DEPARTMENT OF NEUROLOGY SERIES OF REPORTS NO 83, 2006

MINNA KOROLAINEN

Proteomic analysis of post-translationally modified proteins in Alzheimer's disease

Doctoral dissertation

To be presented with assent of Medical Faculty of the University of Kuopio
for public examination in Auditorium L1, Canthia Building of the University of Kuopio,
on Friday 16th June 2006, at 12 noon

Department of Neurology
University of Kuopio and
Kuopio University Hospital

Brain Research Unit
Clinical Research Centre
Mediteknia
University of Kuopio

Distributor:
Department of Neurology
University of Kuopio
P.O.Box 1627
FIN-70211 Finland
Tel. +358 17 162 682
Fax. +358 17 162 408

Author's address:
Department of Neurology
University of Kuopio
P.O.Box 1627
FIN-70211 Finland
Tel. +358 17 162 459
Fax. +358 17 162 408
E-mail: Minna.Korolainen@uku.fi

Supervisors:
Professor Tuula Pirttilä, M.D., Ph.D
Department of Neurology
University of Kuopio
Kuopio University Hospital

Docent Gundars Goldsteins, Ph.D
Department of Neurobiology
A.I.Virtanen Institute for Molecular Sciences
University of Kuopio

Doctor Tuula Nyman, Ph.D
Finnish Institute of Occupational Health
Helsinki

Reviewers:
Professor Kaj Blennow, M.D., Ph.D
The Sahlgrenska Academy at Göteborg University
Department of Clinical Neuroscience
Section of Experimental Neuroscience
Sahlgrenska University Hospital
Mölndal
Sweden

Professor Michael Fountoulakis, Ph.D
F. Hoffmann-La Roche Ltd.
Center for Medical Genomics
Basel
Switzerland

Opponent:
Docent Marc Baumann, Ph.D
Institute of Biomedicine
Biomedicum Helsinki
University of Helsinki

ISBN 951-781-375-9
ISBN 951-27-0213-4 (PDF)
ISSN 0357-6043

Kopijyvä
Kuopio 2006
Finland

Korolainen, Minna. Proteomic analysis of post-translationally modified proteins in Alzheimer's disease. Series of Reports, No 83, Department of Neurology, University of Kuopio 2006, 108 p.

ISBN 951-781-375-9
ISBN 951-27-0213-4 (PDF)
ISSN 0357-6043

ABSTRACT

Alzheimer's disease (AD) is a complex and heterogeneous age-related disorder, and the most common form of dementia. Typical abnormalities in brain include neurodegeneration, gliosis, chronic inflammation, excitotoxicity, oxidative stress, as well as accumulation and abnormal post-translational modification of proteins. Despite extensive research for decades, the exact primary and secondary pathogenetic causes of the disorder still remain largely unknown. As a consequence of rapid demographic aging, AD has become one of the most devastating socioeconomical challenges of the present-day. Now, the new hope of unraveling the secrets of AD is the so-called "New technologies" i.e. proteomics, which have been suggested to represent a breakthrough in improving our understanding, diagnosis, and treatment of AD. The present studies were undertaken in order to develop methodologies in proteomic analysis of differentially post-translationally modified proteins, and to compare brain and cerebrospinal fluid proteomes in AD patients as compared to age-matched controls. In addition, the aim was to examine whether changes in post-translationally modified proteins were linked to the stage of AD as well as to the extent of brain pathology.

Proteomic analysis aims at simultaneous separation, quantification and identification of protein isoforms over the largest possible scale. We performed proteomic analysis using two-dimensional gel electrophoresis in combination with immunoblotting and mass spectrometry. In addition to the quantification, modifications, which may crucially influence the functional output of a protein in a cell, are accessible to study by slightly modifying proteome analysis techniques. We examined oxidatively modified carbonylated proteins in brain and cerebrospinal fluid of AD patients by two-dimensional multiplexed oxyblotting, a method which was developed during this thesis work. Glycosylation and phosphorylation of glial fibrillary acidic protein were studied by removing these modifications enzymatically prior to two-dimensional immunoblotting. When compared to native two-dimensional images, any disappearing groups are indicative of the presence of the modification in question.

We detected about 150 proteins and more than 100 oxidised proteins in both AD and control brains. Cytosolic malate dehydrogenase and glutamate dehydrogenase were characterized as being less oxidised proteins in AD brain as compared to age-matched controls. Moreover, the changes were associated with the duration of AD but not with brain pathology. These findings may be interpreted as compensatory changes occurring in response to compromised metabolism in AD.

Twentytwo oxidatively modified proteins were characterised in cerebrospinal fluid of AD patients and controls. The levels of identified cerebrospinal fluid proteins were generally decreased whereas the degree of oxidation tended to remain either unchanged or increased in AD as compared to controls. None of the brain-specific proteins exhibited significant changes in the extent of oxidative modification in AD.

About 46 isoforms and degradation products were separated and quantified by two-dimensional immunoblotting. The statistical analysis revealed a 60% increase in the amount of more acidic isoforms of glial fibrillary acidic protein in AD when compared to controls. These isoforms were both phosphorylated and N-glycosylated whereas O-glycosylated basic isoforms revealed no quantitative differences between the groups. The data suggest that glial fibrillary acidic protein may be another abnormally phosphorylated and glycosylated protein related to the pathogenesis of AD.

To conclude, the results emphasize the importance of studying the modifications occurring to proteins in neurodegenerative disorders. By better understanding modifications of proteins in AD patients, new aspects of the pathogenesis and therapeutic targets may be discovered.

National Library of Medicine Classification: WT 155, QU 58.5, QU 475, WL 203
Medical Subject Headings: Alzheimer Disease; Alzheimer Disease/etiology; Proteins; Protein Processing, Post-Translational; Proteomics; Brain; Cerebrospinal Fluid; Electrophoresis, Gel, Two-Dimensional; Immunoblotting; Spectrum Analysis, Mass

Believe those who are seeking the truth; doubt those who find it.

-André Gide-

ACKNOWLEDGEMENTS

This work was carried out in the Department of Neurology, University of Kuopio, during the years 2000-2006.

I owe my deepest gratitude to my principal supervisor Professor Tuula Pirttilä for her excellent professional guidance and encouragement throughout this thesis work. She is such an excellent example, teaching me the importace of scientific and non-scientific values of real life including altruism, self-confidence, honesty, and a healthy ambition to achieve one's best. She is truly one of the pioneers in "down-to-earth" school of science.

I wish to thank my supervisor Docent Gundars Goldsteins especially for providing the initial impetus in teaching me proteomics. I also wish to thank my supervisor Dr. Tuula “mass spectrometry” Nyman for her advice, critical opinions and friendship during these years.

I wish to express my gratitude to Professor Hilkka Soininen, the Head of the Department of Neurology, for all the support and for providing such excellent facilities to allow me to carry out this work. I also wish to thank Professor Jari Koistinaho for the possibility to practice proteomics as “an apprentice” at the group of Molecular Brain Research, A.I.Virtanen Institute for Molecular Sciences.

I extend my special thanks to the official reviewers of this thesis, Professors Kaj Blennow and Michael Fountoulakis, for careful examination and useful comments that greatly helped to improve the manuscript.

I am thankful to my co-authors Docent Irina Alafuzoff for irreplaceable help and support in neuropathology; Docent Seppo Auriola for his collaboration with the mass spectrometry; Samuel Hartikainen, for providing excellent CSF gels for protein identification; and Paula Nyyssönen for fruitful collaboration and friendship during these years. I also thank Maarit Heimonen and Seija Hynynen for their excellent technical assistence.

And then, the 1.30 p.m. ”coffee team”…Nilla Karjalainen, Tarja Kauppinen, Esa Koivisto, Sari Palvianen, and Tuija Parsons. Actually, without them, you would not be reading this book right now! Thank you, this department, including me, would collapse without your efforts. Don’t worry, wherever I'll be, I still know where to find you…and I’ll be back.

I wish to thank Ewen MacDonald for enriching linguistic revisions during the thesis work. I am also grateful for the Statistical and Mathematical Services at the University of Kuopio, especially Pirjo Halonen.

My most sincere thanks belong to the whole personnel at the Department of Neurology, Brain Research Unit, Clinical Research Centre at Mediteknia, and A.I. Virtanen Institute for Molecular Sciences for creating such a homely and innovative atmosphere to carry out this work. I thank you all for sharing ideas at work and for splendid off-work times! Especially, I wish to thank Mikko Laakso for being like an unofficial supervisor by introducing me to the literary shenanigans in the scientific world. I am also grateful to the co-ordinator of PhD-students, Pauliina Korhonen, for always maintaining such a cheerful spirit. I thank Antero Salminen and Johanna Ojala for stimulating ideas and support in research.

The life of a PhD student is not only limited to work; she has to find time for a private life. It is a period of extensive intellectual and spiritual metamorphosis. I have been fortunate to be able to share these challenging times with friends. Particularly, I want to mention Taneli Heikkinen, Anna Hicks, Jouni Ihalainen, Miia Kivipelto, Petri Kerokoski, Erkki Kuusisto, Susanna Narkilahti, Maija Pihlajamäki, Susanna Tervo and Iain Wilson. Last, but not least, I wish to thank “Misukat” including Sanna-Kaisa Herukka, Anne Hämäläinen, Hennariikka Iivonen, Mari Oksman and Mia Pirskanen for the unforgettable times spent together. I hope that this may well be a beginning of a lifelong friendship!

Warm thanks also belong to the people involved in our organised social activities including Aivopesula, den Svenska Kluppen av Neurovetenskapare i Kuopio, Kyökkipiiat, and the final outcome, de Neurovetenskapliga Kökspigor i Kuopio.

I have known Laura Parkkinen as a friend and colleague, as a waitress and as a PhD student, for more than a decade. I feel that it was more like destiny rather than a coincidence that we both ended up in Kuopio at the same time. Thank you for your sisterhood. I owe my love to “the family” that in those early days only incorporated Anna Kiialainen, Marika Malinen, and Linda Kontula. "The family" seems to be "growing" gradually, so I wish to thank you all who now feel like belonging to it! We have experienced caring companionship over the years and enjoyed living at “the Alppimaja of Ajurinkatu”. In fact, these "Alps" do not depend on the geographical location! We’ve spend great times all over the world, but especially in Helsinki, Kuopio and Savonlinna.

I thank my beloved godchildren Joanna Ahteela, Jesse Huittinen, Kirsikka Karpansalo, and Milla Teräväinen. Of course, I cannot forget their wonderful siblings Joonatan Ahteela, Olli Ekblad, Roope Huittinen, Saga and Saska Karpansalo. It is impossible to be in the bad mood after seeing you!

I am indebted to my all dear old, but not aged, Savonlinna friends. Especially, I want to mention Jonna Halinen, Mari Halonen, Paavo Halonen, Pekka Huittinen, Niina Jäppinen, Mikko “ Pode” Poutanen, and Mira Teräväinen. My warmest thanks also belong to my friends in Kuopio and Sweden, especially, Krista Karpansalo and Lisette Marjavaara, respectively. Without you I would not have survived!

I wish to thank the members of the following former and present households: Hurme, Ikonen, Kontinen, Kuronen and Kärkkäinen. I also owe my gratitude to my scientific step-mother Sari Tikanoja.

I thank all my family. I owe my dearest thanks to my parents Anneli and Reima Korolainen who have been supporting me in all activities and education from the day one. It is rare to have such loving and unselfish parents to bring you up. My special thanks belong to my sister Hanna-Kaisa Korolainen and her husband Benjamin Goursot for always being willing to offer me an ear to share both laughter and sorrow. I thank Pasi Tuppurainen for his love and understanding. I also wish to thank my cousin Erja, her husband Ari Ahteela, my aunt Ritva Väyrynen, and the eldest of the family, Sirkka Honka, for their endless support. You all have always been there for me when needed!

My grandmother, Aino Väyrynen, sent me a letter only two weeks before she died in spring 1996: “Minna, I am so proud of you, you have always had the courage to direct yourself headlong to new challenges, and with that attitude of yours, I know that you will have success and be happy in your life.” This sentence comforts and guides me in life!

And finally, all my dear friends and contributors that have not been mentioned, you are worthy of a whole book that may never be finished. You are not forgotten, thank you!

The study was supported by the National Technology Agency of Finland (grant 201495), the Academy of Finland, Kuopio University Hospital (EVO grant 5772722), University of Kuopio, the Kuopio Doctoral Program of Medical Sciences, the Nordic Centre of Excellence, the Kuopio University Foundation, the Finnish Cultural Foundations of Northern and Southern Savo, the Farmos Research Foundation, Helsingin Sanomain 100-vuotissäätiö Foundation, Maire Taponen Foundation, and the Alzheimer Research Society of Finland.

Minna Korolainen

ABBREVIATIONS

AAT	aspartate aminotransferase
Aβ	amyloid-beta
AD	Alzheimer’s disease
α-1-AT	alpha-1-antitrypsin
α-kg	alpha-ketoglutarate
α-KGDHC	alpha- ketoglutarate dehydrogenase complex
AM	activated microglia
ApoE	apolipoprotein E
APP	amyloid precursor protein
AUC	area under curve
BBB	blood-brain-barrier
BPB	bromphenol blue
CA	carbonic anhydrase
CAs	carrier ampholytes
CDRs	Clinical Dementia Rating score
CERAD	Consortium to Establish a Registry for Alzheimer’s Disease
CNS	central nervous system
CSF	cerebrospinal fluid
DNP	2,4-dinitrophenylhydrazone
DNPH	2,4-dinitrophenylhydrazine
DS	Down´s syndrome
DTT	dithiothreitol
ER	endoplasmic reticulum
ESI	electrospray ionization
GDH	glutamate dehydrogenase
GFAP	glial fibrillary acidic protein
HBPP	Human Brain Proteome Project
HLA-DR	human leukocyte antigen
HSV	herpes simplex virus
HUPO	Human Proteome Organisation
IAA	iodoacetamine
IEF	isoelectric focusing
IL	interleukin

IPG	immobilized pH gradient
(HP)LC	(high performance) liquid chromatography
MALDI	matrix-assisted laser desorption/ionization
MCI	mild cognitive impairment
MDH	malate dehydrogenase
MHC	major histocompatibility complex
MMSE	Mini Mental State Examination
Mr	molecular weight
MRI	magnetic resonance imaging
MS	mass spectrometry
NFT	neurofibrillary tangle
NIA	National Institute of Aging-Reagan Institute
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association
NMDA	N-methyl-D-aspartate
NP	neuritic plaque
PHF-tau	paired helical filaments of hyperphosphorylated tau protein
pI	isoelectric point
PMD	post-mortem delay
PMF	peptide mass fingerprinting
PTMs	post-translational modifications
RA	reactive astrocyte
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOD1	Cu/Zn-superoxide dismutase
TCA	tricarboxylic acid
TOF	time-of-flight
1-D, 1-DE	one-dimensional, one-dimensional gel electrophoresis
2-D, 2-DE	two-dimensional, two-dimensional gel electrophoresis

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that are referred to in the text by Roman numerals **I-IV**.

I **Korolainen MA**, Goldsteins G, Alafuzoff I, Koistinaho J, Pirttilä T (2002). Proteomic analysis of protein oxidation in Alzheimer's disease brain. *Electrophoresis* 23(19):3428-33.

II **Korolainen MA**, Goldsteins G, Nyman TA, Alafuzoff I, Koistinaho J, Pirttilä T (2006). Oxidative modification of proteins in the frontal cortex of Alzheimer's disease brain. *Neurobiology of Aging* 27(1):42-53. Epub 2005 Jan 23.

III **Korolainen MA**, Auriola S, Nyman TA, Alafuzoff I, Pirttilä T (2005). Proteomic analysis of glial fibrillary acidic protein in Alzheimer's disease and aging brain. *Neurobiology of Disease* 20(3):858-70. Epub 2005 Jun 27.

IV **Korolainen MA**, Nyman TA, Nyyssönen P, Hartikainen ES, Pirttilä T. Oxidation and levels of cerebrospinal fluid proteins in aging and Alzheimer’s disease, submitted

TABLE OF CONTENTS

1. INTRODUCTION

The population is aging, particularly in the Western world, and society is confronted by new challenges in understanding, diagnosing, and treating complex and heterogeneous age-related disorders such as dementia. The most common cause of dementia is Alzheimer's disease (AD) that is characterised by a progressive decline in memory and cognitive functions. The classical neuropathological hallmarks of AD brains are extracellular deposits of amyloid-beta (Aβ) containing plaques and intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein. However, these changes are not specific for AD and have also been reported in brains from cognitively unimpaired elderly individuals (Gold et al. 2000, Davies 2001, Riley et al. 2002). Other essential pathological abnormalities are neuronal death, gliosis, chronic inflammation, excitotoxicity and oxidative stress (Pereira et al. 2005). All known pathological alterations in AD complement each other and are undoubtedly involved in the vicious circle of the pathogenesis. Nonetheless, their role in explaining the diverse clinical symptoms and progression of AD is still largely unclear.

AD is a pathologically complex and aetiologically multifactorial disease. There are three causative genes which have been linked to the small proportion of patients with early-onset familiar AD. In addition, a number of other candidate genetic risk factors predisposing for AD have been identified (Rocchi et al. 2003). However, it has become evident that genes alone cannot explain the late-onset sporadic AD. In addition, many environmental factors contribute significantly to the development of the disease. The word proteome means the entire protein complement expressed by a genome, or by a cell or a tissue type at any given time (Wilkins et al. 1996). Unlike the genome that is relatively static, the proteome is extremely dynamic and constantly changing in response to any internal (e.g. aging) or external stimuli (e.g. toxic exposure). The dynamical complexity of proteins may be easily appreciated, i.e. they are composed of a variety of combinations of twenty amino acids, and thereafter modified co- and post-translationally by i.e. phosphorylation, glycosylation, and oxidation (Anderson et al. 2001). It is these modifications that influence the activity state, folding, location, function, and interactions of proteins. Alterations in the expression and post-translational modification of proteins may have functional consequences and lead to a variety of pathophysiological conditions. Indeed, proteome analysis has been suggested to hold the key to improving

understanding, diagnosing, and treating AD, as well as to follow-up of the progression of the disease.

The goal of proteomics is the identification and quantification of as many proteins and their isoforms as possible. The traditional approach in expression proteomics involves separation of proteins by two-dimensional gel electrophoresis (2-DE) (Klose 1975, O'Farrell 1975, Bjellqvist et al. 1982) followed by identification of proteins using immunoblotting or mass spectrometry (Henzel et al. 1993, Mann and Wilm 1994, Yates et al. 1995). In the 2-DE technique, proteins are separated according to their isoelectric point (pI) in the first dimension and then according to their molecular weight (Mr) in the second dimension. Two-dimensional (2-D) images are visualized and scanned followed by image analysis with specific software. It is possible to find differences in proteomes by comparing 2-D maps in healthy and diseased states. In addition to protein quantification, post-translational modifications (PTMs) may be studied by using slightly modified 2-DE techniques.

The development of proteomics techniques to the current stage has emerged for tens of years (Righetti 2004). However, until very recently, there have been both technical and computational limitations restricting the use of proteomics, and it is still considered as a relatively new phenomenon in science (Hochstrasser et al. 1998, Fountoulakis 2004). Therefore, the present study was focused on developing clinical expression proteomic applications. Levels and modifications of proteins were examined in brain and cerebrospinal fluid (CSF), and protein profiles of AD patients were compared to those of defined controls. It is hoped that the present series of reports will contribute to both methodological developments in proteomics, as well as to deepening our understanding of the pathogenesis of AD.

2. REVIEW OF LITERATURE

2.1. ALZHEIMER'S DISEASE

2.1.1. The societal challenge

Alois Alzheimer described neuropathological changes in a middle-aged woman, Auguste D, suffering from early-onset dementia 100 years ago (Alzheimer 1907, Maurer et al. 1997). The autopsy revealed the presence of peculiar protein aggregates, referred to as senile plaques and NFTs in the patient's brain. This disorder was named after the inventor himself as “Alzheimer's disease” but was long considered to be a rare disease affecting middle-aged individuals. It took more than 50 years to recognize that AD is also the most common cause of senile dementia (Blessed et al. 1968). Already in 1978, Katzman predicted that senile dementia of the Alzheimer type was becoming one of the most common malignant diseases and great challenges of our aging society (Katzman 1978). Thereupon, AD became "the silent epidemic' of the 20th century" and was considered uniquely as to be a problem of the economically advanced countries. Nowadays, AD is recognized as the most common form of dementia affecting an estimate of 24.3 million people worldwide, 40% of them living in less developed countries (Ferri et al. 2005). The prevalence of dementia in Western Europe is about 4.5 million, and its total annual costs amount to 55 billion euros (Andlin-Sobocki et al. 2005, Ferri et al. 2005, Jönsson and Berr 2005). It has been estimated that the number of patients with dementia will double every 20 years; e.g. to 42.3 million in 2020 and to 81.1 million by 2040 (Ferri et al. 2005). If nothing is done, an exponential increase in the costs of dementia will be experienced during the next few decades. As a consequence of rapid demographic ageing, AD has become one of the most severe progressive socio-economical and medical burdens facing countries all over the world.

At the present time, there is no cure for AD, and the disease inevitably causes severe and progressive decline in daily activities, eventually resulting in institutionalisation and death. The pathological brain changes may occur as early as 20 to 30 years prior to the onset of clinical symptoms, and the symptomatic phase of AD can last from about 5 up to 12 years (Figure 1)(DeKosky and Marek 2003). Currently available drugs are able to delay the symptom progression of the disease and to positively influence the quality of everyday life of

patients and caregivers (Connell et al. 2001). Unfortunately, their efficacy is limited in magnitude and length. The treatment should be initiated as early as possible to achieve the best benefit. However, the diagnosis of early AD remains problematic. Moreover, the available drugs do not influence the progression of brain pathology in AD. Many diverse mechanisms are claimed to have a role in the pathogenesis of AD but their exact contributions remain largely unknown, and their interactions have proved tenuous, resulting in difficulties in discovering new therapeutic targets.

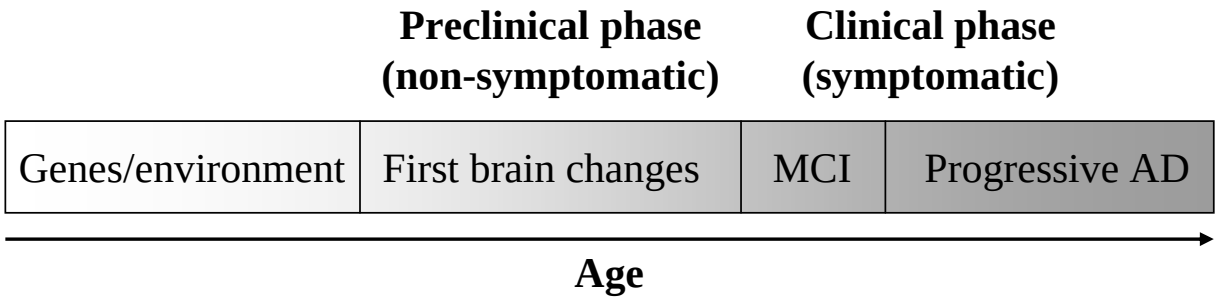


Figure 1. AD is considered to be a result of a sum of complex antecedent events involving both genetic and environmental factors. The initial brain changes may occur decades prior to the onset of clinical symptoms. The first symptomatic clinical phase is called mild cognitive impairment (MCI). However, MCI may have several causes (i.e. normal aging, cerebrovascular pathology etc.) and the clinical syndrome defined as MCI does not necessarily mean that the patient has AD (Chong and Sahadevan 2005). Due to recent developments in the treatment, the diagnostic focus has shifted to the earliest phases of the disorder and to the detection of those MCI patients who will convert to AD. The ideal goal in the future would be to find interventions that prevent the development or progression of AD. (Modified from DeKosky and Marek 2003)

2.1.2 Causative and risk factors

AD can be classified into early onset (<65 years) and late onset (>65 years) forms (Terry and Katzman 1983). Already during the 1950's, it was clear that there were families suffering from dominantly inherited AD, indicating that genetic factors were causing the disorder, at least in a small proportion of early-onset patients. Finally, at the end of 1980's, the first gene causing the familial early-onset AD was found (Goate et al. 1991, Price et al. 1998). It was located on chromosome 21 and encoded for amyloid precursor protein (APP), which is the

precursor protein for Aβ. This finding also explained why all patients with Down's syndrome (trisomy of the chromosome 21) (DS) having chromosomal triplication and showing overexpression of APP gene, develop neuropathological features of AD and most of them become demented after 50 years of age (Schupf and Sergievsky 2002). To date, all mutations underlying familial autosomal dominant AD in three genes seem to influence the production and/or metabolism of APP and thus amyloid pathology in the brain (Rocchi et al. 2003, Thinakaran and Parent 2004). In addition to the APP gene, two other disease-causing genes are presenilin-1 and -2 on chromosomes 14 and 1, respectively (Levy-Lahad et al. 1995, Rogaev et al. 1995, Sherrington et al. 1995). Presenilins have been suggested to be a part of the γ-secretase complex and thus be crucially involved in cleavage of APP, resulting in the generation of Aβ peptides. Mutations in genes coding for presenilins are thought to be responsible for more than 80% of all familial AD cases (Rocchi et al. 2003). Interestingly, none of the genetic determinants for inherited AD have been directly linked to neurofibrillary pathology.

Sporadic AD is the most common form of both early- and late-onset AD. It is considered to be a result of complex genetic and environmental risk factors. Apolipoprotein E (ApoE) polymorphism is the most thoroughly studied genetic risk factor in AD. There are three ApoE alleles ε2, ε3 and ε4 on chromosome 19 that result in six different phenotypes in humans (Zannis et al. 1981, Olaisen et al. 1982). The ε4 allele is the most important genetic risk factor for sporadic AD in all ages and most races, whereas ε2 is thought to be protective for the disease (Corder et al. 1993, Raber et al. 2004). The ApoE ε4 allele has been suggested to result in earlier onset of AD when compared to other phenotypes, and to be related to more pronounced Aβ, and neurofibrillary pathology (Polvikoski et al. 1995, Polvikoski et al. 2001, Raber et al. 2004). Recent studies have also revealed several other genetic risk genes and chromosome locuses that contribute to the susceptibility for AD (Rocchi et al. 2003).

The increased longevity now experienced by many humans does not come without a price. It has been accompanied by a variety of chronic and degenerative illnesses e.g. age itself is the major risk factor for sporadic AD. The prevalence rate is about 7% for all individuals over 65 years of age, although the risk of AD increases exponentially with age (Rocca et al. 1991, Breteler et al. 1992, McDowell 2001). Women, including female ApoE ε4 carriers, have been suggested to be at a higher risk for developing AD than men (Jorm et al. 1987, Payami et al.

1996, Bretsky et al. 1999, Turner, 2001). The impact of hormones on the pathogenesis of AD is not well understood i.e. the claims for beneficial effects of postmenopausal estrogen replacement therapy as a protective factor for AD remain highly controversial (Tang et al. 1996, Zandi et al. 2002, Mulnard et al. 2004, Almeida and Flicker 2005)

Elevated blood pressure and serum cholesterol in middle-age have been recently recognized as modifiable vascular risk factors for AD (Kivipelto et al. 2001, Kivipelto et al. 2002). They are known to be involved in vascular disorders (i.e. atherosclerosis, cardiovascular and cerebrovascular disease, type II diabetes) that are also considered to increase the risk of developing AD. Brain infarcts lower the threshold for clinical symptoms (Snowdon et al. 1997) and they also seem to influence the development of AD brain pathology (Jellinger 2004). Head trauma is considered as a risk factor for clinical AD, particularly if it occurs in close proximity to the beginning of the clinical symptoms (van Duijn et al. 1992, Plassman et al. 2000). It is possible that this association is due to similar threshold effect as hypothesized for brain infarcts, although experimental studies have shown that head injury influences APP metabolism (Uryu et al. 2002).

Use is fundamental for the development and maintenance of brain functioning. The saying “use it or lose it” refers to the necessity of action in protection of our brains from harmful effects of various factors. For example, a low level of education and a passive life style are considered to predispose an individual to AD (Stern et al. 1994, Qiu et al. 2001). In addition, loneliness and history of depression are possible risk factors for AD and cognitive decline (Speck et al. 1995, Berger et al. 1999, Jorm 2000, Tilvis et al. 2004). Both of these characteristics are closely associated with each other and with aging. In contrast, a rich social network and many leisure activities are considered to be protective for AD (Fratiglioni et al. 2004). The risk of AD may also be diminished by diet, moderate alcohol consumption and avoidance of vitamin deficiency (Letenneur 2004, Luchsinger and Mayeux 2004). Indeed, lifestyle does seem to matter, and it may be possible to avoid or postpone the onset of AD by taking care of both mental and physiological activities to sustain systemic health.

Infectious agents have been suspected to contribute to cognitive impairment in the elderly as well as to AD. Some postmortem studies have detected signs of *Chlamydia pneumoniae* and herpes simplex type 1 virus (HSV-1) more often in AD brain as compared to control patients (Balin et al. 1998, Itzhaki et al. 2004, Mattson 2004a). In 2004, Strandberg et al., examined

bacterial and viral seropositivity among 383 home-dwelling elderly individuals. No significant associations were observed between bacterial burden and cognition, not even for *Chlamydia pneumoniae*. However, viral burdens of cytomegalovirus, HSV 1- and -2 were associated with cognitive decline. In addition, other factors such as hypothyroidism, metals, pesticides and electromagnetic fields have been suggested to be risk factors for AD but these associations need to be replicated (Brown et al. 2005).

2.1.3. The pathogenetic puzzle of AD

As was already described one century ago, AD brains are characterized by the presence of extracellular deposits of Aβ containing plaques and intracellular NFTs composed of paired helical filaments of hyperphosphorylated tau protein (PHF-tau). It has become obvious that these changes are not specific for AD and are also found in brains of cognitively unimpaired individuals (Davis et al. 1999, Gold et al. 2000, Riley et al. 2002). Other central pathological abnormalities are gliosis, chronic inflammatory reactions, excitotoxic damage, and oxidative stress that all appear to be involved in neurodegenerative processes occurring in the AD brain (Figure 2). Extensive research conducted over decades has failed to pinpoint the key processes resulting in neuronal death, synaptic loss and finally in the symptoms of AD. Research into AD can be likened to inserting random pieces into an enormous jigsaw hoping that the actual picture will emerge sometime in the future.

2.1.3.1. The amyloid cascade hypothesis

APP is a ubiquitously expressed transmembrane glycoprotein that is produced by neurons, astrocytes and microglia in the central nervous system (CNS) (Kang et al. 1987, Weidemann et al. 1989, LeBlanc et al. 1996). There are a total of eight isoforms of APP, arising from alternative splicing (Hartmann et al. 1996). The three major isoforms of APP vary in size containing 695, 751 or 770 amino acids. APP_{695} is the isoform mostly present in neurones (LeBlanc et al. 1991), with the major difference as compared to the other isoforms, of not having a serine protease inhibitor domain (also called as Kunitz protease inhibitor domain). The proposed physiological functions of APP include regulation of neuronal survival, synaptic plasticity and cell adhesion (Mattson 2004b).

