cell or type of tissue as well as to their identity, quantity and placement [128].

Hence, metallomics is defined as the study of metallome, of the functional connections of metal ions and their species with genes, proteins, metabolites and other biomolecules in organisms and ecosystems [129].

Metal elements and metalloids are essential components of biological systems which regulate and take part in numerous cellular processes. Thus, correlations are established between the elemental concentrations and the genome statistically, structurally or functionally [130].

Recently, different sub-disciplines have emerged in the context of metallomics, the most relevant being metalloproteomics, metallometabolomics and ionomics [131].

Unlike the other omic sciences, the study field of metallomics presents a high chemical and functional heterogeneity due to the wide range of existing metallo species [132]. Among these metallo species, metalloproteins must be highlighted. They can be linked to a hetero element or they can contain a metal core joined by coordination.

Metalloproteins are the proteins to which a metal confers a certain function. Among the different functions one can find the catalytic activity, the implication in an electronic transfer reaction, or even the stabilization of its tertiary or quaternary structure. When a metal is bound to the protein by high affinity interactions, that is, the union is not broken when it is manipulated, it is called the metalloprotein; But when the binding occurs through low affinity interactions, and breaks easily, they are called metal-protein complexes [133].

Due to the specificity of metalloproteomic studies, it is vital to describe the binding sites of metal proteins, metal stoichiometry and the structural changes that appear from the metal-protein linkage. In the study of metalloproteins it is necessary to preserve the link between the metal and the protein, develop methodologies that allow for the specific detection of metalloproteins in a sample, and purification and/or preconcentration of proteins containing metals [112].

As the levels of metalloproteins in biological samples are at trace levels, this scientific field needs to combine high-resolution techniques for the separation of proteins with sensitive enough techniques to detect metals. Among the wide range of options to separate proteins, gel electrophoresis, capillary electrophoresis and high-resolution chromatography must be highlighted [113]. The **inductively coupled plasma mass spectrometry** (ICP-MS) is the most used analytical technique in metallomics for the detection of metals associated to proteins. Among the numerous advantages of mass spectrometry, its use as detector (atomic masses) with ICP has the exceptional advantage of differentiating between metal linked to protein and free metal, previous separation.

This technique in not only used in liquid samples, but it has been and is also used in solid samples and even to measure isotope ratios [134].

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2. SPECTROMETRIC BASED TECHNIQUES FOR METAL-BINDING PROTEIN ASSESSMENT IN CLINICAL, ENVIRONMENTAL AND FOOD SAMPLES

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II. OBJECTIVES

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Objectives

Current limitations in early diagnosis of the Alzheimer's disease (AD) have led to an increasing research on discovering sensitive and specific biomarkers. In addition, the potential applicability of biomarkers should be also used for diagnosing AD at different stages as well as people suffering mild cognitive impairment (MCI).

Therefore, highly sensitive and robust metallomic and proteomic techniques have been developed/applied for charactering biochemical disturbances associated to the evolution of AD in the current research.

Recent data have confirmed the influence of essential metals (Fe, Cu, Zn, etc.) in neurodegeneration processes, and levels of these metals in blood serum and cerebrospinal fluid have been found to be altered in AD patients.

The main objective of this Doctoral Thesis has been the discovery of serum biomarkers (metals, minor proteins and metalloproteins) for allowing an accurate discrimination among healthy people (controls), MCI patients and AD patients.

The specific objectives of the Doctoral Thesis are therefore as follows:

1. Study of trace metals as potential biomarkers in serum micro-samples.

1.1 Development and optimization of a method based on serum dried spots (20 µL of serum sample dried on paper) and further laser ablation (LA) and inductively couple plasma – mass spectrometry (ICP-MS) as determination technique (**Chapter 1 Experimental Part**).

1.2 Development of discrete sampling based-flow injection procedures that allow low sample consumption by exploring the

possibilities of new and advanced sample introduction systems such as SeaFast for ICP-MS measurement (**Chapter 2 Experimental Part**).

2. Proteomic study for the identification of minor serum proteins (qualitatively and quantitatively) altered in healthy people and MIC and AD patients.

2.1 Developments of sample pretreatments for major/minor proteins depletion before applying proteomic platforms.

2.2 Development and application of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) methods for minor proteins separation before identification by mass spectrometry (MS) (**Chapter 3 Experimental Part**).

2.3 Development and application of advanced Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH) methods for minor proteins determination/identification by MS techniques (**Chapter 4 Experimental Part**).

3. Study of the levels of trace metals associated with proteins in serum samples.

3.1. Development of LA-ICP-MS methods for assessing trace metals in selected proteins spots after 2D-PAGE (**Chapter 5 Experimental Part).**

3.2 Study of the possibilities of the levels of metals in metal-protein complexes as potential biomarkers of AD diagnosis (**Chapter 5 Experimental Part**).

III. RESULTS AND DISCUSSION

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CHAPTER 1

DEVELOPMENT OF DRIED SERUM SPOT SAMPLING TECHNIQUES FOR THE ASSESSMENT OF TRACE ELEMENTS IN SERUM SAMPLES BY LA-ICP-MS

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CHAPTER 2

DISCRETE SAMPLING BASED-FLOW INJECTION AS AN INTRODUCTION SYSTEM IN ICP-MS FOR THE DIRECT ANALYSIS OF LOW VOLUME HUMAN SERUM SAMPLES

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CHAPTER 3

SERUM PROTEIN-BASED BIOMARKERS IN MILD COGNITIVE IMPAIRMENT AND ALZHEIMER´S DISEASE

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Serum protein-based biomarkers in mild cognitive impairment and Alzheimer's disease

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Abstract

Low abundant proteins in serum from patients suffering mild cognitive impairment (MCI) and Alzheimer's disease (AD) have been explored as potential biomarkers for early AD diagnosis. After protein depletion by affinity chromatography, low abundant proteins were separated by 2-DE, and were further identified by MALDI-TOF. SameSpots software was used to discover increased/decreased proteins in two patient groups (MCI and AD). Statistically significant differences were found for pigment epithelium-derived factor (PEDF), ficolin-3 (FNC3), serotransferrin (TRFE), and haptoglobin (HP), all of them increase in AD patients. Other serum proteins, previously reported to be decreased in AD patients, and to be related to other illnesses, were also assessed. In addition, some increased proteins (PEDF, TRFE, and HP), as well as many decreased proteins, were found to undergo post-translational modifications (phosphorylation). Serum PEDF and HP have been found as potential biomarkers of early AD. PEDF has also been reported to be increased in cerebrospinal fluid (CSF) in AD patients; whereas, serum HP has been reported to be increased in AD patients compared to healthy people.

3.1 INTRODUCTION

Alzheimer's disease (AD), first described by a German neurologist Alois Alzheimer, is a physical disease affecting the brain [1]. AD is the most common cause of dementia and implies a progressive neurodegenerative disorder that usually appears sporadically. AD neuropathological hallmarks are senile plaques (SP) which contain deposits of β−amyloid peptide (Aβ), and neurofibrillary tangles (NFT), which result from the aggregation of hyperphosphorylate Tau protein [2]. Mild cognitive impairment (MCI) has been proposed to be an early phase of cognitive decline that precedes dementia. MCI patients may progress to AD, vascular disease and other kinds of dementia. MCI is an intermediate stage between normal cognitive decline with aging and dementia. During this stage, patients have a greater cognitive decline than expected for their age and educational level [3]. Rates of annual conversion from MCI to dementia are reported to range from 2.7% to 15%. In addition, the percentage of MCI patients who finally suffer AD in the first four years is 56%. A longitudinal study showed that people with MCI were 6.7 times more likely to develop AD than cognitively normal individuals. MCI may therefore be a useful AD prodromal phase in which to test putative biomarkers for early AD diagnosis [4].

There is an urgent need for biomarkers of AD, especially to detect the early stages of the disease. Strategies for biomarker discovery in AD are based on advanced neuroimaging assays, and certain candidate proteins (β-amyloid and tau protein) in cerebrospinal fluid (CSF) [5]. However, these methods may not be widely available for use in large, community based, multicenter studies, or in routine clinical care of large numbers of frail elderly people [6]. Although CSF represents the most suitable biological fluid to study neurodegenerative diseases because it can point to biochemical changes occurring in the brain, CSF analysis is not always feasible because the costs involved are enormous; furthermore, CSF procedures are invasive, uncomfortable and unsafe for the patient. For a full screening and early diagnosis, biomarkers easily detectable in biological samples, such as plasma/serum, are needed. Blood-based biomarkers sampling is less invasive than CSF,

and could increase diagnostic accuracy and be useful for prognosis and in monitoring therapeutic interventions, especially for large scale studies and for repeated measures. The search for reliable AD biomarkers in peripheral blood is very challenging due to difficulties with the standardization of the analysis methods and the low reproducibility of the results [7].

Human serum proteins originate in a variety of tissue and blood cells as a result of secretion or leakage. Numerous biomedical studies have demonstrated that plasma protein levels reflect human physiological or pathological states, and they can be used for disease diagnosis and prognosis. Serum proteome analysis has been hampered by the predominance of several highly abundant proteins including albumins, immunoglobulins, alpha-1-antitrypsin, fibrinogen, and haptoglobin, and their isoforms and fragments [8]. As the presence of abundant proteins in most biofluids used for diagnostic purposes decrease the capacity of analytical methods to detect low-abundance proteins or peptides, several approaches have been developed to reduce the total amount of protein. Depletion of these highly abundant proteins is often desired prior to protein analysis [9].

Proteomics is the branch of science that characterizes the entire proteome (the entire set of proteins expressed by a genome) by analyzing the structure and functions of all proteins present in cells or body fluids [10]. The primary aim of proteomic analysis is to separate, identify and characterize proteins, and understand their interactions with other proteins. Proteomic analysis can be used on tissue samples and also on body fluids such as serum, plasma, urine and saliva [11]. Recent progress in proteomics provides a new basis for a better understanding of plasma and serum proteins as potential biomarkers of several diseases. Valuable information about the process of pathogenesis of a disease can be obtained by comparing the different protein expression profiles between plasma samples from healthy and patients. There are several analytical approaches for proteomic studies. Two-dimensional gel electrophoresis (2-DE) is one of the most effective and widely used methods to separate proteins; whereas, mass

spectrometry (MS) is commonly used for protein identification and characterization [10].

The aim of the current research has been to explore the possibilities of certain serum proteins as potential biomarkers of early AD. The expression levels of serum proteins were analyzed and compared between AD and MCI patients. Protein separation was achieved by 2-DE; whereas, protein identification by peptide mass fingerprinting using a MALDI-TOF/TOF (matrix-assisted laser desorption ionization time of flight) [12].

3.2 MATERIAL AND METHODS

3.2.1 Clinical samples

Serum samples from healthy volunteer adults and patients were supplied by the *Servicio de Neurología* at the University Clinical Hospital of Santiago de Compostela (Santiago de Compostela, Spain). The developed research has been ascribed to the approved and in force expert opinion from the *Comité de ética de la investigación con medicamentos* – CEIm-G (Ethics Committee for the Research with medicines) of Galicia (Registration Code: CEIm-G 2018/575).

For serum collection, a total of 2 mL of venous blood was collected in Vacutainer blood collection tubes with silicone-coated interiors (BD Diagnostics, Franklin Lakes, NJ, USA) by a standard venipuncture method. The collected serum samples were stored at room temperature to allow for blood clotting, and then centrifuged at 1800 g for 10 min at 4 °C in order to remove the fibrin clot and other cellular elements. Serum samples were then immediately frozen at –80 °C.

3.2.2 Preparation of serum proteins

Six most abundant plasma proteins (albumin, transferrin, immunoglobulins G and A, haptoglobin and antitrypsin) were immunodepleted from serum pools using the Multiple Affinity

Removal System Hu6 column and buffer kit on an Infinity1260 HPLC system (Agilent, Santa Clara, CA, USA) according to manufacturer's instructions. The fractions were them concentrated using a Centricon 3K (Millipore, Burlington, MA, USA), followed by precipitation (standard MeOH/$CHCl_3$ protocol) before their separation by 2DE.

Samples were re-suspended in the 2-DE buffer (65 mM DTT, 65 mM CHAPS, 5M urea, 2M thiourea, 0.15M NDSB-256, 200nM tributylphosphine, 100nM NaF, 1M Na_3VO_4, and 1M benzamidine) and stored at –20°C until further use. All reagents were of high purity and were purchased from Sigma-Aldrich (St. Louis, MO, USA). The re-suspended proteins were centrifuged at 13000g for 5 min, and quantified before 2-DE for total protein content (RC DC Protein Assay, BioRad Lab, Hercules, CA, USA).

3.2.3 2-DE

Isoelectric focusing (IEF) was performed in pH 4-7 immobilized pH gradient (IPG) strips of 17 cm (GE Healthcare, Uppsala, Sweden). 500 µg of protein were re-suspended in 300 µL of 2-DE buffer supplemented with ampholytes (0.1% servalyte 3–10 and 2-4, 0.05 % servalyte 9-11 (SERVA, Heidelberg, Germany), and subjected to solubilization during 2 h. After solubilization, the mixture was centrifuged at 13000g for 20 min. Samples were applied to each strip and submitted to active rehydration at 50 V during 12 h before IEF (performed in a Protean IEF Cell focusing unit (BioRad) until 20000 V/total h were reached). After IEF, the IPG strips were equilibrated for 15 min in 4M urea, 2M thiourea, 50mM Tris pH 6.8, 2% sodium dodecyl sulphate (SDS), 12mM dithiothreitol (DTT) and 30% glycerol plus 1% DTT and 15 min in 4M urea, 2M thiourea, 50mM Tris pH 6.8, 2% sodium dodecyl sulphate (SDS), 12mM dithiothreitol (DTT) and 30% glycerol plus 4% iodoacetamide (all reagents from Sigma-Aldrich). SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12% polyacrylamide gels using a ProteanPlusDodeca Cell (BioRad) at 15 mA/gel and 18 °C constant temperature for 12 h, or until the dye front reached the bottom of the gel. The 2-DE gels were stained with

Sypro Ruby (Lonza, Basel, Switzerland) following manufacturer's instructions.

3.2.4 Image acquisition and software analysis of 2-DE gel

The Sypro Ruby stained 2-DE gels were scanned using a Typhoon fluorescence scanner (GE Healthcare). The scanned images were processed using the ProgenesisSameSpots software v.4.5 (Nonlinear Dynamics, Durham, NC, USA). Both manual and automatic alignments were used to align the images. All gels were compared, and the fold-changes (FC) and p-values of all spots were calculated using the SameSpots software with 1-way ANOVA analysis. The differential protein expression was considered significant when the FC was at least 1.8, and the p-value was lower than 0.05.

3.2.5 Tryptic digestion

Digestion of the spots from 2-DE was manually performed according to the protocol established by Shevchenko et al. [13], with minor modifications. The spots selected from representative 2-DE gels were excised and washed with a solution containing 50 mM NH_4HCO_3 and 50% MeOH HPLC grade (Scharlau, Barcelona, Spain). The proteins were reduced with 10 mM DTT in 50mM NH_4HCO_3 and alkylated with 55mM iodoacetamide in 50mM NH_4HCO_3. Subsequently, the proteins were rinsed with 50 mM NH_4HCO_3 in 50% MeOH, dehydrated through the addition of acetonitrile (ACN) (HPLC grade, Scharlau) and dried in a Speed Vac (Thermo Scientific, Waltham, MA, USA). Modified porcine trypsin (Promega, Madison, WI, USA) prepared in 20mM NH_4HCO_3 (concentration of 20 g/μL) was added to the dried gel slices, followed by incubation at 37°C for 16 h. The peptides were extracted three times by incubation in 40 μL of a solution containing 60% ACN and 0.5% formic acid (HCOOH) for 20 min. The resulting peptide extracts were pooled, concentrated in a Speed Vac and stored at −20°C.

3.2.6 Protein identification through MALDI-TOF

MALDI-TOF analysis of the peptides digested from spots was performed by mixing equal volumes (0.5 μL) of peptides and matrix solution (3 mg of α-cyano-4-hydroxycinnamic acid (CHCA) dissolved in 1 mL of 50% ACN in 0.1% trifluoroacetic acid (TFA)). The mixture was deposited onto a 384 Opti-TOF MALDI plate (Applied Biosystems, Foster City, CA, USA) using the thin layer method. Mass spectrometric data were obtained in an automated analysis loop using 4800 MALDI-TOF/TOF analyser (Applied Biosystems). MS spectra were acquired in reflector positive-ion mode with a Nd:YAG, 355 nm wavelength laser, averaging 1000 laser shots, and at least three trypsin autolysis peaks were used as internal calibration. All MS/MS spectra were performed by selecting the precursors with a relative resolution of 300 (FWHM) and metastable suppression. Automated analysis of mass data was achieved using the 4000 Series Explorer Software V3.5. MS and MSMS spectra data were combined with the Protein Pilot Explorer Software v4.5 using Mascot software search engine v2.1 (Matrix Science, Boston, MA, USA).

Searches were performed against a non-redundant database (release version 2016-05; February, 551193 entries) with 100 ppm precursor tolerance, 0.35Da MS/MS fragment tolerance, and allowing only missed cleavage. All spectra and database results were manually inspected in detail using the previously mentioned software. Protein scores greater than 56 were accepted as significant ($p<0.05$), considering the identification positive when the protein score (CI%, Confidence Interval) was above 98. In the case of MS/MS spectra, the total ion score CI% was above 95.

3.3 RESULTS

Currently, AD diagnosis is based on several valuations, such as medical history, physical examination, neurophychological tests, and brain scans, the latter being the most important diagnosis tool. However, a brain scan is only useful (brain imaging appears abnormal

compared to a typical healthy brain) when AD is in middle or late stages. There is therefore no single test that can diagnose early AD with 100% accuracy [1]. Current techniques predict the disease when it is already advanced. The development of early biomarkers is therefore necessary. Serum is one of the most representative biofluids, and AD could cause characteristic changes in the concentrations of some biomolecules such as serum proteins. Post transcriptional modifications of signaling proteins could generate a detectable disease-specific protein partner [14].

3.3.1 High abundance protein depletion

The presence of high abundant proteins in most biofluids decreases the capacity of the analytical methods by a factor from 5 to 10 to detect low-abundance proteins. High-abundance proteins removal, therefore, allows improvements when assessing low-abundance proteins.

Several approaches have been developed to deplete high abundant proteins [9], and affinity chromatography is one of the most appealing strategies. Affinity columns are based on dye ligands or antibodies for albumin removal, and on protein A or G for removing immunoglobulins (IgGs). New multiple affinity chromatographic columns allow several high-abundance proteins (albumin, IgG, IgA, transferrin, haptoglobin, and α1-antitrypsin) depletion at the same time, and have been found to be the most effective protein depletion systems, providing more reproducible results (retention times and peak areas) during LC-MS analysis than previously proposed methods [8].

Serum samples (200 µL) have been diluted five times with buffer A, and high abundant proteins were depleted using a Multiple Affinity Removal System Hu6 chromatographic column following the manufacturer's instructions. *Figure 1* shows a chromatogram with two high chromatographic signals corresponding to low abundance proteins (retention times between 2 and 5 min) and high abundance proteins (retention times between 12 and 13.5 min). The presence of low abundance proteins was, however, checked along the whole

chromatographic run by dividing the chromatogram into nine fractions at different retention times as shown in *Figure 1a* (fractions 2 and 4 encompass the two high chromatographic signals related to low abundant and high abundant proteins, respectively). Each fraction was further loaded/run in a 10 % SDS-gel, and results show that low abundant proteins are also present in fraction 3 (retention times between 5 and 12 min). Therefore, the low abundance proteins fraction was fixed between 2 and 12 minutes (fraction 2 and 3 in *Figure 1b*); whereas, high abundance proteins were fixed between 12 and 13.5 min (fraction 4 in *Figure 1b*).

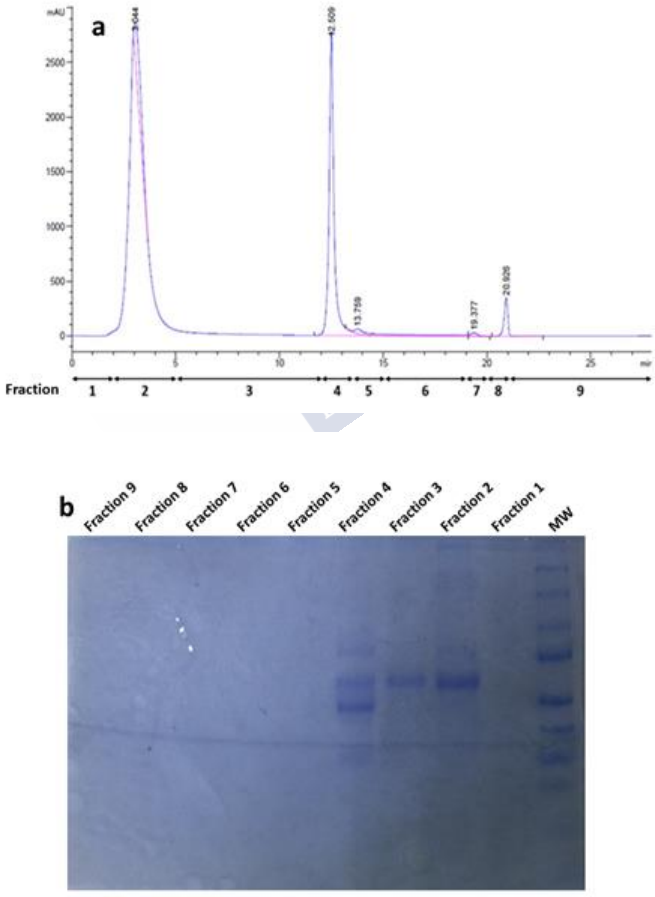

Figure 1. a) Serum chromatogram. b) SDS-gel of 9 fractions.

3.3.2 2-DE and gel image analysis

Proteins (low and high abundance proteins) from seven control (CTR) and seven patient samples (three AD and four MCI patients) were separated according to their isoelectric point and molecular weight by 2DE (17 cm strips pH 4-7, 10 % SDS gel), and images of the gels were compared with the SameSpots software (*Figure 2*). Most studies regarding low abundant proteins involved in AD compare only control (CTR) and AD patients [1, 12, 15-18]. However, MCI patients have been included in our study, and we have performed CTR/MCI, and CTR/AD (data not given), and MCI/AD comparisons (more novel and reliable results for early AD diagnosis).

Gel images from AD and MCI patients were obtained for low abundance and high abundance protein fractions. Results show differences between AD and MCI patients when performing analysis of high and low abundance proteins. Regarding the high abundance proteins fraction, sixteen statistically significant spots (p-value <0.05 and a Fold Change >1.3) have been obtained. Seven spots showed to be increased in AD patients; whereas, nine spots were found to be decreased in AD patients. There were more statistically significant differences in spots of the low abundance proteins fraction. Twenty-four spots showed p-values lower than 0.05 and Fold Change ratios higher than 1.7. Seven of these spots were increased (*Figure 3a*) and 17 spots were decreased in advanced AD patients (*Figure 3b*).