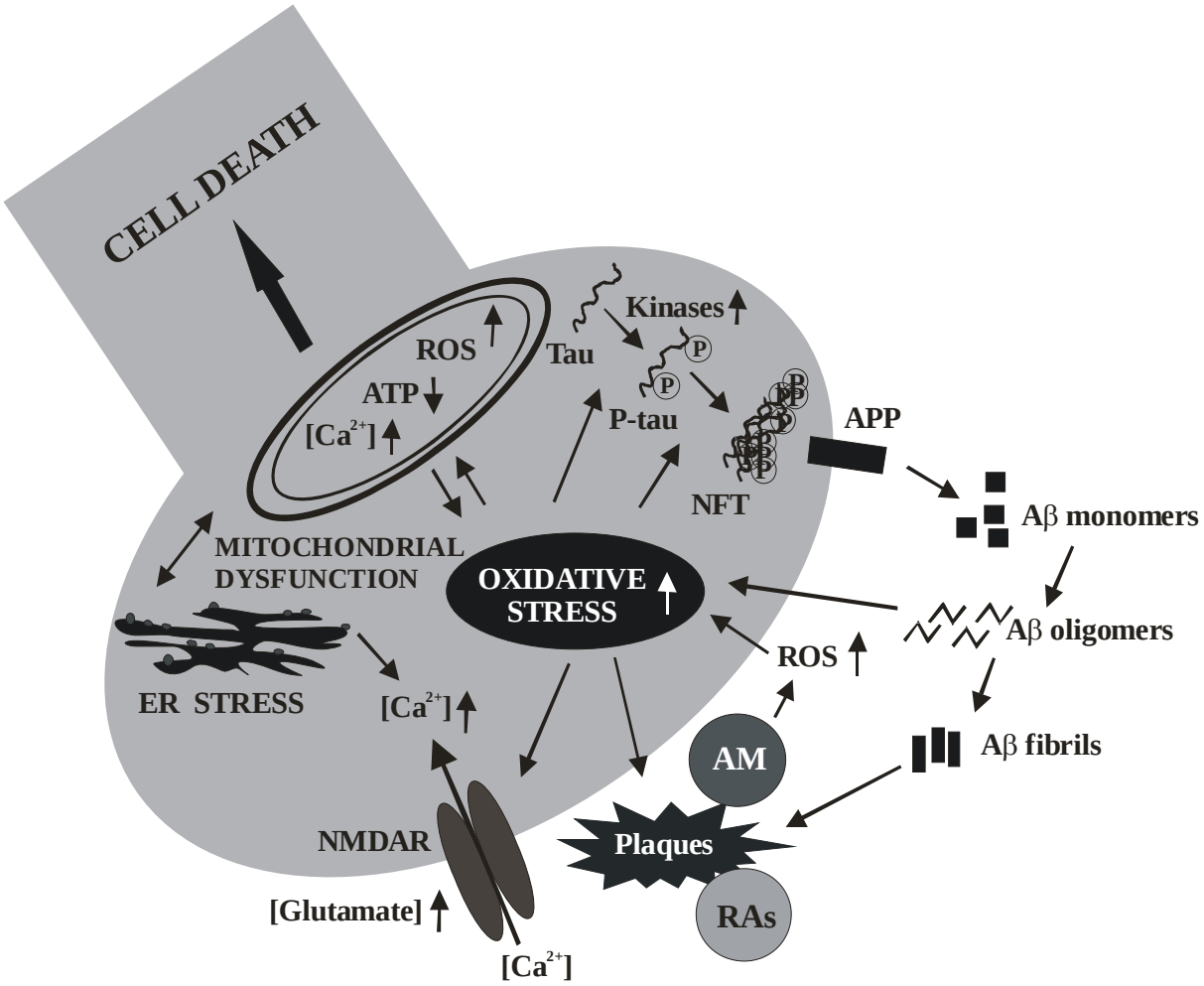


Figure 2. Schematic and simplified representation of the main pathogenetic events in AD including plaque formation, neurofibrillar pathology, gliosis, inflammation, excitotoxicity and oxidative stress. All complement each other and are undoubtedly involved in the vicious circle of the pathogenesis to synaptic dysfunction eventually leading to cell death. (Modified from Pereira et al. 2005)

Aβ, amyloid-beta; AM, activated microglia; APP, amyloid precursor protein; ER, endoplasmic reticulum; NFT, neurofibrillary tangle; NMDAR, N-methyl-D-aspartate receptor; P-tau, hyperphosphorylated tau; RA, reactive astrocytes; ROS, reactive oxygen species

The processing of APP and the formation of Aβ plaques are well characterised and involve a series of enzymes called α-, β-, and γ-secratases (Nunan and Small 2000). APP matures through the secretory pathway. It is post-translationally modified in the endoplasmic reticulum (ER) by N-glycosylation, and in Golgi apparatus by O-glycosylation (Cook et al. 1997, Katayama et al. 2004). Cleavage of APP occurs on the plasma membrane when it is transported to or from the cell surface via either secretory or endocytic pathways (Hartmann

1999). The cleavage of APP by α-secretase occurs in the middle of Aβ-peptide and leads to the generation of non-amyloidogenic fragments of APP. In contrast, the cleavage of APP by β-secretase followed by γ-secretase creates potentially amyloidogenic peptides that are released into extra- or intracellular space (Mattson 2004b).

The most common amyloidogenic Aβ-peptides contain 40 or 42 amino acids depending on the γ-cleavage. Both are formed during normal metabolism but Aβ42 is considered to be more critical and to be overproduced in AD (Haass et al. 1992, Younkin 1995). Aβ42 aggregates more rapidly as compared to Aβ40 (Burdic et al. 1992, Irie et al. 2005). The initial parenchymal extracellular non-filamentous deposits of Aβ are composed mainly of Aβ42. As the deposits grow, Aβ40 starts to aggregate onto Aβ42 deposits. Diffuse and neuritic plaques (NPs) are the two major amyloid deposit types in AD brain. Diffuse plaques contain mainly nonfibrillar amyloid. Further developed NPs contain dense bundles of amyloid fibrils and are surrounded by dystrophic neurites, astrocytes and microglia. Other components associated with plaques include serum amyloid P, α_1-antichymotrypsin, sulphated glycosaminoglycans, α_1-antitrypsin, apolipoproteins E and D, and the neurotrophic factor midkine (Desai et al. 2005, Frey et al. 2005).

The role of amyloid deposits in brain as the triggering factor for AD has been extensively studied since its characterisation in 1984 (Glenner et al. 1984). However, this “amyloid cascade hypothesis” was challenged by the fact that plaques are also found in brains of cognitively unimpaired elderly people (Keller 2006). Despite considerable effort, there seems to be no correlation between the extent of plaques and the severity of cognitive decline (Nagy et al. 1995, Neve and Robakis 1998). In addition, Aβ deposition is not necessarily accompanied by neurogeneration, and vice versa (Suh and Checler 2002). It has now become acknowledged that protein aggregates alone are not sufficient to cause neurodegeneration, and that the protein aggregates may even be protective against other harmful events in brain. Currently, the “amyloid cascade hypothesis” has evolved into the “Aβ cascade hypothesis”, which suggests that soluble Aβ oligomers, Aβ-derived diffusible ligands (ADDLs) and Aβ protofibrils, and their intraneuronal accumulation, play crucial neurotoxic roles in the pathogenesis of AD (Hardy and Selkoe 2002, Klein 2002, Golde 2003) (Figure 2). The recent evidence suggests that these soluble Aβ intermediates are involved in causing synaptic dysfunction, which may be an early event in memory decline and AD (Pereira et al. 2005).

“The Aβ cascade hypothesis" as the primary trigger is supported by the facts that all known mutations causing familiar AD target APP processing and that plaque formation has also been estimated to be a relatively early event in AD brain. Additional evidence has been found by examining a Swedish family having the “Arctic” mutation that causes atypical AD. These patients have decreased plasma levels of both Aβ40 and Aβ42, and less severe cerebral amyloid angiopathy, but their clinical features resemble aggressive early-onset AD. In particular, the formation of soluble protofibrils is enhanced by “Arctic” mutation, leading to accelerated intracellular accumulation of Aβ. “Arctic” mutation may help to confirm the role of the "Aβ cascade hypothesis", and Aβ oligomerisation in the early pathogenesis of AD (Nilsberth at al. 2001).

Despite the evidence supporting the role of “Aβ cascade hypothesis” in the pathogenesis of AD, there are also data arguing against amyloid alone causing AD. For example, transgenic mouse models bearing familiar AD mutations do not show evidence of significant neuronal loss, excessive tau phosphorylation or tangle formation (Suh and Checler 2002). There are also studies that have failed to demonstrate the toxicity of Aβ in vivo and in some culture conditions Aβ has even promoted neurite outgrowth (Suh and Checler 2002). The clinical picture and the morphological end-stage in the brain appear to be the same in familiar and sporadic AD. Even though there is an extensive body of evidence supporting the role of Aβ in AD, especially in its familiar forms, the etiology of AD, particularly that of the sporadic form, remains unresolved.

2.1.3.2. From tau to neurofibrillary tangles

Tau is a phosphoprotein that is mainly synthesized by neurons but which is also present in astrocytes and oligodendrocytes in brain, and in peripheral tissues (Kosik et al. 1989, Papasozomenos 1989). Tau is coded by a gene located on chromosome 17 and alternatively spliced into six isoforms ranging from 352 to 441 amino acids in length (Brandt et al. 2005, LaFerla and Oddo 2005). Tau is the major microtubule-associated protein promoting the assembly and stability of microtubules, which play vital structural and functional roles in neurons. The main difference between the six isoforms is due to the presence of three or four repeats of domains that are important in microtubule binding. It is now three decades ago when tau was first identified to be the protein facilitating microtubule assembly (Weingarten

et al. 1975). Since that discovery, little interest was paid on tau until the middle of 1980's when it was found to be the major protein present in PHFs that make up NFTs in AD (Grundke-Iqbal et al. 1986a, Grundke-Iqbal et al. 1986b, Kosik et al. 1986).

Tau is functionally regulated by phosphorylation, and in its phosphorylated form cannot stabilise microtubules (Lee et al. 2005). Tau is a remarkable phosphoprotein since it has about 30 potential phosphorylation sites (Geschwind 2003). The longest isoform has been suggested to have phosphorylations attached to up to 20% of all amino acids (Stoothoff and Johnson 2005). In AD, tau is hyperphosphorylated, aggregated into PHFs and NFTs inside the neurons (Figure 2), and as a consequence, microtubules can no longer be stabilised by tau. When the neuron dies, NFTs become extracellular ghost tangles. Tau phosphorylation has been suggested to play a key role in AD pathogenesis since it has been shown to correlate with the severity of dementia. In 1991, Braak and Braak proposed a model of pathological evolution of AD. According to this model, NFTs appear in the entorhinal cortex during the preclinical phase, spreading to hippocampus in the middle phase and, finally, progress to the neocortex during the late stages of AD (Braak and Braak 1991b).

Although NFTs are a characteristic hallmark in AD brain, these intracellular aggregates have been proposed to be a secondary process in the pathogenesis. Some studies suggest that tau is not required for normal cell function, and that neurons may survive with NFTs for more than 20 years (Lee et al. 2005). The underlying event that leads to hyperphosphorylation has been suggested to be a result of an imbalance between the activities of kinases and phosphatases although the actual mechanisms remain very obscure. There is evidence that phosphatase activity may be deficient in AD (Gong et al. 1993, Gong et al. 2004). Another hypothesis suggests that an increase in oxidative stress results in activation of kinases resulting in hyperphosphorylation of tau (Zhu et al. 2000, Lee et al. 2005). Phosphorylated tau is vulnerable to oxidative stress and becomes carbonylated followed by rapid aggregation. Notably, tau phosphorylation has been suggested to be protective for cell survival during increased oxidative stress in AD. Other studies suggest that tau is essential for Aβ aggregation (Pereira et al. 2005).

NFTs are found in cognitively unimpaired elderly individuals (Price and Morris 1999) and in a number of other "tauopathies" including DS, progressive supranuclear palsy, corticobasal degeneration, and frontotemporal dementia as well as in other neurodegenerative diseases

such as Parkinson’s disease. Mutations in the gene coding for tau are causative for frontotemporal dementia with parkinsonism (FTDP-17) (Buée et al. 2000).

2.1.3.3 Gliosis and chronic inflammation

In 1910, another pioneer of AD research, Oskar Fisher, failed to prove his hypothesis of inflammatory reactions being involved in the pathogenesis of senile dementia (Fisher 1910, Stuchbury and Münch 2005). One of the reasons may have been that brain has traditionally been considered as an immunologically privileged organ and Fisher was unable to detect any typical signs of inflammation. Today, it has become clear that CNS possesses an extensive immune system although it is quite different from that in the periphery i.e. brain does not exhibit "the four cardinal signs" of peripheral inflammation; calor (heat), rubor (redness), tumor (swelling), and dolor (pain) (McGeer and McGeer 2001). Inflammatory reactions in CNS are mainly mediated by brain-specific glial cells, notably by microglia and astrocytes, and to a lesser degree by neurons. Under physiological conditions, glial cells support and protect neurons and their functions, and have many critical roles in the homeostasis and activity of the brain (Tuppo and Arias 2005). In AD, inflammation has recently been confirmed as an essential feature, particularly in association with neuritic plaques (Figure 2). The inflammatory reaction is characterized by activation of glial cells, gliosis and the appearance of inflammatory proteins such as complement factors, acute phase proteins, and proinflammatory cytokines (Akiyama et al. 2000).

In brain, microglial cells are considered to represent the equivalent of monocytes and macrophages (Ling and Wong 1993, Rock et al. 2004). Under normal conditions, microglial cells exist in a resting stage, where they are inactive, and show low expression of surface receptors, secretory activity, proliferatory and phagocytic function, and minimal migration (Giulian et al. 1995, Färber and Kettenmann 2005). Microglia is the first cell population showing activity in response to any kind of insult in brain such as trauma, infection or inflammation. Activated microglia (AM) do not only exhibit phagocytic and scavenger activities but also release reactive oxygen species (ROS), nitrogen compounds, excitatory amino acids (i.e. glutamate), proteases and cytokines (i.e. interleukins (IL) -1, -6, tumor necrosis factor alpha), and macrophage colony stimulating factor (Akiyama et al. 2000, Blasko et al. 2004). AM are characterised by their increased expression of human leukocyte antigen (HLA-DR), a class II major histocompatibility complex (MHC) cell-surface antigen.

Astrocytes are the most common cells found in the brain. Until recently, they were thought to be mostly rather passive and solely involved with connective tissue and skeletal function in the brain. They were known to participate in the formation and maintenance of the blood-brain-barrier (BBB) that isolates the brain from peripheral influences and protects it from a number of inflammatory factors (Ballabh et al. 2004). Molecules that are small or highly lipophilic can pass into the CNS under normal physiological conditions. Today, it has become evident that astrocytes play an active role in the functioning of brain although their complete contribution has yet to be revealed. Under physiological conditions, astrocytes regulate extracellular pH and ion levels, take up and detoxify harmful compounds such as amino acids (i.e. glutamate-glutamine cycling), ammonia, free radicals and xenobiotics (Blasco et al. 2004). In addition, astrocytes are involved in neuronal migration, neurite outgrowth, synaptic plasticity, modulation of inflammatory responses and the formation of glial scars (Norenberg 1994). During inflammation, AM can activate astrocytes (i.e. by secreting IL-1 and -6), which are then transformed into reactive astrocytes (RAs). During gliosis, RAs increase in size and/or number, and are characterised by overexpression of glial fibrillary acidic protein (GFAP) (Eng et al. 2000, Eikelenboom and Van Gool 20004).

The role of inflammation in neurodegenerative diseases remains controversial since it may be both neuroprotective and neurotoxic, like a double-edged sword (Marchetti and Abbracchio 2005). Inflammation is almost always a secondary desirable response to a primary injury. However, when inflammation becomes chronic in its nature, it will likely evoke more damage than the initial injury. Both AM and RAs are found to be associated with Aβ depositions in AD brain (Nagele et al. 2004) (Figure 2). It has been hypothesised that Aβ is indirectly toxic, activating microglia to produce neurotoxins including ROS and cytokines that further activate astrocytes. Activated glial cells are classically thought to remove and degrade Aβ depositions in AD brain (De Felice and Ferreira 2002, Nicoll and Weller 2003, Streit 2005). However, the homeostatic control may fail and the clearance of Aβ may become altered in AD patients. As a consequence, the crosstalk between neurons, astrocytes and microglia may become disturbed. Thus, inflammation may contribute to neurodegeneration via increased oxidative stress and neuropathological changes.

2.1.3.4. Excitotoxicity

Excitotoxicity is also considered to play an important role in the pathogenesis of AD. It is triggered by excessive stimulation of glutamate receptors (e.g. N-methyl-D-aspartate also known as NMDA) due to either increased release or decreased uptake of excitatory amino acids, mostly glutamate (Hynd et al. 2004) (Figure 2). As a result, perturbations occur in cellular ion homeostasis (Ca^{2+}, K^{+}, Na^{+}) and metabolic activities. The most crucial disturbance is considered to be the overload in the intracellular level of Ca^{2+} that may originate from the extracellular space and/or ER, resulting in diverse neurotoxic effects due to calcium dysregulation and alterations in Ca^{2+}-signaling pathways (Siesjö 1981, Paschen 2000). Calcium dysregulation may also be caused by ER stress, which is characterized by inhibition of protein glycosylation and an increase in unfolded proteins in the ER (Katayama et al. 2004, Schröder and Kaufman 2005). Under pathological conditions, an increase in intracellular calcium results in overload of Ca^{2+} in mitochondria, causing mitochondrial dysfunction manifested by increased production of ROS, decreased energy metabolism, release in cytochrome c, and eventually apoptosis (Rego and Oliveira 2003). In brain, calcium dysregulation is considered to be capable of eliciting increased Aβ formation and tau phosphorylation (LaFerla et al. 2002, Pereira et al. 2005). It must be remembered that calcium dysregulation, ER stress, mitochondrial dysfunction, and defective energy metabolism may also be initiated by other factors that are unrelated to excitotoxicity.

2.1.3.5. Oxidative stress and accumulation of redox-active metals

The free radical theory of aging was first presented by Harman in 1956. Aging was described as a progressive, inevitable process partially related to the accumulation of biomolecules damaged by radiation. Oxidative damage of biological systems occurs when the production of oxygen radicals and ROS exceeds the cell's antioxidant capacity, leading to oxidative imbalance in a system (Figure 3) (Dalle-Donne et al. 2003a, Mariani et al. 2005). The main intracellular source of ROS is the electron transport chain in the oxidative phosphorylation processes occurring within mitochondria, which is the “energy factory of the cell”. Moreover, deterioration of mitochondria itself, as a consequence of oxidative stress, is thought to play a key role in aging processes (Miquel et al. 1980; Sastre et al. 2003). The main cellular source of ROS in the brain is AM, linking oxidative stress closely with inflammatory processes. The brain is considered to be particularly vulnerable to the deleterious effects of ROS since the

CNS consumes high amounts of oxygen to produce energy. In addition, the brain contains high amounts of polyunsaturated fatty acids that are susceptible to lipid peroxidation, as well as metals that can catalyse the reactions leading to the production of ROS. Furthermore, the brain is thought to have a relatively low antioxidant capacity (Mariani et al. 2005).

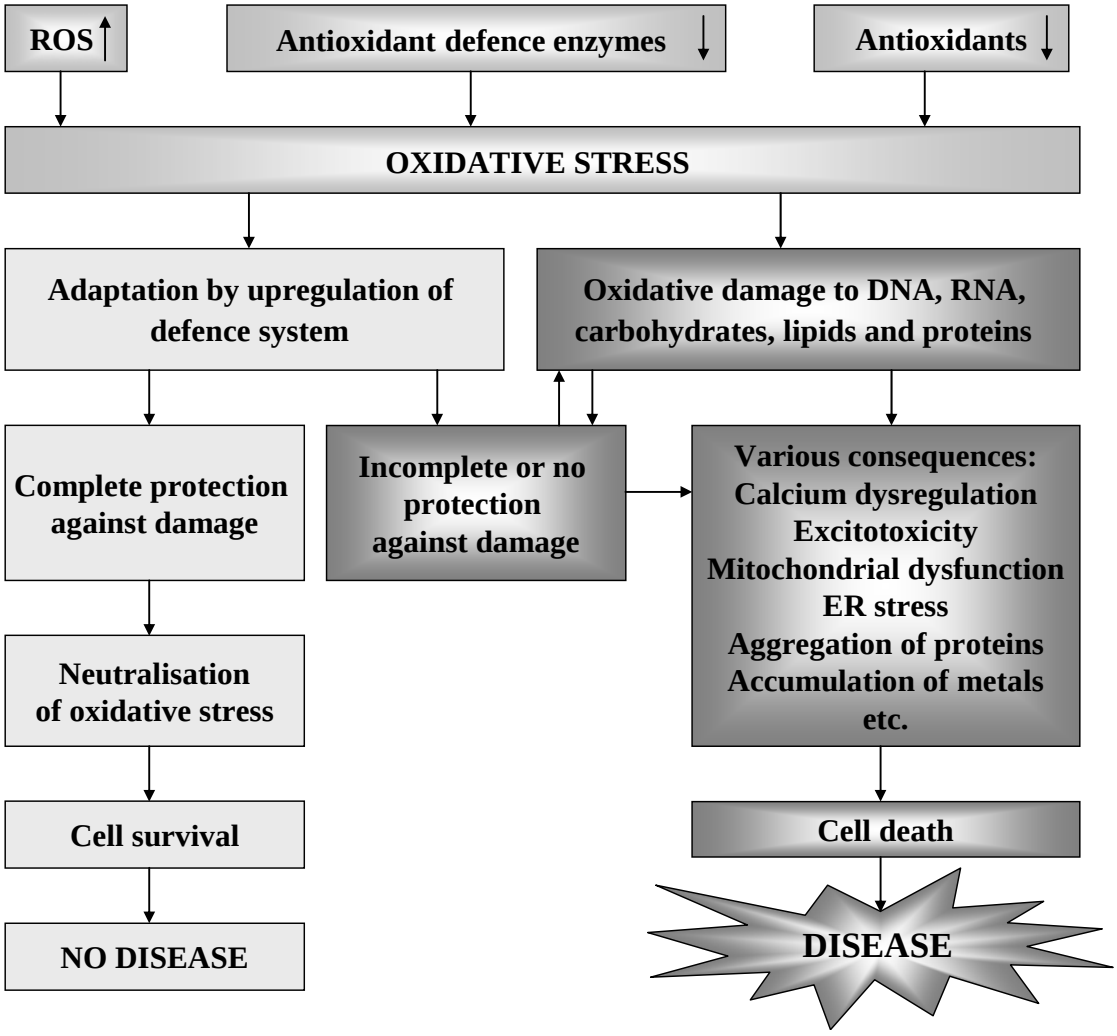


Figure 3. Oxygen is both necessary and toxic to organisms living in an aerobic environment. Oxidative stress plays a central role in both "normal aging" and in the pathogenesis of AD. Origins of oxidative stress in aging and disease arise from a failure of an organism to adapt to increased production of ROS and/or diminished antioxidant defence systems. Oxidative stress may have a variety of functional consequences that lead to cell death. (Modified from Dalle-Donne et al. 2003a)

ER, endoplasmatic reticulum; ROS, reactive oxygen species.

The concept of oxidative stress in AD was originally derived from the free radical theory of aging. In the last 10 years, an increasing number of studies have been published reporting a variety of results on the role of oxidative stress in AD (Praticò 2005). There is evidence of increased oxidation of DNA, RNA, protein, lipids and carbohydrates in AD brain (reviewed by Sayre et al. 2005). Oxidative stress markers have also been found to be increased in CSF, blood and urine of AD patients (Irizarry 2004, Praticò 2005). In addition, oxidative stress is associated with altered metal ion homeostasis in AD, leading to enhanced accumulation of redox-active metals and thus to increased production of ROS (Atwood et al. 1999, Pereira et al. 2005, Sayre 2005). Accumulation of metals is also associated with enhanced aggregation of proteins. Although oxidative stress can be easily associated with other pathological features of AD, it has been considered as being secondary to the pathogenesis. Oxidative stress is clearly an important neurodegenerative element that contributes to neuronal loss, and therefore plays a crucial role in disease processes. Furthermore, oxidative damage is also considered to be one of the earliest signs in AD but then to decrease with disease progression and NFT formation (Nunomura et al. 2001, Praticò et al. 2002, Lee et al. 2005). Additionally, protein aggregation, including Aβ, has been suggested to be protective against increased oxidative stress.

2.1.3.6. Other pathological features

Synaptic dysfunction in the absence of loss of synapses and neuronal cell death has been suggested to be an early event in AD and to occur prior to plaque formation (Selkoe 2002, Pereira et al. 2005). The synaptic loss is the best current pathologic correlate of memory and cognitive decline (Coleman and Yao 2003). On the other hand, some studies indicate that it is soluble Aβ which correlates significantly with the synaptic pathology in AD (Lue et al. 1999, McLean et al. 1999). A variety of mechanisms appear to contribute to synaptic dysfunction and have diverse consequences such as alterations in synaptic proteins, membrane lipids, vesicular function and loss of plasticity (Coleman et al. 2004). The initial triggers may be Aβ toxicity or disrupted intracellular transport of aggregated tau (Pereira et al. 2005). In addition, excitotoxicity, oxidative stress, and apoptosis have all been claimed to contribute to synaptic dysfunction.

It is evident that the final crucial event in AD pathogenesis is cell death. In 1972, Kerr et al described two types of cell death called necrosis or apoptosis (Kerr et al. 1972, Sastry and Rao 2000). Necrosis is characterised as "passive atrophy" that is a result of injury and it is a process that triggers inflammation. In contrast, apoptosis is described as "active energy-dependent degeneration" that is required for normal development and involves activation of a complex intrinsic cellular suicide program. Increasing evidence suggests that apoptosis is the major type of cell death involved in neurodegenerative disorders although inflammation is also involved in the pathogenesis (Loo et al. 1993, Su et al. 1994, Smale et al. 1995). In CNS, potential inducers of apoptosis include neurotrophic factors, changes in potassium concentration, calcium dysregulation, modulators of protein phosphorylation, DNA damage, neurotransmitters (i.e. glutamate), peptides and proteins (i.e. Aβ), oxidative stress, nitric oxide, lipids (i.e. retinoic acid), irradiation, and certain neurotoxins (i.e. ethanol) (Sastry and Rao 2000).

2.1.4. Diagnosis

2.1.4.1. Clinical diagnosis

There is no laboratory test for the diagnosis of AD. Today, the diagnosis of AD is mainly based on clinical and neuropsychological findings, combined with the exclusion of other possible causes for dementia. The most commonly used clinical criteria were outlined two decades ago by the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA) (McKhann et al. 1984). Patients with probable AD manifest a gradual onset and progression of memory and cognitive decline, and do not show signs or findings of other causes for dementia. In contrast, the diagnosis of possible AD is more complex and given in the presence of i) dementia syndrome in the absence of other neurologic, psychiatric, or systemic disorders than AD with variations in the typical course of the clinical picture, ii) secondary brain disorder/s sufficient to cause dementia although AD is suspected to be the major cause of dementia, and iii) gradually progressive severe cognitive deficit in the absence of other identifiable causes. According to NINCDS-ADRDA, the diagnosis of possible and probable AD can be confirmed to be definite only by neuropathological examination at either biopsy or autopsy. The diagnosis made by NINCDS-ADRDA has high accuracy (80-90%) according to the data obtained from patients with severe AD and repeated examinations (Galasko et al. 1994,

Kosunen et al. 1996). However, the accuracy is acknowledged to be lower when diagnosing patients with early stages of AD or with mild cognitive impairment (MCI), and in the absence of repeated examinations (Blennow and Hampel 2003). The earliest cases of AD can be found among individuals suffering from MCI. Currently, the most commonly used criteria for the diagnosis of MCI has been forwarded by the Mayo Clinic Alzheimer's Disease Research Center (MCADRC) (Petersen et al. 1999, Petersen 2004, Chong and Shavadevan 2005). The clinical criteria for amnestic MCI (MCI with memory impairment) require the presence of memory complaint, objective memory impairment for age, preserved general cognitive function, normal functional activities, and absence dementia. However, MCI is an aetiologically heterogeneous condition and its prognosis varies in different studies and populations. Conversion rates from MCI to dementia up to 12% annually, and 80% at six years' follow-up have been described (Petersen 2004).

The current diagnosis of AD is far from straightforward and the accuracy often varies between health care centers. Brain imaging, particularly magnetic resonance imaging (MRI) can strengthen the diagnosis of AD. The decrease in volumes of hippocampus and entorhinal cortex detected by MRI has been suggested to be useful in the diagnosis of AD, and even in predicting MCI patients who will develop AD (Laakso 2002, Chong and Shavadevan 2005). Still, there is a great need for an equivocal diagnostic test for AD and progressive MCI that could be routinely applied in laboratories. Until now, candidate biomarkers have been sought from the CSF of AD patients. Since 1992, a large number of research groups have examined the suitability of total tau, phosphorylated tau, total Aβ, Aβ40 and Aβ42 as possible biomarkers for AD. Consistent findings have included a reduction of Aβ42 or the ratio of Aβ42/Aβ40, and an increase of total and phosphorylated tau in CSF in AD patients as compared to controls (Blennow and Hampel 2003). Today, the best outcome in early diagnosis and prediction of progressive MCI has been reached by combining these Aβ and tau markers (Herukka et al. 2005). In general, the current tests achieve good specificity but unsatisfactory sensitivity (Frey et al. 2005). Nonetheless, they are suitable for routine use in specialized units.

2.1.4.2. Neuropathological diagnosis

As is the case with clinical diagnosis, several classifications also exist when performing neuropathological examinations for AD. The foundations for the currently used criteria were laid by Khachaturian in 1976, and were based on detecting both senile plaques of any type and

tangles (Khachaturian 1985). In 1991, new criteria were introduced by the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) (Mirra et al. 1991), which are based on the presence or absence of plaques, clinical dementia, and possibly other neuropathological lesions that might influence memory or cognition. CERAD divides AD into four possible classifications being normal, possible, probable, and definite. In 1991, Braak and Braak, suggested a pattern for progression of AD depending on the presence of NFTs in hippocampal brain regions. The severity of AD was thus assessed as entorhinal (I-II), limbic (III-IV), and isocortical (V-VI) stages (Braak and Braak 1991b). The most recent criteria were established by the National Institute on Aging- Reagan Institute (NIA) (1997), which follow the guidelines of both CERAD and Braak and Braak, supplemented with some additional immunohistochemical approaches.

It is quite common that cognitively intact subjects possess sufficient levels of brain pathology to fulfill the criteria of AD. For example, 49% of dementia free subjects have been diagnosed with AD when following the Khachaturian criteria, 18-25% met the CERAD criteria for possible AD; and 10-12% fullfilled the NIA criteria for intermediate likelihood for dementia (reviewed by Keller 2006). One dilemma is the long preclinical phase in AD. Although the subjects are free of clinical symptoms, it is not possible to determine whether their brain changes are due to the preclinical phase of AD. It is unclear whether significant amyloid pathology can develop during disease-free aging.

2.2. THE ERA OF PROTEOMICS

2.2.1. From genome to proteome

The human genome sequencing was completed in 2002. One of the most surprising findings in the project was that humans have hardly any more genes than a fly or a worm (Southan 2004). As a consequence, it was recognised that human complexity cannot be solely explained by the genome, and the attention turned to examining the changes of proteins in a given genome at a given time (Anderson et al. 2001). The word proteome (=the entire PROTein complement expressed by a genOME, or by a cell or a tissue type at a given time) was first coined by Wilkins et al. in 1995 (Wilkins et al. 1996). Between individuals, the proteome differs not only depending on the genome but also dynamically due to any internal and external stimuli i.e. physiological state, health, disease, age, stress, and drugs. After

transcription, proteins can be modulated at many points prior to PTMs that finally determine the functional consequences inside the cell (Figure 4) (Banks et al. 2000). Ultimately, proteome analysis is like taking a photograph of the expression pattern of modified proteins and their isoforms at a particular time.