The value of log normalized volume, expressed as the mean value and standard deviation of the same spot in gels of different patients (also given by the SameSpots software), has further been used to know the extension of the increase/decrease of certain spots. Results after comparing two sets of gels (AD and MCI patients) classify the spots into increased (*Figure 4a*) or decreased in AD patients (*Figure 4b*).

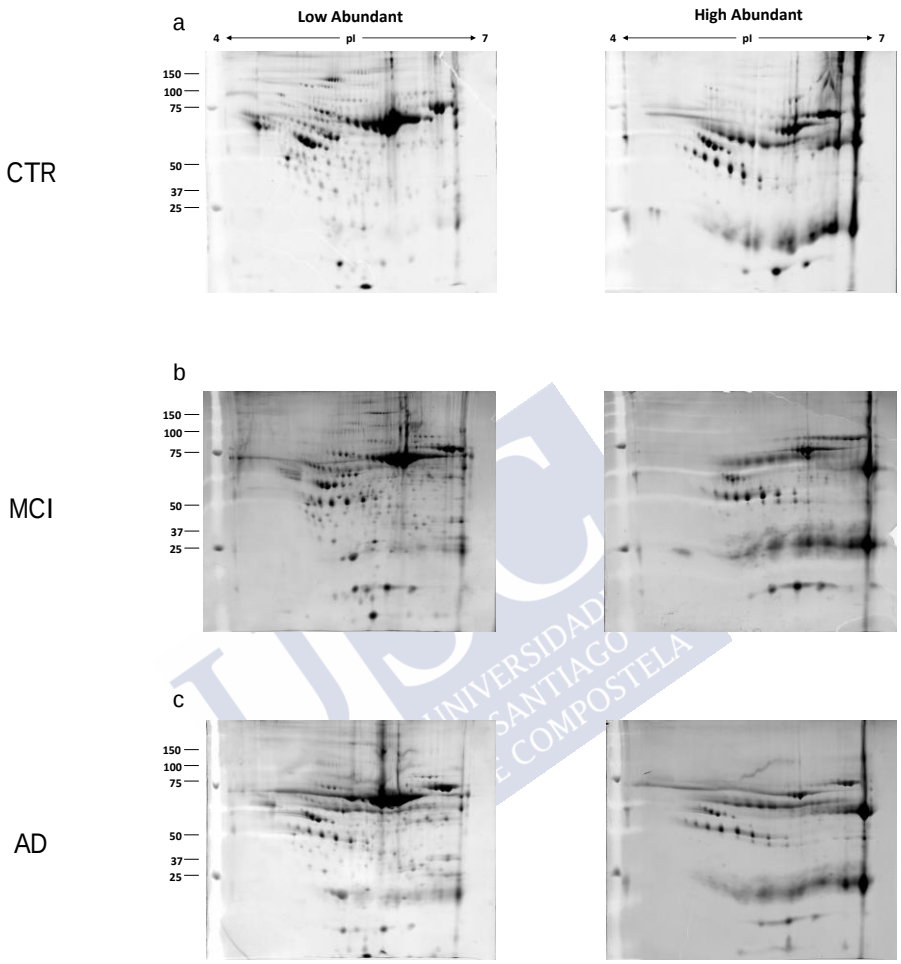

Figure 2. Typical image of a Sypro stained 2-DE map of serum. a) Image of CTR; b) Image of MCI patient; c) Image of AD patient.

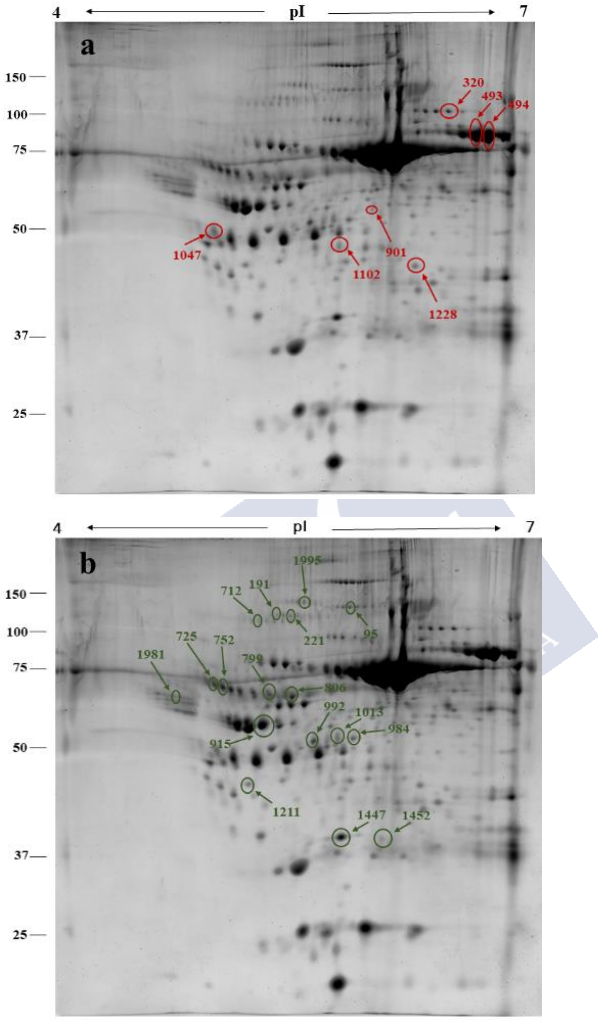

Figure 3. a) Protein spots increases in AD patients versus MCI patients. b) Protein spots decreased in AD versus MCI patients. Analysis performed with SameSpots software. The spots were considerated significative when $P < 0.05$ **and Fold Change ≥ 1.3.**

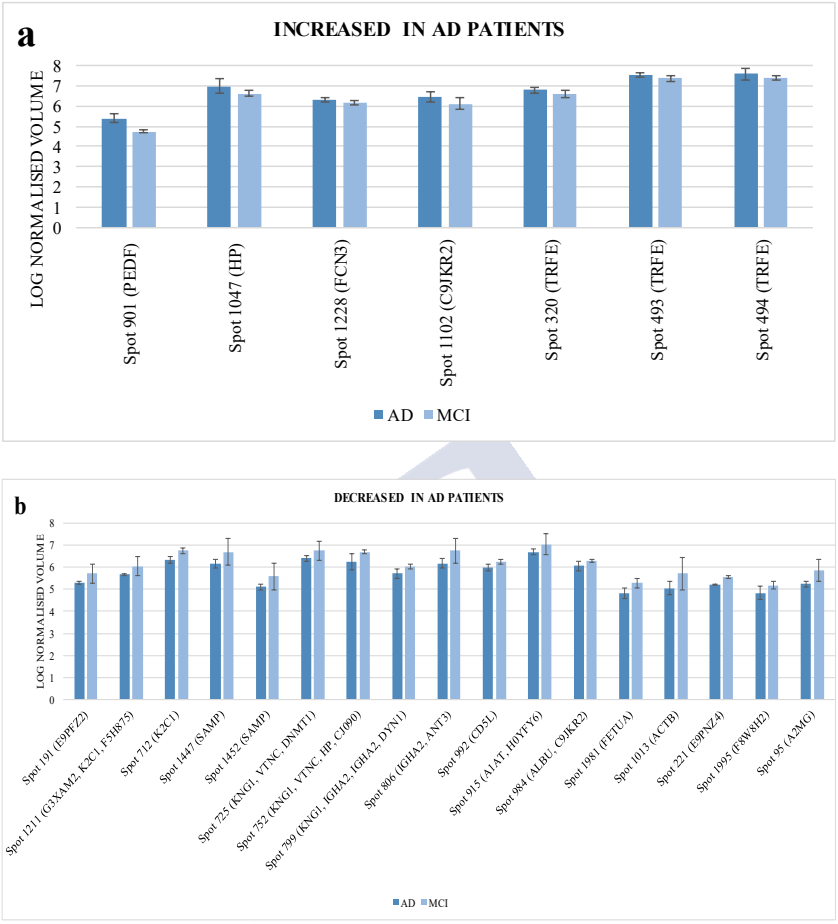

Figure 4. log normalized volume of spots (a) increased and (b) decreased in AD patients.

3.3.3 MALDI-TOF identification of increased and decreased proteins in AD patients

Statistically significant low abundant protein spots (gel images analysis) have been cut, digested, and identified by MALDI-TOF. Results (score values, percentage of coverage of sequence, size and theoretical isoelectric point) are shown in *Table 1*.

Table 1. MALDI-TOF/TOF identification of serum proteins from AD and MCI patients.

Protein	Spot No.	Uniprot ID	Score	Sequence coverage (%)	Theorical MW(Da)/pI
Increased low abundant proteins in AD					
Pigment epithelium-derived factor	901	PEDF	69	16	46283.30/5.97
Haptoglobin	1047	HP	215	51	31387.87/8.48
Ficolin-3	1228	FCN3	132	18	32881.99/6.20
Albumin, isoform CRA_k	1102	C9JKR2	304	37	47256.70/5.97
Serotransferrin	320, 493, 494	TRFE	191	18	77013.63/6.81
Decreased low abundant proteins in AD					
Ceruloplasmin	191	E9PFZ2	60	5	108751.41/5.49
Complement factor I	1211	G3XAM2	82	19	65016.27/7.72
Keratin, type II cytoskeletal 1	1211, 712	K2C1	75	25	65999.0/8.15
Charged multivesicular body protein 1ª	1211	F5H875	62	38	14304.15/4.69
Serum amyloid P-component	1447, 1452	SAMP	366	30	25371.13/6.10
Kininogen-1	725, 752, 799	KNG1	183	18	71912.15/6.34
Vitronectin	725, 752	VTNC	138	16	54271.17/5.55
DNA (cytosine-5)-methyltransferase 1	725	DNMT1	60	12	183049.81/7.99
Ig alpha-2 chain C region	799, 806	IGHA2	183	15	14743.35/4.93
Ig alpha-1 chain C region	799	IGHA1	182	15	37731.56/5.63
Dynamin-1	799	DYN1	62	17	97408.25/6.73
Haptoglobin	752	H3BS21	68	30	24780.65/6.08
Centrosomal protein C10 or F90	752	CJ090	64	13	73349.21/8.88
CD5 antigen-like	992	CD5L	99	22	38062.94/5.28
Antithrombin-III	806	ANT3	208	36	52568.86/6.32

Table 1. MALDI-TOF/TOF identification of serum proteins from AD and MCI patients (Continued).

Protein	Spot No.	Uniprot ID	Score	Sequence coverage (%)	Theorical MW(Da)/pI
Decreased low abundant proteins in AD					
Alpha-1-antitrypsin	915	A1AT	275	45	46707.02/5.37
Nuclear mitotic apparatus protein 1	915	H0YFY6	60	14	107345.79/9.16
Serum albumin	984	ALBU	174	22	69321.49/5.92
Albumin, isoform CRA-k	984	C9JKR2	117	23	47256.70/5.97
Alpha-2-HS-glycoprotein	1981	FETUA	92	21	39299.71/5.43
Actin, cytoplasmic 1	1013	ACTB	231	32	41709.73/5.29
Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	221	E9PNZ4	61	10	230947.41/5.20
Intermediate filament family orphan 1	1995	F8W8H2	67	29	29399.77/4.41
Alpha-2-macroglobulin	95	A2MG	63	7	163187.89/6.03

Similarly, data were launched against post-translational modifications such as phosphorylation (Table 2). As listed in Table 2, there are few identified proteins that undergo phosphorylation, and in these cases, the position of the protein modification is shown. Post-translational modifications are very important because they can be indicators of the state or progression of many diseases [19]. Changes in the pattern of protein phosphorylation of different brain regions have been suggested to promote AD transition from a presymptomatic to a symptomatic state in response to amyloid β-peptide (Aβ) accumulation. In addition, phosphorylation changes contribute to disturbance of multiple signaling pathways and to transitions to a pathological state postulated to be necessary for cognitive decline [20].

As can be seen in *Table 2*, proteins were identified with high score and good sequence coverages. Identified proteins (proteins increased in AD patients) were pigment epithelium-derived factor (PEDF), haptoglobin (HP), ficolin-3 (FCN3), serum albumin (ALBU), CRA_k isoform (C9JKR2) and serotransferrin (TRFE). Comparisons with reported data are difficult because previously published papers deal with proteins in healthy people and AD patients [21-24], while differences found in the current work (increased or decreased proteins) focus on serum proteins in patients at two different AD stages (early and advance).

PEDF is a protein that has been previously found to be increased in CSF samples from patients with neurodegenerative diseases such as frontotemporal dementia, vascular dementia, multiple sclerosis, and AD [25]. Our study shows that this protein is also increased in serum samples from AD patients. Similarly, FCN3 is a protein reported by other authors as a possible early biomarker in serum samples from multiple sclerosis patients [26]. In addition, other authors associate this protein with severe traumatic brain injury, and variation in the FCN3 concentration is related to the mortality of patients suffering from this disease [27]. TRFE is another protein found to be increased in serum samples from AD patients. Our findings agree with those previously reported by other authors [28-32]. In addition, variations in iron

concentration and Fe-based proteins, such as TRFE and ferritin (FT) have also been linked to AD [15,33]. Regarding HP, increased serum HP in AD patients agrees with some findings reported for increased HP levels in patients with sepsis, and also with the pathogenesis of neurodegenerative disorders, which involves inflammation and oxidative stress (HP exhibits antioxidant and anti-inflammatory activities and the ability to participate in immune system regulation). However, evidence of a direct association between serum HP levels and AD is lacking [2, 16]. In our study, we have found this protein both increased and decreased, but in different spots. This fact is due to the presence of post-transcriptional modifications. The UniProt database suggests that HP can exhibit glycosylation and phosphorylation as post-transcriptional modifications. Our results show that serum HP in AD patients exhibits phosphorylation sites that may modify both molecular weight and pH (*Table 2*). These findings agree with those previously reported by other authors [34].

Table 2. Protein phosphorylation: phosphorylate peptide sequence and phosphorylation positions.

Protein	Spot No.	Phosphorilate peptide sequence	STY Phosphorilation
Increased low abundant proteins in AD			
Pigment epithelium-derived factor	901	ITGKPIK	T2
		IAQLPLTGSMSIIFFLPLK	T7/S9
Haptoglobin	1047	ISQMTAARSPPR	S2
		YVMLPVADQDQCIR	Y1
Albumin, isoform CRA_k	1102	RHPDYSVVLLLR	Y5/S6
Serotransferrin	493, 494	SASDLTWDNLKGK	S1
Decreased low abundant proteins in AD			
Charged multivesicular body protein 1ª	1211	VQTAVTMK	T3
		VQTAVTMK	T3/T6
		VQTAVTMKGVTK	T3/T6
Keratin, type II cytoskeletal 1	712	NKYEDEINK	Y3
		SRQFSSRSGYR	S1
		LLRDYQELMNTK	Y5/T11
		SSGGSSSVKFVSTTYSGVTR	S1/S2/S5
		EDLARLLRDYQELMNTK	Y10/T16
Kininogen-1	752	ITYSIVQTNCSK	T2
Haptoglobin	752	VVLHPNYSQVDIGLIK	Y7
		AVGDKLPECEAGATLINEQWLLTTAK	T14
Centrosomalprotein C10 or F90	752	SLTLQEALEVR	S1
		AVHTKVFSGSSK	T4/S8
		KYWVTHADDK	Y2/T5
		SVLSLNLNCSSHR	S1/S4
		MISSIVISQMIDENKSR	S3/S4
		ETSFSPDTPLSGKSPLVFSSCVHLR	T1/S3

Table 2. Protein phosphorylation: phosphorylate peptide sequence and phosphorylation positions (Continued).

Protein	Spot No.	Phosphorilate peptide sequence	STY Phosphorilation
Decreased low abundant proteins in AD			
DNA (cytosine-5)-methyltransferase 1	725	IYISK	Y2
		R.LRSQTK.E	S3
		R.TYSKFK.N	T1
		FNSRTYSK	S3
		QTTITSHFAK	T2
		KEELSEEGYLAK	S5
		QTTITSHFAKGPAK	T2/T3
		DHICKDMSALVAAR	S8
		VGMADANSPPKPLSKPR	S8/S14
		SQGFPDTYRLFGNILDK	S1/T7/Y8
		LTCSFVYCKHGHLCPIDTGLIEK	T2/S5
Ig alpha-2 chain C region	799	QEPSQGTTTYAVTSILR	S4
CD5 antigen-like	992	QLGCGRAVLTQK	T10
		ELGCGAASGTPSGILYEPPAEK	S8
		HQNQWYTVCQTGWSLR	Y6/T7/T11/S14
Alpha-1-antitrypsin	915	KLSSWVLLMK	S3/S4
Actin, cytoplasmic 1	1013	RGILTLK	T5
Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	221	EARLYEGK	Y5
		LECTNLYR	T4/Y7
		QHTILMSDK	T3/S7
		SSVASLVGRSK	S1/S2/S5/S10
		EETVAKMYISELK	T3/Y8
		TRYTALVTLTTQHVK	T1
		SLRPAVERLELLLQIANK	S1
		LYEGKMSQNFHTSYAETLGK	Y2

Table 2. Protein phosphorylation: phosphorylate peptide sequence and phosphorylation positions (Continued).

Protein	Spot No.	Phosphorilate peptide sequence	STY Phosphorilation
Decreased low abundant proteins in AD			
Intermediate filament family orphan 1	1995	SNNLSELDTKIQEK	S1/S5/T9
Alpha-2-macroglobulin	95	MLERSNHVSR	S5
		KLSFYYLIMAK	S3
		TGTHGLLVKQEDMK	T1/T3
		LSFYYLIMAKGGIVRTGTHGLLVK	S2

Finally, several decreased proteins in AD patients have been identified (Table 1). Results agree with several authors that have identified most of these proteins in CSF (also in postmortem brain studies) from patients with several degenerative diseases [6, 12, 35-45].

3.4 DISCUSSION

Most studies based on Proteomics to find AD biomarkers have focused on CSF [46, 47]. There are few studies regarding plasma/serum. In addition, plasma/serum biomarkers for MCI assessment have not yet been explored, and neuroproteomics can be therefore considered as an emerging technology. Identified plasma proteins by proteomic techniques are proteins associated with transportation of lipids and other molecules, and participating in immune regulation and inflammation [22]. Recently, proteomics strategies have been used to search for unique biochemical changes in peripheral tissues that may represent putative biomarkers and may allow early AD diagnosis and prognosis. One of the major challenges in this field is to identify specific alterations at the systemic level that may point to brain damage. Different authors have recently demonstrated that impairment of heme degradation pathways, due to oxidative modifications of the main components, occurs in postmortem brains from MCI and AD patients as well as in plasma samples [48].

Although high abundance proteins could be useful for finding AD biomarkers (our results show that there are several high abundant proteins that are increased or decreased for samples from MCI and AD patients) the current research has focused on low abundance proteins differences. Therefore, results regarding increased and decreased low abundant proteins in serum from patients suffering AD at several progression stages of the disease have been fully discussed.

3.4.1 Increased low abundant proteins in AD patients

3.4.1.1 Pigment epithelium-derived factor (PEDF)

PEDF is a non-inhibitory member of the serpin class of proteins with various biological functions including anti-angiogenesis, anti-vasopermeability, anti-tumor, and neurotrophic activities [49]. High PEDF levels have been recently proposed as a CSF biomarker for AD. However, the presence of PEDF in CSF (whether derived from the brain or from the systemic circulation) and the specificity of this finding remains unclear. As PEDF has neuroprotective and anti-inflammatory functions, the increase of PEDF in AD patients may be a response to brain injury [25]. The exact role of PEDF in AD physiopathology remains unknown, but recent work highlights the implication of PEDF in the regulation of proliferation in hippocampal progenitor cells and thus in memory consolidation [2]. PEDF has also been found to be increased in serum from AD patients. Therefore, and as PEDF is also increased in CSF in AD patients [50], PEDF could be a potential biomarker for AD diagnosis.

3.4.1.2 Ficolin-3 (FCN3)

FCN3 contributes to the complement independent inflammatory processes of traumatic brain injury. Lower serum FCN3 levels have been demonstrated to be highly associated with unfavorable outcome after ischemic stroke [27]. Therefore, this protein is commonly associated with degenerative diseases such as multiple sclerosis (MScl), and FCN3 has been proposed as a possible MScl biomarker [26]. The presence of high levels of FCN3 in serum from AD patients suggests that increased FCN3 is related to neurodegenerative processes.

3.4.1.3 Serotransferrin (TRFE)

TRFE has been reported to be increased in plasma samples from AD patients [33, 49]. These studies have suggested that iron oxidation promotes the accumulation of this protein, which is an iron-transport

protein [48]. Our findings also confirm that serum TRFE is increased in AD patients compared to MCI patients (early AD stage). As previous reports have shown that plasma TRFE is increased in AD patients compared to healthy people, further research is needed with healthy (control) / MCI patient comparison.

3.4.1.4 Haptoglobin (HP)

Since HP in CSF is a useful marker of AD progress, a reliable analytical method of measuring HP concentrations in AD patients is needed. HP is an acute-phase protein produced by the liver that functions to scavenge cell-free hemoglobin and its by-product. HP exhibits several functional properties, including antioxidant and anti-inflammatory activities, and the ability to participate in immune system regulation. Furthermore, serum HP levels have been shown to be increased in humans with sepsis, and the pathogenesis of neurodegenerative disorders such as AD, and it has been reported to involve inflammation and oxidative stress. In addition, significantly higher serum HP levels in the patients with AD and Parkinson's disease (PD) compared to those observed in control patients have also been reported for serum HP, and no significant differences have been observed between AD and PD groups. Our findings also show that serum HP is increased in AD compared to MCI patients, a fact that is in good agreement with those shown by Song et al. when comparing healthy people and AD patients [16].

3.4.2 Decreased serum proteins in AD patients

There are several different (nature and function) low abundant proteins that have been found to be decreased in AD patients compared to MCI patients. Most of them have been reported as related with AD, but they were described as increased or decreased in many other diseases such as diabetes, eye diseases, and even MScl. Some serum proteins found to be decreased in AD patients compared to MCI patients (*Table 1*) have been related to neurodegenerative processes when comparing healthy people and AD patients. These proteins are

ceruloplasmin (CP), complement factor 1 (CF1), charged multivesicular body protein 1A (CHMP1A), kininogen-1 (KNG1), DNA (cytosine-5)-methyltransferase 1 (DNMT1), CD5 antigen-like (CD5L), alpha-1-antitrypsin (SERPINA1), antithrombin-3 (SEPINC1), alpha-2-HS-glycoprotein (AHSG), actin cytoplasmatic 1 (ACTB), and alpha-2-macroglobulin (A2M).

3.4.3 FunRich and String analysis

Characterization of identified decreased low abundant proteins was performed by FunRich software (functional analysis). In this case, the images of the gels of patients with MCI *versus* patients with AD were compared. As shown in *Figure 5*, two groups, one related to brain central nervous systems diseases (*Figure 5a*), and another to peripheral organ disease (*Figure 5b*), have been obtained. Proteins involved in memory loss, progressive dementia, and neurological disease have been found as proteins related to the central nervous system. In addition, proteins involved in diabetes, retinal degeneration and achalasia have been found to belong to the peripheral organ disease group (*Figure 5b*). Finally, there have also been identified proteins related to certain diseases that cause alterations in serum metal levels, such as hemosiderosis, anemia, and aceruloplasminemia. Several studies regarding trace metal levels have pointed out that metal ions play an important role in the promotion of these diseases [51]. Metals such as iron, copper, zinc, manganese and aluminum have therefore been found to be related to this disease [52]. Because metal ions are essential cofactors for many proteins, and can compete with each other for binding to proteins, they help preserve neuronal function. Some heavy metals may worsen the progression of the disease due to their high neurotoxicity and their ability to induce epigenetic changes [53].

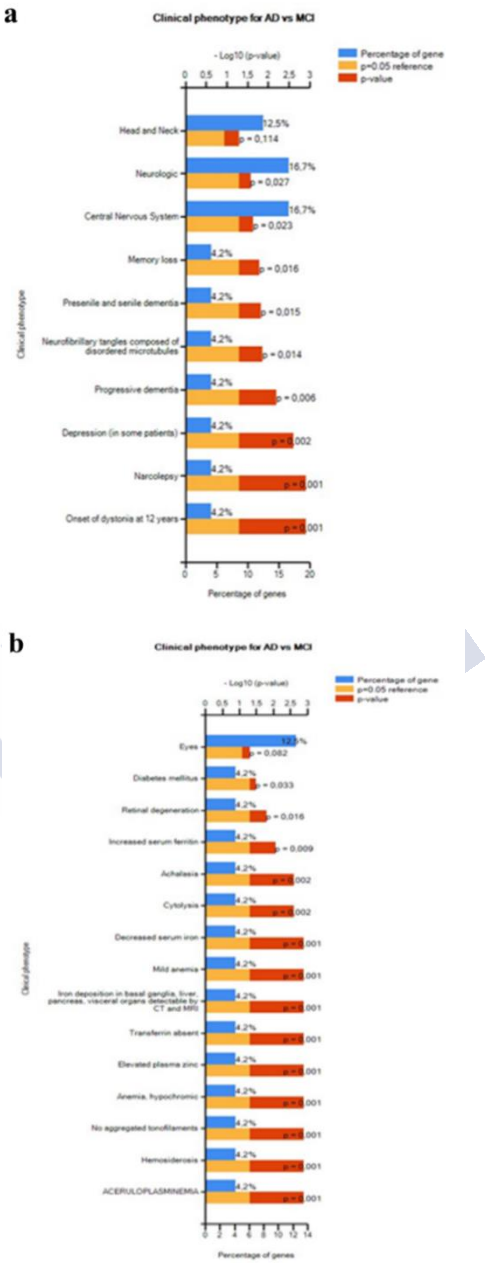

Figure 5. Functional analysis using FunRich software. a) Diseases associated with the brain and central nervous system. b) Other diseases.

Identified proteins are related not only to neurodegenerative diseases but also to other diseases that are often associated with diseases such as AD. String software was used to investigate possible interactions between all the proteins identified in order to highlight predominant networks, pathways and connections to pathophysiological processes. Findings show that thirteen proteins are connected in a network related to mediated transport, seven proteins are involved in negative regulation of endopeptidase activity, eight proteins are related to a negative regulation of hydrolase activity, eight proteins are related to a regulation of response to wounding, and seven proteins affect the regulation of the inflammatory response network. From the results shown in *Figure 6*, three increased proteins (HP, TRFE and FCN3) in patients with AD are related among each other, and are also transport proteins.

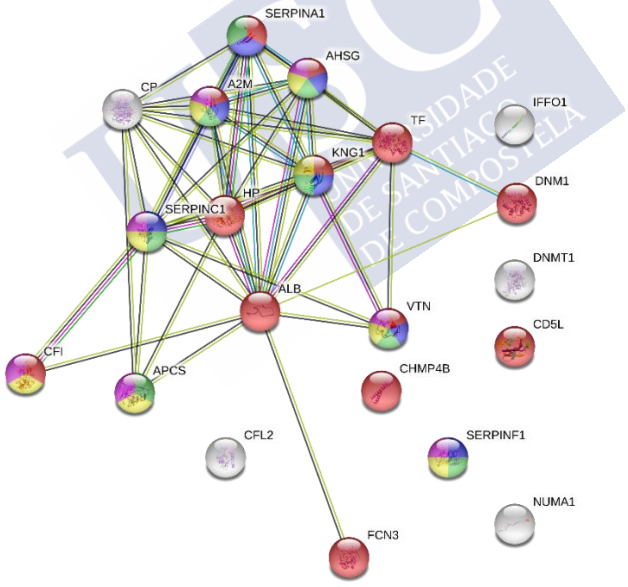

Figure 5. STRING interaction network analysis. In grey, general interaction network of all proteins identified; in red, protein network related to mediated transport; in blue, protein network related to negative regulation of endopeptidase activity; in green, regulation of response to wounding; in violet, protein network related to regulation of inflammatory response.

3.5 CONCLUSIONS

CSF has typically been proposed as a potential biofluid for detecting protein-based biomarkers. In addition, most studies follow a strategy based on comparing healthy (control) people and advanced AD patients. However, more available clinical samples such as serum/plasma and oral fluid are preferred for performing proteomic studies. Moreover, a direct comparison between MCI and AD patients is also preferable for early AD diagnosis.

Our results confirm some previous findings regarding certain serum proteins such as TRFE, which has been reported to be increased in AD patients compared to healthy people (serum TRFE is also increased when comparing with TRFE values in MCI patients). Some potential biomarkers in CSF such as PEDF have also been found to be increased in serum from AD patients compared to MCI patients. In addition, increased serum HP has also been observed in AD patients compared to MCI patients. High levels of serum FCN3 in AD patients compared to MCI patients suggest this protein could be related to neurodegenerative illnesses because it is also increased in serum from MScl patients. Finally, several serum proteins have been found to be decreased in AD patients compared to MCI patients. These proteins (serum and CSF) have also been found to be decreased in AD patients compared to healthy people; however, they have also been reported to be related to other illnesses.

As conclusion, serum PEDF and HP could be potential biomarkers of early AD diagnosis. These two proteins have also been reported at high levels in CSF (PEDF) and serum (HP) in AD patients compared to healthy people.

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CHAPTER 4

EXPLORING BIOMARKERS FOR ALZHEIMER´S DISEASE BY A QUALITATIVE AND QUANTITATIVE PROTEOMIC APPROACH

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Exploring biomarkers for Alzheimer´s disease by a qualitative and quantitative proteomic approach

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Abstract

Since blood is more easily accessible than cerebrospinal fluid (CSF), finding reliable blood (serum or plasma) biomarkers for Alzheimer's disease (AD) is highly desirable. Therefore, a qualitatively (using LC-MS/MS) and quantitatively (using SWATH-MS) proteome analysis have been developed for serum samples from 8 healthy people (control) and 22 patients [(7 patients with mild cognitive impairment (MCI) and 15 patients with AD)].

After qualitative analysis, a total of 36, 36 and 120 proteins were exclusively identified in the control, MCI and AD groups, respectively.

Based on proteins' functionality, the most of these proteins have been found to be related with a transcriptional activity in both AD and MCI.

Quantitative analysis showed that 19 differentially proteins were found after the comparison of patients with MCI and AD; whereas, 31 and 42 proteins were found when comparing controls against patients with AD and MCI, respectively.

The expression levels of Apoliprotein A-II (Apo A-II) were found to be significantly decreased in serum from AD patients in comparison with MCI. In addition, the expression levels of this protein were found to be significantly increased in serum samples from MCI patients when comparing to controls. These findings means that Apo A-II may play an important role in the progression of AD, and this protein could thus be a susceptibility biomarker for the early diagnosis of this disease. Finally, serotransferrin (TRFE) immunoglobulin kappa constant (IGKC) could be also considered as protein biomarkers at an early stage of AD neurodegeneration.

4.1 INTRODUCTION

Alzheimer's disease (AD) is an age-dependent neurodegenerative disorder that impairs cognitive and memory function progressively and is the most common form of dementia in the elderly [1]. Pathologically, AD is characterized by neuronal loss and the accumulation of neurofibrillary tangles and amyloid plaques [2]. Currently, effective diagnostics are costly and treatments and preventative strategies are lacking, thus making the validation of AD biomarkers imperative [3].

Biomarkers that have been examined can play a critical role in diagnostics and drug development. The research for AD biomarker has taken many directions, includes measuring of proteins in blood, cerebrospinal fluid (CSF), and urine. Studies involving proteomics to discover biomarkers have been developed for decades; however, these attempts have met less success samples which was used to be a clinical biomarker for AD. Validation of a clinical biomarker is a challenge due to there are many critical steps from the biomarker discovery to the

biomarker validation, and also due to the complexity of the body fluids, and the low abundance of the potential biomarkers,. Despite of the challenges exist, there are some "-omics" areas leading us into the future study for AD biomarkers, such as peptidomics, modification-specific proteomics, and metabolomics. An approach based on combining all these "-omics" techniques could offer new insights to discover biomarkers [4].

CSF is a biological fluid which is in direct contact with the extracellular space of the brain. Therefore, biochemical changes in the brain are reflected in the CSF. Since AD pathology is restricted to the brain, CSF has been the focus in research on diagnostic biomarkers for AD [5]. Due to the difficulty of sampling CSF from a patient with AD, recent studies have been focused on samples such as blood, serum or saliva. Since blood is more easily accessible than CSF, finding reliable blood biomarkers for AD is desirable. Due to the blood–brain barrier, the concentration of brain-derived proteins in the blood is lower than in the CSF, which makes this task a challenge [6].

Furthermore, examined AD serum samples have shown increased levels of interleukin-1 beta (IL-1β), interleukin-8 (IL-8), tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), granulocyte-macrophage colony-stimulating factor (GMCSF), soluble CD40 ligand (sCD40L), and vascular endothelial growth factor (VEGF) [7,8]. Recently, chemokines have been found to serve novel roles in the pathophysiology of psychiatric disorders [9], and the most important proteins have been identified as CXCL10 (interferon gamma-induced protein 10, IP10) and CX3CL1 (fractalkine) [10]. Other contributing factors to cognitive impairment include metabolic disorders, such as obesity and hyperglycemia, and cellular dysfunction such as oxidative stress [11-13]. Furthermore, AD patients were found to have higher serum levels of adiponectin and insulin, and significantly lower serum levels of leptin compared to age- and gender-matched control subjects [14]. Thus, cytokines/chemokines and other metabolic factors related to both neuro-inflammatory and peripheral inflammatory processes are of increasing interest when searching for AD biomarkers [15].

The high complexity and large dynamic range of serum proteins can be a problem when using the proteins profile as a biomarker of a disease because the more useful information (differences between healthy and patients) is currently obtained for minor serum proteins. To circumvent these technological limitations, it is describe here a new two-stage strategy for the mass spectrometry (MS) assisted discovery, verification and validation of disease biomarkers [16]. New advances in MS based proteomics that combine developments in instrumentation, sample preparation and computational analysis, could help to fill the gap in the development of non-invasive and easy-to-implement AD biomarkers [17].

Despite of rapid development in MS based proteomics over last 20 years, up to date the US Food and Drug Administration (FDA) approved only around 109 unique protein markers including over 20 protein-based cancer biomarkers. The majority (88 of 109) are measured by immunoaffinity assays, and the remaining targets (21 of 109) by other assays including MS/MS [18]. Paradoxically, only 20% of the currently approved protein assays were introduced since the application of MS/MS to measure proteins, thus on average 1.5 new protein assays were approved by the FDA per year [19].

These figures suggest that the current protein biomarker discovery pipeline is inefficient and suffering from relatively high false-positive rates, which hampers identification and validation of true biomarkers.

In the post-genome era, a wide range MS-based proteome analysis method have been developed, which provide valuable insight about proteins and their expression-levels directly from complex tissue and body fluid samples [20, 21]. Exact identification and quantitation of proteins is essential for a better understanding of biological processes in health and disease [22]. Moreover, precise quantification of specific proteins in tissues and body fluids provides valuable insights, and validation of potential biomarkers for diseases stages [23].

The progress on biomarker discovery and validation will be markedly accelerated by the use of more robust and quantitative protein assays, based on targeted reaction monitoring and possibly other emerging techniques [24, 25].

Selected reaction monitoring (SRM), sometimes also referred to as multiple reaction monitoring (MRM), is currently the method of choice for sensitive protein analyses. SRM is suited to quantify from molar to millimolar amounts of a specific protein in a body fluid, [26] or equivalent to measuring in the range of 50 to > 1 million copies [27]. SRM methods rely on using a triple-quadrupole (QQQ) mass spectrometer as a dual mass filter to allow passage and analyses of only predefined targeted proteotypic peptides, by specifically selecting precursor ions in Q1 and their specific fragment ions in Q3 as predefined mass to charge (m/z) values [28]. The signal intensities of SRM transitions (precursor/fragment ion pairs) of the unique peptide can be monitored over time, and they are efficient as surrogate measures of a specific proteins quantity. Due to its high sensitivity, reproducibility and broad dynamic range, SRM has become a powerful tool used in absolute and relative quantification in several biological samples, especially in the area of biomarkers research.

Depending on sample type and instrument methods, only ten to fifty proteins can be quantified within each analytical run of a complex sample. More recently, with the introduction of ultra-fast scanning high-resolution Q-TOF instruments, the Sequential Window Acquisition of all Theoretical spectra (SWATH) has been introduced as a novel SRM-like analysis method but based on a Data Independent Acquisition (DIA) strategy [24].

SWATH-MS presents a new and faster alternative to SRM-MS (*Figure 1),* and like SRM-MS, SWATH-MS operates by collecting time-resolved data from peptides and their fragments, an allows identification and quantification of specific proteins in complex tissue sample. SWATH-MS can theoretically collect all MS/MS fragment ion spectra for all precursor ions in a complex sample by using stepped m/z

windows, which can be used for biomarker discovery with parallel and consistent detection of 30,000-40,000 peptides from 4000-5000 targeted proteins in large sets of samples [24, 29].

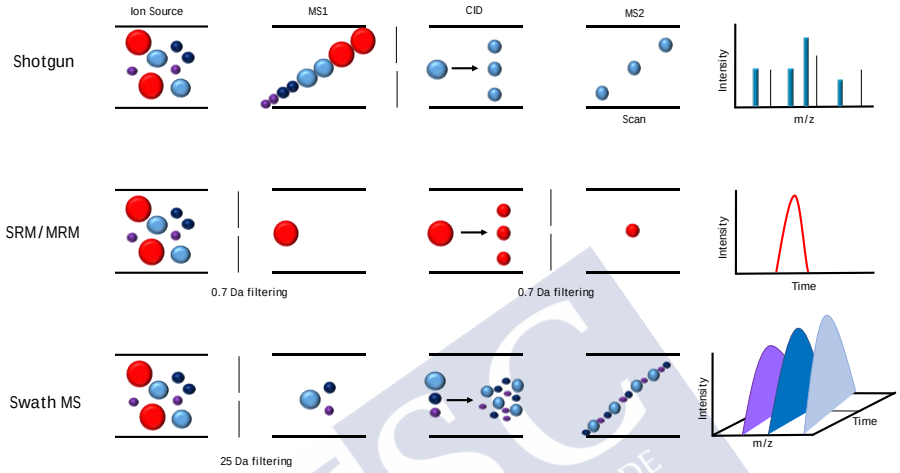

Figure 1. Mass spectrometry acquisition methods.

Both SRM and SWATH methods for quantification of human proteins have been developed at an amazing pace over the past decade, and today we have open access to accurate and validated MS methods for every known human protein [30, 31]. But, such MS-based methods are species- and tissue-specific because parameters as linearity and limit of quantification for a certain protein and/or peptide, depends on the complexity of the biological sample. The development of MS-based protein quantification methods is a challenge because there are not many applications for specific tissue/targets [32].

The objective of the present study is the identification of novel potential serum biomarkers of AD using a combination of next-generation proteomics and machine-learning algorithms for feature selection.

4.2 MATERIALS AND METHODS

4.2.1 Clinical samples

Serum samples from healthy volunteer adults and patients (Table 1) were supplied by the *Servicio de Neurología* at the University Clinical Hospital of Santiago de Compostela (Santiago de Compostela, Spain). The developed research has been ascribed to the approved and in force expert opinion from the *Comité de ética de la investigación con medicamentos* – CEIm-G (Ethics Committee for the Research with medicines) of Galicia (Registration Code: CEIm-G 2018/575).

Table 1. Characteristics of healthy subjects and patients with MCI and AD.

Demographic variables	Control	MCI	AD
Number of subjects (M/F)	8 (2/6)	7 (4/3)	15 (8/7)
Age (years)	64.3 ± 8.3	75.4 ± 7.2	79.5 ± 10.2
Total cholesterol (mg/dl)	235 ± 32	184 ± 20	198 ± 42
Triglycerides (mg/dl)	137 ± 73	99 ± 35	96 ± 48

Data are represented as mean ± SD. M/F = male/female. N/A = not applicable. FAST = Functional Assessment Staging. MMSE = Mini Mental State Examination.

Venous blood samples (2.0 mL) samples were collected in Vacutainer blood collection tubes with silicone-coated cores (BD Diagnostics, Franklin Lakes, NJ, USA) by a standard venipuncture method. The collected serum samples were stored at room temperature to allow for blood clotting and were centrifuged (1800 g, 4 °C, 10 min). Serum samples were then immediately frozen at –80 °C.

4.2.2 Depletion of multiple high abundant proteins

Serum aliquots were filtered with Miller-GP® Filter Unit (Millipore) with a size of 0.22 µm. Each aliquot of human serum (30 µL) was depleted with dithiothreitol (DTT) according to the protocol described by Warder el al. [33, 34]. Fresh DTT 500 mM (3.3 µL) was mixed with 30 µL of human serum and vortex briefly. Samples were

then incubated until a viscous white precipitate persisted (60 min), followed by centrifugation at 14000 rpm for 20 min. Supernatants were transferred to a clean tube and total dry (30 ºC, 45 min).

4.2.3 One dimensional SDS-PAGE (1-DE)

Samples were reconstituted in 24 µL of ultrapure water and mixed with 4 µL of SDS-PAGE loading buffer (10% w/v SDS, Tris-Base 40 mM, pH 6.8, 50% v/v glycerol, 0.1% v/v bromophenol blue, 10% v/v β-mercaptoethanol). Then, all samples were denatured by heating at 100 °C for 5 min and loaded into a 10% acrylamide/ bis-acrylamide, stacking gel / 12.5% acrylamide/bis-acrylamide running gel, of 1 mm thickness, and separated at 80 V (constant voltage) and the run was stopped as soon as the front had penetrated 3 mm into the resolving gel. The gels were stained with Coomassie Blue for 2 hours at room temperature under agitation and distained with methanol / acetic acid (45% / 7.5%) for 12 hours, also under continuous agitation. Gels were then washed with ultrapure water and scanned.

4.2.4 Tryptic digestion

Protein bands were excised and washed with a solution containing 50 mM NH_4HCO_3 and 50 % MeOH HPLC grade (Scharlau, Barcelona, Spain). The proteins were reduced with 10 mM DTT in 50 mM NH_4HCO_3 and alkylated with 55 mM iodoacetamide in aqueous 50 mM NH_4HCO_3. Subsequently, the proteins were rinsed with 50 mM NH_4HCO_3 in 50 % MeOH, dehydrated by adding acetonitrile (ACN) (HPLC grade, Scharlau), and dried in a Speed Vac. Modified porcine trypsin (Promega, Madison, WI, USA) prepared in 20 mM NH_4HCO_3 (concentration of 20 g/µL) was added to the dried gel slices, followed by incubation at 37°C for 16 h. The peptides were extracted three times by incubation in 40 µL of a solution containing 60% ACN and 0.5% formic acid (HCOOH) for 20 min. The resulting peptide extracts were pooled, concentrated in a Speed Vac and stored at −20°C.

4.2.5 Protein identification by mass spectrometry (LC-MS/MS) and data analysis

Digested peptides of each sample were separated using Reverse Phase Chromatography. Gradient was developed using a micro liquid chromatography system (Eksigent Technologies nanoLC 400, Sciex) coupled to high speed Triple TOF 6600 mass spectrometer (Sciex) with a micro flow source. The analytical column used was a silica-based reversed phase column Chrom XP C18 150 × 0.30 mm, 3 mm particle size and 120 Å pore size (Eksigent, Sciex). The trap column was a YMC-TRIART C18 (YMC Technologies, Teknokroma) with a 3mm particle size and 120 Å pore size, switched on-line with the analytical column. The loading pump delivered a solution of 0.1% formic acid in water at 10 µL/min. The micro-pump provided a flowrate of 5 µL/min and was operated under gradient elution conditions, using 0.1% formic acid in water as mobile phase A, and 0.1% formic acid in acetonitrile as mobile phase B. Peptides were separated using a 25 min gradient ranging from 2% to 90% mobile phase B (mobile phase A: 2% acetonitrile, 0.1% formic acid; mobile phase B: 100% acetonitrile, 0.1% formic acid). Injection volume was 4 µL.

Data acquisition was performed in a TripleTOF 6600 System (Sciex, Foster City, CA) using a Data dependent workflow. Source and interface conditions were as follows: ion spray voltage floating (ISVF) 5500 V, curtain gas (CUR) 25, collision energy (CE) 10 and ion source gas 1 (GS1) 25. Instrument was operated with Analyst TF 1.7.1 software (Sciex, USA). Switching criteria was set to ions greater than mass to charge ratio (m/z) 350 and smaller than m/z 1400 with charge state of 2–5, mass tolerance 250 ppm and an abundance threshold of more than 200 counts (cps). Former target ions were excluded for 15 s. Instrument was automatically calibrated every 4 h using as external calibrant tryptic peptides from PepcalMix (Sciex).