Proteomics has become an expanding multidisciplinary technology-driven area of science focused on studying structures, functions and levels of proteins (Fountoulakis 2004). These disciplines are called structural, functional, and expression proteomics. The latter is currently the most advanced and most widely studied of the known disciplines in clinical proteomics (Aldred et al. 2004). Although proteomics as an entity is relatively new, the methodological and theoretical foundations have been under development for more than three decades (Anderson et al. 2001, Righetti 2004). Methodologically, expression proteomics aims at i) separation of complex protein mixtures and ii) quantification and identification of these proteins. Theoretically, the goal of proteomics is to study changes of multiple proteins simultaneously. In fact, this concept was already introduced in 1981 by Clark as "towards the total protein map" who forecast the separation and characterization of thousands of proteins simultaneously. Though there have been major methodological and theoretical developments in proteomics, it is still considered to be in its infancy, and to belong to so called "New Technologies" (Fountoulakis 2004).

In the future, downstream steps after genomics and proteomics will aim at understanding functional consequences of biomolecule interactions in different biological pathways in a system. This will involve other overlapping “–omics” such as peptidomics, metabolomics, interactomics etc. The main goal is to understand how living organisms function as a whole. The knowledge of all “–omics” gathered together is called systems biology.

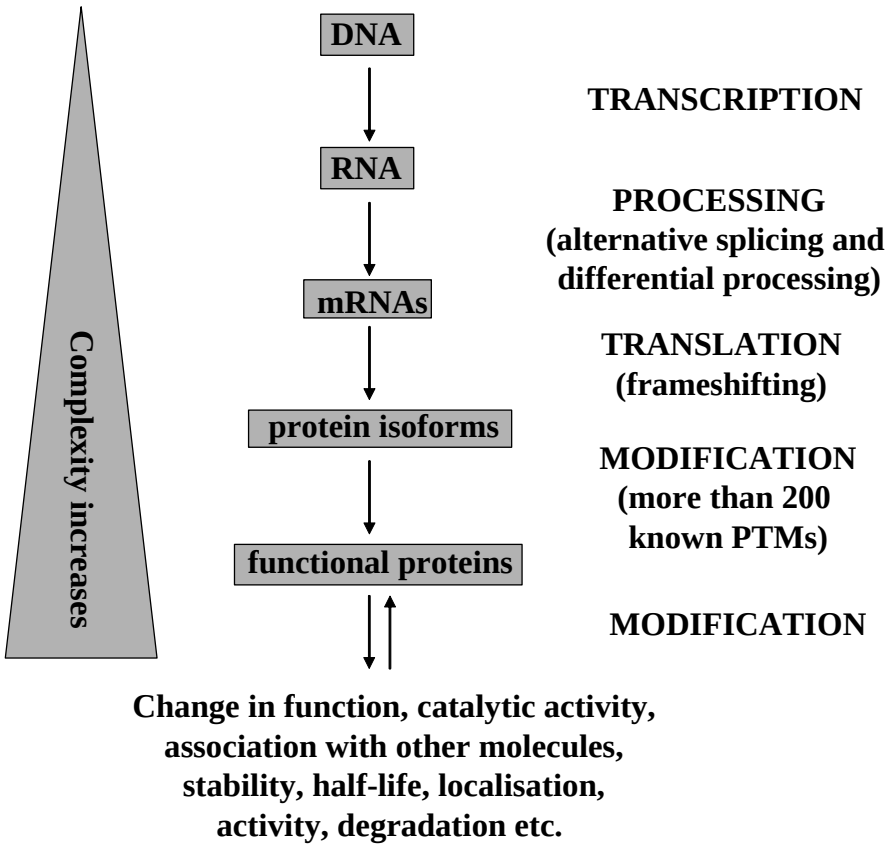


Figure 4. The complexity increases from the genetic code which is made up of four nucleic acids in DNA up to proteins created from twenty amino acids. The size of human genome consist of about 3000 megabasepairs, giving rise to an estimate of about 25 000 to 30 000 genes (Southan 2004). Messenger RNA can be spliced in various ways but the amount of RNA does not necessarily correlate with the amount of active protein in a cell. The number of protein isoforms per gene has been estimated to be greater than ten. Moreover, after being synthesised, proteins are subject to a variety of modifications that take place in different cellular compartments including nucleus, cytosol, endoplasmic reticulum, and Golgi apparatus. In other words, modified proteins are the time-dependent output of a cell, and thus the functional output cannot be predicted from analysis of nucleic acids alone. The function of a protein may also vary during its lifespan; depending on modifications and cellular localisations i.e. proteins can be multifunctional. Despite the existence of tight regulatory controls, it is reasonable to assume that alterations in processes from genes to functional proteins may lead to a variety of pathological conditions. (Modified from Banks et al 2000)

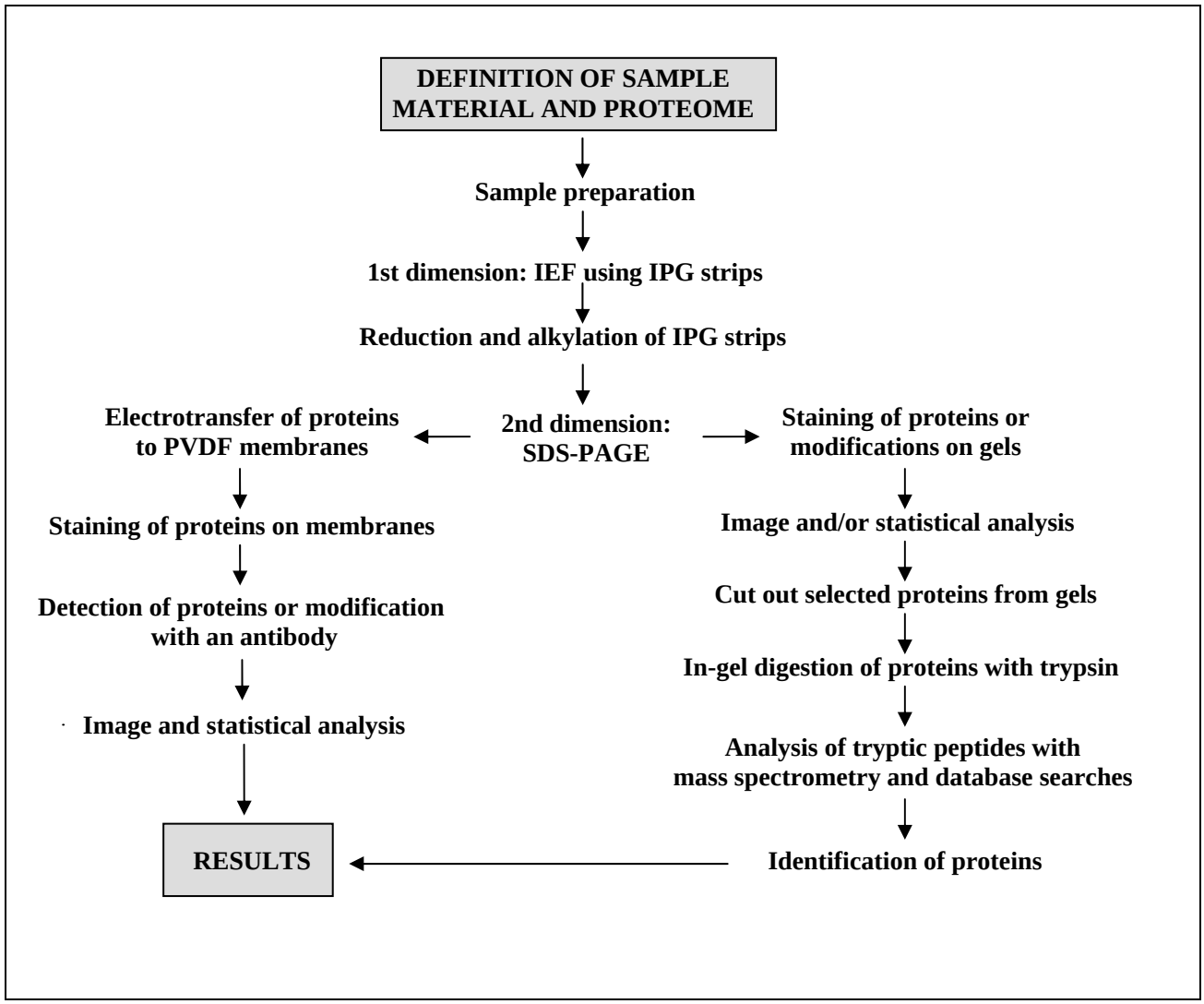


Figure 5. Common workflows in two-dimensional gel electrophoresis in combination with immunoblotting and mass spectrometry. After preparation, samples are applied to immobilized pH gradient (IPG) strips by either in-gel rehydration or by cup-loading and then the proteins are separated according to their isoelectric points by isoelectric focusing (IEF) in the first dimension. Proteins on the IPG strips are reduced and alkylated, and laid on the top of a gel. In the second dimension, proteins are separated usually by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to their molecular weight. Proteins are visualised normally by Coomassie brilliant blue, silver staining or fluorescent dyes followed by scanning in order to convert the gels into a digital format. The levels and modifications of proteins on two-dimensional (2-D) gels are then determined by image analysis. Alternatively, levels of proteins and selected modifications can be studied by specific stains, immunoblotting and image analysis. Stained proteins on 2-D gels can be cut out for in-gel digestion and identification by mass spectrometry and database searches.

2.2.2. Proteome analysis

Though there have been promising alternative and new techniques introduced into proteomics (Graham et al. 2005), 2-DE in combination of mass spectrometry and/or immunoblotting still remains the most widely used methodological approach in diffrential and quantitative proteome analyses. The traditional workflow usually includes i) sample preparation and protein solubilisation, ii) separation of proteins by 2-DE, iii) protein detection, iv) 2-D image analysis and quantification, v) identification of selected proteins (Figure 5).

2.2.2.1. Sample preparation for electrophoresis

Definition of sample material and solubilisation of proteins, are some of the most important steps in 2-DE. Currently, it has been emphasized that it is important to define what type and kind of a proteome population is intended to be studied i.e.: is it the proteome of one particular type of cell, a subcellular organelle, a cytosolic or a membranic fraction? Understandably but unfortunately, there is no single powerful method for sample preparation that can be applied to all kinds of samples to be analysed by 2-DE, since the chemical nature of proteins is extremely diverse in different tissues and localisations. The three fundamental steps in sample preparation are cell disruption, protein inactivation plus removal of interfering substances, and solubilisation of proteins of choice. Additionally, in some cases, proteins need to be enriched or prefractionated. It is desirable that the sample preparation should be as simple and reproducible as possible (Görg et al. 2004).

2.2.2.2. Two-dimensional gel electrophoresis

2-DE is based on the separation of proteins, first according to their pI in the first dimension and then according to their Mr in the second dimension. It has taken decades for 2-DE to develop as the fundamental base of proteomics. The use of polyacrylamide in electrophoretical separations was first introduced by Raymond and Weintraub in 1959 whereas the basic theory of IEF in combination with carrier ampholytes (CAs) as a way to obtain an isoelectric distribution of proteins took place during the 1960’s (Svensson 1961, Vesterberg and Svensson 1966, Dale and Latner 1969).

In the 1970's, the basis for the current 2-DE technology was established (Klose 1975, O'Farrell 1975). In the early studies, isoelectric focusing (IEF) was performed using CAs that have certain limitations i.e. poor reproducibility, cathodic drift and batch to batch variability. To overcome the limitations in conventional CAs, immobilized pH gradient (IPG) strips were introduced by Bjellqvist et al. in 1982. These IPG strips clearly improved the reproducibility and resolution of IEF. A stable pH gradient was created by using ampholyte molecules co-polymerised in the polyacrylamide gel. One of the most beneficial features of IPG strips as compared to CAs is that when an electric field is applied, the gradient does not drift. In addition, IPG strips have proved to be easily commercialised and currently there is a great variety of different pH gradients available for researchers i.e. 7-24 cm long IPG strips with wide (i.e. pH 3-12), medium (i.e. pH 4-7), narrow (i.e. pH 5.5-6.5) and ultra-narrow (i.e. pH 4.9.-5.3) pH ranges (Görg et al. 2000, Görg et al. 2004). Despite the technical improvements in IEF thanks to introduction of IPG strips, they have not supplanted conventional CAs that are still successfully used in several laboratories. To date, the largest 42 x 33 cm 2-D gels have been made using CAs and, remarkably, more than 10 000 polypeptides can be detected (Klose and Zeindl 1984). In addition, carrier ampholyte-based IEF still possesses several technical advantages as compared to IPG strips such as high tolerance to chemicals used for solubilisation of proteins and, thus it is more suitable for studying i.e. hydrophobic and membrane proteomes.

The latest innovation in 2-DE was the development of the differential in-gel electrophoresis (DIGE) technology (Ünlü et al. 1997, Lilley and Friedman 2004). It is based on labelling proteins with fluorescent Cy2, Cy3, and Cy5 dyes. All of these dyes have identical Mr and pI but differ in excitation and emission wavelengths. Therefore, up to three different protein mixtures can be labelled individually but separated and analyzed on a single gel. After scanning one gel, three separate images are obtained. The major advantages when considering clinical proteomics are that an internal protein standard may be labelled with one of the dyes, leading to better control of gel to gel variation and thus to better quantitative and qualitative image analysis of proteins.

2.2.2.3. Image analysis

One of the several reasons why 2-DE has become popular in clinical expression proteomics during the last decade has been the development of algorithms for convenient data acquisition to be able to compare 2-D maps and to quantify the proteins. Already in 1975, O'Farrell detected about 1100 *E.coli* proteins on 2-D images (O'Farrell 1975, Righetti 2004). Obviously, the analysis of the proteins by eye was comparable to looking at stars on the sky. The development of the first image analysis software began during the 1980's (Righetti 2004), and since then these programs have continuously improved. Currently, there are several 2-D image analysis software packages commercially available. However, the existing programs still need to be improved into faster and more automated analyses that do not require manual interventions by the user (Görg et al. 2004). The traditional workflow in image analysis includes spot detection, matching of proteins to corresponding spots on a reference gel, background subtraction, and quantification.

2.2.2.4. Protein identification by mass spectrometry

The new era in proteomics began during 1990's also thanks to the development of mass spectrometry (MS) techniques as well as the appearance of sequence databases on the World Wide Web. In electrophoresis-MS-based identification, proteins are first separated electrophoretically and excised from gels followed by proteolytical in-gel digestion e.g. with trypsin. The resulting peptides are analysed by MS and the proteins identified by database searches (Thiede et al. 2005).

The breakthrough in biological MS took place in the late 1980's in conjunction with the invention of electrospray ionization (ESI) (Fenn et al. 1989) and matrix-assisted laser desorption/ ionization (MALDI) (Tanaka et al. 1988, Karas and Hillenkamp 1988). The discovery and development of these currently widely used two ionization methods was recognized by the award of the 2002 Nobel Prize in Chemistry. ESI ionizes analytes out of solution and is often coupled to a liquid separation tool such as liquid chromatography (LC). MALDI is based on laser pulses bombarding samples mixed with matrix, attached to a steel probe, leading to desorption and ionisation of the peptides. A mass spectrometer consists of either ESI or MALDI ion source coupled to an analyser/s. There are several different types of

analysers that are all very different from each other, with their own strengths and weaknesses (Yates 1998, Aebersold and Mann 2003).

Protein identification can be divided into two categories: peptide mass analysis and partial peptide sequencing (Yates 1998). The simplest and fastest way to identify proteins is still considered to be the peptide mass fingerprinting (PMF) also called mass analysis. In PMF, the experimentally obtained masses of peptides are matched with the theoretical peptide masses generated from protein sequences stored in databases. Robust and high-throughput MALDI in combination with a time-of- flight (TOF) analyser is often used for PMF with a limitation that it can only handle quite simple peptide mixtures i.e. proteins isolated from a 2-D gel. In contrast to PMF, peptide sequencing using tandem MS (MS/MS), can be applied to identify proteins in more complex mixtures. In addition to peptide masses, the MS/MS spectra gives information about a part of the amino acid sequence of the peptide. LC-ESI-MS/MS is often used for peptide sequencing.

2.2.2.5 Gel-free proteome analysis

Although IPG-based 2-DE is currently the most commonly used technique in expression proteomics, and has recently experienced methodological improvements including DIGE technology, it is still time- and labour-consuming, and suffers from several limitations. Therefore, researchers are trying to develop quantitative proteomics methods towards alternative more automated, high-throughput, and gel-free systems (David et al. 2005, Graham et al. 2005, Schmidt et al. 2005). The most promising new gel-free systems that have been developed are based on LC-MS applications. Traditionally, these applications are able to answer the question: which proteins are present in a sample, but not: how much of a protein is in a sample (Reinders et al. 2004). In order to be able to obtain relative abundances of proteins in a sample using gel-free MS-based technical approaches, novel techniques for stable isotope protein labelling (i.e. by using isotope-coded affinity tagging of proteins called ICAT or peptides using iTRAQ^{TM}) have been introduced recently (Schneider and Hall 2005). Generally, in these approaches, two or more samples are labelled with distinguishable chemically identical reagents having slightly different molecular weights, proteins are digested, separated, and analysed by MS. The labelled peptides in each sample have defined size difference in their masses leading to analysis of quantitative differences of proteomes between groups. Moreover, proteins can be identified from MS/MS data or peptide

sequencing. There are also other approaches that may be able to resolve some of the problems in differential 2-DE i.e. antibody arrays and surface enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS) (Davis et al. 2005). Despite overcoming a number of problems in proteomics, none of the new approaches can yet substitute for 2-DE in separating and quantifying proteins in terms of the numbers of proteins, followed by robust and reliable identification by MS. In other words, there is no single powerful method in proteomics since all techniques are still considered to be complementary to each other.

2.2.2.6. Detection of protein modifications

The analysis of protein modifications represents a challenging task in clinical research. Modifications may be structurally extremely heterogeneous i.e. glycosylations where there are many carbohydrates that are difficult to analyze (Reinders et al. 2004). They can also be highly unstable e.g. as in the case of phosphorylation, which is an important modification involved in the activation and deactivation of about 50% of all proteins (Reinders and Sickmann 2005).

Some modifications can be deduced by examining protein patterns on a stained 2-DE gel or a membrane. For example, in 2-DE, phosphorylation and glycosylation are known to affect the charge carried by the proteins leading to a pearl-like horizontal trail of protein spots, and they may also affect the size of the protein (Mann and Jensen 2003). There are several commercially available staining kits and antibodies that can be used to stain or detect specifically modified proteins, respectively (Talent et al. 1998, Kaufmann et al. 2001, Patton 2002). Proteins can also be enriched based on the affinity of the modifications prior to analysis i.e. by using lectins to separate glycoproteins (Hage 1999, Mann and Jensen 2003). Alternatively, proteins can be separated by 2-DE before and after the enzymatic removal of the modifications (Mann and Jensen 2003). "Disappearing" groups indicate the presence of modified proteins.

If modifications of intact proteins are to be studied on a large-scale in more detail, 2-DE in combination with other methods is currently the approach of choice. Moreover, a multiplexed proteomics platform has recently been introduced and allows the parallel determination of levels and modifications of proteins. The multiplexed proteomics technology utilizes fluorophores with different emission and/or excitation wavelengths to study multiple targets

on a single gel or a membrane (Patton 2002). Modifications of proteins and their sites on the peptide backbone can be studied by a variety of traditional MS approaches but the protein usually needs to be isolated and digested prior to analysis. However, the levels and modifications of multiple proteins can be studied after digestion using MS-based stable isotope protein labelling technologies (i.e. ICAT or iTRAQ^{TM}) but these large-scale approaches still remain troublesome partially due to the lack of sophisticated software and databases for data analysis.

2.3. 2DE-BASED EXPRESSION PROTEOMICS AND AD

2.3.1. Protein changes

Even if expression proteomics is still considered to be methodologically in its infancy, the technology has been claimed to represent an efficient tool in finding new biomarkers or a panel of markers for more accurate diagnosis of complex human diseases. Until now, most efforts in screening for new biomarker candidates have been concentrated in cardiovascular, infectious, and neurological disorders as well as in several types of cancers (Banks et al. 2000). When considering neurological disorders, one good example for the usability of 2-DE in exploring new biomarkers, was the discovery of two unknown protein isoforms p130 and p131 that were suggested to be able to discriminate Creutzfeld-Jakob disease from other types of dementia (Harrington et al. 1986). Subsequently, these proteins were identified as 14-3-3 proteins and further studies confirmed the usefulness of this finding. Notably, the measurement of 14-3-3 has now been included in the diagnostic criteria of Creutzfeld-Jakob disease (Zerr et al. 2000, Ingrosso et al. 2002). Expression proteomics has also been suggested to be the solution in improving the diagnosis of AD, even in its early stages, as well as to enable the follow-up of the progression of the disease. AD has clearly been one of the major focuses of research in this field. Yet, the current findings represent the tip of the iceberg in the search for diagnostic markers and uncovering the secrets of AD pathogenesis.

2.3.1.1. Brain

Most efforts in understanding the pathogenesis using 2-DE-based expression proteomics have been made by comparing brain proteomes of AD patients and controls. Some of the first 2-DE studies examined the levels of AD brain proteins (Comings 1982c, Comings 1982b, Comings

1982a, Smirnov et al. 1991, Burbaeva 1992, Mattila and Frey 1994). Initial studies revealed alterations in the levels of a number of proteins i.e. GFAP, tubulin, and creatine kinase. Subsequently, the number of 2-DE studies has multiplied and at present changes in the levels of more than 100 brain protein isoforms have been identified in neurodegenerative disorders (Butterfield et al. 2003, Fountoulakis 2004, Vercauteren et al. 2004). Despite the multiplicity of isoform specific protein changes, the findings still remain rather fragmented, for example, they have not revealed unified or even novel hypothesis related to the pathogenesis of AD.

2.3.1.2. Cerebrospinal fluid

Although the brain is the target organ in AD, it is obvious that post-mortem studies have several drawbacks which complicate any elucidation of the pathology in living patients. CSF is considered as a window into brain metabolism since it is in contact with extracellular space in brain, and thus its contents partially reflect cerebral metabolic changes. Approximately 20% of CSF proteins are derived from brain. The first work on 2-D distribution of CSF proteins in AD was carried out already 1973 by Latner but the methodology in use then was very different from the current 2-DE (Dale and Latner 1969). Later, several of the early 2-DE studies failed to identify AD-specific changes in the CSF proteome (Harrington and Merril 1984, Harrington et al. 1985, Alafuzoff et al. 1986, Harrington et al. 1986, Townsend et al. 1987). In the early 1990's, an isoform of haptoglobin-alpha-1 chains was found to be present in the majority of 2-D maps in AD vs. age-matched controls. These findings were considered to reflect an altered blood-brain-barrier function in AD (Mattila et al. 1994). Recent studies have reported various differences in the CSF proteome by comparing AD patients and controls (Hesse et al. 2001, Choe et al. 2002, Davidsson et al. 2002, Puchades et al. 2003). In the studies of Davidsson et al. 2002 and Puchades et al. 2003, a large number of protein isoforms with altered levels were identified in AD. One common finding in these studies was that the levels of most proteins tended to be decreased in AD vs. controls although some proteins showed increased levels. These findings may reflect brain pathology since several of the identified proteins are involved in inflammatory events and, are known to be associated with the protein aggregates present in the AD brain.

2.3.1.3. Blood

Although the lumbar puncture needed to obtain CSF is relatively safe, it is still an invasive and painful procedure that is not routinely performed, except in specialized centres involved in research trials. An increasing amount of evidence has revealed numerous abnormalities in peripheral tissues e.g. in blood and skin cells in AD (Blass and Zemcov 1984, Scott 1993). Therefore, some studies have focused on the possible AD-specific proteome changes in blood and other peripheral tissues. The first 2-DE study of peripheral changes in AD identified an abnormal expression of actin in lymphocytes (Jabbour et al. 1992). Thereafter, variations in the proteomes of red blood cell membrane, platelets and lymphocytes were detected between AD patients and controls (Mattila and Frey 1995). One of the proteins consistently identified was the cytoskeletal protein, actin, which exhibited reduced levels in AD platelets and lymphocytes as compared to controls.

When considering the availability of blood samples for research and the current state of art in proteomics, it may seem surprising that there are only few published studies that have mined biomarkers in plasma and sera of AD patients (Alafuzoff et al. 1986, Ai et al. 1989, Ueno et al. 2000, Zhang et al. 2004). However, in blood, the most abundant proteins i.e. albumin and immunoglobulins are considered to obliterate the less abundant ones. In fact, the dynamic range of plasma protein concentrations may comprise ten orders of magnitude. The disease-specific biomarkers are suspected to be seen in concentrations of the less abundant proteins i.e. in the "deep proteome" consisting of only 1% of proteins in the whole blood proteome (Righetti et al. 2005). In order to study the less abundant proteins, several prefractionation methods and steps are required. Moreover, the removal of high abundance proteins may concomitantly remove associated proteins. The problem of the "deeper proteome" is not limited to blood since biomarker research and expression proteomics using other body fluids also suffer from the wide range of protein concentrations.

2.3.2. Post-translational modifications

Despite the recent developments in the detection of modified proteins, these techniques have rarely been used to study proteomes of AD patients. In 2-DE studies, modifications are still mostly reflected on the basis of isoform distribution. Oxidation, phosphorylation and glycosylation are a few examples of modifications that have been studied in AD.

2.3.2.1. Oxidation

Carbonylation as a sign of oxidation is currently the most common modification of proteins which has been studied in AD using 2-DE based proteomics. It is based on ROS oxidising amino acid residue side-chains into ketone or aldehyde derivatives called protein-bound carbonyls (Levine et al. 1990, Berlett and Stadtman 1997, Dalle-Donne et al. 2003b). Carbonyl groups can also be introduced into proteins by lipid peroxidation or glycation. Carbonylated proteins can be derivatised, and studied by two-dimensional (2-D) oxyblotting based on separation of proteins by 2-DE followed by immunodetection of the derivatised carbonylated proteins (Shacter et al. 1994, Nakamura and Goto 1996). Several carbonylated proteins have recently been characterised by 2-D oxyblotting in AD plasma and brain (Aksenov et al. 2001, Castegna et al. 2002a, Castegna et al. 2002b, Choi et al. 2002, Choi et al. 2004, Choi et al. 2005, Sultana et al. 2005) (Table 1).

2.3.2.2. Phosphorylation and glycosylation

Phosphorylation is the most widely studied modification related to AD. Therefore, it is surprising that no large-scale clinical 2-DE studies on phosphoproteomes of AD patients have been conducted, though there are 2-D immunoblotting studies on tau phosphorylation (Ksiezak-Reding et al. 1990, Sergeant et al. 1997, Tolnay et al. 2002). Also, increased phosphorylation of elongation factor 2 has been demonstrated in AD brain by the 2-D approach (Johnson et al.1992).

Table 1. Oxidised proteins and their levels in AD patients as compared to controls as revealed by 2-DE and 2-D oxyblotting.

No. of patients		*Name of protein*	*Tissue*	*Deregulated levels in AD*	*Deregulated oxidation in AD*	*Participates in*	*References*
Control	*AD*						
6	6	Beta actin	SMT	NK	increase	cytoskeletal component	Aksenov et al. 2001
6	6	Creatine kinase BB	SMT		increase	stress response	Aksenov et al. 2001
5	5		IPL		increase		Castegna et al. 2002a
7	7		H	decrease			Schonberger et al. 2001
5	5	Glutamine synthase	IPL		increase	glutamate-glutamine cycle,	Castegna et al. 2002a
6	6		CC	decrease (total)		astrocytic	Robinson 2000
5	5	DRP-2	IPL		increase	neuronal protein, synaptic	Castegna et al. 2002b
6	6		H	decrease	increase	transmission	Sultana et al. 2005
7	7		CG	decrease			Schonberger et al. 2001
5	5	HSC 71	IPL		increase	stress response	Castegna et al. 2002b
11	10		TP	decrease			Yoo et al. 2001
5	5	Alpha enolase	IPL		increase	glycolysis	Castegna et al. 2002b
6	6		H	increase	increase		Sultana et al. 2005
7	7		H	increase			Schonberger et al. 2001
7	7		CG	increase			Schonberger et al. 2001
5	5	UCH-L1	IPL		increase	proteolytic degradation of	Castegna et al. 2002a
5	5		FC	decrease	increase	proteins	Choi et al. 2004

6	6		H	increase	increase		Sultana et al. 2005
7	7		EC	decrease			Schonberger et al. 2001
5	5	SOD 1	FC	increase (total)	increase	stress response, antioxidant enzyme	Choi et al. 2005
7	7		H	increase			Schonberger et al. 2001
9	9	Alpha-1-antitrypsin	plasma		increase	acute phase protein	Choi et al. 2002
7	7	precursor	CSF	increase			Puchades et al. 2003
9	9	Fibrinogen-γ-chain	plasma	NK	increase	polymerises into fibrin and involved in platelet aggregation	Choi et al. 2002
6	6	Pin-1	H	decrease	increase	cell cycle, chaperone	Sultana et al. 2005
6	6	PGM1	H	decrease	increase	glycolysis	Sultana et al. 2005
6	6	Gamma-SNAP	H	increase	increase	secretory vesicular transport, neurotransmittor release	Sultana et al. 2005
6	6	TPI	H	increase	increase	glycolysis	Sultana et al. 2005
6	6	Carbonic anhydrase II	H	increase	increase	acid-base balance	Sultana et al. 2005

DRP-2, dihydropyriminidase-related protein; Gamma-SNAP, gamma synaptosomal protein like soluble A-ethylmaleimide-sensitive factor attachment proteins; HSC 71, heat shock cognate 71; PGM1, Phosphoglycerate mutase; Pin-1, peptidyl prolyl cis-trans isomerase; SOD1, TPI, triosephosphate isomerase; UCH-L1, Ubiquitin carboxyl-terminal hydrolase isozyme L1
CC, cerebral cortex; CG, cingulate gyrus; FC, frontal cortex; EC, entorhinal cortex; H, hippocampus; IPL, interior parietal lobe; SMT, superior and middle temporal gyri,;NK, not known

There are few clinical 2-DE-based studies into glycosylation. An interesting approach was applied by Yu et al. 2003 who examined native and oxidised glycoproteins in AD plasma. They first isolated glycoproteins by affinity chromatography with heparin-agarose and Concavalin A. Thereafter, 2-D oxyblotting and evaluation of the levels of glycoproteins were carried out. They found increased amounts of glycosylated transferrin, hemopexin and α-1-antitrypsin (α-1-AT) in AD patients as compared to controls. Both glycosylated hemopexin and transferrin also exhibited increased levels of oxidation in AD. Moreover, in 2003, Desrosier et al. reported using 2-immunoblotting that the levels of serum melanotransferrin or affinity purified glycosylated isoforms are not suitable diagnostic markers for AD. Another type of approach was applied by Kanninen et al. 2004 using multiplexed proteomics. They separated the most abundant cytosolic brain proteins by 2-DE and stained glycoproteins with Pro-Q Emerald 300, and measured the levels of proteins after Sypro Ruby staining. About 50 glycoproteins and 150 proteins were detected on 2-D gels, suggesting that more than 30% of all proteins may be regulated by glycosylation. Altered levels of glycosylation were found for collapsin response mediator protein 2 and an unidentified protein. GFAP tended to be more glycosylated in AD, and heat shock protein 71 and creatine kinase BB were identified as glycoproteins.

2.4. MULTIPLE USES OF CLINICAL PROTEOMICS

Clinical proteomics aims at both individual patient monitoring and at achieving a better understanding of disease processes manifested by typical symptoms and biochemical alterations. From the diagnostic point of view, the aims are to find early disease stage markers, to be able to evaluate the risk of developing a disease, to follow-up the progression of a disease, to predict the prognosis of a disease, and to be able to monitor the effects of treatment. From the point of view of drug discovery and pharmacology, proteomics may assist in faster development and validation of molecules for medical intervention in disease processes.

3. AIMS OF THE STUDY

Proteomics is attracting an increasing amount of interest in its applications to better understand disease processes, to search for new biomarkers, and to accelerate drug development. Accordingly, the initial aim of the present studies was to establish 2-DE and 2-D immunoblotting methods for studying both the levels and post-translational modification of individual proteins. In addition, our working hypothesis was that proteomic analysis would reveal new protein changes that are related to the pathogenesis of AD, and could be potential biomarkers for the diagnosis of AD.

The specific aims of the present study were:

1) To develop a multiplexed proteomics approach for simultaneous examination of the levels and oxidation of multiple proteins in AD. (**I**)

2) To apply the multiplexed proteomics approach for quantification of oxidatively modified proteins in brain and CSF of AD patients. (**I, II, IV**)

3) To develop other approaches to study PTMs such as glycosylation and phosphorylation in AD; as an example we chose GFAP. (**III**)

4) To analyse the relationship of protein changes between clinical and neuropathological data. (**II**-**III**)

4. MATERIALS AND METHODS

4.1. STUDY SUBJECTS

4.1.1. Brain samples

The post-mortem tissue was obtained from the Kuopio Brain Bank, Kuopio University Hospital. The permission for sampling of brain tissue was granted by the Finnish National Board of Medical Legal Affairs, and the study was approved by the Ethical Committees of the Kuopio University Hospital and University of Kuopio. All patients were institutionalised and evaluated clinically by a neurologist prior to death. The clinical diagnosis of probable AD was based on the consensus criteria of NINCDS-ADRDA (McKhann et al. 1984) and the DSM-III-R criteria (American Psychiatric Association 1987). For the assessment of AD pathology, subjects were classified into neuropathological groups as recommended by the guidelines established by Braak & Braak (1991a, 1991b) and by CERAD. AD patients were selected based on the severity of dementia (Braak stages V-VI) and the absence of histological changes other than those related to AD. Control cases were age-matched and classified as normals according to CERAD and represented Braak and Braak stages I-IV (Mirra et al. 1991). Further, the retrospective evaluation of medical records of controls did not indicate any signs of cognitive impairment or other neurological deficits. APO E genotype was characterised as previously described (Hixson et al. 1990, Tsukamoto et al. 1993). Patient demographics are presented in Table 2.

Table 2. Patient demographics

Study	*Diagnosis*	*n*	*Women/ Men (n)*	*Apo E ε4+ (n)*	*Age (years)*	*Duration of CI (years)*	*PMD (h)*	*Brain weight (g)*
I , II	Control	9	2/7	1	82±2	-	13±3	1322±7
	AD	10	9/1	7	83±2	12±4	5±1	1111±3
III	Control	12	4/8	2	80±3	-	20±5	1320±5
	AD	12	11/1	9	83±2	11±1	5±1	1113±4

AD, Alzheimer's disease; Apo E ε4+, number of subjects having one or two copies of apolipoprotein E ε4 allele; Duration of CI, duration of clinical impairment; PMD, post-mortem delay

4.1.2. Cerebrospinal fluid samples

CSF samples were collected as a part of the ongoing biomarker project, and obtained from eleven AD patients and from nine neurological controls showing no cognitive decline (Table 3). Informed consent of participation in the study was obtained from all subjects and caregivers of demented patients. The study was approved by the local ethics committees of University of Kuopio and Kuopio University Hospital. The diagnosis of probable AD was established according to NINCDS-ADRDA criteria (McKhann et al. 1984). Mini Mental State Estimation (MMSE) is a commonly used short test battery for screening of cognitive status and it was used to assess the severity of AD (Folstein et al. 1975). Lumbar puncture was performed using a standardised protocol followed by immediate freezing and storage of CSF samples at -70^{o}C until use.

Table 3. Patient demographics.

Study	*Diagnosis*	*n*	*Women /Men (n)*	*Apo E ε 4+ (n)*	*Age (years)*	*Duration of CI (years)*	*MMSE (score)*
IV	Control	9	3/6	4	65±3	-	24±1
	AD,MCI/AD	11	8/3	8	74±3	2±0	21±1

AD, Alzheimer's disease; Apo E ε4+, number of subjects having one or two copies of apolipoprotein E ε4 allele; Duration of CI, duration of clinical impairment; MMSE, scores obtained in Mini Mental State Examination

4.2. NEUROPATHOLOGY AND IMMUNOHISTOCHEMISTRY

According to the dissection protocol used in the Kuopio University Hospital, the brains were weighed, evaluated for grossly detectable lesions and vessel abnormalities. For electrophoreses, tissue samples were obtained from the left hemisphere, frontal cortices (Broadman area 9). The fresh brain specimens were stored immediately after dissection at -70 ^{o}C until use. The right hemisphere was fixed in 10% buffered formalin for at least one week and cut in coronal slices of 1 cm thickness. For diagnostic purposes, brain specimens were taken from 15 standard cortical and subcortical regions, embedded in paraffin and cut into 7μm-thick sections that were stained routinely applying haematoxylin and eosin and modified Bielschowsky silver impregnation. In brain proteome studies **II** and **III**, data of immunohistochemistry were taken into consideration in order to further assess the quantitative pathology in relation to the proteomics data. The quantification of Aβ aggregates, PHF-tau, GFAP labelled RAs and HLA DR labelled AM was performed as previously described (Kraszpulski et al. 1998, Alafuzoff et al. 1999, Overmyer et al. 1999b, Overmyer et al. 1999a)

4.3. SAMPLE PREPARATION FOR ELECTROPHORESIS

In studies **I**−**III**, brain samples were homogenised in 50mM Tris-HCl, pH 7.4, 1mM EDTA and protease inhibitor cocktail Complete (Roche Molecular Systems, Almeda, CA, USA). Soluble fractions containing equal amounts of Tris-HCl soluble proteins were separated by centrifugation at 13 800xg at 4^{o}C for 15 minutes prior to precipitation of proteins with 10% TCA on ice for 30 minutes. Precipitates were then washed three times with ethanol-ethyl acetate (1:1, V/V). In study **IV**, individual CSF samples containing equal amounts of protein were precipitated with ice cold acetone (1:2, V/V) and incubated overnight at -20 ^{o}C followed by a centrifugation at 5000 x g at 4^{o}C for 15 minutes. After precipitation, proteins were dissolved in the appropriate sample buffer for either one-dimensional gel electrophoresis (1-DE) or 2-DE experiments.

4.4. TWO-DIMENSIONAL GEL ELECTROPHORESIS

Precipitated proteins were dissolved in sample buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 20 mM dithiothreitol (DTT), and a trace of bromophenol blue (BPB). Additionally, in studies **I** and **II**, 0.5% of IPG buffer was added to the sample buffer prior to IEF. In studies **III** and **IV**, the amount of IPG buffer was increased to 1% since it seemed to improve the resolution of proteins on 2-D images. Isoelectric focusing was performed using IPG strips (Amersham Biosciences) and an IPGphor focusing unit (Amersham Biosciences, Uppsala, Sweden). A more detailed description of the IEF conditions is presented in Table 4.

Table 4. Summary of IEF conditions in each study.

Study	*Tissue*	*pI range*	*Amount of IPG buffer (%)*	*IPG strip length (cm)*	*Duration of IEF (Vhr)*
I, II	Brain	3-10 NL	0.5	7	11 500
III	Brain	4-7	1	7	11 500
IV	CSF	3-10 NL	1	18	32 500

IEF, Isoelectric focusing; IPG, immobilised pH gradient; pI, isoelectric point

Before the 2nd dimension SDS-PAGE separation, IPG strips were removed from the IPGphor apparatus, reduced and alkylated with a solution containing 50mM Tris-HCl (pH 8.8), 6M urea, 30% (v/v) glycerol, 2% SDS, and a trace of BPB. Additionally, the reduction step contained 1% DTT, and the alkylation step 3.5% iodoacetamide (IAA). In brain proteome studies **I-III**, proteins were separated according to their Mr on 10% SDS-PAGE with Mini Protean II (Bio-Rad, Hercules, CA, USA). In the CSF study **IV**, proteins were separated by 12.5% SDS-PAGE with Protean II (Bio-Rad, Hercule, CA, USA).

4.5. ONE-DIMENSIONAL GEL ELECTROPHORESIS

Precipitates of brain protein extracts were first dissolved in Laemmli sample buffer (Laemmli 1970) and separated according to their Mr using 10% SDS-PAGE with Mini Protean II (Bio-Rad, Hercules, CA, USA) (**II-III**).

4.6. IMMUNOBLOTTING

After either 1-DE or 2-DE, proteins were electrotransferred to Hybond-P (Amersham Biosciences) PVDF membranes in Towbin transfer buffer by using Mini TransBlot (Bio-Rad) (**I-III**) or Protean II Transfer apparatus (Bio-Rad) (**IV**). Membranes were then blocked with 5% skimmed milk in PBS-Tween, and incubated with the primary antibody overnight at 2-8 ^{o}C. Membranes were washed with PBS-Tween and incubated with the secondary Cy5-labelled antibody for two hours at room temperature followed by washing with PBS-Tween. All secondary antibodies were purchased from Jackson Immunoresearch Laboratories, West Grove, PA, USA. Fluorescence signals were detected by fluoroimager Storm 860 (Amersham Biosciences, Uppsala, Sweden) at the wavelength of 635nm. The primary antibodies used for immunoblottings are listed in Table 5.

Table 5. Primary antibodies for immunoblotting.

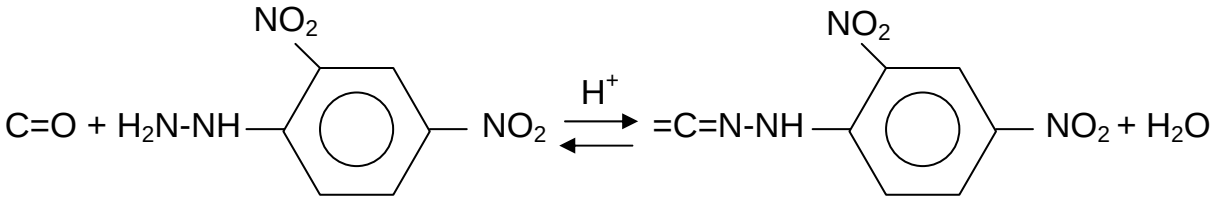
Study	*Name*	*Application*	*Dilution*	*Source*
I-IV	Rabbit anti-DNP	2-D	1:1000	Dako, Glostrup, Denmark
II,III	Rabbit anti-cow GFAP	1-D	1:3000	Dako
III	Rabbit anti-cow GFAP	2-D	1:1500	Dako
II	Sheep anti-pig heart MDH1	1-D	1:1500	Rockland, Gilbertsville, PA, USA
II	Rabbit anti-bovine liver GDH	1-D	1:1500	Rockland

Application, indication of 1-D or 2-D immunoblotting; DNP, dinitrophenyl; GFAP, glial fibrillary acidic protein; MDH1, cytosolic malate dehydrogenase, GDH, glutamate dehydrogenase

4.7. TWO-DIMENSIONAL MULTIPLEXED OXYBLOTTING

In order to detect carbonylated brain proteins, tissue extracts were derivatised with 2mM 2,4-dinitrophenylhydrazine (DNPH) (Sigma, St. Louis, MO, USA) in 2N HCl prior to TCA precipitation during sample preparation (**I-III**) (Figure 6). In study **IV**, the derivatisation of CSF proteins took place after IEF, IPG strips were incubated in 10 mM DNPH in 2N HCl and equilibrated according to the "in-strip derivatization" method described by Conrad et al. 2001.

Otherwise, brain and CSF proteins were separated by 2-DE and immunoblotted essentially as described in sections 4.4 and 4.6. However, in order to detect protein levels, PVDF membranes were first stained with Sypro Ruby fluorescent protein stain according to the manufacturer's instructions (Bio-Rad), and scanned at 450nm by fluoroimager Storm 860 (Amersham Biosciences). Thereafter, membranes were incubated with anti-dinitrophenyl (DNP) primary antibody followed by visualization of protein-bound carbonyls with fluorescent Cy5-labelled secondary antibody.



Protein-bound carbonyl | 2,4-dinitrophenylhydrazine (DNPH) | 2,4-dintrophenylhydrazone (DNP)

Figure 6. Demonstration of the derivatization of protein-bound carbonyls with DNPH, which becomes covalently attached to the peptide backbone. The resulting DNP-group is detected with an antibody as a marker of oxidative modification of the protein.

4.8. DEGLYCOSYLATION AND DEPHOSPHORYLATION OF GFAP

In study **III**, brain proteins were deglycosylated and dephosphorylated prior to sample preparation. Deglycosylations of N-linked oligosaccharides and complex O-linked carbohydrates having the Gal-β(1-3)-GalNAc core structure were performed using a commercial enzymatic protein deglycosylation kit E-DEGLY (Sigma, Saint Louis, MI, USA) according to the manufacturer's instructions under denaturing conditions. Lambda Protein Phosphatase system (New England Biolabs Inc, Beverly, MA, USA) was used to remove phosphorylations attached to serine, threonine and tyrosine residues according to the manufacturer's instructions. Protein samples were then separated by 2-D immunoblotting. The existence of glycosylations and phosphorylations was determined by comparing treated and untreated 2-DE patterns of GFAP.

4.9. PROTEIN IDENTIFICATION

After 2-DE, proteins were stained with Sypro Ruby gel stain according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Proteins of interest were manually cut out on a UV-table, reduced and alkylated, in-gel digested with trypsin and desalted as previously described (Rosenfeld et al. 1992, Shevchenko et al. 1996, Nyman et al. 2000). The detailed protocols for in-gel digestion and desalting can be found in the web-pages, http://www3.btk.utu.fi: 8080/Genomics/ Proteomics/ Protocols. The resulting peptides were analysed by MALDI-TOF MS or by nanoscale liquid chromatography tandem mass spectrometry (LC-MS/MS). Database searches were performed using the Mascot Search engine (http://www.matrixscience. com) or with BioAnalyst 1.0 software and database searches with PepSea Server version 2.2.1.7.

Alternatively, proteins were in-gel digested as described by Kanninen et al. 2004, and the resulting peptides were analysed using high performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) (**III**). The proteins were identified with peptide molecular weight and MS/MS data using Sequest search program (Thermoquest, San Jose, CA).

4.10. IMAGE ANALYSIS

4.10.1. Two-dimensional images

Fluorescence intensities of detected proteins on membranes were analysed by using ImageMaster 2D Elite software (Amersham Biosciences). One of the membranes of a control sample was chosen to serve as the reference gel during the image analysis. Protein spots were detected and matched among 2-D membranes in order to compare and correlate differences between AD patients and age-matched controls. Averaging was not performed during image analysis. This means that the membranes were not grouped according to the diagnosis during the image analysis, but the values of light intensities of individual spots were taken separately from each membrane to the statistical analysis. In studies **I-II,** oxidised brain proteins detected by anti-DNP immunoblotting, and proteins stained by Sypro Ruby fluorescent stain, were analyzed separately. In studies **I-II**, both the levels and oxidation of brain proteins were

given as normalised values. Also in study **IV**, the levels of CSF proteins were given as normalised values. For the comparison of the levels of proteins, the normalised value was justified since the total amount of protein loaded to each gel was the same. However, the carbonylation of CSF proteins were given as light intensity values after realising that the equal amount of total protein cannot be expected to exhibit the same degree of modification in individuals. We also tested the analysis of carbonylation of CSF proteins (**IV**) using normalised values and carbonylation of brain proteins (**II**) using intensity values. Fortunately and unexpectedly, these differences in analyses did not influence the results. The levels of GFAP were analysed as intensity values, and not as normalised values, since it was assumed that individuals did not have equal amounts of the immunodetected protein (**III**). Measures of light intensities were transferred to SPSS for statistical analysis.

4.10.2. One-dimensional images

In brain proteome studies **II** and **III**, the light intensities of immunoblotted proteins including cytosolic malate dehydrogenase (MDH1), glutamate dehydrogenase (GDH), and GFAP were measured by using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). Values of light intensities were transferred to SPSS for statistical analysis.

4.11. STATISTICAL ANALYSIS

Mann-Whitney's U test (SPSS Inc., Chicago, IL, USA) was used to compare light intensities of detected proteins. The degree of oxidation was calculated as the ratio of protein-bound carbonyls to the amount of protein. Furthermore, in brain proteome studies **II** and **III**, the relationships between post-mortem delay (PMD), duration of the clinical impairment, age at onset, age at death, brain pathology and protein changes were calculated by Pearson's correlation test (SPSS Inc.). In study **III,** a curve diagram was created from the average values of the amounts of GFAP isoforms, the area under curve (AUC) was calculated, and the curves were further fitted into a log normal mathematical equation (SigmaPlot).

5. RESULTS

5.1. IDENTIFICATION OF PROTEINS

The multiplexed quantitative 2-D oxyblotting approach for the simultaneous measurement of the amounts and carbonylation of proteins on a single membrane was developed in study **I**. Thereafter, in study **II**, brain proteins exhibiting oxidative changes in AD patients as compared to age-matched controls were further characterised. In study **IV**, the multiplexed 2-D oxyblotting method was adapted to study oxidatively modified CSF proteins in AD patients as compared to controls. In study **III,** the levels of GFAP isoforms, and their phosphorylation, glycosylation, and oxidation were studied in AD brain. As a consequence of the results obtained by 2-D immunoblottings, a total of 14 brain and 30 CSF proteins of interest were identified by MS and database searches. These proteins are presented in Figures 7 and 8 and Tables 6 and 7. The numbering in the figures and tables is followed hence when presenting the results.

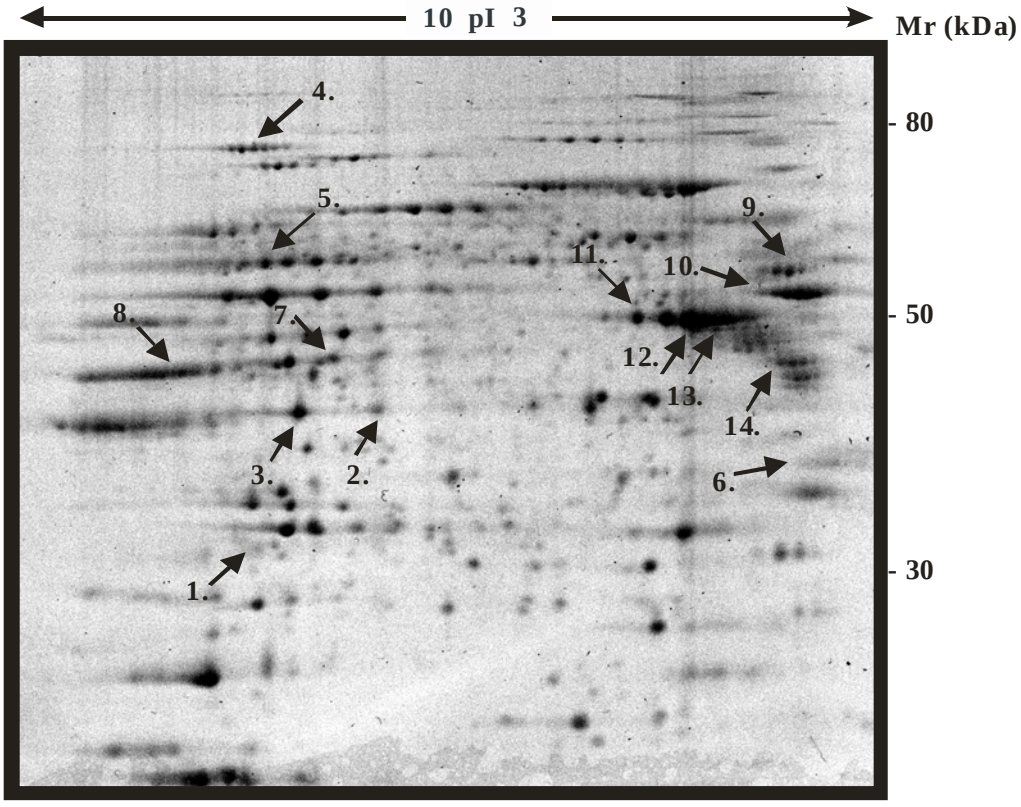


Figure 7. 2-DE image of the most abundant human frontal cortex proteins highlighting proteins selected for identification. Proteins were separated first according to their pI using 18 cm 3-10 NL IPG gel. In the second dimension, proteins were separated according to their Mr using 12.5% SDS-PAGE gel. Proteins were visualized with Sypro Ruby fluorescent stain and cut out manually for in-gel digestion and identification.

Table 6. Identified human brain proteins

#	*Protein*	*Acc. No.*	*No. of peptides*	*Seq. cov. (%)*	*Study*
1	Carbonic Anhydrase I	P00915	5	25	**II**
2	Malate dehydrogenase 1 a	P40925	5	19	**II**
3	Malate dehydrogenase 1 b	P40925	20	49	**II**
4	Aconitase, mitochondrial	Q99798	9	14	**II**
5	Glutamate dehydrogenase	P00367	10	22	**II**
6	14-3-3 protein zeta/delta	P29312	14	55	**II**
7	Aldolase C	P09972	10	35	**II**
8	Aldolase A	P04075	7	17	**II**
9	ATP synthase beta chain, mitochondrial	P06576	10	24	**II**
10	Gamma-enolase	P09104	2	6	**III**
11	Creatine kinase, B chain	P12277	5	18	**III**
12	Beta-actin	P02570	1	5	**III**
13	Gamma-actin	P02571	6	19	**III**
14	Glial fibrillary acidic protein	P14136	3	7	**III**

#, number of protein; Acc. No, identification of proteins is indicated by SWISS-PROT accession numbers; No. of peptides, number of peptides matched; Seq. cov., sequence coverage of matched peptides.

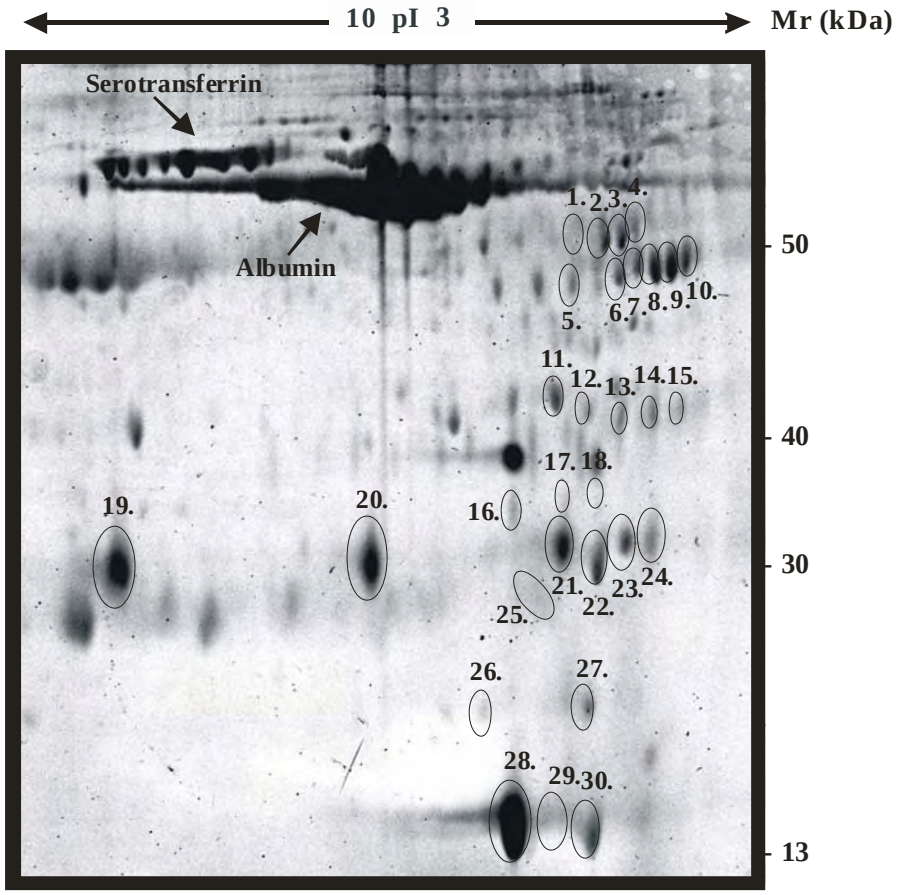


Figure 8. 2-DE image of the most abundant human CSF proteins. A total of 30 proteins were selected to be identified in study **IV**. Proteins were separated using 18 cm long 3-10 NL IPG gel and 12.5% SDS-PAGE gel. Proteins were visualized with Sypro Ruby fluorescent stain and cut out manually for in-gel digestion and identification.

Table 7. Identified proteins in human cerebrospinal fluid (study IV).

#	*Name*	*Acc. No.*	*Score*
1	Vitamin D binding protein	21730549	46
2	Vitamin D binding protein	22219267	67
3	Vitamin D binding protein	22219267	72
4	Vitamin D binding protein	72105	54
5	Alpha-1-antitrypsin precursor	1942629	65
6	Alpha-1-antitrypsin precursor	7245932	54
7	Alpha-1-antitrypsin polymer	7245932	80
8	Alpha-1-antitrypsin precursor	P01009	94

9	Alpha-1-antitrypsin precursor	P01009	67
10	Alpha-1-antitrypsin precursor	P01009	91
11	Apolipoprotein E3	178853	85
12	Apolipoprotein E3	1942471	84
13	Apolipoprotein E	338305	75
14	Apolipoprotein E	178853	63
15	Apolipoprotein J	338305	79
16	Unidentified	-	
17	Lambda chain precursor	33395	54
18	Unidentified	-	-
19	Beta-trace (N-terminal peptide from human CSF)	410564	48
20	Beta-trace (N-terminal peptide from human CSF)	18028972	77
21	Beta-trace (N-terminal peptide from human CSF)	18028972	125
22	Proapolipoprotein	178775	94
23	Proapolipoprotein	178775	125
24	Proapolipoprotein	178775	106
25	Beta-trace	18028972	53
26	Cu/Zn-superoxide dismutase	1237406	62
27	Unidentified	-	-
28	Transthyretin	339685	68
29	Transthyretin	14719497	80
30	Transthyretin	999653	80

#, number of proteins; Acc.No., identification of proteins indicated as NCBI or SWISS-PROT accession numbers; Score, probability based mowse score where individual ions scores > 47 indicate identity or extensive homology ($p<0.05$).

5.2. OXIDATIVELY MODIFIED PROTEINS

5.2.1. Brain

The 2-D oxyblot application used was developed in study **I**, in which we detected about 150 cytosolic proteins and 100 oxidised proteins. In study **II**, proteins exhibiting significant changes in oxidation status in AD brains were identified (Table 8). All oxidised proteins were not always quantified since the same spots were sometimes detected in only a few of the membranes or did not achieve the sufficient resolution to be analyzed by the image analyses software. Proteins exhibiting differences in the levels and oxidation status between AD patients and controls are presented in Table 8. Since MDH1 and GDH were characterised as less oxidised proteins in AD brains, we also measured the total levels of the proteins by one-dimensional (1-D) immunoblotting. No difference in the amount of MDH1 was found between AD patients and controls. However, the total soluble level of GDH was significantly increased by 60% in AD patients.

5.2.2. Cerebrospinal fluid

The oxidation status of proteins did not generally differ significantly between AD patients and controls showing no cognitive decline (Figure 9). Only one of the identified proteins, lambda chain precursor, was found to exhibit increased oxidation in AD patients. In contrast, the levels of proteins tended to be decreased in AD patients as compared to control. Furthermore, the oxidation status of proteins generally did not differ significantly between ApoE ε4 carriers as compared to non-carriers (Figure 10). Lambda chain precursor was the only identified protein exhibiting increased oxidation in ApoE ε4 carriers. Again, the levels of several proteins were decreased in ApoE ε4 carriers as compared to non-carriers. Protein levels did not generally differ between men and women but the oxidation status of several proteins was increased in men (Figure 11).

Table 8. Oxidation and levels of brain proteins in AD patients when compared to controls.

#	Protein	Protein-bound carbonyls (LI)			Amount of protein (LI)			Degree of oxidation		
		Control (n=9)	AD (n=10)	AD vs controls	Control (n=9)	AD (n=10)	AD vs controls	Control (n=9)	AD (n=10)	AD vs controls
1	Carbonic anhydrase	10.96±2.56	26.76±7.13	↑	165.73±49.2	234.77±49.63	x	0.12±0.03	0.21±0.10	x
2	Malate dehydrogenase 1a	21.54±7.97	8.58±3.12	↓	142.16±35.54	390.75±72.62	↑↑	0.22±0.08	0.03±0.01	↓↓
3	Malate dehydrogenase 1b	32.91±10.70	6.44±1.80	↓↓	346.73±67.16	579.77±127.61	x	0.16±0.06	0.01±0.00	↓
4	Aconitase	299.24±42.50	156.88±37.08	↓	451.10±129.90	254.00±61.74	x	37.11±36.14	2.24±1.59	x
5	Glutamate dehydrogenase	128.81±25.85	88.63±20.42	x	117.37±24.78	227.59±52.84	x	1.61±0.42	1.41±0.91	↓
6	14-3-3 protein zeta/delta	32.38±15.92	3.17±0.90	↓	636.75±134.63	414.46±77.48	x	0.06±0.03	0.01±0.00	x
7	Aldolase C	127.33±27.44	58.55±11.58	↓	441.67±81.07	363.24±62.01	x	0.36±0.08	0.46±0.30	x
8	Aldolase A	97.11±27.27	72.95±41.18	↓	457.89±107.50	179.48±36.01	x	0.23±0.03	0.73±0.56	x
9	ATP synthase beta chain, mitochondrial	588.15±139.74	600.01±82.93	x	795.56±205.85	1544.12±240.01	↑	2.59±1.66	0.44±0.06	x

#., number of the protein spot, degree of oxidation, light intensities of protein-bound carbonyls divided by light intensities of the amount of corresponding protein spots; LI, light intensity; AD, Alzheimer's disease; vs, versus

Arrows indicate the levels in AD when compared to controls: x, no difference; ↓ or ↑ ($p \leq 0.05$);↓↓ or ↑↑ ($p \leq 0.01$).