4.2.6 Protein quantification by SWATH (Sequential Window Acquisition of all Theoretical Mass Spectra)

4.2.6.1 Creation of the spectral library

In order to prepare MS/MS spectral libraries, the peptide solutions were analyzed by a shotgun data-dependent acquisition (DDA) approach by micro-LC-MS/MS. Pooled vials of samples from each group (control, MCI and AD) were prepared using equal mixtures of the original samples to get a good representation of the peptides and proteins present in all samples. A volume of 4 µL (4mg) of each pool was separated into a micro-LC system Ekspert nLC425 (Eksigen, Dublin, CA, USA) using a column Chrom XP C18 150 × 0.30 mm, 3 mm particle size and 120 Å pore size (Eksigent, Sciex) at a flow rate of 5µL/min. Water and ACN, both containing 0.1 % formic acid, were used as solvents A and B, respectively. The gradient run consisted of 5% to 95 % B for 30 min, 5 min at 90 % B, and finally 5 min at 5 % B for column equilibration (total run time of 40 min). Eluted peptides were directly injected into a hybrid quadrupole-TOF mass spectrometer Triple TOF 6600 (Sciex, Redwood City, CA, USA) operated with a data-dependent acquisition system in positive ion mode. A Micro source (Sciex), operated at 2600 V voltage, was used for the interface between microLC and MS. The acquisition mode consisted of a 250 ms survey MS scan from 400 to 1250 m/z followed by an MS/MS scan from 100 to 1500 m/z (25 ms acquisition time) of the top 65 precursor ions from the survey scan (total cycle time of 2.8 s). The fragmented precursors were then added to a dynamic exclusion list for 15 s; any singly charged ions were excluded from the MS/MS analysis.

The peptide and protein identifications were performed using Protein Pilot software (version 5.0.1, Sciex) over Human specific Uniprot database (iodoacetamide as Cys alkylation). The false discovery rate (FDR) was set to 1 for both peptides and proteins. The MS/MS spectra of the identified peptides were then used to generate the spectral library for SWATH peak extraction using the add-in for PeakView Software (version 2.2, Sciex) MS/MSALL with SWATH

Acquisition MicroApp (version 2.0, Sciex). Peptides with a confidence score above 99% (as obtained from Protein Pilot database search) were included in the spectral library).

4.2.6.2 Relative quantification by SWATH acquisition

SWATH–MS acquisition was performed on a TripleTOF® 6600 LC-MS/MS system (Sciex). Samples from control, MCI and AD were analysed using a data-independent acquisition (DIA) method (30 total samples). Each sample 4 µL (from a 1 mg/ml solution) was analysed using the LC-MS equipment and LC gradient described above for building the spectral library but using the SWATH-MS acquisition method. The method consisted of repeating a cycle that consisted of the acquisition of 65 TOF MS/MS scans (400 to 1500 m/z, high sensitivity mode, 50 ms acquisition time) of overlapping sequential precursor isolation windows of variable width (1 m/z overlap) covering the 400 to 1250 m/z mass range with a previous TOF MS scan (400 to 1500 m/z, 50 ms acquisition time) for each cycle. Total cycle time was 6.3 s. For each sample set, the width of the 65 variable windows was optimized according to the ion density found in the DDA runs using a SWATH variable window calculator worksheet from Sciex.

4.2.6.3 Data analysis

The targeted data extraction of the fragment ion chromatogram traces from the SWATH runs was performed by PeakView (version 2.2) using the SWATH Acquisition MicroApp (version 2.0). This application processed the data using the spectral library created from the shotgun data. Up to ten peptides per protein and seven fragments per peptide were selected, based on signal intensity; any shared and modified peptides were excluded from the processing. Five-minute windows and 30 ppm widths were used to extract the ion chromatograms; SWATH quantitation was attempted for all proteins in the ion library that were identified by ProteinPilot with an FDR below 1%.

The retention times from the peptides that were selected for each protein were realigned in each run according to the iRT peptides spiked in each sample and eluted along the whole-time axis. The extracted ion chromatograms were then generated for each selected fragment ion. The peak areas for the peptides were obtained by summing the peak areas from the corresponding fragment ions. PeakView computed an FDR and a score for each assigned peptide according to the chromatographic and spectra components. Peptides with an FDR below 5% were used for protein quantitation. Protein quantitation was calculated by adding the peak areas of the corresponding peptides.

The integrated peak areas (processed. mrkvw files from PeakView) were directly exported to the MarkerView software (Sciex) for relative quantitative analysis. The export will generate three files containing quantitative information about individual ions, the summed intensity of different ions for a particular peptide and the summed intensity of different peptides for a particular protein. MarkerView has been used for analysis of SWATH-MS data reported in other proteomics studies [17, 35-37] because of its data-independent method of quantitation. MarkerView uses processing algorithms that accurately find chromatographic and spectral peaks direct from the raw SWATH data. Data alignment by MarkerView compensates for minor variations in both mass and retention time values, ensuring that identical compounds in different samples are accurately compared to one another.

We have performed a global normalization based on the total sum of all peak areas extracted from all peptides and transitions from the replicates of each sample. By this way, possible uneven sample loss during the sample preparation process is controlled. Unsupervised multivariate statistical analysis using principal component analysis (PCA) was performed to compare the data across the samples, using range scale. The average MS peak area of each protein was derived from the biological replicates of the SWATH-MS of each sample followed by Student's *t*-test analysis (MarkerView software) for comparison among the samples based on the averaged area sums of all the transitions derived for each protein. The *t*-test will indicate how well

each variable distinguishes the two groups, reported as a *p*-value. For each library, the *t*-test set of differentially expressed proteins (*p*-value < 0.05) with a 1.5 fold in- or decrease was selected.

Functional analysis was performed by FunRich (Functional Enrichment analysis tool) open access software for functional enrichment and interaction network analysis (http://funrich.org/index.html).

4.3 RESULTS AND DISCUSSION

A total of 22 patient and 8 healthy people (Control) samples were studied in the current study. The group of patients was divided into 7 patients with MCI and 15 patients with AD. All samples have been recruited, processed and analyzed in the same way. The result has been the identification of a large number of proteins, which give us differences between these groups.

4.3.1 Proteins identified in serum samples by shotgun proteomic techniques

Protein analysis was carried out qualitatively and quantitatively using two different technologies based on 2-DE-MALDI / TOF /TOF (Chapter 3) and LC / ESI / MS / MS. Proteins were selected according to the technology and the type of assay. The function and interactions of the proteins were analyzed using databases (*Figure 2*).

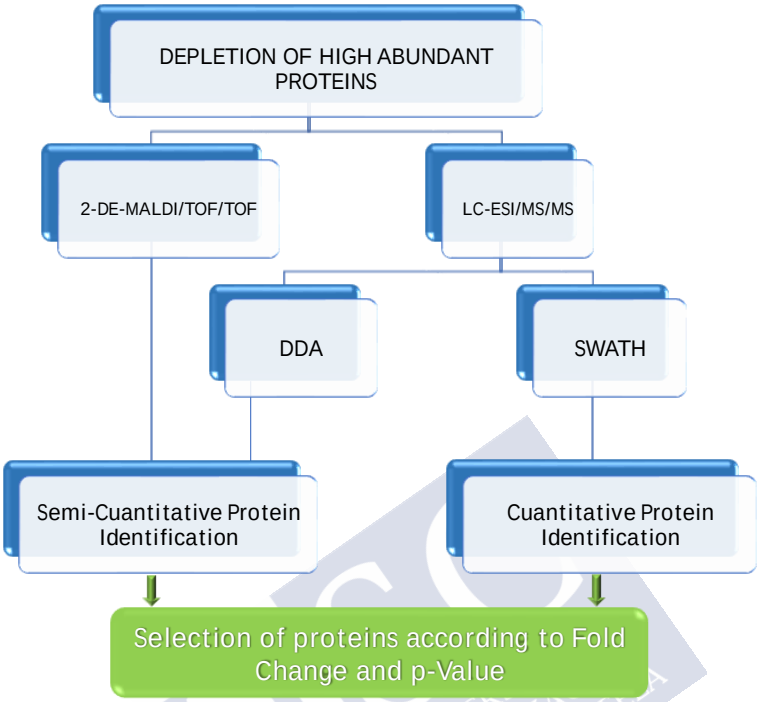

Figure 2. Diagram of the workflow carried out for the differential proteome analysis.

The first step in this study was to identify the proteins present in all samples using shoutgun and DDA. After the identification of these proteins, a list of the statistically significant proteins present in each group has been compiled. The number of identified proteins in control samples was 274; whereas, 229 and 358 proteins were identified in samples for patient suffering MCI and AD, respectively (*Table 1*).

Table 2. Number of Proteins identified by LC-ESI/MS/MS by DDA analysis.

	DDA
CONTROL	274 (*P*-VALUE ≤ 0.05)
MCI	229 (*P*-VALUE ≤ 0.05)
AD	358 (*P*-VALUE ≤ 0.05)

Once all proteins of each group were identified, they were compared using the FunRich software. A Venn diagram, such as that show in *Figure 3*, was obtained. A list of common or non-common proteins can be obtained among the several groups to be compared.

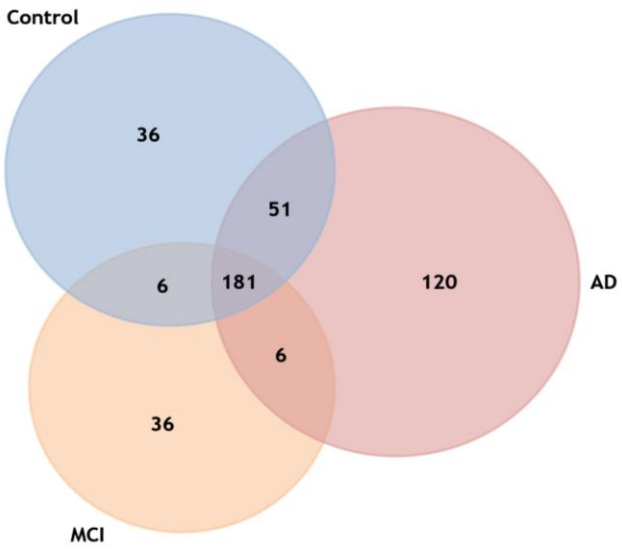

Figure 3. Venn diagram indicating the overlap between the identified protein groups (1% FDR) in each group of samples.

It was observed that 181 proteins are common among the three groups of samples, most of them major proteins. It is very important to obtain the list of proteins that are only present in a group, that is, those that are not identified in any of the two remaining groups. A total of 36 proteins were only found in Control group; whereas, this number was equal to 36 and 120 in the MCI and AD groups, respectively (*Table 3*).

Table 3. List of unique proteins from each group of samples.

	Protein	UniProt ID	Peptides(95%)	% Cov(95%)
CONTROL	Immunoglobulin lambda constant 2	IGLC2	159	96.2
	Immunoglobulin heavy variable 3-33	HV333	35	70.9
	Immunoglobulin heavy variable 3-11	HV311	33	65.8
	Immunoglobulin heavy variable 3-53	HV353	32	40.5
	Immunoglobulin heavy variable 4-59	HV459	23	55.2
	Immunoglobulin kappa variable 1D-17	KVD17	10	27.3
	Complement factor H-related protein 2	FHR2	4	17.4
	Immunoglobulin heavy variable 1-45	HV145	3	25.6
	Cartilage acidic protein 1	CRAC1	2	2.7
	L-lactate dehydrogenase A-like 6A	LDH6A	2	5.2
	KAT8 regulatory NSL complex subunit 1	KANL1	2	0.6
	Brefeldin A-inhibited guanine nucleotide-exchange protein 2	BIG2	1	0.4
	Complement component C8 alpha chain	CO8A	1	2.9
	Integrator complex subunit 10	INT10	1	1.1
	Proteoglycan 4	PRG4	1	0.6
	DNA-dependent protein kinase catalytic subunit	PRKDC	1	0.2
	Dystrophin	DMD	1	0.2
	Putative Polycomb group protein ASXL3	ASXL3	1	0.5

Table 3. List of unique proteins from each group of samples (Continued).

	Protein	UniProt ID	Peptides(95%)	% Cov(95%)
CONTROL	E3 ubiquitin-protein ligase TRIM4	TRIM4	1	1.4
	Vitamin K-dependent protein C	PROC	1	1.7
	Transmembrane protein 131-like	T131L	1	0.4
	Nischarin	NISCH	1	1.0
	Testis-expressed sequence 37 protein	TEX37	1	4.4
	Nance-Horan syndrome protein	NHS	1	0.4
	Secreted frizzled-related protein 5	SFRP5	1	3.2
	PDZK1-interacting protein 1	PDZ1I	1	9.6
	Bis(5'-nucleosyl)-tetraphosphatase [asymmetrical]	AP4A	1	4.1
	Motor neuron and pancreas homeobox protein 1	MNX1	1	2.5
	Nuclear pore complex-interacting protein family member B15	NPB15	1	3.8
	IQ motif and SEC7 domain-containing protein 1	IQEC1	1	0.8
	Xaa-Pro dipeptidase	PEPD	1	2.2
	Complement component C7	CO7	1	2.4
	Serine/threonine-protein kinase NIM1	NIM1	1	3.9
	FERM, ARHGEF and pleckstrin domain-containing protein 1	FARP1	1	0.7
	Ketimine reductase mu-crystallin	CRYM	1	3.2
	Ras suppressor protein 1	RSU1	1	3.2

Table 3. List of unique proteins from each group of samples (Continued).

	Protein	UniProt ID	Peptides(95%)	% Cov(95%)
MCI	Immunoglobulin kappa variable 1D-16	KVD16	22	39.3
	Immunoglobulin heavy variable 3-9	HV309	17	61.9
	Immunoglobulin heavy variable 4-39	HV439	17	36.8
	Hemoglobin subunit gamma-2	HBG2	10	45.6
	Immunoglobulin kappa variable 2-24	KV224	6	59.2
	Immunoglobulin lambda variable 2-23	LV223	2	14.1
	AT-rich interactive domain-containing protein 5B	ARI5B	1	0.8
	PDZ domain-containing protein 2	PDZD2	1	0.3
	UPF0606 protein KIAA1549	K1549	1	0.5
	Structural maintenance of chromosomes protein 4	SMC4	1	0.5
	Protein NYNRIN	NYNRI	1	0.6
	Coiled-coil domain-containing protein 8	CCDC8	1	2.8
	Dedicator of cytokinesis protein 7	DOCK7	1	0.7
	RNA-binding protein 40	RBM40	1	2.1
	Melanoma-associated antigen D4	MAGD4	1	1.1
	Tonsoku-like protein	TONSL	1	1.1
	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial	ACADV	1	3.4
	Inositol polyphosphate 1-phosphatase	INPP	1	4.5

Table 3. List of unique proteins from each group of samples (Continued).

	Protein	UniProt ID	Peptides(95%)	% Cov(95%)
MCI	5-oxoprolinase	OPLA	1	0.8
	Ornithine decarboxylase antizyme 3	OAZ3	1	4.3
	Cip1-interacting zinc finger protein	CIZ1	1	1.3
	Box C/D snoRNA protein 1	BCD1	1	2.1
	Actin-related protein 5	ARP5	1	1.5
	O-acetyl-ADP-ribose deacetylase MACROD1	MACD1	1	2.8
	Glomulin	GLMN	1	2.9
	ATR-interacting protein	ATRIP	1	2.1
	Putative ankyrin repeat domain-containing protein 20A12 pseudogene	A2012	1	4.6
	Activating signal cointegrator 1 complex subunit 1	ASCC1	1	2.7
	Oxidoreductase-like domain-containing protein 1	OXLD1	1	10.2
	Coiled-coil domain-containing protein 153	CC153	1	5.7
	Protein NPAT	NPAT	1	0.5
	NACHT, LRR and PYD domains-containing protein 11	NAL11	1	1.2
	Carbonic anhydrase 2	CAH2	1	3.5
	Apolipoprotein L3	APOL3	1	2.7
	Mitochondrial ribonuclease P catalytic subunit	MRPP3	1	1.2
	Zinc finger protein 609	ZN609	1	0.8

Table 3. List of unique proteins from each group of samples (Continued).

	Protein	UniProt ID	Peptides(95%)	% Cov(95%)
AD	Immunoglobulin kappa variable 1-8	KV108	50	50.4
	Immunoglobulin heavy variable 4-61	HV461	45	63.6
	Immunoglobulin lambda variable 3-25	LV325	17	53.6
	Immunoglobulin heavy variable 3-73	HV373_	12	65.5
	Immunoglobulin lambda variable 3-1	LV301	11	51.3
	Keratin, type II cytoskeletal 6A	K2C6A	8	12.2
	Immunoglobulin lambda variable 3-16	LV316	8	26.1
	Keratin, type I cytoskeletal 17	K1C17	6	11.3
	Extracellular superoxide dismutase [Cu-Zn]	SODE	5	30.4
	Immunoglobulin lambda variable 5-39	LV539	5	20.3
	Properdin	PROP	4	10.7
	Fibrinogen gamma chain	FIBG	4	12.8
	Transferrin receptor protein 1	TFR1	3	4.1
	Mannan-binding lectin serine protease 2	MASP2	3	4.8
	Adipocyte plasma membrane-associated protein	APMAP	3	7.2
	Cysteine-rich secretory protein 3	CRIS3	3	18.0
	L-lactate dehydrogenase B chain	LDHB	2	6.6
	Protein S100-A9	S10A9	2	24.6

Table 3. List of unique proteins from each group of samples (Continued).

	Protein	UniProt ID	Peptides(95%)	% Cov(95%)
AD	Platelet basic protein	CXCL7	2	19.5
	Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA	MA1A1	2	3.8
	Insulin-like growth factor-binding protein complex acid labile subunit	ALS	2	3.8
	Sperm flagellar protein 2	SPEF2	2	1.5
	RNA-binding protein 25	RBM25	2	1.1
	Complement factor D	CFAD	2	8.3
	E3 ubiquitin-protein ligase HACE1	HACE1	2	1.8
	Interleukin-12 receptor subunit beta-2	I12R2	2	0.9
	Rab GTPase-binding effector protein 1	RABE1	2	1.2
	Calcium-binding protein 39-like	CB39L	2	2.7
	Homeobox protein DLX-6	DLX6	2	8.0
	Glutaredoxin domain-containing cysteine-rich protein 2	GRCR2	2	3.6
	Protein S100-A8	S10A8	1	11.8
	EGF-containing fibulin-like extracellular matrix protein 1	FBLN3	1	4.7
	Uncharacterized protein KIAA1958	K1958	1	1.1
	von Willebrand factor	VWF	1	0.5
	HEAT repeat-containing protein 4	HEAT4	1	0.9
	Hydrocephalus-inducing protein homolog	HYDIN	1	0.1

Table 3. List of unique proteins from each group of samples (Continued).

	Protein	UniProt ID	Peptides(95%)	% Cov(95%)
AD	Helicase SRCAP	SRCAP	1	0.3
	LIM domain and actin-binding protein 1	LIMA1	1	1.4
	Cathepsin Z	CATZ	1	5.3
	Dynein heavy chain 2, axonemal	DYH2	1	0.2
	Liprin-beta-2	LIPB2	1	1.1
	C-Jun-amino-terminal kinase-interacting protein 4	JIP4	1	1.1
	Cytosolic carboxypeptidase 2	CBPC2	1	1.2
	Complement component C6	CO6	1	1.7
	Coagulation factor XIII B chain	F13B	1	1.8
	Integrator complex subunit 4	INT4	1	1.0
	Gamma-glutamyl hydrolase	GGH	1	4.4
	Methylcytosine dioxygenase TET1	TET1	1	0.3
	FERM and PDZ domain-containing protein 3	FRPD3	1	0.7
	DNA repair protein RAD50	RAD50	1	0.8
	Spectrin alpha chain, erythrocytic 1	SPTA1	1	0.3
	LIM domain only protein 7	LMO7	1	0.7
	Pre-mRNA-splicing factor SYF1	SYF1	1	0.8
	Heparan sulfate glucosamine 3-O-sulfotransferase 3A1	HS3SA	1	2.0

Table 3. List of unique proteins from each group of samples (Continued).

	Protein	UniProt ID	Peptides(95%)	% Cov(95%)
AD	Intraflagellar transport protein 140 homolog	IF140	1	0.7
	Uncharacterized protein C17orf47	CQ047	1	1.9
	Probable E3 ubiquitin-protein ligase MARCH10	MARHA	1	1.2
	Protein WWC2	WWC2	1	1.3
	Low-density lipoprotein receptor-related protein 2	LRP2	1	0.2
	Collagen alpha-1(IX) chain	CO9A1	1	2.5
	Angiomotin-like protein 2	AMOL2	1	2.3
	AF4/FMR2 family member 4	AFF4	1	0.9
	Serine/threonine-protein kinase H2	KPSH2	1	2.3
	Zinc finger protein 28 homolog	ZFP28	1	1.0
	Retinoic acid-induced protein 1	RAI1	1	0.9
	Protein EFR3 homolog A	EFR3A	1	1.7
	Calmodulin-regulated spectrin-associated protein 2	CAMP2	1	1.1
	Anthrax toxin receptor 2	RED1	1	1.2
	Anthrax toxin receptor 2	ANTR2	1	1.8
	Serpin B4	SPB4	1	3.6
	Prostaglandin-H2 D-isomerase	PTGDS	1	3.7
	3-hydroxy-3-methylglutaryl-coenzyme A reductase	HMDH	1	1.2

Table 3. List of unique proteins from each group of samples (Continued).

	Protein	UniProt ID	Peptides(95%)	% Cov(95%)
AD	Obscurin-like protein 1	OBSL1	1	0.7
	Interferon-inducible protein AIM2	AIM2	1	2.3
	Coiled-coil domain-containing protein 69	CCD69	1	2.7
	T-box transcription factor TBX22	TBX22	1	1.7
	UPF0160 protein MYG1, mitochondrial	MYG1	1	2.9
	(E3-independent) E2 ubiquitin-conjugating enzyme	UBE2O	1	0.6
	Collectin-11 OS=Homo sapiens	COL11	1	4.1
	Noelin	NOE1	1	1.4
	Down syndrome critical region protein 8	DSCR8	1	14.4
	UBX domain-containing protein 10	UBX10	1	3.6
	Radical S-adenosyl methionine domain-containing protein 2	RSAD2	1	2.2
	Partitioning defective 3 homolog	PARD3	1	0.7
	Uncharacterized protein C7orf57	CG057	1	3.1
	UPF0160 protein MYG1, mitochondrial	LS14A	1	2.4
	Leucine-rich repeat LGI family member 2	LGI2	1	1.8
	Synaptotagmin-like protein 1	SYTL1	1	1.8
	Piwi-like protein 3	PIWL3	1	1.0
	Ras association domain-containing protein 6	RASF6	1	2.7

Table 3. List of unique proteins from each group of samples (Continued).

	Protein	UniProt ID	Peptides(95%)	% Cov(95%)
AD	Putative zinc finger protein 66	ZNF66	1	1.7
	Condensin-2 complex subunit H2	CNDH2	1	3.0
	Transmembrane protein 198	TM198	1	2.5
	Membrane primary amine oxidase	AOC3	1	1.0
	Laminin subunit alpha-4	LAMA4	1	0.3
	Polypeptide N-acetylgalactosaminyltransferase 1	GALT1	1	1.8
	Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial	MMSA	1	1.9
	Methionine--tRNA ligase, cytoplasmic	SYMC	1	0.8
	Hepatocyte nuclear factor 3-alpha	FOXA1	1	2.8
	Ephrin type-B receptor 4	EPHB4	1	1.7
	Coatomer subunit delta	COPD	1	1.4
	G protein-coupled receptor kinase 6	GRK6	1	1.6
	60S ribosomal protein L13	RL13	1	4.3
	Bile salt-activated lipase	CEL	1	1.2
	Pantetheinase	VNN1	1	1.8
	Testis-expressed protein 46	TEX46	1	7.4
	Putative uncharacterized protein DNAJC9-AS	DAS1	1	5.4
	LRP2-binding protein	LR2BP	1	2.9

Table 3. List of unique proteins from each group of samples (Continued).