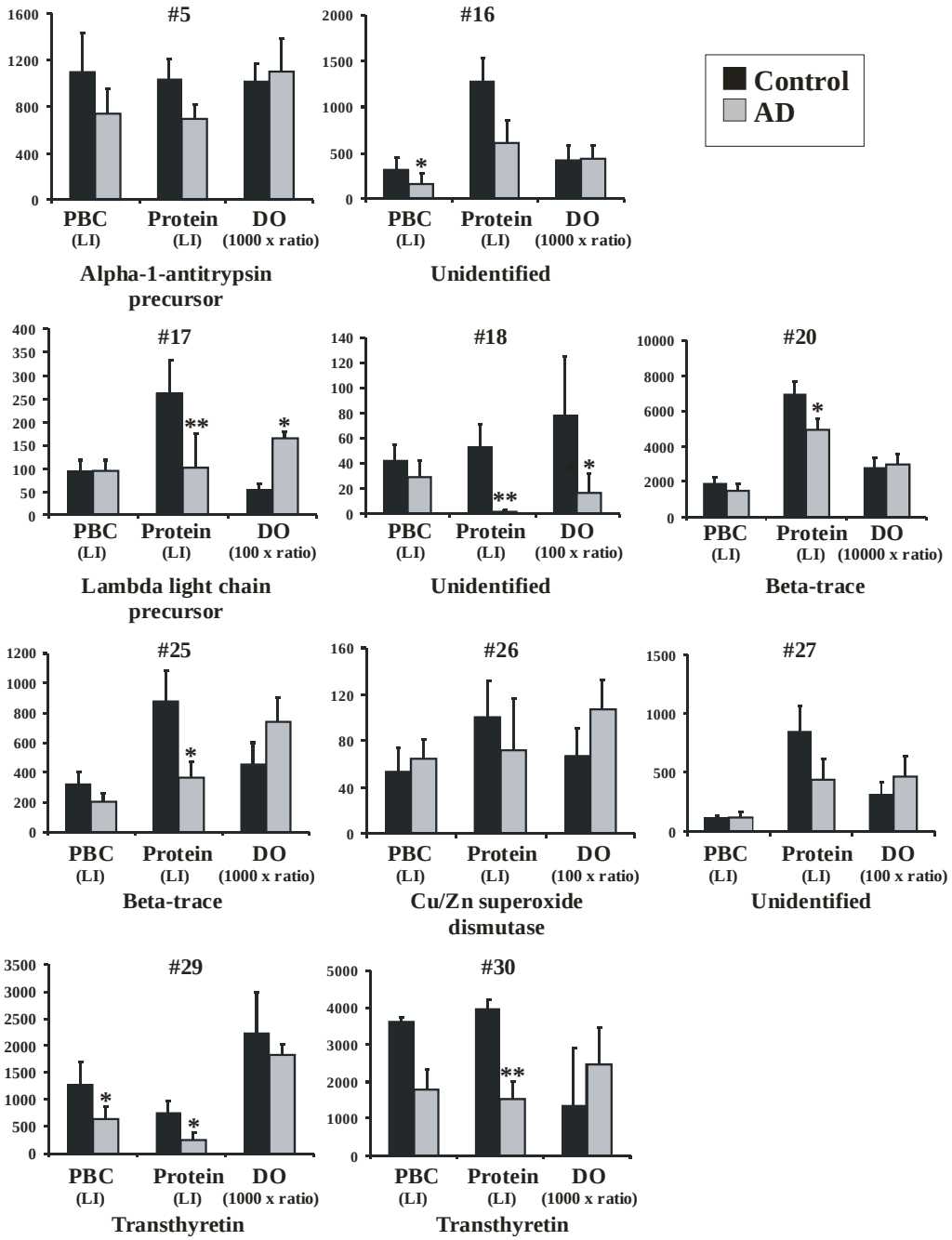


Figure 9. Oxidation and the levels of CSF proteins in AD patients (n=11) as compared to age-mathced controls (n=9). The degree of oxidation of the spot #18 appeared to be decreased in AD. However, the protein spot was detected in only one patient indicating that the protein spot was qualitatively more oxidised in AD CSF.

*#, number of the protein spot corresponding to numbering in figure 8 and table 6; PBC, protein-bound carbonyls; DO, degree of oxidation ratio, protein-bound carbonyls divided by the amount of protein; LI, light intensity; 100 x, 1000 x or 10000 x, degree of oxidation multiplied in order to fit all the numeric data of one spot onto the same scale; *, $p \leq 0.05$; **, $p \leq 0.01$.*

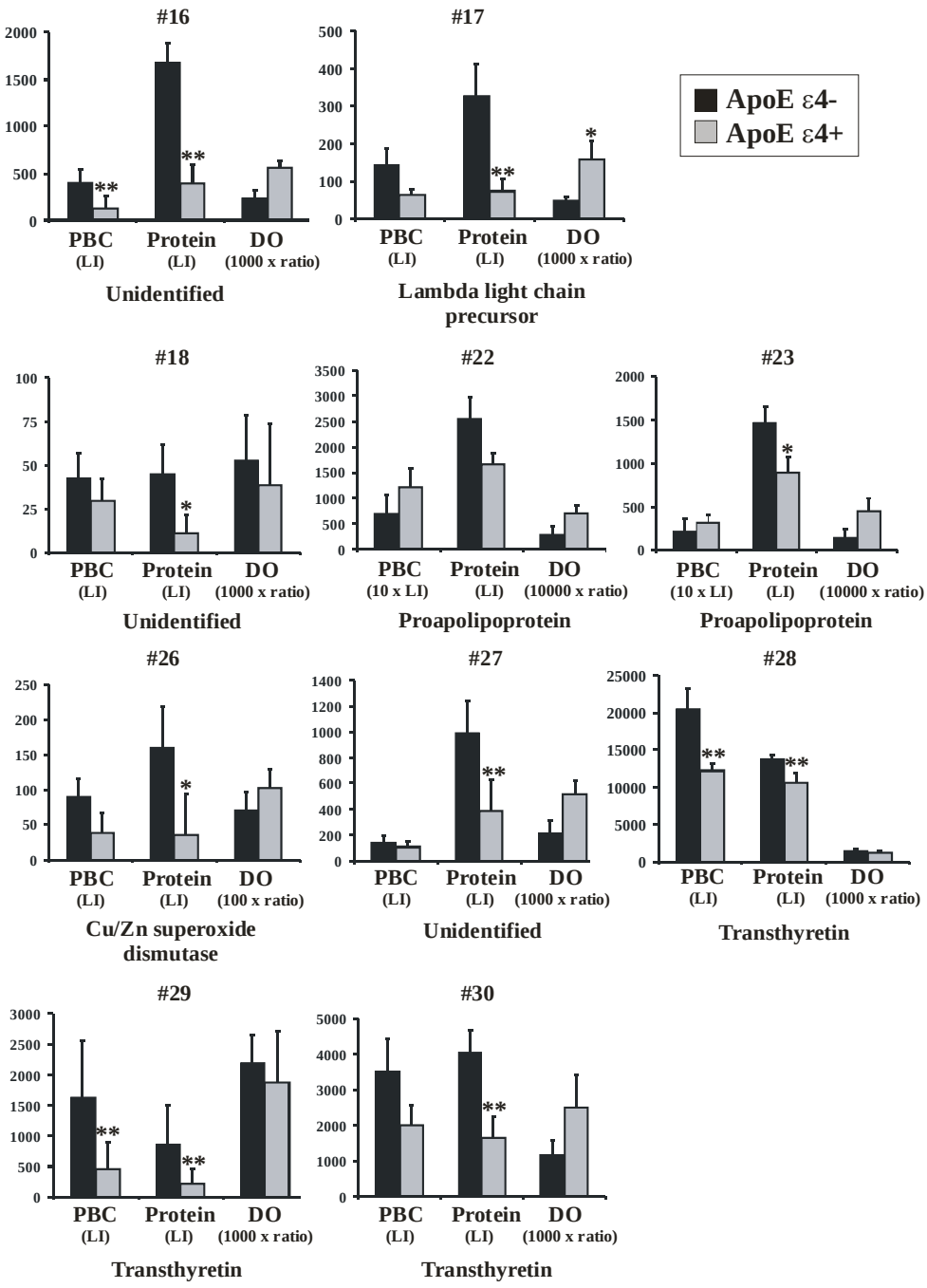


Figure 10. Oxidation and the levels of proteins in Apolipoprotein E ε4 carriers (n=12) as compared to non carriers (n=8).

*#, number of the protein spot corresponding to numbering in figure 8 and table 6; PBC, protein-bound carbonyls; DO, degree of oxidation, ratio, protein-bound carbonyls divided by the amount of protein; LI, light intensity; 100x, 1000x or 10000x, light intensities or the degree of oxidation multiplied in order to fit all the numeric data of one spot onto the same scale; *, p≤0.05; **, p≤0.01.*

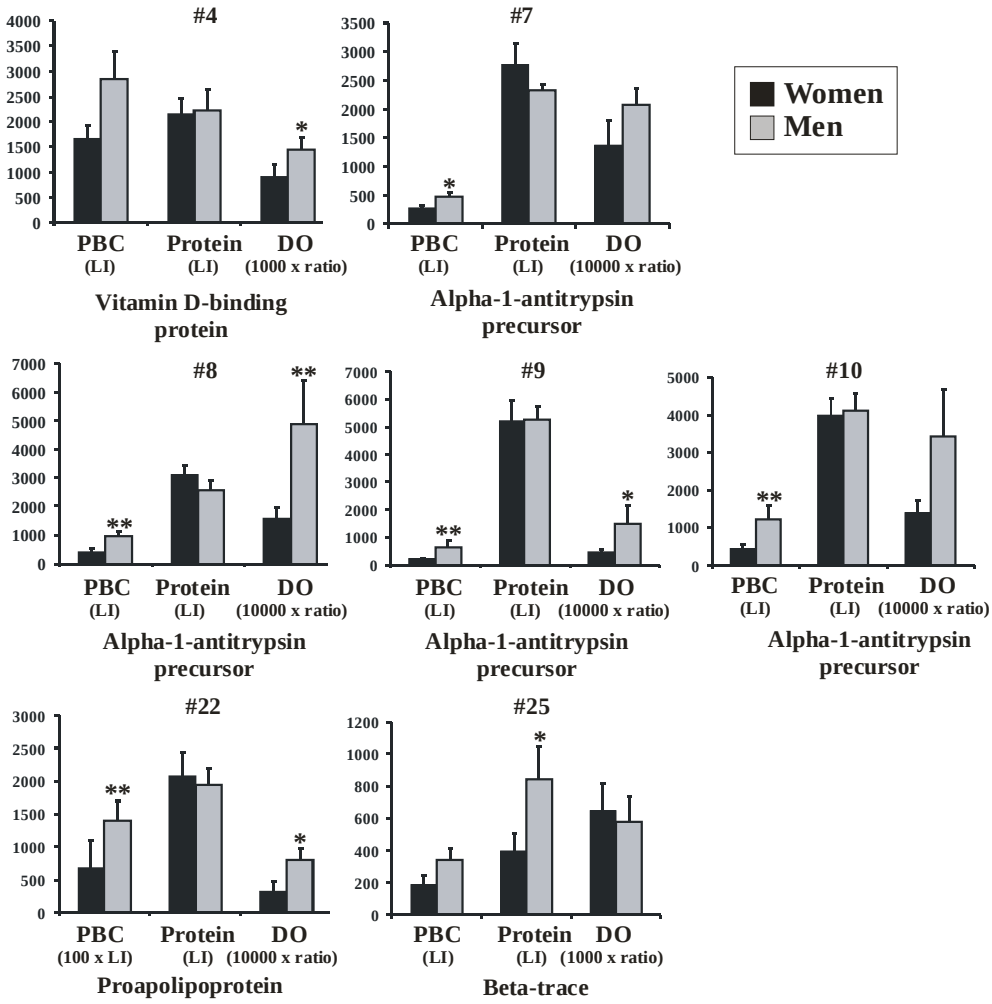


Figure 11. Oxidation and the levels of proteins in women (n=11) as compared to men (n=9).

*#, number of the protein spot corresponding to numbering in figure 8 and table 6; PBC, protein-bound carbonyls; DO, degree of oxidation, ratio, protein-bound carbonyls divided by the amount of protein; LI, light intensity; 100x, 1000x or 10000x, light intensities or the degree of oxidation multiplied in order to fit all the numeric data of one spot onto the same scale; *, $p \leq 0.05$; **, $p \leq 0.01$.*

5.3. POST-TRANSLATIONAL MODIFICATION OF GFAP

The total amount of soluble GFAP was increased by 95% in AD brains as compared to the age-matched controls (Figure 12A) (**III**). A total of 46 soluble isoforms of GFAP were detected by 2-D immunoblotting (Figure 12B, C). The amounts of only 11 distinct isoforms of GFAP were increased in AD as compared to controls. Moreover, nine of them were among the higher Mr isoforms 1-11 and 16-27 (Figure 12C), and the quantitative distribution seemed to have undergone an acidic shift in AD. In order to further assess the isoelectric shift, a curve was created from the average values of light intensities of isoforms 1-11 and 16-27, and the AUCs were calculated. The curves were fitted into a four-parametric log normal equation.

$$y = y_0 + ae^{\left[-0.5\left(\frac{\ln\left(\frac{x}{x_0}\right)}{b}\right)^2\right]}$$

Spot	*Diagnosis*	x_0	y_0	*a*	*b*	R^2
1-11	Control	4.2665	744851	4131934	0.3073	95.5
1-11	AD	5.0321	239741	7085167	0.3517	99.6
16-27	Control	3.2760	883867	5287100	0.4118	99.1
16-27	AD	4.1503	880828	7463607	0.4523	99.7

All curves created from average values and from the equation are presented in Figure 12D and indicate a 60% increase in the amount of more acidic isoforms of GFAP in AD for isoform trails 1-11 and 16-27. PTM analysis verified that the more acidic isoforms were both phosphorylated and N-glycosylated, while the more basic isoforms were O-glycosylated and exhibited no quantitative differences between post-mortem AD and control brains.

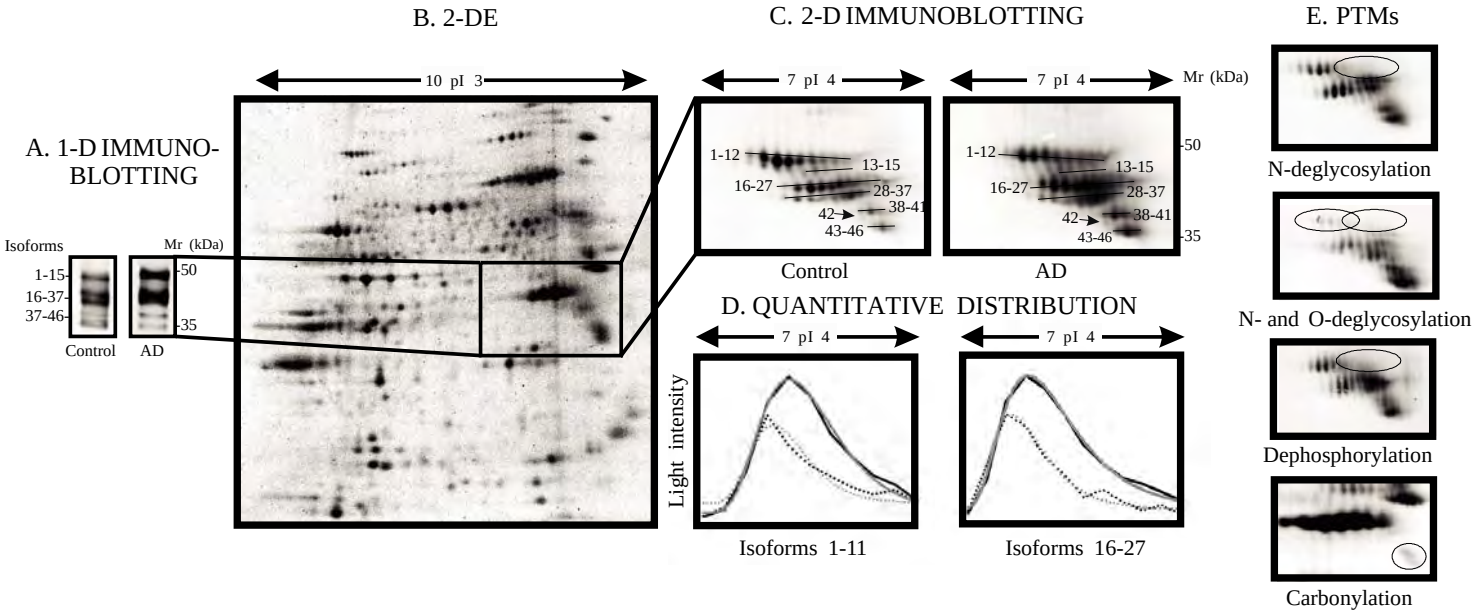


Figure 12. The box in the Sypro Ruby stained 2-DE image indicates the location of the GFAP isoforms **(B)**. An average of five bands and 46 isoforms were detected by 1-D **(A)** and 2-D **(C)** immunoblotting, respectively. Light intensities of individual isoforms were measured by 2-D image analysis. Overlapping curves **(D)** formed by the mean values of light intensities of distinct isoforms (black) and by the four-parametric equation (grey) revealed an excellent fit with each other. A 60% increase was found for both isoform trails 1-11 and 16-27 in AD. The increase was restricted to the more acidic isoforms and the isoform distribution had undergone a qualitative acidic shift in AD (lines) when compared to controls (dotted lines). The enzymatic cleavages resulted in changes in the 2-D patterns **(E)** (oval circles) indicating that the more acidic isoforms were N-glycosylated and phosphorylated whereas the more basic isoforms were O-glycosylated. The smallest isoforms of GFAP at around 35 kDa exhibited immunoreactivity by 2-D oxyblotting (circle) suggesting oxidative modification of these isoforms. *GFAP, glial fibrillary acidic protein; PTMs, post-translational modifications; 1-D, one-dimensional; 2-D, two-dimensional; 2-DE, two-dimensional gel electrophoresis*

5.4. PROTEIN CHANGES AND NEUROPATHOLOGY

In study **II,** individual correlations were calculated between brain protein changes and the neuropathological data in AD (Tables 12 and 13).

Table 9. Significant correlations between levels of brain proteins, symptoms and neuropathological data in AD.

#	*Protein*	*Duration of CI*	*Aβ load*	*AM*	*RA*	*PMD*
1	Carbonic anhydrase Ii	+	+			
5	Glutamate dehydrogenase	+				
6	14-3-3 protein zeta/delta			+		
7	Aldolase C				-	+
8	Aldolase A					+

#, number of the protein spot; Duration of CI, duration of clinical impairment; Aβ, amyloid-beta; AM, activated microglia; RA, reactive astrocytes; PMD, post-mortem delay, +, significant positive relationship;-significant negative relationship

Table 10. Degrees of oxidation of brain proteins correlated significantly with symptoms and neuropathological data in AD.

#.	*Protein*	*Duration of CI*	*Aβ load*	*AM*	*Braak*	*PMD*
1	Carbonic anhydrase Ii				+	
3	Malate dehydrogenase 1b	+				
5	Glutamate dehydrogenase	+				
6	14-3-3 protein zeta/delta			+		
8	Aldolase A					+

#, number of the protein spot; Duration of CI, duration of clinical impairment; Aβ, amyloid-beta; AM, activated microglia; Braak, Braak and Braak staging; PMD, post-mortem delay +, significant positive relationship

In study **III**, the total amount of soluble GFAP exhibited a positive relationship with AM in AD whereas nine distinct isoforms correlated with the extent of astrogliosis in controls. Moreover, positive correlations were noted between Aβ load and five mainly basic isoforms in controls. Numeric data of the relationships are presented in the original publications.

6. DISCUSSION

6.1. SIGNIFICANCE IN RELATION TO ALZHEIMER'S DISEASE

In the present thesis work, proteomic applications were developed for studying both the levels and modifications of brain and CSF proteins in AD. In studies **I**, **II,** and **IV**, differences in the levels and oxidative modification of brain and CSF proteins were examined between AD patients and age-matched controls. In study **III**, glycosylated, phosphorylated, and oxidised GFAP isoforms were studied in post-mortem AD and control frontal cortices. Several alterations in the levels and modifications of proteins were found in AD patients as compared to the age-matched controls. Moreover, some protein changes were related to the neuropathology and duration of clinical impairment. These results may deepen our understanding of the pathogenesis of AD.

6.1.1. Oxidatively modified proteins

About 150 cytosolic brain proteins and more than a hundred oxidatively modified proteins were detected on 2-D oxyblots using the multiplexed proteomics approach that was developed in study **I**. A total of nine brain proteins were shown to be oxidatively modified and exhibited protein changes in AD brains (**II**). In contrast to the expectations, five of these proteins (aconitase, GDH, MDH1a and b, 14-3-3 protein zeta-delta and ATP synthase beta chain) tended to exhibit decreased, and only three (CAI, aldolases A and C) increased oxidation statuses in AD. In study **III**, GFAP was identified to be an oxidatively modified protein in AD and control brains but there were no differences between the groups.

We found oxidative changes in two mitochondrial enzymes, aconitase and GDH, which are intimately involved in the tricarboxylic acid (TCA) cycle (figure 13). Aconitase is considered to be the most sensitive enzyme in the TCA cycle to be subjected to H_2O_2 mediated oxidative stress (Tretter and Adam-Vizi 2000) and it is known to be a target of oxidative stress during aging (Yan et al. 1997). We found a significant decrease in the amount of aconitase-bound carbonyls whereas the amount and degree of oxidation of aconitase exhibited a nonsignificant tendency of being lower in AD brains when compared to controls. Since increased oxidation is normally related to dysfunction of proteins, these findings are in accordance with the unaltered

activity of aconitase in AD frontal cortex as compared to age-matched controls (Bubber et al. 2005). The second most sensitive enzyme to H_2O_2 mediated oxidative stress in the TCA cycle is the rate-limiting alpha-ketoglutarate dehydrogenase complex (α-KGDHC) (Tretter and Adam-Vizi 2000), an enzyme exhibiting reduced activity in AD (Butterworth and Besnard 1990, Gibson et al. 1998, Gibson et al. 1999, Gibson et al. 2000). As a consequence, the whole enzymatic cascade in the TCA cycle becomes sluggish. It can be postulated that GDH may compensatorily replace α-KGDHC, and thus catalyse the reaction between α-ketoglutarate (α-kg) and glutamate (Figure 13) (Erecinska and Nelson 1990, McKenna et al. 2000). It is generally assumed that a low level of oxidation of proteins is associated with enhanced enzymatic functional status, whereas an increase of oxidation enhances degradation or accumulation of proteins (Shringarpure and Davies 2002). Indeed, we found that the degree of oxidation was decreased while the total amount of GDH was increased in AD. The latter finding was confirmed recently by Burbaeva et al. (2005). The functional consequences of the changes in the levels and oxidation of GDH remain to be clarified but they may be compensatory, and related to compromised cellular energy metabolism in AD.

The major findings of study **II** were that the degrees of oxidation of not only GDH but also two isoforms of another oxido-reductase, cytosolic MDH1, were significantly decreased in AD. This is the first time when oxidatively less modified proteins have been identified in AD as compared to previous studies (Table 1). Both of these enzymes are present in neurons and astrocytes. GDH is co-localised in cells with aspartate aminotransferase (AAT), which participates in the malate-aspartate shuttle with MDHs (Yudkoff et al. 1994, McKenna et al. 2000, Kimmich et al. 2002) (Figure 13). In addition to GDH, AAT is the only other known enzyme catalysing the reaction between α-kg and glutamate (Cooper 1985, McKenna et al. 2000). The activities of i) GDH have been suggested to be unchanged in temporal cortex of AD patients (Butterworth and Besnard 1990), ii) MDH increased in AD frontal cortex (Bubber et al. 2005) and iii) AAT increased in CSF of AD patients (Riemenschneider et al. 1997, Tapiola et al. 1998). Moreover, enhanced activity of the malate-aspartate shuttle involving both MDH1 and AAT has been associated with the late-stages of oxidative stress and to occur as a consequence of increased mitochondrial membrane permeability (Atlante et al. 2001). In addition, GDH, AAT and MDH are considered to be mostly active during conditions of cellular energy deprivation. Thus, these changes may point to the presence of alternative metabolic routes in AD since both GDH and MDH may be involved in the production of

NADH for the electron transport chain. In addition, decreased oxidation of GDH and MDH1 may also reflect alterations in neurotransmittor release and thus glutamate-related excitotoxicity in AD. Finally, our results indicate the possibility of MDH1 being involved in the stress response in AD since this enzyme has been proposed to be identical with alpha-keto acid reductase, which is thought to be involved in detoxification of carbonyl species (Friedrich et al. 1988, O'Connor et al. 1999, Picklo et al. 2002).

Compromised energy metabolism plays a crucial role in the evolution of AD and it is believed to be related to clinical symptoms (Blass et al. 2002). Our results indicated that changes in oxidation of MDH1 and GDH were positively associated with the duration of the clinical impairment but not with brain pathology in AD. The activity of MDH has been suggested to correlate positively with clinical dementia rating scores (CDRs) (Bubber et al. 2005). There are no previous data which have correlated the levels or oxidation of GDH with the duration of the clinical impairment. However, AD frontal cortex activities of dehydrogenases appeared to correlate positively whereas the dehydrogenases with decarboxylase activity negatively with CDRs (Bubber et al. 2005).

Abnormalities in the oxidative glycolysis have been reported earlier in AD (Bigl et al. 1996, Bigl et al. 1999). Increased oxidations of glycolytic proteins in AD have been found for phosphoglycerate mutase and triosephosphate isomerase in hippocampus; as well as for alpha-enolase in hippocampus and inferior parietal lobe. Our results showed a decrease in the levels of protein-bound carbonyls for aldolase A and C (Figure 13). The degree of oxidation of these proteins exhibited a non-significant tendency of being higher in AD when compared to controls, possibly since also the amounts of these proteins were lower in AD. Our results are in accordance with previous studies that have found no changes in the levels or activity of aldolases in brains of AD and DS patients (Bigl et al. 1999, Kitzmueller et al. 2001). It is possible that aldolases may be modified after death in AD since the degrees of oxidation correlated positively with PMD. No such correlations were found in control brains, despite the longer PMD. Post-mortem changes may occur in relation to the physiological or to the pathological biochemical environment i.e. variations in ante-mortem conditions such as tissue pH, may have a variety of unpredictable consequences.

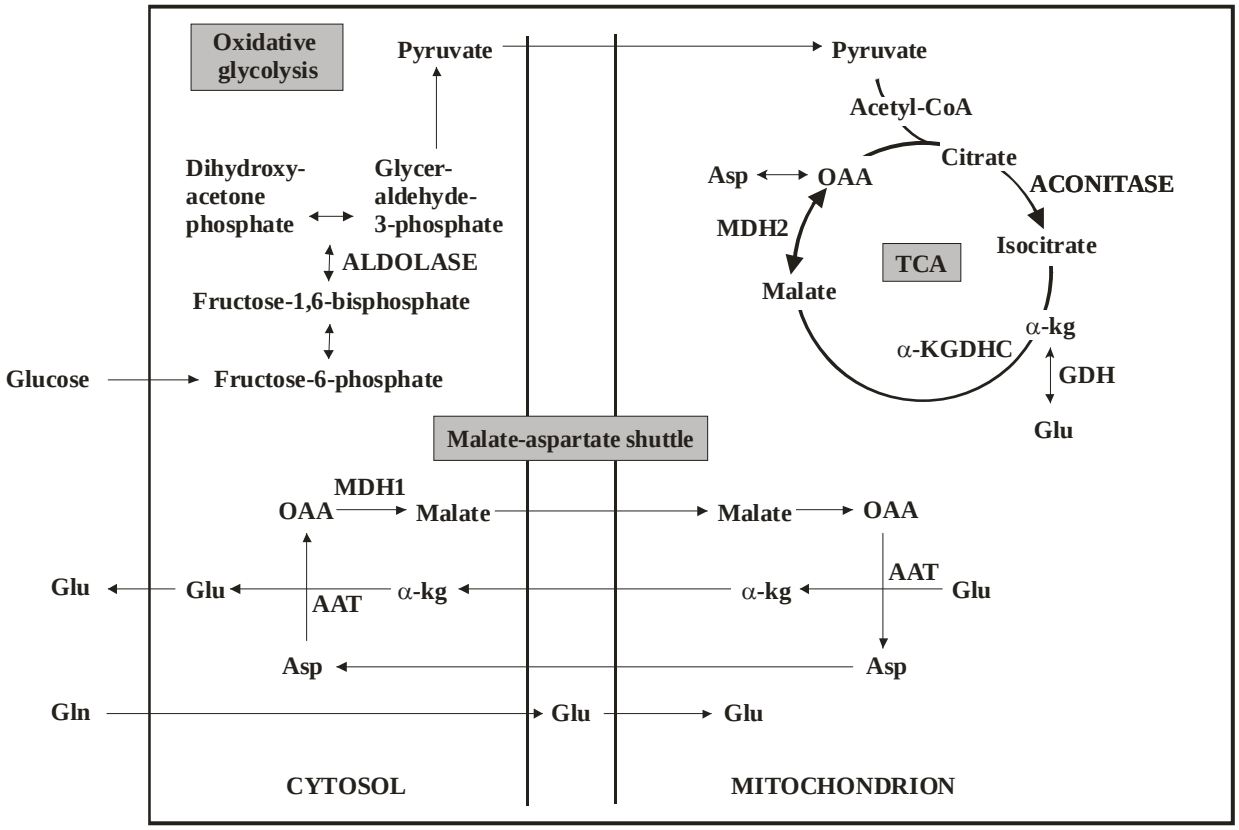


Figure 13. The basic neuronal metabolic routes are demonstrated with respect to the results. Aldolase is a cytosolic protein involved in oxidative glycolysis. Aconitase catalyses a step in the TCA cycle and glutamate dehydrogenase (GDH) a reversible reaction between α-ketoglutarate (α-kg) and glutamate (Glu). Malate dehydrogenase 1 (MDH1) is a cytosolic enzyme involved in the malate-aspartate shuttle, catalysing the reaction between oxaloacetate (OAA) and malate. In the malate-aspartate shuttle, malate is taken into the mitochondrion in exchange for α-kg and glutamate is taken into the mitochondrion in exchange for aspartate (Asp). Aspartate aminotransferase (AAT) functions co-operatively with the malate-aspartate shuttle. Both reactions support the release of glutamate as a neurotransmitter and thus also influence the glutamate/glutamine (Gln) shuttle between neurons and astrocytes. (Yudkoff et al. 1994, Kimmich et al. 2002)

Isoforms of CA catalyse the reversible hydration of carbon dioxide, and participate in many biological processes including respiration, acid-base balance, and are involved in the formation of CSF (Dodgson 1991). The activity of CA has generally been suggested to be decreased in AD brain (Meier-Ruge et al. 1984). We found that the levels of CAI-bound carbonyls were increased in AD, perhaps reflecting the increase in the total amount of CAI in AD brains since the degree of oxidation was not significantly increased. Another isoform, CAII, was recently shown to exhibit both increased levels and increased oxidation in hippocampus of AD patients (Sultana et al. 2005). Interestingly, the levels of CAI correlated positively with the duration of clinical impairment and Aβ-load in AD brains and the degree of oxidation exhibited a positive relationship with Braak staging. These correlations may reflect pH changes occurring in the AD brain.

Another protein exhibiting a decrease in the carbonyl levels in AD was 14-3-3 protein zeta/delta. Human 14-3-3 proteins are highly conserved multifunctional proteins playing an important role in various cell-signalling events (Aitken et al. 1992, Yaffe 2002). The amount and the degree of oxidation correlated inversely with the extent of activated microglia in AD. The only difference between 14-3-3 zeta and delta is that the delta isoforms is phosphorylated. A number of cytotoxic agents and cytokines released by activated microglia regulate the phosphorylation of 14-3-3 proteins. It is thus possible that the amount and oxidation status of 14-3-3 protein zeta/delta in someway reflect the extent of AM in AD brain. Although we found no significant changes in the amount of any single isoform of zeta/delta, previous studies have shown increased levels in several brain regions of some isoforms of 14-3-3 gamma and epsilon in AD and DS patients (Fountoulakis et al. 1999).

Our findings of decreased or unchanged oxidation of brain proteins do not nullify the hypothesis of increased oxidative stress in AD. One reason for decreases in the oxidation statuses may be that we studied easily soluble/cytosolic proteins, which may inversely correlate with unsoluble aggregates of oxidised proteins in AD brain. Some proteins may also be functional in a soluble form, and oxidation statuses and levels of proteins may also reflect metabolic changes in AD. Moreover, our results represent protein changes occurring during severe end-stage AD in contrast to some studies that have examined protein oxidation in patients with moderate AD dementia (Sultana et al. 2005). Some studies have indicated that oxidative stress diminishes and the levels of some proteins vary during the progression of AD (Nunomura et al. 2001, Lee et al. 2005). Therefore, due to the stage of AD studied here, it is

not surprising that our results may differ from other published studies. Changes in the protein oxidation status and the amount of protein may be strongly dependent on the type of proteome under investigation, the stage and the duration of the clinical impairment as well as on the brain pathology of the patient.

In study **IV**, differences were not only observed in AD patients vs. controls but also in women vs. men, and in ApoE ε4 carriers vs. non-carriers. The decrease in the levels of CSF proteins seemed to be more closely related with the ApoE genotype than with the clinical diagnosis of AD. The material was, however, too small to allow us to draw any definite conclusions. Moreover, the statistically most significant differences in oxidation statuses of proteins were found between men and women. In studies **I**-**III**, there was an imbalance between the genders i.e. most AD patients were women and most control subjects were men. In addition, most AD patients represented ApoE ε4 carriers and most controls were non-carriers. Due to the small number of samples, we were not able to analyse the impact of these imbalances on the brain proteins. Imbalances in gender and ApoE genotype are usual in AD studies since both are risk factors for the disorder. In the future, it will be important to aim at gender- and ApoE-matching of patients in proteomic studies.

There are no previous 2-D oxyblot studies on oxidatively modified proteins in AD CSF, and therefore the changes observed in oxidative modification cannot be directly compared with other proteomic studies. We analysed the levels and oxidation of 22 abundant CSF proteins in AD and controls. In general, the extent of oxidation did not vary between AD patients and controls. Only two proteins exhibited a significantly higher extent of oxidation in AD patients as compared to controls. One protein was identified as blood-derived lambda chain precursor protein, which is involved in systemic and localised primary immunoglobulin-related amyloidosis (Hamidi Asl et al. 1999, Schröder and Linke 1999). However, since lambda chain precursor is not a brain-specific protein, its origin in CSF is difficult to establish. It is possible that lambda chain precursor infiltrates into CSF directly from blood. Therefore, changes in oxidation and levels of lambda chain precursor may also reflect peripheral changes or some phenomenon not related to the pathogenetic processes going on in the brain.

CSF is composed mainly of soluble proteins, and our result of the prevalent trend of decreases in the levels of proteins was consistent with previous studies (Davidsson et al. 2002, Puchades et al. 2003). A decrease in the amount of several brain proteins in CSF maybe due to

decreased production or increased aggregation in brain that may occur during the early stages of the disease. Our results confirmed the decreases of beta-trace and transthyretin in AD although we were not able to confirm the prominent decreases in the levels of proapolipoproteins. This is possibly due to differences in the selection of study subjects (Davidsson et al. 2002, Puchades et al. 2003). Instead, we detected decreases in the levels of proapolipoprotein in ApoE ε4 carriers vs. non-carriers. The amount of Cu/Zn-superoxide dismutase (SOD1), a vital antioxidant enzyme, was also decreased in ε4 carriers. Proapolipoprotein and SOD1 have been found in association with amyloid deposits in AD brain and both proteins are susceptible to oxidative stress (Harr et al. 1996, Kivatinitz et al. 1997, Choi et al. 2005). The decreases in the levels of proteins generally tended to be more pronounced in ApoE ε4 carriers vs. non-carriers than between AD patients and controls. Several proteins exhibited also a tendency of being more extensively oxidised in ε4 carriers. These results agree with previous studies that have shown enhanced aggregation of proteins and increased vulnerability to oxidative stress in ε4 carriers when compared to the other phenotypes (Ramassamy et al. 1999, Ramassamy et al. 2001).

We did not study the overall level of increased oxidative stress in our subjects instead we examined the level of carbonylation of individual CSF and brain proteins. The reasons and functional consequences for the extent of oxidative modification of these proteins remain unknown. Oxidation precedes degradation of proteins and is required for elimination of the misfolded proteins. Proteins that are active may be more resistant to oxidation and carbonylated proteins may be associated with dysfunctional proteins. Further, carbonyl formation under oxidative stress is not the primary event but it is believed to occur after other protein damaging modifications such as oxidation of methionine (Dalle-Donne et al. 2003a). Since carbonylation is thought to attack already inactivated proteins, it cannot be ruled out that protein-bound carbonyls may even represent beneficial changes that are necessary for normal physiological functions.

In conclusion, we found new potentially important changes in the levels and oxidation of proteins in brain and CSF of AD patients. Some of these changes were associated with clinical and neuropathological data, indicating that they reflect functionally relevant changes. The results need to be replicated in further studies since the material is too small to allow one to draw definite conclusions and to exclude other possible confounding factors. The results emphasize that protein isoforms may exhibit a distinct, characteristic response to oxidative

stress. Therefore, it may be beneficial to examine the oxidation of proteins more profoundly i.e. immunoprecipitate the protein of choice prior to 2-D oxyblotting. The effects of other post-translational modifications influencing the susceptibility of protein isoforms to oxidation are also far from being understood. In the future it will be important to determine at what stage of the disease process the changes in the oxidation status of proteins start to occur.

6.1.2. Post-translational modification of GFAP

In study **II** we observed that GFAP-labelled astrocytes showed a negative correlation with the amount and the degree of oxidation of aldolase C in AD whereas the cytosolic fraction of GFAP that was measured by 1-D immunoblotting exhibited a positive correlation with the degree of oxidation of aldolase C and GDH in control brains. These results attracted our attention because it was already well established that GFAP was an excellent candidate for 2DE analysis due to its several isoforms (Comings 1982c, Greber et al. 1999, Porchet et al. 2003), and therefore we decided to examine GFAP and its modifications in detail in study **III**.

Consistent with the previous studies, we detected an overall increase in the amount of GFAP in the frontal cortex of AD patients when compared to controls (Arnold et al. 1996, Bigl et al. 1999, Overmyer et al. 1999a, Ross et al. 2003). However, only eleven out of 46 distinctive isoforms exhibited significant increase. Surprisingly, we were able to fit the average values of the quantities into a mathematical equation. According to the profile of isoforms created using the equation, the significant increase was limited to the acidic isoforms of GFAP. Further analyses revealed that the acidic isoforms were both phosphorylated and N-glycosylated whereas the basic isoforms seemed to be O-glycosylated. Therefore, the results indicated that not only the amount of GFAP was increased in AD but also that the way GFAP became modified may have been shifting the quantitative isoelectric distribution towards the phosphorylated and N-glycosylated isoforms of GFAP. Our results emphasize the importance of analysis of dynamic isoform-specific levels of proteins in pathophysiological conditions. It remains to be determined whether quantities of other AD-related protein isoforms obey a similar mathematical distribution as detected for GFAP. The methodology used in study **III** can be adopted to permit examination of the isoelectric distribution of other protein isoforms.

It is well-established that GFAP is a phosphoprotein (Noetzel 1990) but it was only recently proposed that GFAP is a glycoprotein (Kanninen et al. 2004). Our results not only supported these findings but also revealed that GFAP is both N-and O-glycosylated. The quantitative acidic shift in the isoform pattern of GFAP may reflect a net effect of changes in both phosphorylation and N-glycosylation, since hyperphosphorylation of tau has been reported to evoke a similar type of acidic shift in the 2-D pattern as we found for GFAP in AD brains (Lefebvre et al. 2003). AD-tau is also known to be N-glycosylated, resulting in an enhanced phosphorylation of tau (Liu et al. 2002a, Liu et al. 2002b) whereas O-glycosylation has been suggested to be protective against tau phosphorylation (Lefebvre et al. 2003). Indeed, GFAP may be another phosphorylated and glycosylated protein involved in the pathogenesis of AD. It is also tempting to speculate that the suggested imbalance in the activities of phosphatases and kinases in AD may not only affect post-translational modification of tau but also other proteins such as GFAP.

The total amount of soluble GFAP, a quantitative marker of RAs, correlated with the counts of microglia in AD. It is well-known that microglia secrete inflammatory mediators leading astrocyte activation (Norenberg 1994). Further, the levels of several isoforms were related to the counts of RAs in controls. These findings indicate that the soluble levels of GFAP may increase concomitantly with astrocyte activation in normal aging brain. The Aβ-load correlated with several O-glycosylated basic isoforms of GFAP in controls, hinting at a connection between astrocytes and βA homeostasis (Guénette 2003, Nicoll and Weller 2003). No correlations were found between GFAP and tau or Aβ pathology in AD. The lack of correlations between GFAP isoforms, RAs and Aβ in AD brains may be due to the fact that the expression of GFAP had reached a ceiling effect due to extensive astrocytosis and neuropathology.

6.2. CORNERSTONES AND PITFALLS WHEN INTERPRETING DATA

In clinical expression proteomics, misintepretations of data can occur at every step, from the clinical information to the results. Although AD proteomes have been scrutinized during the last decade, the published studies are currently difficult, even impossible to be compared with each other due to insufficient characterisation of the study material, the small number of patients involved in studies and the variations in experimental designs. Results may thus be

interpreted to be falsely controversial between proteomic studies although they are simply due to logical differences in study designs. Biological and methodological variation is a part of random and unbiased nature of proteome analysis.

6.2.1. Study material

One of the current focuses in clinical proteomics is concentrated on the characteristics of patients since they substantially influence the results. The patients and samples need to be as well characterised as possible since the proteomes are dynamic and the number of patients recruited into proteomic studies will commonly remain small due to the methodological and computational limitations inherent in the technique (i.e. Table 1 and **I-IV**). In addition to individual proteomic heterogeneity, post-mortem studies are influenced by complex ante- and post-mortem events (reviewed by Lewis 2002, Hynd et al. 2003). For example, in studies **I-III**, most AD patients died from pneumonia with prolonged agonal stage probably accompanied by fever, inflammation and hypoxia; while the controls died from sudden cardiac death. It cannot be ruled out that these differences in agonal stages may in some way have influenced expression and modification of proteins. There are a number of factors that are important confounders in the interpretation of the differences in 2-D-maps between healthy and disease states. Insufficient reporting of patient demographics such as duration of the disease, severity of dementia or the brain area for sampling is common in many proteomics studies. This results in difficulties in the comparison of the studies.

In brain proteome studies **I-III**, we selected AD patients with severe dementia, short PMDs and relatively pure AD-neuropathology without additional pathologies such as vascular lesions or synuclein pathology. One problem that is common to the most studies is the selection of control subjects. The main selection criteria for the controls in the present study were age and cognition. The controls were independently living individuals who experienced sudden cardiac death at home. This is one reason for the longer PMDs of controls as compared to AD patients (3-64 hours vs. 2-12 hours). Moreover, because assessment of cognition was based on retrospective evaluation of the medical records, the possibility of mild cognitive changes cannot be completely excluded. The availability of completely "healthy" elderly individuals wihout age-related diseases is limited. In addition, it is common that controls often have AD-related brain changes to some extent even if they are cognitively

unimpaired. This was also the case with our studies since some control patients exhibited a limited degree of AD-related neuropathology.

Selection and characterization of the tissue and body fluid to be studied is also important in proteomics. Different regions in brain have distinct cellular compositions and functions; and thus most likely express variations in their proteomes. Morever, progression and duration of AD are also relevant issues in the selection of the tissue. AD is a stage- and region-specific disease that evolves following a characteristic pattern of symptoms and pathology in the brain. Neurodegeneration starts anatomically and histologically from the entorhinal cortex and spreads progressively via hippocampus to the cortical areas (Braak and Braak 1991b, Reddy and McWeeney 2005). Therefore, the pathogenic process in AD influences the proteomes in brain regions in a time-dependent manner. Our AD cases represented the end-stage of the disease (Braak and Braak stages V-VI), and therefore the findings cannot be generalized to reveal the changes occurring in the early phases of the disease. We used the frontal cortex tissue from Broadman area 9. Since the cell structures vary in different Brodman areas (Carpenter 1976, England and Wakely 1991), our results are best comparable with proteomic studies performed using the frontal cortex specimens from AD patients with a long-standing disease. Control subjects fell into Braak and Braak stages I-II (except one control who had stage IV) and did not have any PHF-tau pathology in their frontal cortices. In addition, controls may have had some amyloid deposits in frontal cortices, which are often found in individuals without cognitive deficits. All controls were classified as normal (a or b) according to the CERAD criteria.

Patients in the study **IV** about oxidatively modified CSF proteins had a clinical diagnosis of probable AD according to the NINCDS-ADRDA criteria (McKhann et al. 1984). They had mild dementia and the duration of clinical impairment was relatively short. The neuropathologically verified accuracy of clinical diagnosis of AD has been reported to be above 90% in our clinic (Kosunen et al. 1996). The control group included age-matched cognitively intact subjects who suffered from neurovegetative or psychiatric symptoms, tremor and memory problems but did not have a chronic neurodegenerative disease. As previously stated, AD-related pathology is common even in cognitively intact subjects, and it cannot be excluded solely on clinical basis. Moreover, patients with a clinical diagnosis of AD may possess additional concomitant brain pathologies that may interfere when aiming at examining disease-specific changes in CSF. These are common limitations in all studies using

clinical patient material.

Unlike genetic determinants such as ApoE genotype and gender, clinical AD is far from being a "static" state. There are no biological markers for reliable follow-up of the progression of AD and pathological changes in biochemical pathways that are dependent on the disease stage. Therefore, proteomic patterns may vary in different disease stages, which need to be taken into account in the interpretation of the results. Although no straightforward test exists for monitoring the progression of AD, it is most likely that patients with a long duration of clinical impairment and low MMSE scores are at a different stage of the disorder as compared to patients with progressive MCI or early AD with higher scores. In addition, the selection of both the control and AD group may influence the results. For example, in the study of Puchades et al. 2003, the levels of CSF proteins were measured in non-age-matched "healthy" cognitively intact elderly people with MMSE scores at 29-30 and older AD patients. It is obvious that the study subject differed from our material in study **IV** where the levels of CSF proteins were measured in age-matched controls who did not show progressive decline (but may have had stable cognitive deficit with MMSE scores between 21 and 29), and in AD/MCI patients suffering from mild dementia (CSF puncture may have been taken prior to the clinical diagnosis of progressive disorder with MMSE scores between 14 and 26).

In all studies **I-IV**, individual proteomes among controls seemed to be more heterogeneous as compared to AD patients. This finding persisted when examining both the levels and oxidation of brain and CSF proteins. This is a fact of life since control groups are often composed of individuals suffering from a variety of heterogeneous age-related health problems. Moreover, oxidation patterns of the proteins may be especially vulnerable to other factors such as genotype (i.e. gender, ApoE ε4 allele), lifestyle (i.e. diet, obesity, exercise), and drug treatments (i.e. vitamin replacement therapy) (Vincent and Taylor 2005). In addition, oxidative stress is always present in chronic-degenerative conditions such as autoimmune diseases (i.e. diabetes, rheumatoid arthritis), vascular disorders (i.e. cardiovascular diseases), chronic inflammatory disorders (i.e. asthma) and in cancers (Galli et al. 2005). It is not known whether peripheral oxidative stress can influence the oxidation level of CNS proteins.

6.2.2. Experimental design

There are several reasons why we chose 2-DE in combination with MS and/or immunoblotting to study differences in AD and control proteomes. Above all, there is currently no single powerful technology for studying quantitative differencies in proteomes in diseased and healthy states. Though there are new promising gel-free techniques in proteomics, 2-DE in combination with MS and/or immunoblotting still remains the most established approach in separating and quantifying multiple proteins in complex mixtures followed by identification. In addition, the ability of 2-DE techniques being easily adaptable to study modifications of proteins served our aims. Indeed, by slightly modifying the 2-DE experiments we were able to study oxidation, glycosylation and phosphorylation of proteins.

As is the case with proteomics in general, neither is there a single powerful 2-DE application for revealing comprehensive changes in AD-related proteomes. The results obtained in 2-D experiments are always influenced by sample preparation protocols, selected IPG strip lengths and pH ranges, sizes of second dimension gels, protein stains, image analysis parameters, bioinformatics, and success of protein identification. Although IPG-based 2-DE is a commonly used protein separation technique in proteomics, it has a several limitations e.g. it is poor at revealing i) low-abundance proteins, ii) hydrophobic proteins i.e. membrane proteins, iii) acidic and basic proteins, iv) low and high Mr proteins, and v) individual protein heterogeneity (reviewed by Fountoulakis 2004). In particular, 2-DE analysis as a whole, is laborous and often requires large amount of sample.

Sample preparation influences the types of proteins to be solubilised and analysed in the selected tissue type (Görg et al. 2004). The chemicals and incubation times influence protein solubilisation and thus the outcome of the proteome in tissues or body fluids. We separated proteins soluble in Tris-HCl buffer for the analysis of brain proteins **(I-III)**. Our protocol solubilizes mostly cytosolic proteins and other chemicals may have dissolved different proteins. We also precipitated brain proteins with TCA and CSF proteins with acetone. Precipitation procedures are important and may lead to differences in 2-D proteome patterns i.e. incubation times and chemicals influence the yield and type of proteins to be precipitated. Therefore, the results can be reliably compared with studies using comparable sample preparation protocols. Sample preparation has improved greatly during recent years and there are currently a variety of commercially available kits and means to separate different types of

proteomes. Sample preparation methods are essential for the reproducibility of 2-D experiments.

The results obtained in 2-D experiments are influenced by IPG gel lengths and pH ranges and sizes of second dimension gels. The same protein may be detected using i) 7 cm long 3-10 NL, ii) 11 cm long 4-7 and iii) 18 cm 5.5-6.5 IPG strips that may resolve the same protein into i) one, ii) two and iii) six spots, respectively. Indeed, the number of resolved protein spots can vary greatly, depending on the resolution attributable to pH gradients and gel lengths. Also the size of the second dimension gel influences the resolution i.e. "the bigger the better". We used the small 7cm IPG gel format with pH gradients of 4-7 and 3-10 in studies **I-III** and the long 18 cm IPG gel format with pH gradient 3-10 in study **IV**. It is very probable that more changes may be detected in AD proteomes using larger gel formats and/or narrower pH gradients. It is common knowledge that interlaboratory qualitative and quantitative differences in the results can be traced to gel sizes. The opposite side of this coin is that the permutations provide an excellent opportunity to reveal more and more protein changes by choosing different gel sizes and pH gradients.

More than hundred proteins were estimated by the naked eye to be present on our 2-D gels. However, not all the proteins were reliably matched to the corresponding protein on a reference gel in the image analysis due to too high or low abundancy, poor resolution or heterogeneity in the individual proteomes. Difficulties in matching and quantification could have been partially overcome by using DIGE technology with an internal protein standard. Moreover, we used ImageMaster 2D Elite software, which detects and quantifies proteins in "two-dimensional space" on 2-D images. In other words, the protein is detected and quantified by the occupied area and the intensity of staining. More protein changes may have been detected using new generation software packages; in these the detection of proteins occurs in "three-dimensional space". This means that proteins are detected by area and the intensity is detected as height leading to transformation of spots into digital mountainlike forms. This improves spot detection and quantification due to improved resolution and better estimation of the spot size, respectively.

Many individual protein isoforms can be observed on 2-D gels. The resolution of these isoforms may differ and there may also be isoform-specific changes in the disease. In theory, the levels of an isoform of a protein called (X_a) may be increased in AD, whereas another

isoform of the same protein (X_b) may not be detected due to poor resolution or low abundance; or else this isoform may exhibit unaltered or decreased levels in AD. In other words, the quantitative amount of one isoform does not necessarily reveal anything about the amount of total protein. Sometimes it is very difficult to determine whether the isoforms detected in different studies are comparable. These issues were clearly demonstrated in study **III**. The acidic shift of isoelectric isoform distribution of GFAP in AD became detectable by 2-D immunoblotting whereas GFAP remained masked by other overlapping proteins in the total protein staining. The decrease and increase of some GFAP isoforms in AD as compared to controls was already described in one previous study using total protein staining (Tsuji et al. 1999). However, it is not possible to determine which isoforms detected here correlate to those reported by Tsuji et al. 1999. In addition, in study **II**, we identified two isoforms of MDH1 but only one of them, the more acidic isoform, exhibited significantly increased levels in AD as compared to controls. It is the case that different isoforms may express unchanged, increased or decreased levels in proteomic studies. However, this does not necessary mean that the results obtained in different studies are in conflict with each other, but may rather reflect methodological and biological variation.

In the present series of reports, experimental assemblies enabled us to study the differences in the levels and modifications of the most abundant brain and CSF proteins. Hereafter, it will be important to go "deeper" into the proteome and to study less abundant proteins. For example, all the CSF proteins that were studied are present in the mg/l range, whereas even Aβ and tau, which are currently the best known biomarker candidates for a diagnostic laboratory test for AD, are present in the ng/l range (Reiber and Peter 2001). To go deeper into the CSF proteome, would require either the removal of the most abundant proteins i.e. albumin and immunoglobulins that account for about 80% of the total protein content in CSF, and/or to prefractionate the sample (Righetti et al. 2005). However, it is challenging to study the "deeper" CSF proteome using 2-DE since CSF has low protein concentration (about 250 mg/l) (Reiber and Peter 2001), and 2-DE currently requires relatively high amounts of protein (up to about 1 mg depending on the methodology). Moreover, the volume of CSF taken per lumbar puncture is limited (5-10 ml/puncture). In contrast to CSF, brain tissue is rich in proteins and the amplitude is smaller to that of body fluids. There are possibilities and ways to study the "deeper" brain proteome using 2-DE i.e. by applying prefractionation steps, narrow pH gradients, and large gel formats.

The general aim in proteomics is to study proteins on a large-scale. However, this is currently rarely the reality since whatever technology is used, proteomic analysis is technically challenging. Also, our studies represent the early days of proteomics and a small-scale approach. Although we started from more than 100 visualised and modified proteins on our 2-D images, we ended up with only few protein changes of identified proteins. Since the beginning of this thesis work, proteomics techniques have improved greatly and the costs of this currently expensive methodology keep diminishing. Undoubtedly, if this work were to be repeated today, more changes would be observed. Therefore, it is of the outmost importance to keep pace with methodological improvements in proteomics. If we understand how the tool works and the limitations of the technology, then the only limits are the limits of human ingenuity.

6.3. FUTURE ASPECTS IN CLINICAL PROTEOMICS

Individual laboratories have already exerted major efforts in examining AD proteomes in order to clarify the pathogenesis of the disease and to screen for new biomarker candidates. Similar to the mapping of human genome (human genome organisation, HUGO), the international research society became united in order to map human proteomes, and established the human proteome organisation (HUPO, www.hupo.org) a few years ago. Currently there are several initiatives under HUPO that all aim at improving and standardising proteomics techniques as well as exploring human proteomes in health and disease states. Human brain proteome project (HBPP, http://www.hbpp.org) is an open international scientific initiative under the umbrella of HUPO (Hamacher et al. 2004). One of the main focuses in HBPP is AD (Hanash 2004). Considering the current state of art in proteomics, it will be interesting to follow future advances in understanding the pathogenesis of AD as well as in biomarker and drug discovery.

7. SUMMARY AND CONCLUSIONS

The major objectives in the series of studies were i) methodological developments in proteome analysis of differentially post-translationally modified proteins, ii) tracing variations in brain and CSF proteomes in AD patients as compared to age-matched controls, and iii) assessment of relationships between proteomes and neuropathological data. The following conclusions related to the pathogenesis and biomarker discovery in AD can be drawn:

1. About 150 proteins and more than 100 oxidised proteins were detected in both AD and control brains using a multiplexed proteomics approach utilizing 2-D oxyblotting. The tendency towards increased levels of proteins was often associated with concomitant decreases in oxidation. The major finding was that two dehydrogenases, MDH1 and GDH, were characterized as less carbonylated proteins according to the degree of oxidation in AD. These findings may be compensatory and occur in response to compromised metabolism in AD. (**I-II**)

2. Twentytwo oxidatively modified proteins were characterised in CSF of AD patients and controls. The levels of identified CSF proteins were generally decreased whereas the degree of oxidation tended to be either unchanged or increased in AD as compared to controls. None of the brain-derived proteins exhibited significant changes in the extent of oxidative modification in AD. (**IV**)

3. PTM and isoform profiles of GFAP have previously remained unclear in AD. Fortysix isoforms of GFAP were separated and quantified. Acidic isoforms of GFAP exhibited a 60% increase in AD as compared to controls. These acidic isoforms were phosphorylated and N-glycosylated whereas the basic isoforms were O-glycosylated and revealed no quantitative differences between the groups. The data suggest that GFAP may be another abnormally phosphorylated and glycosylated protein involved in the pathogenesis of AD. (**III**)

4. Protein changes were dependent on the stage and clinical impairment in AD. (**II-III**)

5. This series of studies emphasizes the importance of studying modifications of proteins in neurodegenerative disorders. Extensive characterisation of PTMs of proteins may help to clarify new pathological processes in AD, and possibly provide new candidate targets for medical intervention. (**I-IV**)

8. REFERENCES

Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature 2003; 422: 198-207.

Ai KZ, Vermuyten K, De Deyn PP, Lowenthal A, Karcher D. A serum protein involved in aging? Mol Chem Neuropathol 1989; 11: 131-41.

Aitken A, Collinge DB, van Heusden BP, Isobe T, Roseboom PH, Rosenfeld G, et al. 14-3-3 proteins: a highly conserved, widespread family of eukaryotic proteins. Trends Biochem Sci 1992; 17: 498-501.

Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, et al. Inflammation and Alzheimer's disease. Neurobiol Aging 2000; 21: 383-421.

Aksenov MY, Aksenova MV, Butterfield DA, Geddes JW, Markesbery WR. Protein oxidation in the brain in Alzheimer's disease. Neuroscience 2001; 103: 373-83.

Alafuzoff I, Adolfsson R, Bucht G, Jellum E, Mehta PD, Winblad B. Isoelectric focusing and two-dimensional gel electrophoresis in plasma and cerebrospinal fluid from patients with dementia. Eur Neurol 1986; 25: 285-9.

Alafuzoff I, Helisalmi S, Mannermaa A, Riekkinen P, Sr., Soininen H. Beta-amyloid load is not influenced by the severity of cardiovascular disease in aged and demented patients. Stroke 1999; 30: 613-8.

Aldred S, Grant MM, Griffiths HR. The use of proteomics for the assessment of clinical samples in research. Clin Biochem 2004; 37: 943-52.

Almeida OP, Flicker L. Association between hormone replacement therapy and dementia: is it time to forget? Int Psychogeriatr 2005; 17: 155-64.

Alzheimer A. Über eine eigenartige Erkrankung der Hirnrinde. Allg Z Psychiat 1907; 64: 146-148.

American Psychiatric Association. Diagnostic and statistical manual of mental disorders. 3rd edn., revised. Washington DC: American Psychiatric Association, 1987.

Anderson NG, Matheson A, Anderson NL. Back to the future: the human protein index (HPI) and the agenda for post-proteomic biology. Proteomics 2001; 1: 3-12.

Andlin-Sobocki P, Jönsson B, Wittchen HU, Olesen J. Cost of disorders of the brain in Europe. Eur J Neurol 2005; 12: 1-27.

Arnold SE, Franz BR, Trojanowski JQ, Moberg PJ, Gur RE. Glial fibrillary acidic protein-immunoreactive astrocytosis in elderly patients with schizophrenia and dementia. Acta Neuropathol (Berl) 1996; 91: 269-77.

Atlante A, Calissano P, Bobba A, Giannattasio S, Marra E, Passarella S. Glutamate neurotoxicity, oxidative stress and mitochondria. FEBS Lett 2001; 497: 1-5.

Atwood CS, Huang X, Moir RD, Tanzi RE, Bush AI. Role of free radicals and metal ions in the pathogenesis of Alzheimer's disease. Met Ions Biol Syst 1999; 36: 309-64.

Balin BJ, Gerard HC, Arking EJ, Appelt DM, Branigan PJ, Abrams JT, et al. Identification and localization of Chlamydia pneumoniae in the Alzheimer's brain. Med Microbiol Immunol (Berl) 1998; 187: 23-42.

Ballabh P, Braun A, Nedergaard M. The blood-brain barrier: an overview: structure, regulation, and clinical implications. Neurobiol Dis 2004; 16: 1-13.

Banks RE, Dunn MJ, Hochstrasser DF, Sanchez JC, Blackstock W, Pappin DJ, et al. Proteomics: new perspectives, new biomedical opportunities. Lancet 2000; 356: 1749-56.

Berger AK, Fratiglioni L, Forsell Y, Winblad B, Backman L. The occurrence of depressive symptoms in the preclinical phase of AD: a population-based study. Neurology 1999; 53: 1998-2002.

Berlett BS, Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. J Biol Chem 1997; 272: 20313-6.

Bigl M, Bleyl AD, Zedlick D, Arendt T, Bigl V, Eschrich K. Changes of activity and isozyme pattern of phosphofructokinase in the brains of patients with Alzheimer's disease. J Neurochem 1996; 67: 1164-71.

Bigl M, Bruckner MK, Arendt T, Bigl V, Eschrich K. Activities of key glycolytic enzymes in the brains of patients with Alzheimer's disease. J Neural Transm 1999; 106: 499-511.

Bjellqvist B, Ek K, Righetti PG, Gianazza E, Görg A, Westermeier R, et al. Isoelectric focusing in immobilized pH gradients: principle, methodology and some applications. J Biochem Biophys Methods 1982; 6: 317-39.

Blasko I, Stampfer-Kountchev M, Robatscher P, Veerhuis R, Eikelenboom P, Grubeck-Loebenstein B. How chronic inflammation can affect the brain and support the development of Alzheimer's disease in old age: the role of microglia and astrocytes. Aging Cell 2004; 3: 169-76.

Blass JP, Gibson GE, Hoyer S. The role of the metabolic lesion in Alzheimer's disease. J Alzheimers Dis 2002; 4: 225-32.

Blass JP, Zemcov A. Alzheimer's disease. A metabolic systems degeneration? Neurochem Pathol 1984; 2: 103-14.

Blennow K, Hampel H. CSF markers for incipient Alzheimer's disease. Lancet Neurol 2003; 2: 605-13.

Blessed G, Tomlinson BE, Roth M. The association between quantitative measures of dementia and of senile change in the cerebral grey matter of elderly subjects. Br J Psychiatry 1968; 114: 797-811.

Braak H, Braak E. Demonstration of amyloid deposits and neurofibrillary changes in whole brain sections. Brain Pathol 1991a; 1: 213-6.

Braak H, Braak E. Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol (Berl) 1991b; 82: 239-59.

Brandt R, Hundelt M, Shahani N. Tau alteration and neuronal degeneration in tauopathies: mechanisms and models. Biochim Biophys Acta 2005; 1739: 331-54.

Breteler MM, Claus JJ, van Duijn CM, Launer LJ, Hofman A. Epidemiology of Alzheimer's disease. Epidemiol Rev 1992; 14: 59-82.

Bretsky PM, Buckwalter JG, Seeman TE, Miller CA, Poirier J, Schellenberg GD, et al. Evidence for an interaction between apolipoprotein E genotype, gender, and Alzheimer disease. Alzheimer Dis Assoc Disord 1999; 13: 216-21.

Brown RC, Lockwood AH, Sonawane BR. Neurodegenerative diseases: an overview of environmental risk factors. Environ Health Perspect 2005; 113: 1250-6.

Bubber P, Haroutunian V, Fisch G, Blass JP, Gibson GE. Mitochondrial abnormalities in Alzheimer brain: mechanistic implications. Ann Neurol 2005; 57: 695-703.

Buée L, Bussière T, Buée-Scherrer V, Delacourte A, Hof PR. Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. Brain Res Brain Res Rev 2000; 33: 95-130.

Burbaeva G. Physiologically active brain proteins as possible markers of mental diseases. Vestn Ross Akad Med Nauk 1992: 51-4.

Burbaeva G, Boksha IS, Tereshkina EB, Savushkina OK, Starodubtseva LI, Turishcheva MS. Glutamate metabolizing enzymes in prefrontal cortex of Alzheimer's disease patients. Neurochem Res 2005; 30: 1443-51.

Burdick D, Soreghan B, Kwon M, Kosmoski J, Knauer M, Henschen A, et al. Assembly and aggregation properties of synthetic Alzheimer's A4/beta amyloid peptide analogs. J Biol Chem 1992; 267: 546-54.

Butterfield DA, Boyd-Kimball D, Castegna A. Proteomics in Alzheimer's disease: insights into potential mechanisms of neurodegeneration. J Neurochem 2003; 86: 1313-27.