	Protein	UniProt ID	Peptides(95%)	% Cov(95%)
AD	HAUS augmin-like complex subunit 2	HAUS2	1	5.1
	Putative sodium-coupled neutral amino acid transporter 7	S38A7	1	1.9
	DNA polymerase epsilon subunit 3	DPOE3	1	15.0
	PR domain zinc finger protein 5	PRDM5	1	1.9
	KAT8 regulatory NSL complex subunit 2	KANL2	1	2.2
	Homeodomain-interacting protein kinase 1	HIPK1	1	1.2
	Glycine N-acyltransferase-like protein 3	GLYL3	1	3.8
	Guanine nucleotide exchange factor MSS4	MSS4	1	6.5
	Nuclear factor NF-kappa-B p105 subunit	NFKB1	1	1.0
	Pulmonary surfactant-associated protein B	PSPB	1	3.2
	Glyceraldehyde-3-phosphate dehydrogenase	G3P	1	2.4
	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit delta isoform	PK3CD	1	0.7

The molecular function of the proteins only present in each group was compared (*Figure 4*). In order to have functional information about the protein we perform an independent analysis in each group using the protein specifically for each one. In AD the majority are proteins related to transcriptional activity and in MCI with transcriptional regulatory activity.

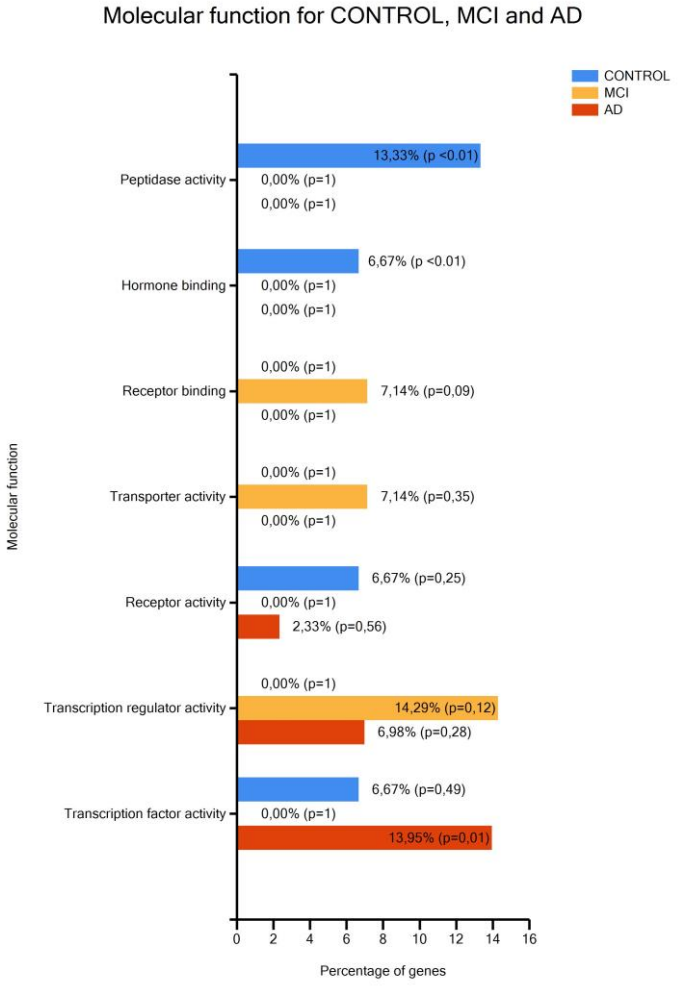

Figure 4. Molecular functions more representative of the proteins present only in each group of samples.

4.3.2 Protein quantification by SWATH analysis

Through this method the identified proteins were obtained, but it is necessary to quantify these proteins and compare them between the samples groups. To this aim, an analysis using SWATH-MS was done to create a library of all samples to be quantified. The generated library consists of 186 proteins that result from the pools of the three different groups of samples. After a comparison between the different groups of samples, it was observed a variation in the number of statistically significant proteins (*Table 4*).

Table 4. Number of Proteins identified by LC-ESI/MS/MS by Swath analysis.

SWATH	
LIBRARY	186
SAMPLES	
AD VS. CONTROL	31 (*P*-VALUE ≤ 0.05)
AD VS. MCI	19 (P-VALUE ≤ 0.05)
MCI VS. CONTROL	42 (P-VALUE ≤ 0.05)

A proteomic study was performed to detect differences between people with mild cognitive impairment (MCI) in early phase versus patients with AD. Moreover, these two stages were compared with samples from healthy people in the same age range. Principal components analysis (PCA) clearly revealed that the samples of each group of patients (*MCI* and *AD*) and healthy people were separated in the PC1 axis, which explains 70.3% of the variance among samples (*Figure 5*).

In this case, the separation between the groups of samples is visible for healthy people (*Control*) and people with MCI. However, the AD samples are statistically located in the two previous groups but separated from them on the X axis (PC1) of the PCA graph. The cause of this result is the high number of samples from patients with AD, with respect to MCI, at the same time, the difficult classification of patients with this type of disease. Thus, in many cases a patient diagnosed with

MCI already develops a much more advanced stage such as AD. Diagnosing this type of disease is very difficult and classifying a patient already diagnosed with this pathology at a certain stage affects a good separation in proteomic studies.

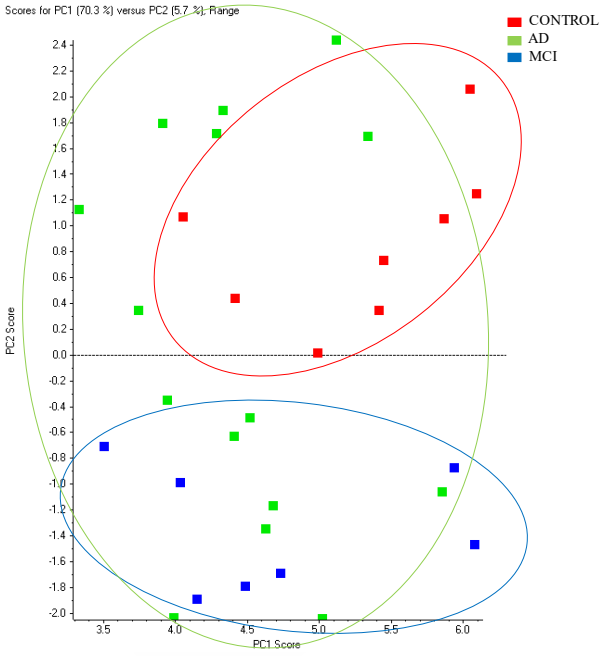

Figure 5. Principal Component Analysis of the log2 transformed SWATH Areas for Control, AD and MCI samples.

4.3.3 Differentially expressed serum proteins

To make this study, a library using pools of the three conditions was first created. Over this library, the swath files were loaded and it was performed the quantitative analysis (*Table 5*).

Graphically we can observe these variations through chart such as the volcano plot. A volcano plot (*Figure 6*) was generated by plotting

the log 2-fold changes for the identified proteins against their corresponding adjusted p-value. In this particular case the volcano plots with significantly upregulated (log 2 (Fold Change) ≥ 0 and -log 10 (p-value) ≥ 0.05) and downregulated (log 2(Fold Change) ≤ 0 and -log 10 (p-value) ≥ 0.05) proteins.

The results obtained show the presence of differentiating proteins in the three comparisons, an interesting fact when the number of samples is so low. When comparing patients with MCI and AD, differentiating proteins were 19, whereas when we compare controls against patients like AD and MCI were 31 and 42, respectively. The number of differentiating proteins among the group of patients is lower since the difference between the number of samples of AD and MCI is very high (15 vs. 7).

With this methodology, we can know how the protein concentration varies when belongs to a certain group or to another, so we can quantify the extent of the increase/decrease variations. In our case, it is very important to know concentration differences among samples belonging to MCI and AD groups.

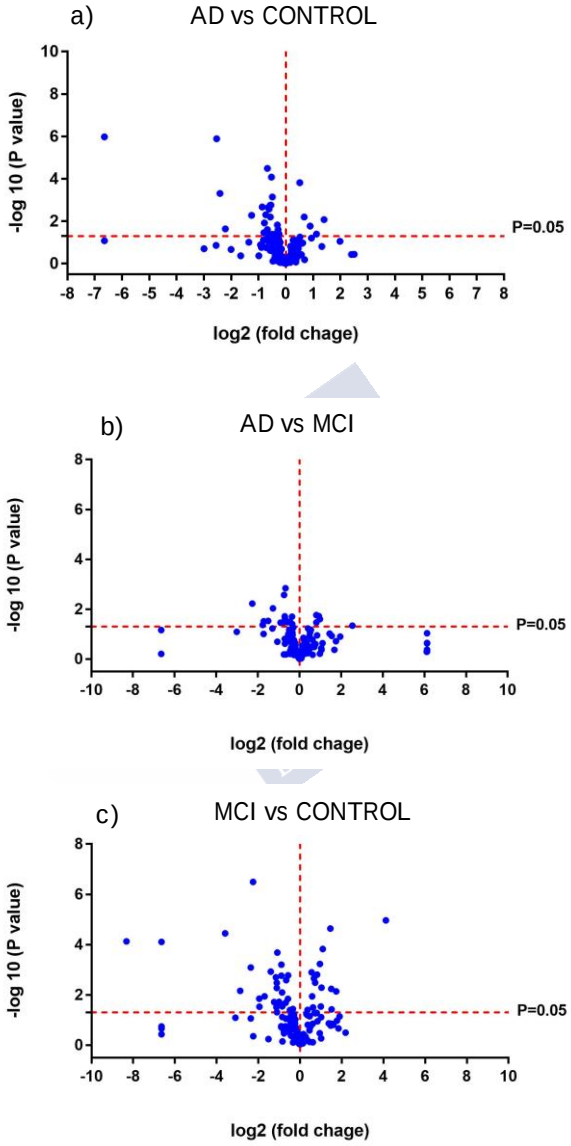

Figure 6. Volcano plot showing differentially abundant proteins identified in SWATH analysis. a) AD vs. Healthy people, b) AD vs. MCI and c) MCI vs. Control.

Table 5. Significant proteins (p-value < 0.05) in comparisons between groups of patients (MCI and AD) and controls.

	Protein	UniProt ID	*p*-value	Fold Change	
AD vs. CONTROL	Alpha-1-antitrypsin	A1AT	0.000150842	1.428064395	↑ AD
	Leucine-rich alpha-2-glycoprotein	A2GL	0.006344712	1.596318104	↑ AD
	Apolipoprotein C-I	APOC1	0.008441469	2.636989575	↑ AD
	Apolipoprotein A-II	APOA2	0.016944023	1.857182466	↑ AD
	Immunoglobulin kappa variable 2D-30	KVD30	0.040196273	2.174683825	↑ AD
	Complement factor H	CFAH	1.29E-06	5.798695127	↑ CONTROL
	Kininogen-1	KNG1	3.24E-05	1.595359954	↑ CONTROL
	Complement C3	CO3	8.24E-05	1.439446029	↑ CONTROL
	Inter-alpha-trypsin inhibitor heavy chain H3	ITIH3	0.000491661	5.311934817	↑ CONTROL
	Fibronectin	FINC	0.000718669	1.406963651	↑ CONTROL
	Complement C2	CO2	0.001725894	1.46266922	↑ CONTROL
	Protein AMBP	AMBP	0.001758244	1.490747413	↑ CONTROL
	Immunoglobulin kappa variable 1D-12	KVD12	0.00212286	1.821266897	↑ CONTROL
	Heparin cofactor 2	HEP2	0.002250161	1.568676809	↑ CONTROL
	Apolipoprotein B-100	APOB	0.002673085	1.530660744	↑ CONTROL
	Vitronectin	VTNC	0.004970623	1.66909992	↑ CONTROL
	Immunoglobulin heavy constant gamma 4	IGHG4	0.005240527	2.380580012	↑ CONTROL
	Complement factor B	CFAB	0.006259083	1.480764183	↑ CONTROL

Table 5. Significant proteins (p-value < 0.05) in comparisons between groups of patients (MCI and AD) and controls (Continued).

	Protein	UniProt ID	*p*-value	Fold Change	
AD vs. CONTROL	Vitamin D-binding protein	VTDB	0.011997727	1.718172847	↑ CONTROL
	Hemopexin	HEMO	0.015162531	1.237937067	↑ CONTROL
	Immunoglobulin lambda constant 7	IGLC7	0.023158812	4.641457012	↑ CONTROL
	Pigment epithelium-derived factor	PEDF	0.024850812	1.215515834	↑ CONTROL
	Immunoglobulin heavy variable 1-18	HV118	0.024875596	1.599633608	↑ CONTROL
	Immunoglobulin kappa variable 1-5	KV105	0.035166522	1.74415005	↑ CONTROL
	Carboxypeptidase B2	CBPB2	0.038993285	1.278737194	↑ CONTROL
	Ficolin-3	FCN3	0.04007809	1.186473438	↑ CONTROL
	Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1	0.040389668	1.237125641	↑ CONTROL
	Vitamin K-dependent protein S	PROS	0.040792118	1.409692252	↑ CONTROL
	Serum albumin	ALBU	0.041157152	1.538937491	↑ CONTROL
	Complement C4-B	CO4B	0.047843604	1.564719953	↑ CONTROL
	Prothrombin	THRB	0.049232372	1.315520959	↑ CONTROL
AD vs. MCI	Complement C5	CO5	0.017168998	1.755079919	↑ AD
	Serotransferrin	TRFE	0.019095969	1.90170588	↑ AD
	Immunoglobulin kappa constant	IGKC	0.025197357	1.968701546	↑ AD

Table 5. Significant proteins (p-value < 0.05) in comparisons between groups of patients (MCI and AD) and controls (Continued).

	Protein	UniProt ID	*p*-value	Fold Change	
AD vs. MCI	Carboxypeptidase N subunit 2	CPN2	0.034451267	1.758305902	↑ AD
	Immunoglobulin heavy variable 3-20	HV320	0.045700741	5.803415614	↑ AD
	Transthyretin	TTHY	0.001454666	1.594812264	↑ MCI
	Lumican	LUM	0.002670538	1.675114157	↑ MCI
	Immunoglobulin heavy constant delta	IGHD	0.006009606	4.833003458	↑ MCI
	Keratin. type II cytoskeletal 1	K2C1	0.009299369	2.42109829	↑ MCI
	Vitamin D-binding protein	VTDB	0.019460924	1.629789887	↑ MCI
	Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1	0.020042686	1.28068723	↑ MCI
	Galectin-3-binding protein	LG3BP	0.023416436	1.620014651	↑ MCI
	Hemoglobin subunit alpha	HBA	0.029631813	2.83759387	↑ MCI
	Apolipoprotein A-I	APOA1	0.031830155	3.291774763	↑ MCI
	Apolipoprotein A-II	APOA2	0.033371384	1.381288291	↑ MCI
	Immunoglobulin kappa variable 3-11	KV311	0.033892277	1.479817159	↑ MCI
	Immunoglobulin lambda-like polypeptide 1	IGLL1	0.03484026	1.880073137	↑ MCI
	Apolipoprotein L1	APOL1	0.039723769	1.277302939	↑ MCI
	Immunoglobulin lambda variable 1-44	LV144	0.043048814	3.402638201	↑ MCI

Table 5. Significant proteins (p-value < 0.05) in comparisons between groups of patients (MCI and AD) and controls (Continued).

	Protein	UniProt ID	*p*-value	Fold Change	
MCI vs. CONTROL	Apolipoprotein A-II	APOA2	2.28E-05	2.748290481	↑ MCI
	Lumican	LUM	0.000150189	2.123310039	↑ MCI
	Leucine-rich alpha-2-glycoprotein	A2GL	0.000586092	1.940647447	↑ MCI
	Apolipoprotein A-I	APOA1	0.001267493	1.469433705	↑ MCI
	Apolipoprotein A-IV	APOA4	0.001564243	1.764189502	↑ MCI
	Apolipoprotein L1	APOL1	0.002214849	1.587071697	↑ MCI
	Transthyretin	TTHY	0.003293402	1.651644513	↑ MCI
	Apolipoprotein C-I	APOC1	0.005147585	2.038582622	↑ MCI
	Keratin. type II cytoskeletal 1	K2C1	0.005759999	2.829818109	↑ MCI
	Serum amyloid A-1 protein	SAA1	0.007293972	3.32895718	↑ MCI
	Alpha-1-antitrypsin	A1AT	0.011435815	1.497934836	↑ MCI
	Immunoglobulin lambda-like polypeptide 1	IGLL1	0.029046511	1.99085618	↑ MCI
	Immunoglobulin lambda variable 4-69	LV469	0.031609644	1.539955317	↑ MCI
	Immunoglobulin kappa variable 2D-30	KVD30	0.037996139	2.860434042	↑ MCI
	Zinc-alpha-2-glycoprotein	ZA2G	0.039731944	1.286912386	↑ MCI
	Immunoglobulin kappa variable 1-5	KV105	3.21E-07	4.761333769	↑ CONTROL
	Complement factor H	CFAH	3.56E-05	12.05833778	↑ CONTROL

Table 5. Significant proteins (p-value < 0.05) in comparisons between groups of patients (MCI and AD) and controls (Continued).

	Protein	UniProt ID	*p*-value	Fold Change	
MCI vs. CONTROL	Immunoglobulin lambda constant 7	IGLC7	7.45E-05	320.4342431	↑ CONTROL
	Carboxypeptidase N subunit 2	CPN2	0.00020751	2.13473544	↑ CONTROL
	Kininogen-1	KNG1	0.000631971	1.856051184	↑ CONTROL
	Immunoglobulin kappa variable 1-6	KV10	0.000823755	5.143184309	↑ CONTROL
	Serotransferrin	TRFE	0.001198066	2.649531958	↑ CONTROL
	Complement C3	CO3	0.001664889	1.483296523	↑ CONTROL
	Prothrombin	THRB	0.001733647	1.864554465	↑ CONTROL
	Immunoglobulin kappa variable 1D-12	KVD12	0.001996502	2.236374253	↑ CONTROL
	Protein AMBP	AMBP	0.002615939	1.584176521	↑ CONTROL
	Complement C5	CO5	0.003318428	2.169251962	↑ CONTROL
	Immunoglobulin heavy constant gamma 3	IGHG3	0.005290911	2.162703993	↑ CONTROL
	Immunoglobulin heavy variable 3-20	HV320	0.006877083	7.321970699	↑ CONTROL
	Immunoglobulin kappa constant	IGKC	0.00801714	1.811098286	↑ CONTROL
	Immunoglobulin heavy constant gamma 4	IGHG4	0.011549245	3.246548248	↑ CONTROL
	Inter-alpha-trypsin inhibitor heavy chain H3	ITIH3	0.014205491	3.874496395	↑ CONTROL
	Complement C2	CO2	0.014397201	1.496975187	↑ CONTROL
	Vitronectin	VTNC	0.018977977	2.005373087	↑ CONTROL

Table 5. Significant proteins (p-value < 0.05) in comparisons between groups of patients (MCI and AD) and controls (Continued).

	Protein	UniProt ID	*p*-value	Fold Change	
MCI vs. CONTROL	Beta-Ala-His dipeptidase	CNDP1	0.019200315	2.367906299	↑ CONTROL
	Complement factor B	CFAB	0.020655338	1.613878547	↑ CONTROL
	Immunoglobulin heavy variable 3-30-5	HV335	0.028507591	1.809828521	↑ CONTROL
	Immunoglobulin kappa variable 1-16	KV116	0.029379335	3.862678459	↑ CONTROL
	Immunoglobulin heavy constant alpha 1	IGHA1	0.031014725	2.196352141	↑ CONTROL
	Apolipoprotein B-100	APOB	0.036646763	1.272142291	↑ CONTROL
	Immunoglobulin heavy variable 1-18	HV118	0.04011809	1.405671966	↑ CONTROL
	CD5 antigen-like	CD5L	0.048651204	2.151042874	↑ CONTROL

4.3.3.1 Apoliprotein A-II

Only one protein [Apoliprotein A-II (Apo A-II)] has been found statistically significant different in the three comparisons (*Figure 7*). This protein is always quantitatively increased in patients *versus* controls (healthy people) with a Fold Change of 1.86 in the case of AD vs. Control and 2.75 in the case of MCI vs Control. This protein is increased in MCI (Fold Change value of 1.48) when comparing MCI/AD.

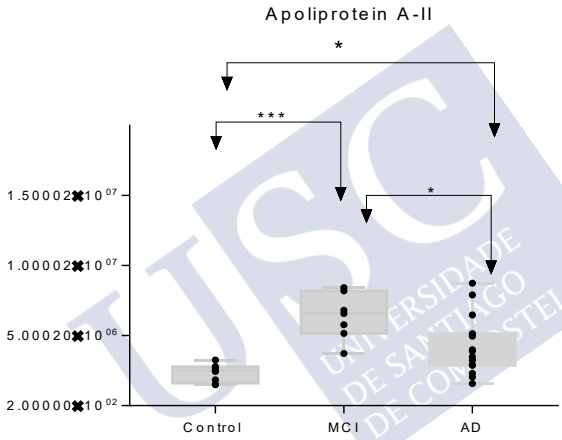

Figure 7. Boxplots representing the mean centered SWATH Areas obtained for Apo A-II in the comparative proteomic analysis of Control, MCI and AD.

Apolipoproteins are a group of proteins related to cholesterol and lipid metabolism. Recent findings indicate that apolipoproteins might also be involved in neurodegenerative processes [38]. The presence of the apolipoproteins accelerates the development of both AD and cerebral amyloid angiopathy [39-41].

We showed that expression levels of Apo A-II were significantly decreased in the plasma of AD patients when compared to MCI. Our results are consistent with a previous report of decreased Apo A-II

concentrations in CSF of neuropathologically confirmed neurodegeneration [42].

Although several functions of Apo A-II correlate with AD pathogenesis, the pathologic role of Apo A-II in AD is still unknown. Our results showed that the expression levels of Apo A-II were significantly increased in plasma samples of MCI patients when compared to controls. The increased expression levels of Apo A-II in the serum of AD patients were previously found to be significant [43]. It is suggested that Apo A-II may play an important role in the progression of AD and could thus be a susceptibility biomarker for the early diagnosis of AD.

4.3.3.2 Other significant proteins

The number of quantified and statistically significant proteins in the comparison between these three groups of samples is high, although the number of samples in the study is small.

Among these proteins, **serotransferrin** (TRFE) was found statistically significant, results that agree with findings reported by authors [44-47]. In our case, this protein was found to be increased in AD samples versus MCI. However, this protein was found in lower concentration in MCI samples when comparing MCI samples versus control (*Figure 8*). Therefore, this protein could be considered a protein biomarker at an early stage of neurodegeneration in diseases such as AD.

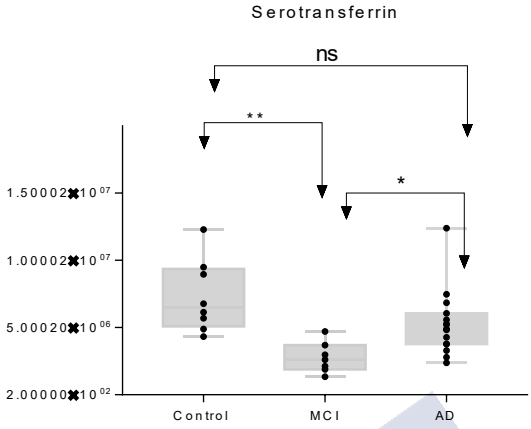

Figure 8. Boxplots representing the mean centered SWATH Areas obtained for serotransferrin in the comparative proteomic analysis of Control, MCI and AD.

This same protein has been identified in the 2DE-MALDI-TOF analysis of Chapter 3, in which serotransferrin appears increased in patients with AD versus MCI.

An extensive list of differentiating proteins has been obtained not only between patient and healthy people, but also between the two stages of the disease. Some of them were also found statistically significant when previously applying 2DE-MALDI-TOF strategies: **Keratin, type II cytoskeletal 1** (K2C1), **Kininogen-1** (KNG1), **CD5 antigen-like** (CD5L), **Alpha-1-antritrypsin** (A1AT), **Vitronectin** (VTNC), **Ficolin-3** (FCN3), **Pigment epithelium-derived factor** (PDEF) and **Serum albumin** (ALBU).

All these proteins vary in concentration in a statistically significant way when comparing controls versus patients in different stages of the disease (*Figures 9 and 10*).

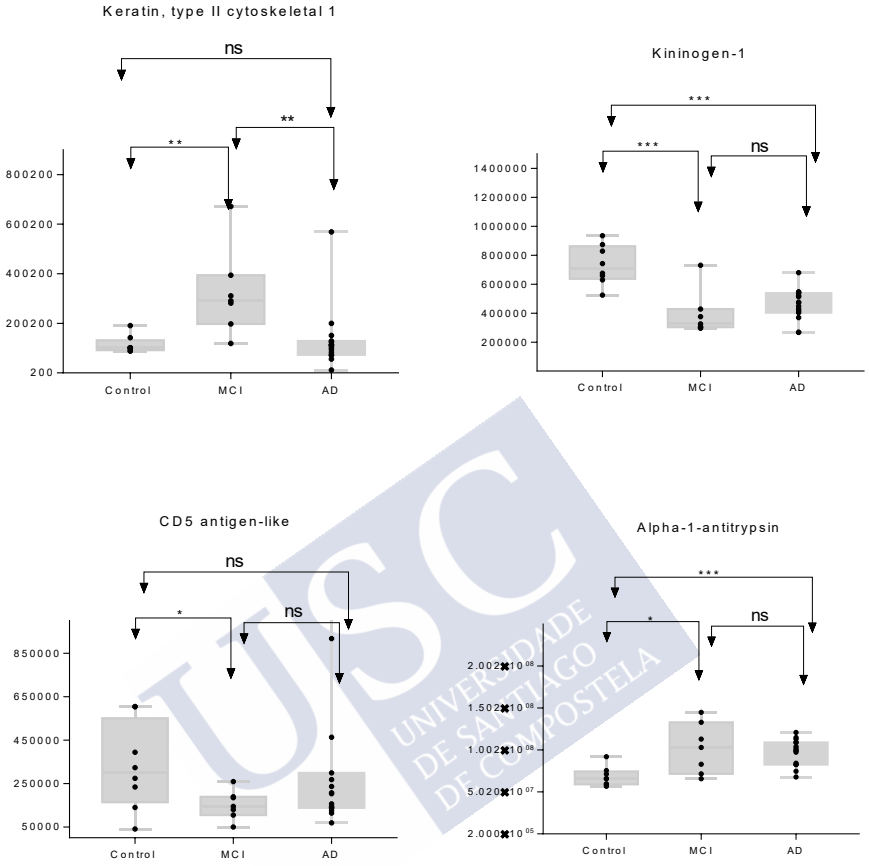

Figure 9. Boxplots representing the mean centered SWATH Areas obtained for K2C1, KNG1, CD5L and A1AT in the comparative proteomic analysis of Control, MCI and AD.

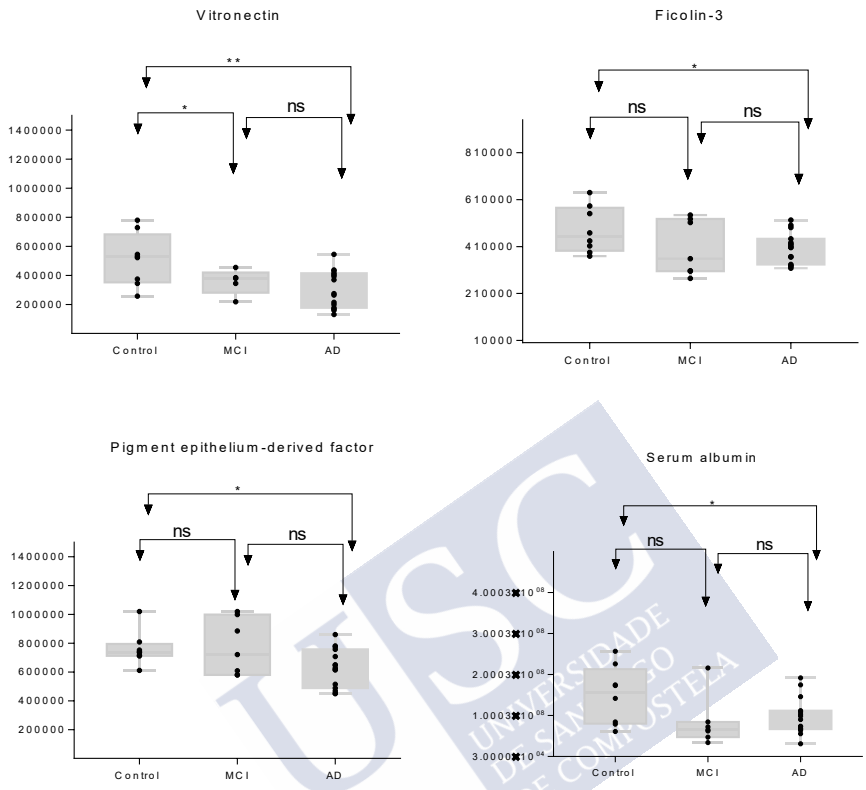

Figure 10. Boxplots representing the mean centered SWATH Areas obtained for VTNC, FCN3, PDEF and ALBU in the comparative proteomic analysis of Control, MCI and AD.

There are several proteins whose concentration decreases with the progression of the disease, although occasionally their quantification is not statistically significant (p-value $\geq$ 0.05). These proteins are **Vitronectin**, **Ficolin-3** and **Pigment ephitellum-derived factor**. Protein Alpha-1-antitrypsin is increased in AD and MCI versus control in both comparisons (statistically significant), but there is no statistical significance when comparing samples from people at the two stages of the disease. Regarding serum albumin, a high decrease in MCI and AD patients respect to control samples was observed.

Immunoglobulin kappa constant (IGKC) has been also identified and quantified, and it was found to be statistically significant not only when comparing AD versus MCI, but also for MCI versus Control comparison (*Figure 11*). This protein offers the same results than those found for serotransferrin, since it increases in AD when compared to MCI and at the same time increased in Controls *versus* MCI. Therefore, this protein decreases in previous stage of neurodegeneration, such as MCI.

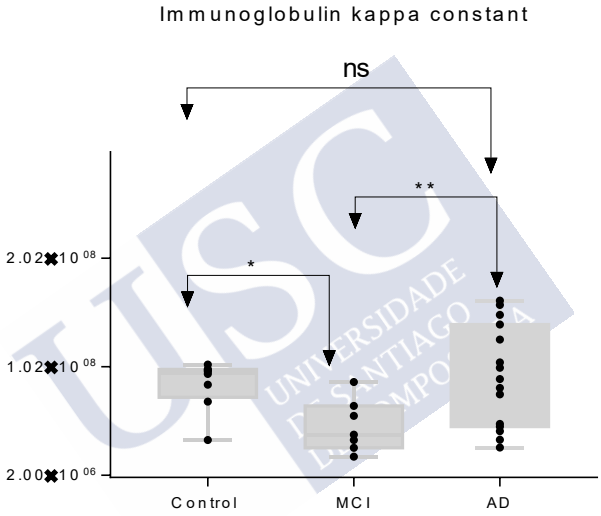

Figure 11. Boxplots representing the mean centered SWATH Areas obtained for serotransferrin in the comparative proteomic analysis of Control, MCI and AD.

Some authors refer IGKC to be a possible biomarker of AD disease [48-51], but in all cases they compare samples from healthy people with patients. In our study we are able to quantify this protein and find concentration differences between different stages of the neurodegeneration disease.

The highest concentration difference was found for **Immunoglobulin lambda constant 7** (IGLC7). This protein is always increased in control samples when comparing with AD and MCI (Fold

Change of 4.64 and 320.43, respectively). Some authors have considered this protein to be a potential biomarker of AD in blood and CSF [51].

4.4 CONCLUSIONS

Because of the serious difficulties to perform brain biopsy, the identification of blood biomarkers that reflect disease in the brain is critical. The new analytical approaches using serum samples for diagnosis are appealing tools for uncovering blood biomarkers that accurately reflect normal/diseased brains. A group of biomarkers could probably reflect the dynamics of disease-perturbed networks, and they could provide early AD diagnosis. Moreover, biomarkers could allow the disease progression monitoring, as well as the establishment of several subtypes of the disease and control the response to a certain therapy. We believe that serum proteins as potential biomarkers could make health care to be more effective, reducing the cost of health care, and promoting innovation in dealing with disease and wellness.

In the current study, serum samples from healthy people and from patients with two different stages of neurodegeneration have been analyzed qualitatively and quantitatively by robust and highly sensitive proteomic techniques. We have found a panel of differentiating serum biomarkers between controls and people suffering AD/MCI, but also between two different stages (AD/MCI). Further studies will be lead to increase the number of samples to confirm that these differentiating proteins could be potential biomarkers of AD. In addition, a group (panel) of biomarkers capable of identifying an early stage of AD will be also addressed.

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CHAPTER 5

SCREENING OF METAL-PROTEIN COMPLEXES TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS AND LASER ABLATION-INDUCTIVELY COUPLED-PLASMA MASS SPECTROMETRY

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Screening of metal-protein complexes two-dimensional polyacrylamide gel electrophoresis and laser ablation-inductively coupled-plasma mass spectrometry

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Abstract

A screening technique using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) after two dimensional (2-D) polyacrylamide gel electrophoresis was developed in order to identify various elements in two separated human serum isoform protein spots (serotransferrin and keratin citoesqueletal) from patients with neurodegenerative disease. The identification of elements (Al, Ba, Ca, Co, Cu, Cr, Fe, Li, Mn, Ni, P, Rb, Sr, V and Zn) in single serotransferrin and keratin citoesqueletal spots was performed with a double-focusing sector field inductively coupled plasma mass spectrometer at medium mass resolution (m/Δm of 4400). A Nd:YAG laser beam (wavelength 213 nm) in lineal scan mode at optimum conditions (laser fluency 3.5 J cm^{-2}, repetition rate 20 Hz, laser spot diameter 200 μm, depth 0 μm, scanning speed 5 μm s^{-1}) was used. Element signal intensities were normalized

with respect to ^{32}S in protein spots. Several elements have been successfully identified in both protein spot.

5.1 INTRODUCTION

Approximately 30% of the proteins and enzymes present in a biological systems contain metal or metalloid ions in their structures, and about 40% of these elements are crucial to maintaining the biological functions of proteins [1].

Metals play an important and essential role as cofactors of proteins in biological systems (e.g., in single cells or cell organelles). The absence or a deficit of essential metals (such as Fe, Cu, Se, Zn) in proteins results in deficiency diseases, but these metals can also catalyze cytotoxic reactions [2,3]. The assessment of metal-containing proteins (metal-protein complexes) is a challenging task in proteomics and includes the protein identification and the determination of the metal concentration associated with the protein. Sensitive analytical techniques are therefore required for these achievements. In addition to the determination of phosphorus, the quantitative determination of zinc-, copper- and iron-protein complexes is of special interest for studying neurodegenerative diseases, e.g., Alzheimer's and Parkinson's disease [4, 5].

Two-dimensional gel electrophoresis (2-DE) is a powerful fractionation technique which allows the separation of thousands of proteins in one run [6]. Proteins are separated according to their isoelectric point in the first dimension (isoelectric focusing, IEF) and then, orthogonally, according to their molecular weight by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins in 2-DE gels are visualized by, e.g. Coomassie Blue or silver staining [7].

Inductively coupled plasma mass spectrometry (ICP-MS) is a sensitive analytical technique which allows the simultaneous determination of metals [8]. Because of metals and metalloids bound to

proteins are present in very low concentrations, an extremely sensitive and selective detector needs to be used for their identification and quantification [9].

ICP-MS fulfills these requirements, due to its particular characteristics: (i) high analytical throughput, (ii) low detection limits for most elements, (iii) minimal matrix effects, (iv) specific response for the heteroatoms (metals, metalloids or nonmetals), (v) capability of up to eight orders of magnitude of linear dynamic range, (vi) information about isotope ratios that can be applied for exact and precise metalloprotein quantification using isotope dilution analysis, and (vii) simple coupling to different separation methods [10, 11].

After PAGE separation, the protein bands or spots can be analyzed by ICP-MS in two ways: (i) as a solution that it is conventionally aspired (a previous microwave-assisted digestion of the gel spots is required), and (ii) in situ, consisting of direct analysis of gels after laser ablation (LA) as a sample introduction system in ICP-MS [12, 13].

LA-ICP-MS is a powerful microanalytical technique that uses a focused laser beam to ablate material from the surface of a solid sample. The sample is mounted on a sample holder in the laser ablation chamber. The photons of the laser beam interact within the laser spot with the material that is ablated and transported by a continuous flow of argon (usually helium may be mixed with argon before the torch injector) to the ICP source [14, 15]. The ions formed are extracted from the atmospheric ion source and subsequently analyzed in the mass spectrometer (quadrupole or time-of-flight mass analyzer, or double-focusing sector field mass spectrometer).

The advantages of this technique are high sensitivity, accuracy and precision. However, the quantification of analytical data using LA-ICP-MS is difficult because adequate standard reference materials are not available [16].

The aim of this article is to optimize the conditions of laser ablation in two-dimensional polyacrylamide gels (2-DE) for the multielement determination of metals associated with proteins. Once the optimal conditions for these determinations have been obtained, the values of essential metals (Al, Ba, C, Ca, Co, Cr, Cu, Fe, Li, Mn, Mo, Ni, P, Rb, S, Se, Si, Sr, V and Zn) have been identified in two different protein spots (serotransferrin (TRFE) y la Keratin type II cytoskeletal 1 (K2C1)) in eight serum samples.

5.2 MATERIALS AND METHODS

5.2.1 Instrumentation

A double-focusing magnetic sector (HR-MS)-based ICP-MS (ELEMENT XR, Thermo Finnigan, Thermo Instruments, Bremen Germany) coupled to a Q-switched Nd:YAG laser ablation system operating at wavelength of 213 nm and equipped with a computer controlled parfocal video microscope (2.6x – 32.5x optical zoom range), and a HelEx II Active 2-Volume Ablation Cell Compatible (LSX-213 G2+ Laser Ablation System, Teledyne CETAC Technologies, Omaha, NE 68144, USA) was used for the microlocal analysis of metals in protein spots in 2-DE gel. The laser beam was focused on separated protein spots, the ablated material was transported by argon as a carrier gas into the inductively coupled plasma (ICP-MS). To separate possible interfering molecular ions from the atomic ions $^{63}Cu^{+}$, $^{56}Fe^{+}$, $^{31}P^{+}$, $^{32}S^{+}$, and $^{64}Zn^{+}$, all LA-ICP-MS measurements were performed at medium mass resolution (m/Δm of 4400).

Power Pac Basic power supply from Bio-Rad (CA, USA) was used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) protein separation. Protein quantification was accomplished by measuring the absorbance at 280 nm with the use of a Qubit™ 4 Quantitation Starter Kit from Thermo Fisher Scientific. Gel image acquisition was carried out with a UVP PhotoDoc-ItTM Imaging System from Analytik Jena.

5.2.2 Reagents and materials

Ultrapure water with a resistance of 18 MΩ-cm was obtained from a Milli–Q® purification device (Millipore Corporation, USA). Multi-element standard solutions were prepared by combining stock standard solutions of 1.000 g L^{-1} (Al, Ba, Ca, Co, Cu, Cr, Fe, Li, Mn, Ni, P, Rb, Sr, V and Zn) supplied by Merck (Poole, Dorset, UK). NIST 612 glass standard from National Institute of Standards and Technology (Gaithersburg, MD) was used to ICP-MS instrumental conditions optimisation.

All reagents and solvents used were HPLC-grade or higher. Dithiothreitol (DTT), Iodoacetamide (IAA), SYPRO Ruby Protein Gel Stain, 2-DE buffer, and the Sigma Marker wide range 6.5-200 KDa were purchased from Bio Rad (Hercules, California, USA). Sodium dodecylsulfate (SDS), Glycerol 86-88%, Tris-base, urea, thiourea, and sodium carbonate were purchased from Sigma-Aldrich (San Luis, Misuri, Estados Unidos). β-mercaptoethanol was purchased from Merck (Hohen-Brunn,Germany) and bromophenol-blue was purchased from Riedel-de Haen (Seelze,Germany). Acrylamide/bis-acrylamide 30% solution (37.5:1) and 3-[(3-Cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS) was purchase from Serva (Heidelberg, Germany).

5.2.3 Serum collection and storage

Serum samples from healthy volunteer adults and patients (Table 1) were supplied by the Servicio de Neurología at the University Clinical Hospital of Santiago de Compostela (Santiago de Compostela, Spain). The developed research has been ascribed to the approved and in force expert opinion from the Comité de ética de la investigación con medicamentos – CEIm-G (Ethics Committee for the Research with medicines) of Galicia (Registration Code: CEIm-G 2018/575).

For serum collection, a total of 2 mL of venous blood was collected in Vacutainer blood collection tubes with silicone-coated interiors (BD

Diagnostics, Franklin Lakes, NJ, USA) by a standard venipuncture method. The collected serum samples were stored at room temperature to allow for blood clotting, and then centrifuged at 1800 g for 10 min at 4 °C in order to remove the fibrin clot and other cellular elements. Serum samples were then immediately frozen at –80 °C.

5.2.4 Depletion of multiple high abundant proteins

Serum aliquots were filtered with Miller-GP® Filter Unit (Millipore) with a size of 0.22 μm. Each aliquot of human serum (30 μL) was depleted with dithiothreitol (DTT) according to the protocol described by Warder el al. [17, 18]. Fresh DTT 500 mM (3.3 μL) was mixed with 30 μL of human serum and vortex briefly. Samples were then incubated until a viscous white precipitate persisted (60 min), followed by centrifugation at 14000 rpm for 20 min. Supernatants were transferred to a clean tube and total dry (30 ºC, 45 min).

Samples were re-suspended in the 2-DE buffer (65 mM DTT, 65 mM CHAPS, 5 M urea, 2 M thiourea, 0.15 M NDSB-256, 200 nM tributylphosphine, 100 nM NaF, 1 M Na_3VO_4, and 1 M benzamidine) and stored at –20°C until further use. All reagents were of high purity. The re-suspended proteins were centrifuged at 13000 g for 5 min, and quantified before 2-DE for total protein content.

5.2.5 2-DE

Isoelectric focusing (IEF) was performed in pH 4-7 immobilized pH gradient (IPG) strips of 7 cm. 100 μg of protein were re-suspended in 125 μL of 2-DE buffer supplemented with ampholytes (0.1% servalyte 3–10 and 2-4, 0.05% servalyte 9-11), and subjected to solubilization during 2 h. After solubilization, the mixture was centrifuged at 13000 g for 20 min. Samples were applied to each strip and submitted to active rehydration at 50 V during 12 h before IEF (performed in a Protean IEF Cell focusing unit until 8000 V/total h were reached). After IEF, the IPG strips were equilibrated for 15 min in 4 M urea, 2 M thiourea, 50 mM Tris pH 6.8, 2% sodium dodecyl sulphate

(SDS), 12 mM dithiothreitol (DTT) and 30% glycerol plus 1% DTT and 15 min in 4 M urea, 2 M thiourea, 50 mM Tris pH 6.8, 2% sodium dodecyl sulphate (SDS), 12 mM dithiothreitol (DTT) and 30% glycerol plus 4% iodoacetamide. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12% polyacrylamide gels using a ProteanPlusDodeca Cell at 10 mA/gel and 18°C constant temperature for 4 h, or until the dye front reached the bottom of the gel. The 2-DE gels were stained with Sypro Ruby following manufacturer's instructions.

Before LA-ICP-MS analysis, gels were vacuum dried at 60 ºC for 4 h. Gels were first covered with a porous cellophane sheet and then placed between two glass holders.

5.2.6 Procedure for simultaneous multi-elemental analysis of protein spots in the 2-DE gels

The 2-DE gel was cut in several pieces, which contains separated protein spots, in an ultraclean vertical laminar air flow hood NU-156 (NUAIRE, Plymouth, USA). The gel pieces were glued to glass microscopic slides using double-sided tape. The separated protein spots in the 2-DE gels (*Figure 1*) were screened using LA-ICP-MS at mass-to-charge ratios of 27, 137, 12, 44, 59, 52, 63, 56, 7, 55, 97, 60, 31, 85, 32, 77, 28, 88, 51 and 66 with respect to the measurement of Al, Ba, C, Ca, Co, Cr, Cu, Fe, Li, Mn, Mo, Ni, P, Rb, S, Se, Si, Sr, V and Zn) ion intensities.

ICP-MS instrumental conditions (radiofrequency power, Ar flow rates, torch alignment and ion optics) and He gas flow rate were optimized at the beginning of each analysis sets by ablating a NIST 612 glass standard (*Table 1*). Helium at an optimum flow rate of 1.0 L min^{-1} was used to transport the ablated material into the ICP torch. Laser ablation of protein spots was performed with an Nd:YAG laser using the optimum conditions shown in *Table 1*. A wash up time of 50 s was inserted between successive ablations to minimize or avoid memory effect. Measurements were carried out in the scan mode at scan speed

of 5.0 μm s^{-1}; spot size of 200 μm; repetition rate of 20 Hz; and ablation depth of 0 μm. Laser was operated at a power of 60%, which corresponds to a fluence of 5.5 J cm^{-2}.