Butterworth RF, Besnard AM. Thiamine-dependent enzyme changes in temporal cortex of patients with Alzheimer's disease. Metab Brain Dis 1990; 5: 179-84.

Carpenter MB. Human neuroanatomy. Baltimore: The Williams and Wilkins Company, 1976.

Castegna A, Aksenov M, Aksenova M, Thongboonkerd V, Klein J, Pierce W, et al. Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. part I: creatine kinase BB, glutamine synthase, and ubiquitin carboxy-terminal hydrolase L-1. Free Radic Biol Med 2002a; 33: 562.

Castegna A, Aksenov M, Thongboonkerd V, Klein JB, Pierce WM, Booze R, et al. Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part II: dihydropyrimidinase-related protein 2, alpha-enolase and heat shock cognate 71. J Neurochem 2002b; 82: 1524-32.

Choe LH, Dutt MJ, Relkin N, Lee KH. Studies of potential cerebrospinal fluid molecular markers for Alzheimer's disease. Electrophoresis 2002; 23: 2247-51.

Choi J, Levey AI, Weintraub ST, Rees HD, Gearing M, Chin LS, et al. Oxidative modifications and down-regulation of ubiquitin carboxyl-terminal hydrolase L1 associated with idiopathic Parkinson's and Alzheimer's diseases. J Biol Chem 2004; 279: 13256-64.

Choi J, Malakowsky CA, Talent JM, Conrad CC, Gracy RW. Identification of oxidized plasma proteins in Alzheimer's disease. Biochem Biophys Res Commun 2002; 293: 1566-70.

Choi J, Rees HD, Weintraub ST, Levey AI, Chin LS, Li L. Oxidative modifications and aggregation of Cu,Zn-superoxide dismutase associated with Alzheimer and Parkinson diseases. J Biol Chem 2005; 280: 11648-55. Epub 2005 Jan 19.

Chong MS, Sahadevan S. Preclinical Alzheimer's disease: diagnosis and prediction of progression. Lancet Neurol 2005; 4: 576-9.

Clark BF. Towards a total human protein map. Nature 1981; 292: 491-2.

Coleman P, Federoff H, Kurlan R. A focus on the synapse for neuroprotection in Alzheimer disease and other dementias. Neurology 2004; 63: 1155-62.

Coleman PD, Yao PJ. Synaptic slaughter in Alzheimer's disease. Neurobiol Aging 2003; 24: 1023-7.

Comings DE. Two-dimensional gel electrophoresis of human brain proteins. I. Technique and nomenclature of proteins. Clin Chem 1982a; 28: 782-9.

Comings DE. Two-dimensional gel electrophoresis of human brain proteins. III. Genetic and non-genetic variations in 145 brains. Clin Chem 1982b; 28: 798-804.

Comings DE. Two-dimensional gel electrophoresis of human brain proteins. IV. Disorders of glial proliferation and a polymorphism of glial fibrillary acidic protein--GFAP Duarte. Clin Chem 1982c; 28: 805-12.

Connell CM, Janevic MR, Gallant MP. The costs of caring: impact of dementia on family caregivers. J Geriatr Psychiatry Neurol 2001; 14: 179-87.

Conrad CC, Choi J, Malakowsky CA, Talent JM, Dai R, Marshall P, et al. Identification of protein carbonyls after two-dimensional electrophoresis. Proteomics 2001; 1: 829-34.

Cook DG, Forman MS, Sung JC, Leight S, Kolson DL, Iwatsubo T, et al. Alzheimer's A beta(1-42) is generated in the endoplasmic reticulum/intermediate compartment of NT2N cells. Nat Med 1997; 3: 1021-3.

Cooper AJL. Metabolic significanse of transamination: Wiley, New York, 1985.

Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, et al. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science 1993; 261: 921-3.

Dale G, Latner AL. Isoelectric focusing of serum proteins in acrylamide gels followed by electrophoresis. Clin Chim Acta 1969; 24: 61-8.

Dalle-Donne I, Giustarini D, Colombo R, Rossi R, Milzani A. Protein carbonylation in human diseases. Trends Mol Med 2003a; 9: 169-76.

Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. Protein carbonyl groups as biomarkers of oxidative stress. Clin Chim Acta 2003b; 329: 23-38.

David DC, Hoerndli F, Gotz J. Functional Genomics meets neurodegenerative disorders Part I: transcriptomic and proteomic technology. Prog Neurobiol 2005; 76: 153-68.

Davidsson P, Westman-Brinkmalm A, Nilsson CL, Lindbjer M, Paulson L, Andreasen N, et al. Proteome analysis of cerebrospinal fluid proteins in Alzheimer patients. Neuroreport 2002; 13: 611-5.

Davies KJ. Degradation of oxidized proteins by the 20S proteasome. Biochimie 2001; 83: 301-10.

De Felice FG, Ferreira ST. Beta-amyloid production, aggregation, and clearance as targets for therapy in Alzheimer's disease. Cell Mol Neurobiol 2002; 22: 545-63.

DeKosky ST, Marek K. Looking backward to move forward: early detection of neurodegenerative disorders. Science 2003; 302: 830-4.

Desai PP, Ikonomovic MD, Abrahamson EE, Hamilton RL, Isanski BA, Hope CE, et al. Apolipoprotein D is a component of compact but not diffuse amyloid-beta plaques in Alzheimer's disease temporal cortex. Neurobiol Dis 2005; 20: 574-82.

Desrosiers RR, Bertrand Y, Nguyen QT, Demeule M, Gabathuler R, Kennard ML, et al. Expression of melanotransferrin isoforms in human serum: relevance to Alzheimer's disease. Biochem J 2003; 374: 463-71.

Dodgson SJ, Tashian R, Gross G, Carter ND. The Carbonic anhydrases: Cellular physiology and molecular genetics. New York: Plenum, 1991.

Eikelenboom P, van Gool WA. Neuroinflammatory perspectives on the two faces of Alzheimer's disease. J Neural Transm 2004; 111: 281-94. Epub 2003 Dec 03.
Eng LF, Ghirnikar RS, Lee YL. Glial fibrillary acidic protein: GFAP-thirty-one years (1969-2000). Neurochem Res 2000; 25: 1439-51.

England MA, Wakely J. Color Atlas of Brain and Spinal Cord: Mosby Year Book, 1991.

Erecinska M, Nelson D. Activation of glutamate dehydrogenase by leucine and its nonmetabolizable analogue in rat brain synaptosomes. J Neurochem 1990; 54: 1335-43.

Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM. Electrospray ionization for mass spectrometry of large biomolecules. Science 1989; 246: 64-71.

Ferri CP, Prince M, Brayne C, Brodaty H, Fratiglioni L, Ganguli M, et al. Global prevalence of dementia: a Delphi consensus study. Lancet 2005; 366: 2112-7.

Fisher O. Die presbyophrene Demenz, deren anatomische Grundlage und klinische Abrenzung. Z Ges Neurol Psychiatry 1910; 3: 371-471.

Folstein MF, Folstein SE, McHugh PR. "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. J Psychiatr Res 1975; 12: 189-98.

Fountoulakis M. Application of proteomics technologies in the investigation of the brain. Mass Spectrom Rev 2004; 23: 231-58.

Fountoulakis M, Cairns N, Lubec G. Increased levels of 14-3-3 gamma and epsilon proteins in brain of patients with Alzheimer's disease and Down syndrome. J Neural Transm Suppl 1999; 57: 323-35.

Fratiglioni L, Paillard-Borg S, Winblad B. An active and socially integrated lifestyle in late life might protect against dementia. Lancet Neurol 2004; 3: 343-53.

Frey HJ, Mattila KM, Korolainen MA, Pirttilä T. Problems associated with biological markers of Alzheimer's disease. Neurochem Res 2005; 30: 1501-10.

Friedrich CA, Ferrell RE, Siciliano MJ, Kitto GB. Biochemical and genetic identity of alpha-keto acid reductase and cytoplasmic malate dehydrogenase from human erythrocytes. Ann Hum Genet 1988; 52: 25-37.

Färber K, Kettenmann H. Physiology of microglial cells. Brain Res Brain Res Rev 2005; 48: 133-43.

Galasko D, Hansen LA, Katzman R, Wiederholt W, Masliah E, Terry R, et al. Clinical-neuropathological correlations in Alzheimer's disease and related dementias. Arch Neurol 1994; 51: 888-95.

Galli F, Piroddi M, Annetti C, Aisa C, Floridi E, Floridi A. Oxidative stress and reactive oxygen species. Contrib Nephrol 2005; 149: 240-60.

Geschwind DH. Tau phosphorylation, tangles, and neurodegeneration: the chicken or the egg? Neuron 2003; 40: 457-60.

Gibson GE, Park LC, Sheu KF, Blass JP, Calingasan NY. The alpha-ketoglutarate dehydrogenase complex in neurodegeneration. Neurochem Int 2000; 36: 97-112.

Gibson GE, Park LC, Zhang H, Sorbi S, Calingasan NY. Oxidative stress and a key metabolic enzyme in Alzheimer brains, cultured cells, and an animal model of chronic oxidative deficits. Ann N Y Acad Sci 1999; 893: 79-94.

Gibson GE, Sheu KF, Blass JP. Abnormalities of mitochondrial enzymes in Alzheimer disease. J Neural Transm 1998; 105: 855-70.

Giulian D, Li J, Bartel S, Broker J, Li X, Kirkpatrick JB. Cell surface morphology identifies microglia as a distinct class of mononuclear phagocyte. J Neurosci 1995; 15: 7712-26.

Glenner GG, Wong CW, Quaranta V, Eanes ED. The amyloid deposits in Alzheimer's disease: their nature and pathogenesis. Appl Pathol 1984; 2: 357-69.

Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, et al. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 1991; 349: 704-6.

Gold G, Bouras C, Kovari E, Canuto A, Glaria BG, Malky A, et al. Clinical validity of Braak neuropathological staging in the oldest-old. Acta Neuropathol (Berl) 2000; 99: 579-82; discussion 583-4.

Golde TE. Alzheimer disease therapy: can the amyloid cascade be halted? J Clin Invest 2003; 111: 11-8.

Gong CX, Liu F, Grundke-Iqbal I, Iqbal K. Post-translational modifications of tau protein in Alzheimer's disease. J Neural Transm 2005; 112: 813-38. Epub 2004 Oct 27.

Gong CX, Singh TJ, Grundke-Iqbal I, Iqbal K. Phosphoprotein phosphatase activities in Alzheimer disease brain. J Neurochem 1993; 61: 921-7.

Graham DR, Elliott ST, Van Eyk JE. Broad-based proteomic strategies: a practical guide to proteomics and functional screening. J Physiol 2005; 563: 1-9. Epub 2004 Dec 20.

Greber S, Lubec G, Cairns N, Fountoulakis M. Decreased levels of synaptosomal associated protein 25 in the brain of patients with Down syndrome and Alzheimer's disease. Electrophoresis 1999; 20: 928-34.

Grundke-Iqbal I, Iqbal K, Quinlan M, Tung YC, Zaidi MS, Wisniewski HM. Microtubule-associated protein tau. A component of Alzheimer paired helical filaments. J Biol Chem 1986a; 261: 6084-9.

Grundke-Iqbal I, Iqbal K, Tung YC, Quinlan M, Wisniewski HM, Binder LI. Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. Proc Natl Acad Sci U S A 1986b; 83: 4913-7.

Guénette SY. Astrocytes: a cellular player in Abeta clearance and degradation. Trends Mol Med 2003; 9: 279-80.

Görg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, et al. The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 2000; 21: 1037-53.

Görg A, Weiss W, Dunn MJ. Current two-dimensional electrophoresis technology for proteomics. Proteomics 2004; 4: 3665-85.

Haass C, Schlossmacher MG, Hung AY, Vigo-Pelfrey C, Mellon A, Ostaszewski BL, et al. Amyloid beta-peptide is produced by cultured cells during normal metabolism. Nature 1992; 359: 322-5.

Hage DS. Affinity chromatography: a review of clinical applications. Clin Chem 1999; 45: 593-615.

Hamacher M, Klose J, Rossier J, Marcus K, Meyer HE. "Does understanding the brain need proteomics and does understanding proteomics need brains?"--Second HUPO HBPP Workshop hosted in Paris. Proteomics 2004; 4: 1932-4.

Hamidi Asl K, Liepnieks JJ, Nakamura M, Benson MD. Organ-specific (localized) synthesis of Ig light chain amyloid. J Immunol 1999; 162: 5556-60.

Hanash S. HUPO initiatives relevant to clinical proteomics. Mol Cell Proteomics 2004; 3: 298-301. Epub 2004 Jan 27.

Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 2002; 297: 353-6.

Harman D. Aging: a theory based on free radical and radiation chemistry. J Gerontol 1956; 11: 298-300.

Harr SD, Uint L, Hollister R, Hyman BT, Mendez AJ. Brain expression of apolipoproteins E, J, and A-I in Alzheimer's disease. J Neurochem 1996; 66: 2429-35.

Harrington MG, Merril CR. Two-dimensional electrophoresis and "ultrasensitive" silver staining of cerebrospinal fluid proteins in neurological diseases. Clin Chem 1984; 30: 1933-7.

Harrington MG, Merril CR, Asher DM, Gajdusek DC. Abnormal proteins in the cerebrospinal fluid of patients with Creutzfeldt-Jakob disease. N Engl J Med 1986; 315: 279-83.

Harrington MG, Merril CR, Torrey EF. Differences in cerebrospinal fluid proteins between patients with schizophrenia and normal persons. Clin Chem 1985; 31: 722-6.

Hartmann T. Intracellular biology of Alzheimer's disease amyloid beta peptide. Eur Arch Psychiatry Clin Neurosci 1999; 249: 291-8.

Hartmann T, Bergsdorf C, Sandbrink R, Tienari PJ, Multhaup G, Ida N, et al. Alzheimer's disease betaA4 protein release and amyloid precursor protein sorting are regulated by alternative splicing. J Biol Chem 1996; 271: 13208-14.

Henzel WJ, Billeci TM, Stults JT, Wong SC, Grimley C, Watanabe C. Identifying proteins from two-dimensional gels by molecular mass searching of peptide fragments in protein sequence databases. Proc Natl Acad Sci U S A 1993; 90: 5011-5.

Herukka SK, Hallikainen M, Soininen H, Pirttilä T. CSF Abeta42 and tau or phosphorylated tau and prediction of progressive mild cognitive impairment. Neurology 2005; 64: 1294-7.

Hesse C, Nilsson CL, Blennow K, Davidsson P. Identification of the apolipoprotein E4 isoform in cerebrospinal fluid with preparative two-dimensional electrophoresis and matrix assisted laser desorption/ionization-time of flight-mass spectrometry. Electrophoresis 2001; 22: 1834-7.

Hixson JE, Vernier DT, Tsukamoto K, Watanabe T, Matsushima T, Kinoshita M, et al. Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with HhaI determination by PCR-RFLP of apo E genotype in a Japanese population. J Lipid Res 1990; 31: 545-8.

Hochstrasser DF. Proteome in perspective. Clin Chem Lab Med 1998; 36: 825-36.
Hynd MR, Lewohl JM, Scott HL, Dodd PR. Biochemical and molecular studies using human autopsy brain tissue. J Neurochem 2003; 85: 543-62.

Hynd MR, Scott HL, Dodd PR. Glutamate-mediated excitotoxicity and neurodegeneration in Alzheimer's disease. Neurochem Int 2004; 45: 583-95.

Ingrosso L, Vetrugno V, Cardone F, Pocchiari M. Molecular diagnostics of transmissible spongiform encephalopathies. Trends Mol Med 2002; 8: 273-280.

Irie K, Murakami K, Masuda Y, Morimoto A, Ohigashi H, Ohashi R, et al. Structure of beta-amyloid fibrils and its relevance to their neurotoxicity: implications for the pathogenesis of Alzheimer's disease. J Biosci Bioeng 2005; 99: 437-47.

Irizarry MC. Biomarkers of Alzheimer disease in plasma. NeuroRx 2004; 1: 226-34.
Itzhaki RF, Wozniak MA, Appelt DM, Balin BJ. Infiltration of the brain by pathogens causes Alzheimer's disease. Neurobiol Aging 2004; 25: 619-27.

Jabbour W, Pouplard-Barthelaix A, Houlgatte R, Emile J. Abnormal expression of actin in lymphocytes of Alzheimer's disease and Down's syndrome patients. J Neuroimmunol 1992; 38: 199-208.

Jellinger KA. Pathology and pathophysiology of vascular cognitive impairment. A critical update. Panminerva Med 2004; 46: 217-26.

Johnson G, Gotlib J, Haroutunian V, Bierer L, Nairn AC, Merril C, et al. Increased phosphorylation of elongation factor 2 in Alzheimer's disease. Brain Res Mol Brain Res 1992; 15: 319-26.

Jorm AF. Is depression a risk factor for dementia or cognitive decline? A review. Gerontology 2000; 46: 219-27.

Jorm AF, Korten AE, Henderson AS. The prevalence of dementia: a quantitative integration of the literature. Acta Psychiatr Scand 1987; 76: 465-79.

Jönsson L, Berr C. Cost of dementia in Europe. Eur J Neurol 2005; 12: 50-3.

Kang J, Lemaire HG, Unterbeck A, Salbaum JM, Masters CL, Grzeschik KH, et al. The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. Nature 1987; 325: 733-6.

Kanninen K, Goldsteins G, Auriola S, Alafuzoff I, Koistinaho J. Glycosylation changes in Alzheimer's disease as revealed by a proteomic approach. Neurosci Lett 2004; 367: 235-40.

Karas M, Hillenkamp F. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal Chem 1988; 60: 2299-301.

Katayama T, Imaizumi K, Manabe T, Hitomi J, Kudo T, Tohyama M. Induction of neuronal death by ER stress in Alzheimer's disease. J Chem Neuroanat 2004; 28: 67-78.

Katzman R. Dementias. Postgrad Med 1978; 64: 119-25.

Kaufmann H, Bailey JE, Fussenegger M. Use of antibodies for detection of phosphorylated proteins separated by two-dimensional gel electrophoresis. Proteomics 2001; 1: 194-9.

Keller JN. Age-related neuropathology, cognitive decline, and Alzheimer's disease. Ageing Res Rev 2006; 5: 1-13. Epub 2005 Aug 9.

Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972; 26: 239-57.

Khachaturian ZS. Diagnosis of Alzheimer's disease. Arch Neurol 1985; 42: 1097-105.

Kimmich GA, Roussie JA, Randles J. Aspartate aminotransferase isotope exchange reactions: implications for glutamate/glutamine shuttle hypothesis. Am J Physiol Cell Physiol 2002; 282: C1404-13.

Kitzmueller E, Greber S, Fountoulakis M, Lubec G. Carbohydrate handling enzymes in fetal Down syndrome brain. J Neural Transm Suppl 2001: 203-10.

Kivatinitz SC, Pelsman MA, Alonso AC, Bagatolli L, Quiroga S. High-density lipoprotein aggregated by oxidation induces degeneration of neuronal cells. J Neurochem 1997; 69: 2102-14.

Kivipelto M, Helkala EL, Laakso MP, Hänninen T, Hallikainen M, Alhainen K, et al. Apolipoprotein E epsilon4 allele, elevated midlife total cholesterol level, and high midlife systolic blood pressure are independent risk factors for late-life Alzheimer disease. Ann Intern Med 2002; 137: 149-55.

Kivipelto M, Helkala EL, Laakso MP, Hänninen T, Hallikainen M, Alhainen K, et al. Midlife vascular risk factors and Alzheimer's disease in later life: longitudinal, population based study. Bmj 2001; 322: 1447-51.

Klein WL. Abeta toxicity in Alzheimer's disease: globular oligomers (ADDLs) as new vaccine and drug targets. Neurochem Int 2002; 41: 345-52.

Klose J. Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals. Humangenetik 1975; 26: 231-43.

Klose J, Zeindl E. An attempt to resolve all the various proteins in a single human cell type by two-dimensional electrophoresis: I. Extraction of all cell proteins. Clin Chem 1984; 30: 2014-20.

Kosik KS, Crandall JE, Mufson EJ, Neve RL. Tau in situ hybridization in normal and Alzheimer brain: localization in the somatodendritic compartment. Ann Neurol 1989; 26: 352-61.

Kosik KS, Joachim CL, Selkoe DJ. Microtubule-associated protein tau (tau) is a major antigenic component of paired helical filaments in Alzheimer disease. Proc Natl Acad Sci U S A 1986; 83: 4044-8.

Kosunen O, Soininen H, Paljärvi L, Heinonen O, Talasniemi S, Riekkinen PJ, Sr. Diagnostic accuracy of Alzheimer's disease: a neuropathological study. Acta Neuropathol (Berl) 1996; 91: 185-93.

Kraszpulski M, Soininen H, Riekkinen P, Sr., Alafuzoff I. Pitfalls in the quantitative estimation of beta-amyloid immunoreactivity in human brain tissue. Histochem Cell Biol 1998; 110: 439-45.

Ksiezak-Reding H, Binder LI, Yen SH. Alzheimer disease proteins (A68) share epitopes with tau but show distinct biochemical properties. J Neurosci Res 1990; 25: 420-30.

Laakso MP. Structural imaging in cognitive impairment and the dementias: an update. Curr Opin Neurol 2002; 15: 415-21.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680-5.

LaFerla FM. Calcium dyshomeostasis and intracellular signalling in Alzheimer's disease. Nat Rev Neurosci 2002; 3: 862-72.

LaFerla FM, Oddo S. Alzheimer's disease: Abeta, tau and synaptic dysfunction. Trends Mol Med 2005; 11: 170-6.

Latner AL. Some clinical biochemical aspects of isoelectric focusing. Ann N Y Acad Sci 1973; 209: 281-98.

LeBlanc AC, Chen HY, Autilio-Gambetti L, Gambetti P. Differential APP gene expression in rat cerebral cortex, meninges, and primary astroglial, microglial and neuronal cultures. FEBS Lett 1991; 292: 171-8.

LeBlanc AC, Xue R, Gambetti P. Amyloid precursor protein metabolism in primary cell cultures of neurons, astrocytes, and microglia. J Neurochem 1996; 66: 2300-10.

Lee HG, Perry G, Moreira PI, Garrett MR, Liu Q, Zhu X, et al. Tau phosphorylation in Alzheimer's disease: pathogen or protector? Trends Mol Med 2005; 11: 164-9.

Lefebvre T, Ferreira S, Dupont-Wallois L, Bussière T, Dupire MJ, Delacourte A, et al. Evidence of a balance between phosphorylation and O-GlcNAc glycosylation of Tau proteins--a role in nuclear localization. Biochim Biophys Acta 2003; 1619: 167-76.

Letenneur L. Risk of dementia and alcohol and wine consumption: a review of recent results. Biol Res 2004; 37: 189-93.

Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, et al. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol 1990; 186: 464-78.

Lewis DA. The human brain revisited: opportunities and challenges in postmortem studies of psychiatric disorders. Neuropsychopharmacology 2002; 26: 143-54.

Levy-Lahad E, Wasco W, Poorkaj P, Romano DM, Oshima J, Pettingell WH, et al. Candidate gene for the chromosome 1 familial Alzheimer's disease locus. Science 1995; 269: 973-7.

Lilley KS, Friedman DB. All about DIGE: quantification technology for differential-display 2D-gel proteomics. Expert Rev Proteomics 2004; 1: 401-9.

Ling EA, Wong WC. The origin and nature of ramified and amoeboid microglia: a historical review and current concepts. Glia 1993; 7: 9-18.

Liu F, Zaidi T, Iqbal K, Grundke-Iqbal I, Gong CX. Aberrant glycosylation modulates phosphorylation of tau by protein kinase A and dephosphorylation of tau by protein phosphatase 2A and 5. Neuroscience 2002a; 115: 829-37.

Liu F, Zaidi T, Iqbal K, Grundke-Iqbal I, Merkle RK, Gong CX. Role of glycosylation in hyperphosphorylation of tau in Alzheimer's disease. FEBS Lett 2002b; 512: 101-6.

Loo DT, Copani A, Pike CJ, Whittemore ER, Walencewicz AJ, Cotman CW. Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. Proc Natl Acad Sci U S A 1993; 90: 7951-5.

Luchsinger JA, Mayeux R. Dietary factors and Alzheimer's disease. Lancet Neurol 2004; 3: 579-87.

Lue LF, Kuo YM, Roher AE, Brachova L, Shen Y, Sue L, et al. Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease. Am J Pathol 1999; 155: 853-62.

Mann M, Jensen ON. Proteomic analysis of post-translational modifications. Nat Biotechnol 2003; 21: 255-61.

Mann M, Wilm M. Error-tolerant identification of peptides in sequence databases by peptide sequence tags. Anal Chem 1994; 66: 4390-9.

Marchetti B, Abbracchio MP. To be or not to be (inflamed)--is that the question in anti-inflammatory drug therapy of neurodegenerative disorders? Trends Pharmacol Sci 2005; 26: 517-25.

Mariani E, Polidori MC, Cherubini A, Mecocci P. Oxidative stress in brain aging, neurodegenerative and vascular diseases: an overview. J Chromatogr B Analyt Technol Biomed Life Sci 2005; 827: 65-75. Epub 2005 Sep 23.

Mattila KM, Frey H. Alzheimer brain proteins investigated by two-dimensional gel electrophoresis with immobilized pH gradients in the first dimension. Electrophoresis 1994; 15: 721-5.

Mattila KM, Frey H. Two-dimensional analysis of qualitative and quantitative changes in blood cell proteins in Alzheimer's disease: search for extraneuronal markers. Appl Theor Electrophor 1995; 4: 189-96.

Mattila KM, Pirttilä T, Blennow K, Wallin A, Viitanen M, Frey H. Altered blood-brain-barrier function in Alzheimer's disease? Acta Neurol Scand 1994; 89: 192-8.

Mattson MP. Infectious agents and age-related neurodegenerative disorders. Ageing Res Rev 2004a; 3: 105-20.

Mattson MP. Pathways towards and away from Alzheimer's disease. Nature 2004b; 430: 631-9.

Maurer K, Volk S, Gerbaldo H. Auguste D and Alzheimer's disease. Lancet 1997; 349: 1546-9.

McDowell I. Alzheimer's disease: insights from epidemiology. Aging (Milano) 2001; 13: 143-62.

McGeer EG, McGeer PL. Innate immunity in Alzheimer's disease: a model for local inflammatory reactions. Mol Interv 2001; 1: 22-9.

McKenna MC, Stevenson JH, Huang X, Hopkins IB. Differential distribution of the enzymes glutamate dehydrogenase and aspartate aminotransferase in cortical synaptic mitochondria contributes to metabolic compartmentation in cortical synaptic terminals. Neurochem Int 2000; 37: 229-41.

McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology 1984; 34: 939-44.

McLean CA, Cherny RA, Fraser FW, Fuller SJ, Smith MJ, Beyreuther K, et al. Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. Ann Neurol 1999; 46: 860-6.

Meier-Ruge W, Iwangoff P, Reichlmeier K. Neurochemical enzyme changes in Alzheimer's and Pick's disease. Arch Gerontol Geriatr 1984; 3: 161-5.

Miquel J, Economos AC, Fleming J, Johnson JE, Jr. Mitochondrial role in cell aging. Exp Gerontol 1980; 15: 575-91.

Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, et al. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. Neurology 1991; 41: 479-86.

Mulnard RA, Corrada MM, Kawas CH. Estrogen replacement therapy, Alzheimer's disease, and mild cognitive impairment. Curr Neurol Neurosci Rep 2004; 4: 368-73.

Nagele RG, Wegiel J, Venkataraman V, Imaki H, Wang KC. Contribution of glial cells to the development of amyloid plaques in Alzheimer's disease. Neurobiol Aging 2004; 25: 663-74.

Nagy Z, Esiri MM, Jobst KA, Morris JH, King EM, McDonald B, et al. Relative roles of plaques and tangles in the dementia of Alzheimer's disease: correlations using three sets of neuropathological criteria. Dementia 1995; 6: 21-31.

Nakamura A, Goto S. Analysis of protein carbonyls with 2,4-dinitrophenyl hydrazine and its antibodies by immunoblot in two-dimensional gel electrophoresis. J Biochem (Tokyo) 1996; 119: 768-74.

Neve RL, Robakis NK. Alzheimer's disease: a re-examination of the amyloid hypothesis. Trends Neurosci 1998; 21: 15-9.

NIA. Consensus recommendations for the postmortem diagnosis of Alzheimer's disease. The National Institute on Aging, and Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's Disease. Neurobiol Aging 1997; 18: S1-2.

Nicoll JA, Weller RO. A new role for astrocytes: beta-amyloid homeostasis and degradation. Trends Mol Med 2003; 9: 281-2.

Nilsberth C, Westlind-Danielsson A, Eckman CB, Condron MM, Axelman K, Forsell C, et al. The 'Arctic' APP mutation (E693G) causes Alzheimer's disease by enhanced Abeta protofibril formation. Nat Neurosci 2001; 4: 887-93.

Noetzel MJ. Phosphorylation of the glial fibrillary acidic protein. J Neurosci Res 1990; 27: 184-92.

Norenberg MD. Astrocyte responses to CNS injury. J Neuropathol Exp Neurol 1994; 53: 213-20.

Nunan J, Small DH. Regulation of APP cleavage by alpha-, beta- and gamma-secretases. FEBS Lett 2000; 483: 6-10.

Nunomura A, Perry G, Aliev G, Hirai K, Takeda A, Balraj EK, et al. Oxidative damage is the earliest event in Alzheimer disease. J Neuropathol Exp Neurol 2001; 60: 759-67.

Nyman TA, Matikainen S, Sareneva T, Julkunen I, Kalkkinen N. Proteome analysis reveals ubiquitin-conjugating enzymes to be a new family of interferon-alpha-regulated genes. Eur J Biochem 2000; 267: 4011-9.

O'Connor T, Ireland LS, Harrison DJ, Hayes JD. Major differences exist in the function and tissue-specific expression of human aflatoxin B1 aldehyde reductase and the principal human aldo-keto reductase AKR1 family members. Biochem J 1999; 343: 487-504.

O'Farrell PH. High resolution two-dimensional electrophoresis of proteins. J Biol Chem 1975; 250: 4007-21.

Olaisen B, Teisberg P, Gedde-Dahl T, Jr. The locus for apolipoprotein E (apoE) is linked to the complement component C3 (C3) locus on chromosome 19 in man. Hum Genet 1982; 62: 233-6.

Overmyer M, Helisalmi S, Soininen H, Laakso M, Riekkinen P, Sr., Alafuzoff I. Astrogliosis and the ApoE genotype. an immunohistochemical study of postmortem human brain tissue. Dement Geriatr Cogn Disord 1999a; 10: 252-7.

Overmyer M, Helisalmi S, Soininen H, Laakso M, Riekkinen P, Sr., Alafuzoff I. Reactive microglia in aging and dementia: an immunohistochemical study of postmortem human brain tissue. Acta Neuropathol (Berl) 1999b; 97: 383-92.

Papasozomenos SC. Tau protein immunoreactivity in dementia of the Alzheimer type. I. Morphology, evolution, distribution, and pathogenetic implications. Lab Invest 1989; 60: 123-37.

Paschen W. Role of calcium in neuronal cell injury: which subcellular compartment is involved? Brain Res Bull 2000; 53: 409-13.

Patton WF. Detection technologies in proteome analysis. J Chromatogr B Analyt Technol Biomed Life Sci 2002; 771: 3-31.

Payami H, Zareparsi S, Montee KR, Sexton GJ, Kaye JA, Bird TD, et al. Gender difference in apolipoprotein E-associated risk for familial Alzheimer disease: a possible clue to the higher incidence of Alzheimer disease in women. Am J Hum Genet 1996; 58: 803-11.