^{32}S (an element present at high level in protein spots) was used for internal standard normalization. The optimized experimental parameters of the LA-ICPMS measurements are summarized in Table 1.

Table 1. Optimized experimental parameters used for LA–ICP-MS.

Operating LA conditions		
Laser fluence / J cm^{-2}		5.5
Laser power / %		60
Scan speed / μm s^{-1}		5.0
Repetition rate / Hz		20
Ablation depth / μm		0
Spot diameter / μm		200
Scan pattern		Lineal scan
Analysis time / s		90
Size of ablated area / mm^2		0.065
He flow rate / mL min^{-1}		1.0
Wash up time / s		50
Operating ICP-MS conditions		
Radiofrequency power / W		1475
Gas flows / L min^{-1}	Cooling	15
Ion optics	Extraction / V	−200 0
	Focus / V	−111 0
	X Deflection / V	6.29
	Y Deflection / V	−1.2 6

5.3 RESULTS AND DISCUSSION

5.3.1 Optimization of laser ablation conditions

Several protein spots (spots a, b and c, *Figure 1*) were found to contain Al, Ba, Ca, Co, Cr, Cu, Fe, K, Li, Mn, Ni, P, Rb, Sr, V and Zn (As, Se and Mo were not detected), and they were selected for further analytical studies on 2-DE gels. Several experiments (univariate

approaches) were performed to choose the optimal laser ablation conditions (best signal-to-noise ratio, best precision and fast data acquisition) for multi-element determination from protein spots. Element signals were normalised using ^{32}S to minimize distortions produced by inhomogeneities and curvature of the gel. Each set of laser ablation conditions was replicated three times. At least two different blanks were performed in each set of laser ablation conditions. Lineal scan pattern was used throughout.

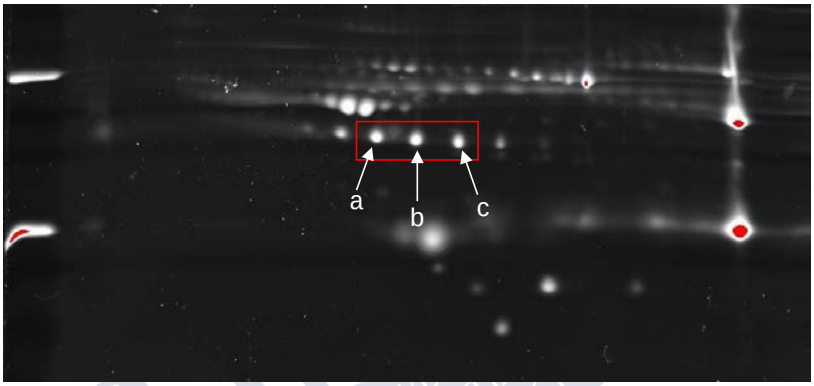

Figure 1. Selected spots for the optimization of the optimal laser ablation conditions.

5.3.1.1 Spot size and ablation depth

The amount of mass ablated and transferred to ICP-MS, and hence sensitivity, is directly dependent on the spot size and ablation depth. Low spot sizes reduce the integrated signals because the laser beam covers a small sample area (small amount of protein spot). Due to the laser used in this research allows spot sizes ranging from 4 to 200 µm and that the use of a narrow beam results in a small ablated surface, a spot size of 200 µm was selected in order to increase the amount of ablated material. Also, due to the use of high ablation depth reduces the transport efficiency from deeper depths (a confined plasma within deep craters leads to a reduction in laser irradiance due to changes in the effective area exposed to the laser beam [19]), an ablation depth of 0 µm was selected.

5.3.1.2 Laser fluency, repetition rate and scan speed

The amount of ablated material is also directly dependent on the laser fluence (laser energy per spot area) and repetition rate. Laser fluency was evaluated within the 1.8 – 5.5 J cm^{-2} range (laser operating at a power of 20 – 60%). Other laser ablation conditions were fixed at 20 Hz for repetition rate, 5.0 µm s^{-1} for scan speed, 0 µm for ablation depth and 200 µm for laser spot size. Target releasing was found to be increased when working at laser fluency up to 5.5 J cm^{-2} as shown in *Figure 2a* for the sum of target element intensities. Repetition rate was evaluated from 5 to 20 Hz (other laser ablation conditions were fixed at 5.5 J cm^{-2} for laser fluency, 5.0 µm s^{-1} for scan speed, 0 µm for ablation depth and 200 µm for laser spot size). Target releasing was increased when working at repetition rate up to 20 Hz (*Figure 2b*). At laser fluency higher than 5.5 J cm^{-2} and repetition rate higher than 2.0 Hz the double-sided tape and glass microscopic slides (where protein spot were applied on) could also be ablated when using high laser powers and repetition rates. In addition, relative standard deviations (N = 3) lower than 10% (for each target element intensity and for the sum of target element intensities) were observed for all laser fluency and repetition rate values tested (*Figure 2a and 2b*). Thus, 5.5 J cm^{-2} and 20 Hz were therefore chosen as optimum values.

Finally, element signal-to-noise ratio and precision also depend on the scan speed. The influence of the scan speed on the integrated signal was assessed within the 5 – 15 µm s^{-1} range (other laser ablation conditions were fixed at 5.5 J cm^{-2} for laser fluency, 20 Hz for repetition rate, 0 µm for ablation depth and 200 µm for laser spot size). Integrated signals for all target elements (and also for the sum of target element intensities, *Figure 2c*) have been found to decrease up to scan speed of 15 µm s^{-1}. If the laser beam, at high fluency and repetition rate, was moved slowly on the gel, an increase of the amount of ablated material is achieved. Similar RSDs (< 10 %) were observed for all target elements and all scan speeds. Therefore, 5 µm s^{-1} were chosen as optimum values.

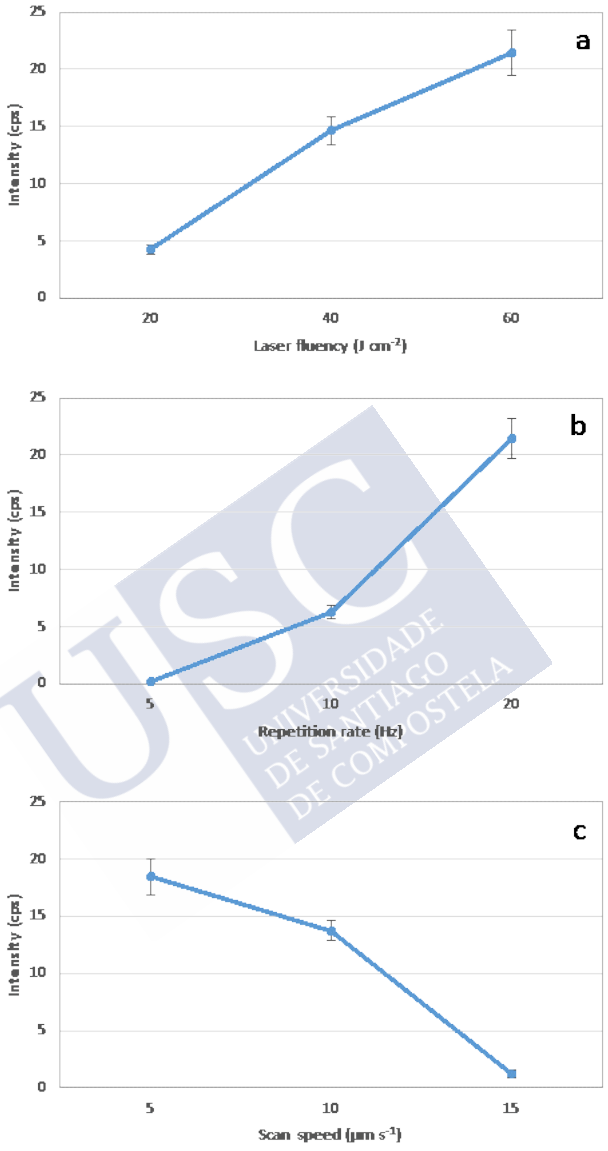

Figure 2. Effect of laser fluency (a), repetition rate (b) and scan speed (c) on the sum of target elements intensities for spots a, b and c (N = 3).

Figure 3 shows typical element signals (normalized using ^{32}S) *vs.* time under optimized conditions. As it can be seen, normalized intensities variations along the analysis shown relative standard deviations lower than 10% for all target element.

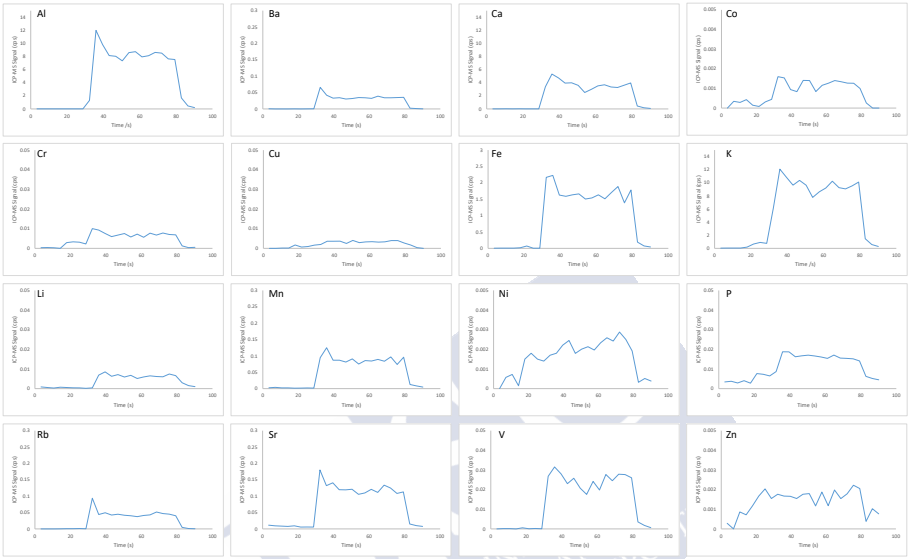

Figure 3. Normalized intensities (by using ^{32}S) vs time profiles for Al, Ba, Ca, Co, Cr, Cu, Fe, K, Li, Mn, Ni, P, Rb, Sr, V and Zn in protein spot a,b and c, under optimized conditions.

5.3.2 Screening of protein spots in two-dimensional gels by LA-ICPMS

Separated protein spots were subjected to a systematic screening for multielement (Al, Ba, Ca, Co, Cr, Cu, Fe, K, Li, Mn, Ni, P, Rb, Sr, V, Zn, Na, Si, As, Se, Mo, and Ag) assessment by LA-ICPMS using the optimized conditions shown in Table 1. A total of two well-separated protein isoforms spots (TRFE and K2C1 spots) (*Figure 4*) from eight serum samples were analyzed.

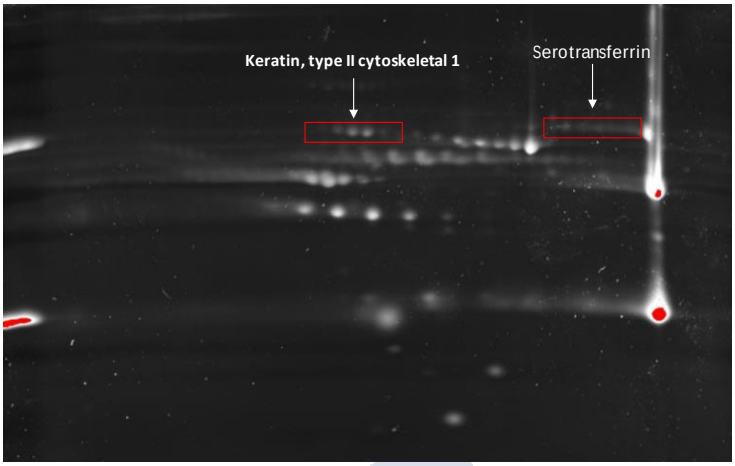

Figure 4. Isoforms of Serotransferrin (spot number 493 and 494, Figure 3 Chapter 3) and Keratin, type II cytoskeletal 1 (spot number 712, Figure 3 Chapter 3) cut to measure metals such as iron, copper or zinc.

5.3.3 Application

The signals were normalized with ^{32}S and the results of these ratios are shown in *Table 2*. There were some metals which show signals similar or even lower than the signals from the same elements in blank gels, and they could not be determined (elements in gray in *Table 2*). The high background signal for some elements in the gels is a common drawback when analyzing protein spots.

Other elements showed signals much higher than those obtained in blank gel and were evaluated adequately.

In both isoforms (TRFE and K2C1) few metals appear in all samples (elements in yellow in *Table 2*). In the case of TRFE, the metals Na, Cr, and Fe are those that appear in all the samples and with values greater than the background (blank). For K2C1 it is the Na, P, Cr, and Fe elements that provide signals in all samples. Although in this case the P can be measured in all the samples, in some of them the value is lower than the blank.

Table 2. Element ratios in protein spots measured by LA-ICP-MS. Relative standard deviation (RSD) of measured element ratios is about 30 %.

Serotransferrin (spot number 493 and 494)									
Metal	*Blank*	*Sample 1*	*Sample 2*	*Sample 3*	*Sample 4*	*Sample 5*	*Sample 6*	*Sample 7*	*Sample 8*
Li	-	-	-	0.022	-	-	-	-	-
Na	5.68	6.04	6.13	6.30	6.38	7.29	7.76	7.79	7.068
Al	-	-	0.01	0.44	-	-	-	-	-
Si	-	-	-	-	-	-	-	-	-
P	0.00017	0.0024	0.0021	0.0053	0.00027	-	-	0.00061	0.00025
K	0.032	0.042	0.049	-	0.0062	-	0.02	0.0045	0.013
Ca	0.0042	0.0082	0.0072	-	0.00046	-	-	-	-
V	-	-	-	0.0015	-	-	-	-	-
Cr	-	0.0021	0.013	0.00031	0.035	0.0081	0.011	0.0066	0.0044
Mn	-	0.00045	0.0039	-	0.0092	0.00082	0.0028	0.0023	0.0025
Fe	-	0.23	0.26	0.48	0.52	0.71	1.13	0.7	0.81
Co	-	0.0022	-	0.0012	0.00021	-	-	-	-
Ni	-	-	-	-	-	-	-	-	-
Cu	-	-	-	0.001	0.00039	-	0.00019	-	0.00014
Zn	-	0.0039	0.0021	0.0017	-	-	-	-	-

Table 2. Element ratios in protein spots measured by LA-ICP-MS. Relative standard deviation (RSD) of measured element ratios is about 30 % (Continued).

Serotransferrin (spot number 493 and 494)									
Metal	*Blank*	*Sample 1*	*Sample 2*	*Sample 3*	*Sample 4*	*Sample 5*	*Sample 6*	*Sample 7*	*Sample 8*
As	-	-	-	-	0.00039	0.00027	0.0012	0.000065	0.00026
Se	-	0.00011	-	-	0.000049	0.0001	0.00018	0.00013	0.00016
Rb	-	-	-	0.011	-	0.0000014	0.000022	-	-
Sr	-	0.00073	0.00035	0.011	-	-	0.0018	-	
Mo	-	-	-	-	0.0000065	-	0.0000065	0.000013	-
Ag	-	-	-	-	-	-	-	-	-
Ba	-	0.0062	0.00057	0.0036	0.0035	-	0.013	-	-
Keratin, type II cytoskeletal 1 (spot number 712)									
Metal	*Blank*	*Sample 1*	*Sample 2*	*Sample 3*	*Sample 4*	*Sample 5*	*Sample 6*	*Sample 7*	*Sample 8*
Li	-	-	-	-	-	-	-	-	-
Na	5.024	6.42	6.49	7.18	7.3	8.04	8.1	7.3	7.52
Al	-	0.0022	0.0029	-	-	-	-	-	-
Si	-	-	-	-	-	-	-	-	-
P	0.00016	0.0009	0.0013	0.0011	0.0002	0.0011	0.00078	0.00028	0.00025
K	0.03	0.025	0.033	0.016	0.0014	0.0062	-	0.023	0.0077
Ca	0.0031	0.0062	0.0052	0.0015	0.0005	0.00052	-	0.0022	-

Table 2. Element ratios in protein spots measured by LA-ICP-MS. Relative standard deviation (RSD) of measured element ratios is about 30 % (Continued).

Keratin, type II cytoskeletal 1 (spot number 712)									
Metal	*Blank*	*Sample 1*	*Sample 2*	*Sample 3*	*Sample 4*	*Sample 5*	*Sample 6*	*Sample 7*	*Sample 8*
V	-	-	-	-	-	-	-	-	-
Cr	-	0.0012	0.0025	0.0067	0.004	0.005	0.0091	0.011	0.0061
Mn	-	0.0012	0.0015	0.006	-	0.007	0.0023	0.0032	0.0017
Fe	-	0.272	0.363	0.59	0.24	0.53	0.79	0.97	0.61
Co	-	0.00026	0.00042	0.00031	-	0.00038	-	-	-
Ni	-	-	-	-	-	-	-	-	-
Cu	-	-	-	-	-	-	-	-	-
Zn	-	0.00023	0.0019	-	-	0.00036	-	-	-
As	-	0.00036	0.00025	-	0.0003	0.00026	-	-	-
Se	-	0.00012	0.00013	0.00016	0.0001	0.000085	-	-	-
Rb	-	-	-	0.000062	-	0.000092	-	-	-
Sr	-	0.00032	0.0018	0.00017	0.00034	0.00064	-	0.00028	-
Mo	-	-	-	-	-	0.000044	-	-	-
Ag	-	-	-	-	-	-	-	-	-
Ba	-	0.0027	0.0016	0.0022	-	0.0018	0.00062	0.00012	-

There are several studies focused on studying the levels of elements bounded to proteins, and some reports have established that the protein-metal ratio in some metal-protein complexes can be critical in the central nervous system. Various neuronal functions, such as neurotransmission systems, maintenance of myelins, enzymatic activities or mitochondrial function imply certain roles of essential metals. The study of the levels of elements such as Fe, Cu, or Zn have been found to be important in patients with a neurodegenerative disease since variations of the levels of these elements are associated with these pathologies [20-22].

In addition, some works have also reported the zinc can be implied in the early development of dementia, because significant lower serum Zn in MCI than in healthy people have been reported [23]. In addition, higher Zn levels in CSF from MCI patients than in AD patients have been also found [24]. This metal malfunction metabolism has been related to an abnormal flux of metals across the blood-brain barrier (BBB) and the choroid plexus, from blood and CSF respectively, which lead to metal accumulation in the brain [25]. Cross-interactions in homeostasis of metals and their uptake into brain is also quite important. As an example, transferrin (Tf) presents the ability to bind several metals, such as Fe, Mn, Zn, Cr, Co, Cd, V and Al [26], and this protein acts as transporter agent via transferrin receptor [27].

Some LA-ICP-MS applications has been focused on generating qualitative and quantitative maps of elemental distribution in thin tissue sections of a variety of biological samples such as brain, cartilage, spinal cord, etc. [28], which helps to discriminate among several disease stages.

Finally, we can say that we have managed to create a preliminary method of measuring metals in metal-protein complexes, which after establishing a suitable quantification method, can be used as discriminating features for classifying serum samples from healthy people and patients.

5.4 CONCLUSIONS

In the present study we demonstrated the possibilities of LA-ICP-MS for multielement determination in separated protein spots after 2-D gel electrophoresis. The most important problem for the determination of the elements in the gel are possible contaminations during sample preparation. The metallomic strategy used provided complementary data that allowed to observe a differentiation in terms of metal bound to proteins with respect to the background (blank). Future work will be focused on minimizing blanks by improving the 2-D gel electrophoretic separation and establishing an adequate quantification method for element determination.

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IV. CONCLUSIONS

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1. Study of trace metals in serum microsamples.

The first steps of this thesis have been focused on setting up a series of techniques for the measurement of metal levels in serum samples. We have optimized two sample introduction methods in ICP-MS instrumentation; the first approach is based on the dried blood spot (DBS) technique, and the second one is based on discrete sample introduction using the possibilities of new autosamplers. Very low sample volumes have been required in both methods, and multi-element determinations in short times and with robust results have been developed.

1.1 Development of dried serum spot sampling techniques for the assessment of trace elements in serum samples by LA-ICP-MS:

Parameters such as laser fluency, repetition rate, ablation depth, ablation speed and laser spot diameter were optimized. Conditions that give the highest signals and the highest repeatability consists of 2.2 J cm^{-2} for laser fluency, 20 Hz for repetition rate, 0 µm for depth, 12 µm s^{-1} for ablation speed, and 90 µm for laser spot diameter.

The sample volume as well as the area of the ablated spot were also investigated, and best performances, mainly repeatability, have been achieved using a volume of 20 µL and ablating ablated the central area of the spot. After the studies of auxiliary oxidizing reagents, it has been found that the best benefits were obtained with 10% acetic acid.

The best calibration method found was based on the use of certified reference material as standard calibrants. Similarly, ^{13}C (naturally present in biological samples) was found to be the best internal standard.

We can conclude that we have optimized a technique for the fast and simultaneous assessment of metals in serum samples over a huge concentrations range. DBS technique offers an additional advantage regarding sample conservation and transportation.

1.2 Discrete sampling based-flow injection as an introduction system in ICP-MS for the direct analysis of low volume human serum samples:

Discrete introduction based on flow injection allows the simultaneous determination of 20 elements using only 200 µL of a sample. Nitric acid at 1% (v/v) has been found as the best diluent for carrying out measurements with a minimum matrix effect. The 1:10 sample dilution with 1% nitric acid (v/v) involves 2 mL of diluted specimen that is suitable for loading a loop of 300 µL in just 3 s, before discrete sampling at a flow rate of 72 µL min^{-1} (flow that allows multi-element determinations in ICP-MS standard and collision cell modes) in 2.5 min.

Matrix effect minimization, together with the low detection limits, has allowed robust multi-element determinations and adequate repeatability and accuracy. On the other hand, the discrete introduction allows increasing the number of samples to be analyzed per unit of time, which is an additional advantage of the developed methodology.

2. Proteomic study for the identification of minor serum proteins altered in healthy people and MIC and AD patients.

2.1 2D gel electrophoresis and MALDI-TOF:

Measurements of protein spots separated by two-dimensional gel electrophoresis, scanning and analysis (ProgenesisSameSpots software v. 4.5), and MALDI-TOF provided a list of qualitatively increased and decreased proteins, and also the identification of post-translational modification in some proteins.

Regarding the discrimination between AD and MCI patients, increased proteins in samples from AD patients have been identified as Pigment epithelium-derived factor (PEDF), Ficolin-3 (FCN3), Serotransferrin (TRFE) and Haptoglobin (HP). These proteins have

been previously described as proteins altered in people suffering neurodegenerative diseases. In addition, some proteins have been found to be decreased in AD patients versus MCI patients: Ceruloplasmin (CP), complement factor 1 (CF1), charged multivesicular Body protein 1A (CHMP1A), Plasma kininogen-1 (KNG1), DNA (cytosine-5)-methyltransferase 1 (DNMT1), CD5 Antigen-like (CD5L), alpha-1-antitrypsin (SERPINA1), antithrombin-3 (SEPINC1), alpha-2-HS-glycoprotein (AHSG), actin cytoplasmatic 1 (TBCA), and alpha-2-macroglobulin (A2M).

2.2 DDA y SWATH:

Qualitatively, a total of 274, 229 and 358 proteins were identified in healthy people, MCI patients and AD patients, respectively. When comparing healthy people and MCI patients, 36 proteins have been found to be different; whereas, 120 proteins were found to be different in samples from AD patients.

From a library consisted of 186 proteins, statistical comparisons based on *p*-value have shown that 31 proteins were quantitatively increased in AD patients vs. healthy people; whereas, 19 proteins were quantitatively higher in AD patients vs, MCI patients. Finally, 42 proteins were found to be increased in MCI patients vs. healthy people. After applying PCA, best discrimination was achieved between healthy people and MCI patients. In the case of AD patients, the separation is not good because of the difference between the number of samples belonging to each group.

It is noteworthy that the protein Apolipoprotein A-II (Apo A-II) was found to be statistically significant (p-value ≤ 0.05) in the three comparisons. This protein was also found to be quantitatively increased at an early disease stage, such as MCI, when comparing to healthy and AD patients. This finding may be a good indicator of an early neurodegeneration stage.

Taking into account results from both proteomic approaches, two proteins [Serotransferrin (TRFE) and Keratin type II cytoskeletal 1 (K2C1)] were found to be modified in MCI and AD patients: Serotransferrin (TRFE) is increased in AD patients compared to MCI patients, and Keratin type II cytoskeletal 1 (K2C1) seems to be decreased in AD patients compared to MCI patients.

3. Study of the levels of trace metals associated with proteins in serum from healthy people and MIC and AD patients.

3.1 Screening of metal-protein complexes two-dimensional polyacrylamide gel electrophoresis and laser ablation-inductively coupled-plasma mass spectrometry:

In this brief section, we have optimized the optimal conditions for laser ablation of acrylamide gels, and the identification of metals linked to specific proteins [Serotransferrin (TRFE) and Keratin type II cytoskeletal 1(K2C1)] which can be increased or decreased is sample from healthy people and AD/MCI patients. However, no definitive conclusions can be obtained because the low number of samples under study.

Therefore, future work will be focused on establishing an efficient quantification method and on increasing the number of samples for statistical studies.

As a global conclusion, the union of two fields such as the analysis of metals and proteomics would offer additional insights for searching biomarker for neurodegenerative diseases. Future work will be focused on the study of metal-protein complexes for differentiating proteins in samples from healthy people and AD/MCI patients.

V. ANNEX I
RESUMEN EN ESPAÑOL

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La enfermedad de Alzheimer (EA) es considerada la nueva epidemia del siglo XXI, ya que con el paso de los años la esperanza de vida aumenta, y esto trae como consecuencia que los casos de pacientes que sufren esta patología aumenten. La EA es una enfermedad de la que no se conoce la etiología exacta, sino que su origen es consecuencia de una lista de diferentes factores. En cuanto a tratamientos, hoy en día no existe una medicación que recupere a los pacientes. De lo que sí se dispone es de una serie de medicaciones que frenan el deterioro, y que en ocasiones es aplicada cuando la enfermedad está muy avanzada.

Es por todo esto que el conocimiento del comportamiento de esta enfermedad en etapas tempranas es una meta a conseguir en el ámbito de la investigación biomédica. Así son múltiples estudios los que comparan personas sanas con pacientes que sufren EA pero actualmente los estudios se centran en conocer la evolución de la enfermedad, es decir, en estudiar las diferencias entre una etapa temprana como es el deterioro cognitivo leve (DCL) y una avanzada como la EA.

Los estudios de muestras biológicas (orina, suero, plasma, saliva, líquido cefalorraquídeo, etc.) han alcanzado en los últimos años gran importancia e interés. Así, hoy en día ha nacido una nueva área científica llamada biopsia líquida, la que pretende complementar estudios en tejidos con estudios en fluidos biológicos, para obtener así resultados complementarios. Las ventajas de estudios en este tipo de muestras son numerosas; entre ellas destacar que es una forma de obtener muestras menos invasiva que las biopsias de tejidos.

Pero no todo son ventajas con este tipo de muestras ya que es muy importante considerar aspectos como toma de muestra, tratamiento antes de ser almacenada, las condiciones de conservación, y los efectos de los ciclos de congelado y descongelado durante la conservación de las mismas. Para ello se debe imponer un protocolo que se aplique a todas las muestras de un mismo estudio de igual forma. Así mismo, es muy importante tener en cuenta que el suero tiene un contenido alto de ciertas sustancias (proteínas mayoritarias) que dificulta el estudio de

moléculas en menor concentración (proteínas minoritarias), y que pueden ofrecer información importante. Es necesario así, establecer un pretratamiento de la muestra que en ocasiones resulta laborioso.

Esta tesis se ha centrado en el estudio de metales y de proteínas minoritarias en muestras de suero de personas sanas (grupo control) y de pacientes con DCL y EA. Los resultados se han agrupado en tres grandes apartados: (i) la puesta a punto de técnicas para el estudio de los niveles de metales en muestras de suero, (ii) el estudio proteómico de muestras de suero y, (iii) el estudio de los niveles de metales asociados a proteínas.

1. Estudio de los niveles de elementos metálicos en muestras de suero.

Los primeros pasos de esta tesis se han centrado en poner a punto una serie de técnicas para la medición de niveles de metales en micromuestras de suero. Para esto se han optimizado dos tipos de introducción de la muestra distintos en la instrumentación ICP-MS. En el primer caso, la introducción ha sido mediante ablación láser (AL), previa inmovilización de la muestra de suero empleando la técnica *dried blood spot* (DBS). El segundo desarrollo se basó en las posibilidades del muestreo discreto con los nuevos automuestreadores. El principal objetivo de esta parte de la tesis ha sido obtener buenos resultados con el mínimo consumo de muestra (20 µL y 200 µL). Ambas técnicas se han optimizado para un número elevado de analitos y se han obtenido buenos resultados de precisión, sensibilidad y reproducibilidad.

1.1 Desarrollo de técnicas de uso de papel como soporte de muestras de suero (DBS) para la evaluación de elementos traza por LA-ICP-MS:

En este apartado de la tesis se han medido directamente una amplia lista de metales, no sólo metales mayoritarios, sino también metales de baja concentración, obteniendo para ambos grupos resultados robustos.

Es de destacar en este capítulo el uso de papel como soporte de la muestra. De esta forma se disfrutan de ventajas como fácil almacenaje y transporte de la muestra e incluso mejor conservación de ella.

El primer paso necesario a la hora de trabajar con ablación láser fue optimizar las condiciones de ablación para obtener la mayor señal posible con una buena reproducibilidad. Se optimizaron parámetros como la influencia del láser en el papel, repetición del láser, profundidad de ablación, velocidad de ablación y el tamaño de la zona a ablacionar. Las condiciones que mayor señal, junto con mayor reproducibilidad, nos han proporcionado son 2.2 J cm^{-2} para la influencia del láser, 20 Hz para la repetición del láser, 0 µm para la profundidad de ablación, 12 µm s^{-1} para la velocidad de ablación, y 90 µm para el tamaño de la zona ablacionada.

Una vez obtenidos los parámetros del láser con los que se trabajará, se han estudiado diferentes volúmenes de muestra e incluso la zona de la mancha (spot) dónde ablacionar, obteniendo que con el volumen tan pequeño como 20 µL es suficiente para obtener buenas señales de los analitos. En cuanto a la posición, debido a la formación de una corona alrededor de la mancha se ha concluido que la mejor reproducibilidad se obtiene ablacionando la zona central de la misma. Se ha estudiado igualmente el efecto de ciertos aditivos (oxidantes) para favorecer la eliminación de la materia orgánica de la muestra y ayudar así a una mejor ablación. En esta tesis se han probado un total de seis oxidantes: ácido fórmico, ácido acético, clorato sódico, azida sódica, BMIMCl y BMIMBr. Además, todos estos oxidantes se han estudiado a tres niveles de concentración diferentes. Tras analizar los resultados obtenidos, se ha concluido que el mayor aumento de la señal se obtiene empleando ácido acético al 10%.

El método de calibración óptimo ha sido mediante el uso de un material de referencia certificado, que en este caso ha sido Seronorm™ level I. Para el estudio de la exactitud se ha analizado otro material de referencia, en este caso Seronorm™ level II, obteniendo valores dentro de los rangos establecidos por el fabricante. Se probaron varios

estándares internos para asegurar la corrección de la deriva instrumental, entre ellos el ^{13}C (materia orgánica presente mayoritariamente en las muestras), y otros elementos como ^{74}Ge, ^{89}Y, ^{103}Rh y ^{130}Te. Los mejores resultados (control de las variaciones de las señales proporcionadas por la ablación láser) se han obtenido con el empleo de ^{13}C.

Esta metodología se aplicó a un total de seis muestras de suero de personas sanas. Elementos mayoritarios como Ca, K, Mg y P se han encontrado en un rango de 20.3-322 mg L^{-1}, y Na entre 3996 y 6634 mg L^{-1}. Elementos como Cu, Fe, Li y Zn ofrecieron valores entre 0.7 y 3 mg L^{-1}, mientras que para elementos traza como Al, Mn, Mo, Rb, Se y V los valores han oscilado entre 0.5 y 120 µg L^{-1}.

Con todo esto podemos concluir que se ha optimizado una técnica robusta y sencilla para medir simultáneamente metales presentes en muestras de suero en diferente rango de concentraciones de forma fácil, económica y rápida. El uso del papel como soporte para deposición de la muestra presenta ventajas importantes como la conservación de la muestra y el fácil transporte.

1.2 Introducción de muestras discretas basada en procedimientos de inyección en flujo:

Al igual que en el capítulo anterior el objetivo principal en este trabajo ha sido determinar un alto número de metales (en diferentes rangos de concentraciones) consumiendo el menor volumen posible de muestra de suero, y obtener una buena exactitud y precisión en los resultados. Se ha procedido así a llevar a cabo un muestreo discreto (introducción de volúmenes pequeños de muestra de forma similar a los sistemas de inyección en flujo, FIA) utilizando las ventajas de los muestreadores automáticos.

Para ello en este capítulo de la tesis se han empezado a hacer pruebas de dos aspectos que pueden variar mucho el consumo de

muestra, como son las condiciones de carga y las condiciones de elución de la muestra. Para el primer aspecto se han desarrollado pruebas con diferentes volúmenes del bucle de carga (300 µL, 500 µL, y 1.0 mL) obteniendo buenos resultados con el bucle más pequeño (300 µL). Relacionado con la carga de la muestra también se han hecho pruebas con el tiempo de aspiración (3.0, 5.0, y 7.0 s para 300 µL, 500 µL, y 1.0 mL, respectivamente). Con el bucle de 300 µL el tiempo de aspiración fue de 3.0 s, y el consumo resultante fue de aproximadamente 1.5 mL. Es por eso por lo que fue necesario que el volumen final de muestra diluida (1% (v/v) HNO_3 como diluyente) fuese de al menos 2.0 mL. En cuanto a la elución, fueron dos las variables a estudiar; por un lado, el diámetro de la tubería, y por otro la velocidad de la bomba. Tras probar dos tuberías de diferente diámetro interno (0.13 y 0.19 mm) y dos velocidades de bombeo (72 y 70 µL min^{-1}), los resultados nos han indicado que el diámetro interno de 0.13 mm necesita de la velocidad de 72 µL min^{-1}, así la velocidad alta con poco diámetro de la tubería elimina pulsos en el sistema.

Todas estas condiciones se aplicaron a tres diferentes diluciones de las muestras (1:10, 1:4 y 1:2) en 1% (v/v) HNO_3 a un volumen final de 2 mL. En los tres casos se obtuvo buena reproducibilidad, por lo que se seleccionó la dilución 1:10 ya que requiere de menor consumo de muestra (200 µL de suero).

En cuanto a los LOD y LOQ, se han obtenido haciendo medidas de blancos disueltos en 1% (v/v) HNO_3, obteniendo valores correctos para poder detectar y cuantificar dichos elementos en muestras de suero. Para el estudio de la repetibilidad y recuperación analítica se han medido once réplicas de cuatro niveles diferentes de concentración de cada elemento encontrándose buena repetibilidad (valores RSDs menores de 15 %). La recuperación analítica ha sido aceptable ya que los valores oscilaron entre 92 y 115 %. La exactitud ha sido estudiada a través del análisis de dos materiales de referencia certificados (Seronorm™ level I y level II). Al comparar estadísticamente los valores de las concentraciones obtenidas con los valores certificados se ha podido concluir que el método es exacto.

Finalmente, este método se ha aplicado a catorce muestras de suero (200 µL) diluídas a un volumen final de 2.0 mL con 1% (v/v) HNO_3 y medidas por triplicado. En el caso del Pb, éste no pudo ser cuantificado en dos muestras ya que los valores se situaban entre el LOD y el LOQ. De la misma forma, en nueve muestras no se ha podido cuantificar Cd y V. Finalmente el Be no pudo medirse en ninguna muestra, siendo su concentración menor que el LOD.

2. Estudio proteómico para la identificación de proteínas séricas minoritarias (cualitativa y cuantitativamente) alteradas en personas sanas y en pacientes con DCL y EA.

En este apartado se ha desarrollado un estudio proteómico que se ha centrado en la búsqueda de proteínas diferenciadoras entre los tres grupos poblacionales (muestras proporcionadas por el Servicio de Neurología del Complejo Hospitalario Universitario de Santiago de Compostela (CHUS)).

Los primeros experimentos en esta parte se han centrado en la eliminación de las proteínas mayoritarias de las muestras de suero, empleando en un primero momento una columna comercial de depleción (cromatografía de afinidad), y en estudios posteriores la incubación con DTT.

Una vez eliminadas las proteínas mayoritarias, el estudio se ha centrado en la búsqueda de proteínas diferenciadoras entre grupos en la fracción de proteínas minoritarias, que son las que proporcionan más información. En este caso son dos las técnicas utilizadas, en una primera parte se utilizó MALDI-TOF, y en una segunda etapa las medidas se hicieron por ESI-LC-MS/MS.

2.1 Búsqueda cualitativa de proteínas diferenciadoras entre pacientes con DCL y EA:

En este capítulo se han llevado a cabo geles bidimensionales de acrilamida (2-DE) de todas las muestras. En una primera dimensión se

separan las proteínas por su punto isoeléctrico (pI) y en la segunda dimensión por tamaños (KDa), obteniendo así spots en los que se pueden identificar varias proteínas a la vez.

Estos geles fueron escaneados, obteniéndose así las imágenes de todos ellos. Dichas imágenes fueron analizadas con el software (ProgenesisSameSpots software v.4.5) a través del cual podemos obtener los spots estadísticamente significativos (p-value < 0.05 y Fold Change > 1.3) y diferenciadores entre cada grupo de muestras (control, DCL y EA). En este estudio se realizan dos comparaciones: controles vs. pacientes, y pacientes con DCL vs. pacientes con EA. Pero es esta segunda comparación en la que nos centramos, cortando así los spots que el programa nos proporciona como diferenciadores, tanto aumentados como disminuidos, que tras la digestión tríptica son analizados por MALDI-TOF.

Las medidas de los spots nos proporcionaron una lista de proteínas aumentadas y disminuidas cualitativamente, de las cuales también pudimos conocer si sufren alguna modificación post-traduccional (como el caso de las fosforilaciones). De la amplia lista de proteínas aumentadas en las muestras de pacientes con EA frente DCL, son destacables las siguientes: Pigment epithelium-derived factor (PEDF), Ficolin-3 (FCN3), Serotransferrin (TRFE) y Haptoglobin (HP). Estas proteínas han sido referidas como significativas en estudios previos relativos a la evolución de enfermedades neurodegenerativas. Al mismo tiempo, entre las proteínas disminuidas en pacientes con EA destancab: Ceruloplasmin (CP), complement factor 1 (CF1), charged multivesicular body protein 1A (CHMP1A), kininogen-1 (KNG1), DNA (cytosine-5)-methyltransferase 1 (DNMT1), CD5 antigen-like (CD5L), alpha-1-antitrypsin (SERPINA1), antithrombin-3 (SEPINC1), alpha-2-HS-glycoprotein (AHSG), actin cytoplasmatic 1 (ACTB), y alpha-2-macroglobulin (A2M).

Como conclusión, se han encontrado distintas proteínas diferenciadoras entre las fases de la enfermedad. Al mismo tiempo se

han observado modificaciones en las proteínas que también son causa del estadío de la enfermedad.

2.2 Identificación cualitativa y cuantitativa de proteínas séricas diferenciadoras entre los tres grupos de muestras (Control, DCL y EA):

En este capítulo la eliminación de las proteínas mayoritarias se ha llevado a cabo con la incubación de la muestra con DTT ya que tras una revisión bibliográfica otros autores confirman que los resultados obtenidos son comparables con otras técnicas comerciales (que son más caras y laboriosas).

Una vez obtenida la fracción de las proteínas minoritarias, éstas fueron preconcentradas en un gel de acrilamida. Tras el corte de esa banda en dónde se encuentra toda la muestra, se desarrolló la digestión en gel como en el capítulo anterior. Las muestras fueron resuspendidas e introducidas en el equipo directamente para su separación por LC e posterior identificación por MS/MS.

Los resultados obtenidos nos proporcionaron las proteínas identificadas cualitativamente (DDA) y cuantitativamente (SWATH).

Cualitativamente se identificaron un total de 274, 229 y 358 proteínas para el grupo control, DCL y EA, respectivamente. Al comparar estas tres listas se observan 181 proteínas comunes entre los tres grupos, pero lo más interesante son aquellas diferenciadoras, es decir, las que tan sólo aparecieron en un grupo. En el caso del grupo control y EDL las proteínas diferenciadoras han resultado ser 36, mientras que en el caso del grupo EA fueron 120 las proteínas que únicamente aparecen en este grupo.

Para el análisis cuantitativo es necesaria la creación de una biblioteca que contenga todas las proteínas presentes en las muestras. En este estudio la biblioteca resultó estar formada por un total de 186 proteínas. Se realizaron comparaciones de los grupos dos a dos y se consideraron estadísticamente significativas aquellas proteínas con un

p-value menor de 0.05. Así, resultaron cuantificadas 31, 19, y 42 proteínas para las comparaciones EA vs. control, EA vs. DCL, y DCL vs. control, respectivamente. Los resultados obtenidos en esta parte muestran una buena separación estadística mediante un análisis de componentes principales (PCA), en el cual la mejor separación es aquella entre el grupo control y los pacientes con DCL. En el caso del grupo de pacientes con EA la separación no es tan robusta debido a la gran diferencia entre el número de muestras de cada grupo.

Entre las proteínas diferenciadoras en dichas comparaciones, cabe destacar la proteína Apoliprotein A-II (Apo A-II) debido a que es estadísticamente significativa (p-value $\leq$ 0.05) en las tres comparaciones realizadas. Esta proteína se encontró aumentada cuantitativamente en una fase temprana (DCL) con respecto a los controles y al grupo EA, por lo que puede ser un indicador de una fase temprana en la neurodegeneración.

Comparando los dos capítulos de esta parte de la tesis, son dos las proteínas que se ven modificadas de la misma forma entre los grupos DCL y EA, tanto cualitativamente como cuantitativamente. Estas dos proteínas son la serotransferrin (TRFE), que aparece aumentada en pacientes de EA en comparación con los pacientes con DCL; y la Keratin. type II cytoskeletal 1 (K2C1) que aparece disminuida en pacientes con EA en comparación con DCL.

3. Estudio de los niveles de metales traza asociados con proteínas en suero de personas sanas y pacientes con DCL y EA.

3.1 Determinación de metales esenciales en isoformas de proteínas separadas mediante geles 2-DE:

En este breve capítulo se han optimizado las condiciones óptimas para la ablación láser (LA) de geles de acrilamida, y así medir metales asociados a las proteínas separadas.

Se han determinado los metales presentes en las isoformas de dos proteínas diferentes, la serotransferrin (TRFE) y la Keratin type II cytoskeletal 1 (K2C1). Estas dos proteínas aparecen en diferentes isoformas debido a que sufren modificaciones post-traducionales. Para todo ello a sido necesario obtener geles 2-DE, recortar la banda en la que se encuentran estas isoformas, secarla a vacío y analizarlas por LA-ICP-MS.

Una vez optimizadas las condiciones y ver que la señal a lo largo de la banda es estable, se han medido un número total de 8 muestras, dónde en cada una han sido ablacionadas las isoformas correspondientes a estas dos proteínas. En todas estas se han medido de forma cualitativa 22 metales, obteniendo buena reproducibilidad entre las réplicas.

Este último trabajo se continuará para establecer el método de cuantificación ya que por ahora es un estudio cualitativo. Se podrá así aplicar la metodología a un mayor número de muestras y ver si existen variaciones en los niveles de metales asociados a las proteínas diferenciadoras entre los diferentes grupos (control, DCL y EA).

Como conclusión global es destacable la unión de dos campos como son el análisis de elementos metálicos y la proteómica. Así, en el futuro el trabajo se centrará en el estudio de los metales unidos a esas proteínas diferenciadoras entres los grupos de muestras, para sí conocer como estos metales varían a lo largo de la evolución de esta patología.

VI. ANNEX II
LIST OF PUBLICATIONS

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

List of publications

During the development of this doctoral thesis have been published in scientific journals the following works:

- Chantada-Vázquez, M. P., Moreda-Pineiro, A., Barciela-Alonso, M. C., & Bermejo-Barrera, P. (2017). Spectrometric-based techniques for metal-binding protein assessment in clinical, environmental, and food samples. *Applied Spectroscopy Reviews*, 52(2), 145-174. https://doi.org/10.1080/05704928.2016.1213736

- Chantada-Vázquez, M. P., Moreda–Piñeiro, J., Cantarero–Roldán, A., Bermejo-Barrera, P., & Moreda-Piñeiro, A. (2018). Development of dried serum spot sampling techniques for the assessment of trace elements in serum samples by LA-ICP-MS. *Talanta*, 186, 169-175. https://doi.org/10.1016/j.talanta.2018.04.049

- Chantada-Vázquez, M. P., Herbello-Hermelo, P., Bermejo-Barrera, P., & Moreda-Piñeiro, A. (2019). Discrete sampling based-flow injection as an introduction system in ICP-MS for the direct analysis of low volume human serum samples. *Talanta*, 199, 220-227. https://doi.org/10.1016/j.talanta.2019.02.050