Pereira C, Agostinho P, Moreira PI, Cardoso SM, Oliveira CR. Alzheimer's disease-associated neurotoxic mechanisms and neuroprotective strategies. Curr Drug Targets CNS Neurol Disord 2005; 4: 383-403.

Petersen RC. Mild cognitive impairment as a diagnostic entity. J Intern Med 2004; 256: 183-94.

Petersen RC, Smith GE, Waring SC, Ivnik RJ, Tangalos EG, Kokmen E. Mild cognitive impairment: clinical characterization and outcome. Arch Neurol 1999; 56: 303-8.

Picklo MJ, Montine TJ, Amarnath V, Neely MD. Carbonyl toxicology and Alzheimer's disease. Toxicol Appl Pharmacol 2002; 184: 187-97.

Plassman BL, Havlik RJ, Steffens DC, Helms MJ, Newman TN, Drosdick D, et al. Documented head injury in early adulthood and risk of Alzheimer's disease and other dementias. Neurology 2000; 55: 1158-66.

Polvikoski T, Sulkava R, Haltia M, Kainulainen K, Vuorio A, Verkkoniemi A, et al. Apolipoprotein E, dementia, and cortical deposition of beta-amyloid protein. N Engl J Med 1995; 333: 1242-7.

Polvikoski T, Sulkava R, Myllykangas L, Notkola IL, Niinistö L, Verkkoniemi A, et al. Prevalence of Alzheimer's disease in very elderly people: a prospective neuropathological study. Neurology 2001; 56: 1690-6.

Porchet R, Probst A, Bouras C, Dráberova E, Dráber P, Riederer BM. Analysis of glial acidic fibrillary protein in the human entorhinal cortex during aging and in Alzheimer's disease. Proteomics 2003; 3: 1476-85.

Praticò D. Peripheral biomarkers of oxidative damage in Alzheimer's disease: the road ahead. Neurobiol Aging 2005; 26: 581-3.

Praticò D, Clark CM, Liun F, Rokach J, Lee VY, Trojanowski JQ. Increase of brain oxidative stress in mild cognitive impairment: a possible predictor of Alzheimer disease. Arch Neurol 2002; 59: 972-6.

Price DL, Tanzi RE, Borchelt DR, Sisodia SS. Alzheimer's disease: genetic studies and transgenic models. Annu Rev Genet 1998; 32: 461-93.

Price JL, Morris JC. Tangles and plaques in nondemented aging and "preclinical" Alzheimer's disease. Ann Neurol 1999; 45: 358-68.

Puchades M, Hansson SF, Nilsson CL, Andreasen N, Blennow K, Davidsson P, et al. Proteomic studies of potential cerebrospinal fluid protein markers for Alzheimer's disease. Brain Res Mol Brain Res 2003; 118: 140-6.

Qiu C, Backman L, Winblad B, Aguero-Torres H, Fratiglioni L. The influence of education on clinically diagnosed dementia incidence and mortality data from the Kungsholmen Project. Arch Neurol 2001; 58: 2034-9.

Raber J, Huang Y, Ashford JW. ApoE genotype accounts for the vast majority of AD risk and AD pathology. Neurobiol Aging 2004; 25: 641-50.

Ramassamy C, Averill D, Beffert U, Bastianetto S, Theroux L, Lussier-Cacan S, et al. Oxidative damage and protection by antioxidants in the frontal cortex of Alzheimer's disease is related to the apolipoprotein E genotype. Free Radic Biol Med 1999; 27: 544-53.

Ramassamy C, Krzywkowski P, Averill D, Lussier-Cacan S, Theroux L, Christen Y, et al. Impact of apoE deficiency on oxidative insults and antioxidant levels in the brain. Brain Res Mol Brain Res 2001; 86: 76-83.

Raymond S, Weintraub L. Acrylamide gel as a supporting medium for zone electrophoresis. Science 1959; 130: 711.

Reddy PH, McWeeney S. Mapping cellular transcriptosomes in autopsied Alzheimer's disease subjects and relevant animal models. Neurobiol Aging 2005; 9: 9.

Rego AC, Oliveira CR. Mitochondrial dysfunction and reactive oxygen species in excitotoxicity and apoptosis: implications for the pathogenesis of neurodegenerative diseases. Neurochem Res 2003; 28: 1563-74.

Reiber H, Peter JB.Cerebrospinal fluid analysis: disease-related data patterns and evaluation programs. J Neurol Sci 2001; 184: 101-122.

Reinders J, Lewandrowski U, Moebius J, Wagner Y, Sickmann A. Challenges in mass spectrometry-based proteomics. Proteomics 2004; 4: 3686-703.

Reinders J, Sickmann A. State-of-the-art in phosphoproteomics. Proteomics 2005; 5: 4052-61.

Riemenschneider M, Buch K, Schmolke M, Kurz A, Guder WG. Diagnosis of Alzheimer's disease with cerebrospinal fluid tau protein and aspartate aminotransferase. Lancet 1997; 350: 784.

Righetti PG. Bioanalysis: its past, present, and some future. Electrophoresis 2004; 25: 2111-27.

Righetti PG, Castagna A, Antonucci F, Piubelli C, Cecconi D, Campostrini N, et al. Proteome analysis in the clinical chemistry laboratory: myth or reality? Clin Chim Acta 2005; 357: 123-39.

Riley KP, Snowdon DA, Markesbery WR. Alzheimer's neurofibrillary pathology and the spectrum of cognitive function: findings from the Nun Study. Ann Neurol 2002; 51: 567-77.

Robinson SR. Neuronal expression of glutamine synthetase in Alzheimer's disease indicates a profound impairment of metabolic interactions with astrocytes. Neurochem Int 2000; 36: 471-82.

Rocca WA, Hofman A, Brayne C, Breteler MM, Clarke M, Copeland JR, et al. Frequency and distribution of Alzheimer's disease in Europe: a collaborative study of 1980-1990 prevalence findings. The EURODEM-Prevalence Research Group. Ann Neurol 1991; 30: 381-90.

Rocchi A, Pellegrini S, Siciliano G, Murri L. Causative and susceptibility genes for Alzheimer's disease: a review. Brain Res Bull 2003; 61: 1-24.

Rock RB, Gekker G, Hu S, Sheng WS, Cheeran M, Lokensgard JR, et al. Role of microglia in central nervous system infections. Clin Microbiol Rev 2004; 17: 942-64.

Rogaev EI, Sherrington R, Rogaeva EA, Levesque G, Ikeda M, Liang Y, et al. Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. Nature 1995; 376: 775-8.

Rosenfeld J, Capdevielle J, Guillemot JC, Ferrara P. In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. Anal Biochem 1992; 203: 173-9.

Ross GW, O'Callaghan JP, Sharp DS, Petrovitch H, Miller DB, Abbott RD, et al. Quantification of regional glial fibrillary acidic protein levels in Alzheimer's disease. Acta Neurol Scand 2003; 107: 318-23.

Sastre J, Pallardo FV, Vina J. The role of mitochondrial oxidative stress in aging. Free Radic Biol Med 2003; 35: 1-8.

Sastry PS, Rao KS. Apoptosis and the nervous system. J Neurochem 2000; 74: 1-20.

Sayre LM, Moreira PI, Smith MA, Perry G. Metal ions and oxidative protein modification in neurological disease. Ann Ist Super Sanita 2005; 41: 143-164.

Schmidt O, Schulenborg T, Meyer HE, Marcus K, Hamacher M. How proteomics reveals potential biomarkers in brain diseases. Expert Rev Proteomics 2005; 2: 901-13.

Schneider LV, Hall MP. Stable isotope methods for high-precision proteomics. Drug Discov Today 2005; 10: 353-63.

Schonberger SJ, Edgar PF, Kydd R, Faull RL, Cooper GJ. Proteomic analysis of the brain in Alzheimer's disease: molecular phenotype of a complex disease process. Proteomics 2001; 1: 1519-28.

Schröder R, Linke RP. Cerebrovascular involvement in systemic AA and AL amyloidosis: a clear haematogenic pattern. Virchows Arch 1999; 434: 551-60.

Schröder M, Kaufman RJ. ER stress and the unfolded protein response. Mutat Res 2005; 569: 29-63.

Schupf N, Sergievsky GH. Genetic and host factors for dementia in Down's syndrome. Br J Psychiatry 2002; 180: 405-10.

Scott RB. Extraneuronal manifestations of Alzheimer's disease. J Am Geriatr Soc 1993; 41: 268-76.

Selkoe DJ. Alzheimer's disease is a synaptic failure. Science 2002; 298: 789-91.

Sergeant N, David JP, Lefranc D, Vermersch P, Wattez A, Delacourte A. Different distribution of phosphorylated tau protein isoforms in Alzheimer's and Pick's diseases. FEBS Lett 1997; 412: 578-82.

Shacter E, Williams JA, Lim M, Levine RL. Differential susceptibility of plasma proteins to oxidative modification: examination by western blot immunoassay. Free Radic Biol Med 1994; 17: 429-37.

Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, et al. Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. Nature 1995; 375: 754-60.

Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 1996; 68: 850-8.

Shringarpure R, Davies KJ. Protein turnover by the proteasome in aging and disease. Free Radic Biol Med 2002; 32: 1084-9.

Siesjö BK. Cell damage in the brain: a speculative synthesis. J Cereb Blood Flow Metab 1981; 1: 155-85.

Smale G, Nichols NR, Brady DR, Finch CE, Horton WE, Jr. Evidence for apoptotic cell death in Alzheimer's disease. Exp Neurol 1995; 133: 225-30.

Smirnov AV, Shevtsov PN, Burbaeva G. Two-dimensional electrophoretic analysis of the protein spectrum of human brain structures in schizophrenia and senile dementia of the Alzheimer type. Zh Nevropatol Psikhiatr Im S S Korsakova 1991; 91: 34-6.

Snowdon DA, Greiner LH, Mortimer JA, Riley KP, Greiner PA, Markesbery WR. Brain infarction and the clinical expression of Alzheimer disease. The Nun Study. Jama 1997; 277: 813-7.

Southan C. Has the yo-yo stopped? An assessment of human protein-coding gene number. Proteomics 2004; 4: 1712-26.

Speck CE, Kukull WA, Brenner DE, Bowen JD, McCormick WC, Teri L, et al. History of depression as a risk factor for Alzheimer's disease. Epidemiology 1995; 6: 366-9.

Stern Y, Gurland B, Tatemichi TK, Tang MX, Wilder D, Mayeux R. Influence of education and occupation on the incidence of Alzheimer's disease. Jama 1994; 271: 1004-10.

Stoothoff WH, Johnson GV. Tau phosphorylation: physiological and pathological consequences. Biochim Biophys Acta 2005; 1739: 280-97.

Strandberg TE, Pitkälä KH, Linnavuori K, Tilvis RS. Cognitive impairment and infectious burden in the elderly. Arch Gerontol Geriatr Suppl 2004: 419-23.

Streit WJ. Microglia and neuroprotection: implications for Alzheimer's disease. Brain Res Brain Res Rev 2005; 48: 234-9. Epub 2005 Jan 15.

Stuchbury G, Münch G. Alzheimer's associated inflammation, potential drug targets and future therapies. J Neural Transm 2005; 112: 429-53. Epub 2004 Jul 28.

Su JH, Anderson AJ, Cummings BJ, Cotman CW. Immunohistochemical evidence for apoptosis in Alzheimer's disease. Neuroreport 1994; 5: 2529-33.

Suh YH, Checler F. Amyloid precursor protein, presenilins, and alpha-synuclein: molecular pathogenesis and pharmacological applications in Alzheimer's disease. Pharmacol Rev 2002; 54: 469-525.

Sultana R, Boyd-Kimball D, Poon HF, Cai J, Pierce WM, Klein JB, et al. Redox proteomics identification of oxidized proteins in Alzheimer's disease hippocampus and cerebellum: An approach to understand pathological and biochemical alterations in AD. Neurobiol Aging 2005; 3: 3.

Svensson H. Isoelectric fractionation, abalysis, and characterization of ampholytes in natural pH gradients I, The differential equation of solute concentration at a steady state and its solution for simple cases. Acta Chem Scand 1961; 15: 325-341.

Talent JM, Kong Y, Gracy RW. A double stain for total and oxidized proteins from two-dimensional fingerprints. Anal Biochem 1998; 263: 31-8.

Tanaka K, Waki H, Ido Y, S. A, Yoshida Y, T. Y. Protein and polymer analyses up to m/z 100,000 by laser ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 1988; 2: 151-153.

Tang MX, Jacobs D, Stern Y, Marder K, Schofield P, Gurland B, et al. Effect of oestrogen during menopause on risk and age at onset of Alzheimer's disease. Lancet 1996; 348: 429-32.

Tapiola T, Lehtovirta M, Pirttilä T, Alafuzoff I, Riekkinen P, Soininen H. Increased aspartate aminotransferase activity in cerebrospinal fluid and Alzheimer's disease. Lancet 1998; 352: 287.

Terry RD, Katzman R. Senile dementia of the Alzheimer type. Ann Neurol 1983; 14: 497-506.

Thiede B, Hohenwarter W, Krah A, Mattow J, Schmid M, Schmidt F, et al. Peptide mass fingerprinting. Methods 2005; 35: 237-47. Epub 2005 Jan 12.

Thinakaran G, Parent AT. Identification of the role of presenilins beyond Alzheimer's disease. Pharmacol Res 2004; 50: 411-8.

Tilvis RS, Kahonen-Vare MH, Jolkkonen J, Valvanne J, Pitkälä KH, Strandberg TE. Predictors of cognitive decline and mortality of aged people over a 10-year period. J Gerontol A Biol Sci Med Sci 2004; 59: 268-74.

Tolnay M, Sergeant N, Ghestem A, Chalbot S, De Vos RA, Jansen Steur EN, et al. Argyrophilic grain disease and Alzheimer's disease are distinguished by their different distribution of tau protein isoforms. Acta Neuropathol (Berl) 2002; 104: 425-34. Epub 2002 Jul 26.

Townsend LE, Gilroy J, LeWitt P, Wolfe DE, Pomara N, Weintraub J, et al. Comparison of methods for analysis of CSF proteins in patients with Alzheimer's disease. Neurochem Pathol 1987; 6: 213-29.

Tretter L, Adam-Vizi V. Inhibition of Krebs cycle enzymes by hydrogen peroxide: A key role of alpha-ketoglutarate dehydrogenase in limiting NADH production under oxidative stress. J Neurosci 2000; 20: 8972-9.

Tsuji T, Shimohama S, Kamiya S, Sazuka T, Ohara O. Analysis of brain proteins in Alzheimer's disease using high-resolution two-dimensional gel electrophoresis. J Neurol Sci 1999; 166: 100-6.

Tsukamoto K, Watanabe T, Matsushima T, Kinoshita M, Kato H, Hashimoto Y, et al. Determination by PCR-RFLP of apo E genotype in a Japanese population. J Lab Clin Med 1993; 121: 598-602.

Tuppo EE, Arias HR. The role of inflammation in Alzheimer's disease. Int J Biochem Cell Biol 2005; 37: 289-305.

Turner RS. Alzheimer's disease in man and transgenic mice: females at higher risk. Am J Pathol 2001; 158: 797-801.

Ueno I, Sakai T, Yamaoka M, Yoshida R, Tsugita A. Analysis of blood plasma proteins in patients with Alzheimer's disease by two-dimensional electrophoresis, sequence homology and immunodetection. Electrophoresis 2000; 21: 1832-45.

Uryu K, Laurer H, McIntosh T, Pratico D, Martinez D, Leight S, et al. Repetitive mild brain trauma accelerates Abeta deposition, lipid peroxidation, and cognitive impairment in a transgenic mouse model of Alzheimer amyloidosis. J Neurosci 2002; 22: 446-54.

Ünlü M, Morgan ME, Minden JS. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 1997; 18: 2071-7.

van Duijn CM, Tanja TA, Haaxma R, Schulte W, Saan RJ, Lameris AJ, et al. Head trauma and the risk of Alzheimer's disease. Am J Epidemiol 1992; 135: 775-82.

Vercauteren FG, Bergeron JJ, Vandesande F, Arckens L, Quirion R. Proteomic approaches in brain research and neuropharmacology. Eur J Pharmacol 2004; 500: 385-98.

Vesterberg O, Svensson H. Isoelectric fractionation, analysis, and characterization of ampholytes in natural pH gradients. IV. Further studies on the resolving power in connection with separation of myoglobins. Acta Chem Scand 1966; 20: 820-34.

Vincent HK, Taylor AG. Biomarkers and potential mechanisms of obesity-induced oxidant stress in humans. Int J Obes 2005; 22: 22.

Weidemann A, Konig G, Bunke D, Fischer P, Salbaum JM, Masters CL, et al. Identification, biogenesis, and localization of precursors of Alzheimer's disease A4 amyloid protein. Cell 1989; 57: 115-26.

Weingarten MD, Lockwood AH, Hwo SY, Kirschner MW. A protein factor essential for microtubule assembly. Proc Natl Acad Sci U S A 1975; 72: 1858-62.

Wilkins MR, Sanchez JC, Gooley AA, Appel RD, Humphery-Smith I, Hochstrasser DF, et al. Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. Biotechnol Genet Eng Rev 1996; 13: 19-50.

Yaffe MB. How do 14-3-3 proteins work?-- Gatekeeper phosphorylation and the molecular anvil hypothesis. FEBS Lett 2002; 513: 53-7.

Yan LJ, Levine RL, Sohal RS. Oxidative damage during aging targets mitochondrial aconitase. Proc Natl Acad Sci U S A 1997; 94: 11168-72.

Yates JR, 3rd. Mass spectrometry and the age of the proteome. J Mass Spectrom 1998; 33: 1-19.

Yates JR, 3rd, Eng JK, McCormack AL, Schieltz D. Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. Anal Chem 1995; 67: 1426-36.

Yoo BC, Kim SH, Cairns N, Fountoulakis M, Lubec G. Deranged expression of molecular chaperones in brains of patients with Alzheimer's disease. Biochem Biophys Res Commun 2001; 280: 249-58.

Younkin SG. Evidence that A beta 42 is the real culprit in Alzheimer's disease. Ann Neurol 1995; 37: 287-8.

Yu HL, Chertkow HM, Bergman H, Schipper HM. Aberrant profiles of native and oxidized glycoproteins in Alzheimer plasma. Proteomics 2003; 3: 2240-8.

Yudkoff M, Nelson D, Daikhin Y, Erecinska M. Tricarboxylic acid cycle in rat brain synaptosomes. Fluxes and interactions with aspartate aminotransferase and malate/aspartate shuttle. J Biol Chem 1994; 269: 27414-20.

Zandi PP, Carlson MC, Plassman BL, Welsh-Bohmer KA, Mayer LS, Steffens DC, et al. Hormone replacement therapy and incidence of Alzheimer disease in older women: the Cache County Study. Jama 2002; 288: 2123-9.

Zannis VI, Just PW, Breslow JL. Human apolipoprotein E isoprotein subclasses are genetically determined. Am J Hum Genet 1981; 33: 11-24.

Zerr I, Pocchiari M, Collins S, Brandel JP, de Pedro Cuesta J, Knight RS, et al. Analysis of EEG and CSF 14-3-3 proteins as aids to the diagnosis of Creutzfeldt-Jakob disease. Neurology 2000; 55: 811-815.

Zhang R, Barker L, Pinchev D, Marshall J, Rasamoelisolo M, Smith C, et al. Mining biomarkers in human sera using proteomic tools. Proteomics 2004; 4: 244-56.

Zhu X, Rottkamp CA, Boux H, Takeda A, Perry G, Smith MA. Activation of p38 kinase links tau phosphorylation, oxidative stress, and cell cycle-related events in Alzheimer disease. J Neuropathol Exp Neurol 2000; 59: 880-8.

APPENDIX: ORIGINAL PUBLICATIONS (I-IV)

I

Proteomic analysis of protein oxidation in Alzheimer's disease brain

Korolainen MA, Goldsteins G, Alafuzoff I, Koistinaho J, Pirttilä T

Electrophoresis 2002; 23(19):3428-33

II

Oxidative modification of proteins in the frontal cortex of Alzheimer's disease brain

Korolainen MA, Goldsteins G, Nyman TA, Alafuzoff I, Koistinaho J, Pirttilä T

Neurobiology of Aging 2006; 27(1):42-53. Epub 2005 Jan 23.

III

Proteomic analysis of glial fibrillary acidic protein in Alzheimer's disease and aging brain

Korolainen MA, Auriola S, Nyman TA, Alafuzoff I, Pirttilä T

Neurobiology of Disease 2005; 20(3):858-70. Epub 2005 Jun 27

IV

Oxidation and levels of cerebrospinal fluid proteins in aging and Alzheimer's disease

Korolainen MA, Nyman TA, Nyyssönen P, Hartikainen ES, Pirttilä T

Submitted for publication

PUBLICATIONS
SERIES OF REPORTS, DEPARTMENT OF NEUROLOGY

1. **Juhani Partanen (1978):** Time-locked phenomena of human motor unit potentials. An electromyographic study of satellites and doubles.
2. **Eeva Leino (1981):** Clinical and biochemical studies on progressive myoclonus epilepsy.
3. **Hilkka Soininen (1981):** Senile dementia. A clinical, neurochemical and etiological study.
4. **Rolf Danner (1982):** Adverse effects of anticonvulsive treatment on peripheral nerve conduction and posterior dominant EEG rhythm.
5. **Markku Saksa (1982):** The autonomic nervous system in experimental allergic neuritis. A functional, morphological and biochemical study.
6. **Juhani Sivenius (1982):** Studies on the rehabilitation, epidemiology and clinical features of stroke in East Central Finland.
7. **Asla Pitkänen (1987):** Somatostatin in epilepsy. An experimental and clinical study.
8. **Esa Mervaala (1987):** Evoked potential in human epilepsy. A neurophysiological study.
9. **Kari Reinikainen (1988):** Neurotransmitters in Alzheimer's disease.
10. **Tapani Keränen (1988):** Epilepsy in adults. An epidemiologic study in Eastern Finland.
11. **Jukka Jolkkonen (1988):** Vasopressin in the central nervous system. A study based on cerebrospinal fluid measurements.
12. **Jouni Sirviö (1989):** The cholinergic system in ageing and dementia. With special reference to acetylcholinesterase.
13. **Hannu Koponen (1989):** Delirium in the elderly. A clinical, neurochemical, neuropsychological and neuroradiological study.
14. **Asla Pitkänen (1989):** Somatostatin in experimental and human epilepsy.
15. **Eeva-Liisa Helkala (1990):** Memory in patients with Alzheimer's disease and demented patients with Parkinson's disease.
16. **-**
17. **Paavo Riekkinen Jr (1990):** Animal models of age-related degeneration of subcortical regulatory systems. With special reference to cholinergic, noradrenergic and serotonergic systems.
18. **Toivo Halonen (1990):** Neurotransmitter amino acids in epileptic convulsions and during vigabatrin treatment.
19. **Ulla Lepola (1990):** Panic disorder. A clinical, neurochemical, neuropsychological, and neuroradiological study.
20. **Kari Murros (1991):** Stress reactions of brain infarction. A prospective study on 105 patients with acute ischemic brain infarction of internal carotid artery territory.
21. **Aarne Ylinen (1991):** Hippocampal reactions and their pharmacotherapy in experimental epilepsy.
22. **Antti Valjakka (1992):** The subcortical deafferentation of the hippocampus and noradrenergic lesions as experimental models of dementia. Hippocampal electrophysiology.
23. **Aimo Rissanen (1992):** Cerebrovascular disease in the Jyväskylä region, Central Finland.
24. **Reetta Kälviäinen (1992):** Newly diagnosed epileptic seizure disorder in adults. A prospective follow-up study on 100 patients.
25. **Maria Mazurkiewicz (1992):** The effects of the enhanced GABAergic transmission on cognitive functions: An experimental study.
26. **Pekka Jäkälä (1992):** Modulation of attention and working memory by noradrenergic, serotonergic and cholinergic systems. An experimental neuropsychopharmacological study.
27. **Kari Alhainen (1992):** Anticholinesterase drug, tacrine (THA), in Alzheimer's disease. Discrimination of responders and nonresponders.
28. **Riitta Miettinen (1993):** Inhibitory circuits and subcortical innervation of the rat hippocampus: Implications for normal function and pathophysiological processes.
29. **Hannele Lahtinen (1993):** Hippocampus in experimental models of temporal lobe epilepsy. Amino acid-mediated neurotransmission and nerve cell injury following the transection of fimbria-fornix and the electrical stimulation of perforant pathway in rat.

30. **Päivi Hartikainen (1994):** Normal ageing. A neurochemical, neurophysiological and neuropsychological study with special reference to Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis.
31. **Outi Heinonen (1994):** Neuropathologic and peripheral markers of Alzheimer's disease with special emphasis on β-amyloid accumulation.
32. **Minna Riekkinen (1994):** The interactions between cholinergic and serotonergic systems in the modulation of spatial navigation and passive avoidance behavior. An experimental neuropsychopharmacological study.
33. **Keijo Koivisto (1995):** Population-based dementia screening program in the city of Kuopio, Eastern Finland: Evaluation of screening methods, prevalence of dementia and dementia subtypes.
34. **Arja Tuunainen (1995):** Evaluation of epileptic patients for temporal lobe surgery and postoperative follow-up. An electrophysiological study with neuropsychological, psychiatric and clinical correlates.
35. **Mervi Pitkänen (1995):** The role and pharmacological modulation of the NMDA receptor/channel on hippocampal synaptic transmission and behavior.
36. **Olli Kosunen (1996):** A neuropathologic study on Alzheimer's disease with a special emphasis on diagnostic accuracy.
37. **Mikko Laakso (1996):** MRI of hippocampus in incipient Alzheimer's disease.
38. **Maarit Lehtovirta (1996):** Familial Alzheimer's disease. A clinical and molecular genetic study.
39. **Tuomo Hänninen (1996):** Age-associated memory impairment. A neuropsychological and epidemiological study.
40. **Vesa Savander (1997):** Organization of intrinsic connections in the rat amygdaloid complex with special emphasis on the lateral, basal and accessory basal nuclei.
41. **Heikki Sorvari (1997):** Neurons containing calcium-binding proteins in the human amygdaloid complex.
42. **Tiina Kotti (1997):** Excitotoxicity-induced neuropathological changes in the rodent hippocampus. Possible functional consequences and drug treatments.
43. **Sirja Ruotsalainen (1997):** Serotonergic system and its interactions with cholinergic receptor mediated mechanisms in the modulation of working memory. An experimental study.
44. **Seppo Helisalmi (1998):** Molecular genetics of Alzheimer's disease with special emphasis on presenilin, amyloid beta precursor protein and apolipoprotein E genes.
45. **Merja Hallikainen (1998):** Age-associated memory impairment, and apolipoprotein E. A population-based clinical, neuropsychological, neurophysiological and neuroimaging study.
46. **Matti Vanhanen (1998):** Cognitive function in glucose intolerance in the elderly: the role of hyperinsulinemia.
47. **Kirsi Juottonen (1998):** MRI-volumes of the entorhinal, perirhinal and temporopolar cortices in normal aging and in Alzheimer´s disease.
48. **Raimo Pussinen (1999):** An experimental study on the role of α_1-adrenoceptors and putrescine in the modulation of hippocampal plasticity and memory encoding - interactions with NMDA receptors.
49. **Tarja Puumala (1999):** Monoamines in the modulation of attention and response inhibition: development of a new animal model of attention deficit and impulsivity.
50. **Mia Mikkonen (1999):** The human entorhinal cortex. Anatomic organization and its alteration in Alzheimer's disease and temporal lobe epilepsy.
51. **Jukka Puoliväli (2000):** An experimental study on the cholinergic modulation of cortical arousal and cognitive functions. With special emphasis on apolipoprotein E.
52. **Kauko Pitkänen (2000):** Stroke rehabilitation in the elderly. A controlled study of the effectiveness and costs of a multidimensional intervention.
53. **Mikko Hiltunen (2000):** A molecular genetic study of factors involved in Alzheimer's disease.
54. **Sami Ikonen (2001):** The role of the septohippocampal cholinergic system in cognitive functions.

55. **Tuuli Salmenperä (2001):** Damage in the hippocampus, amygdala, entorhinal and perirhinal cortex of adults with partial epilepsy.
56. **Zinayida Bezvenyuk (2001):** Multiple pathways of DNA disintegration during neuronal apoptosis.
57. **Tero Tapiola (2001):** Biological markers for Alzheimer's disease. With special emphasis on cerebrospinal fluid ß-amyloid and tau.
58. **Kirsi Puurunen (2001):** The effects of pharmacotherapy and training on functional recovery after global and focal cerebral ischemia in rats.
59. **Maaria Ikonen (2001):** Apoptosis-associated changes in neuronal gene expression. With special emphasis on the insulin-like growth factor system.
60. **Inga Kadish (2002):** Plasticity in the entorhinal-hippocampal pathway. Influences of gene mutations and hormones.
61. **Pauliina Korhonen (2002):** Gene regulation in neuronal degeneration - Role of mSin3 and YY1 factors.
62. **Miia Kivipelto (2002):** Vascular risk factors in Alzheimer's disease and mild cognitive impairment. A longitudinal, population-based study.
63. **Margit Overmyer (2002):** Gliosis in relation to Alzheimer's hallmark lesions in aging and Alzheimer's disease. A postmortem immunohistochemical study.
64. **Marja Äikiä (2002):** Verbal memory in newly diagnosed partial epilepsy. A neuropsychological study.
65. **Li Liu (2003):** Cholinergic neurotransmission, amyloid-β peptide and the pathogenesis of Alzheimer's Disease. A study in the APP and PS1 double transgenic mouse model.
66. **Jun Wang (2003):** The role of Aβ-peptide on spatial memory, EEG, auditory evoked potentials and nicotinic cholinergic receptors in A/P transgenic mice.
67. **Juhana Aura (2003):** Interaction of muscarinic acetylcholine and N-methyl-D-aspartate –type glutamate receptors in the regulation of spatial learning and memory.
68. **Johanna Kuhmonen (2003):** Neuroprotection in experimental acute cerebral ischaemia: α2-adrenoreceptor agonism, MAO-B inhibition, and enhancement of GABAergic neurotransmission as neuroprotective strategies.
69. **Jaana Autere (2003):** Genetics of Parkinson's Disease in the Finnish Population.
70. **Erkki Kuusisto (2004):** Role of the p62 protein in the formation of neuropathological cytoplasmic inclusions.
71. **Maija Pihlajamäki (2004):** Functional MRI studies on human declarative memory.
72. **Chuan-sheng Zhao (2005):** Psychotropic medication and functional recovery following cortical stroke in aged rats.
73. **Dimitrije Jakovljević (2005):** The roles of chronobiological and socioeconomic factors in the occurrence of cerebrovascular diseases.
74. **Sinikka Peurala (2005):** Rehabilitation of gait in chronic stroke patients.
75. **Laura Parkkinen (2005):** Impact of α-synuclein pathology on aging.
76. **Iain Wilson (2005):** Hippocampal place cells as a window into cognitive aging.
77. **Susan Iivonen (2005):** Genetic and expressional studies of Alzheimer's disease candidate genes. Emphasis on CYP19, seladin-1 and HSPG2 genes.
78. **Jouni Ihalainen (2005):** Regulation of dopamine release in the forebrain by alpha2-adrenoceptors and NMDA glutamate receptors - a microdialysis study.
79. **Giedrius Kalesnykas (2005):** Cholinergic neurons of the rodent basal forebrain and their content of estrogen receptor alpha.
80. **Marina Boccardi (2006):** MRI studies in frontotemporal dementia.
81. **Anne Koivisto (2006):** Genetic components of late-onset Alzheimer's disease with special emphasis on ApoE, IL-6, CYP46, SERPINA3 and PPARγ.
82. **Taneli Heikkinen (2006):** Cognitive effects of estrogen in ovariectomized, aged and transgenic mice modeling Alzheimer's disease.